Mutational Analysis of the QRRQ Motif in the Yeast Hig1 Type 2 Protein Rcf1 Reveals a Regulatory Role for the Cytochrome c Oxidase Complex

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The yeast Rcf1 protein is a member of the conserved family of proteins termed the hypoxia-induced gene (domain) 1 (Hig1 or HIGD1) family. Rcf1 interacts with components of the mitochondrial oxidative phosphorylation system, in particular the cytochrome bc_{1} (complex III)-cytochrome c oxidase (complex IV) supercomplex (termed III-IV) and the ADP/ATP carrier proteins. Rcf1 plays a role in the assembly and modulation of the activity of complex IV; however, the molecular basis for how Rcf1 influences the activity of complex IV is currently unknown. Hig1 type 2 isoforms, which include the Rcf1 protein, are characterized in part by the presence of a conserved motif, (Q/I)LX_{3}(R/H)XXR_{3}Q, termed here the QRRQ motif. We show that mutation of conserved residues within the Rcf1 QRRQ motif alters the interactions between Rcf1 and partner proteins and results in the destabilization of complex IV and alteration of its enzymatic properties. Our findings indicate that Rcf1 does not serve as a stoichiometric component, i.e. as a subunit of complex IV, to support its activity. Rather, we propose that Rcf1 serves to dynamically interact with complex IV during its assembly process and, in doing so, regulates a late maturation step of complex IV. We speculate that the Rcf1/Hig1 proteins play a role in the assembly and modulation of complex IV, to support its activity. Rather, we propose that Rcf1 influences the activity of complex IV is currently unknown. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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§ The abbreviations used are: OXPHOS, oxidative phosphorylation; AAC, ADP/ATP carrier; CL, cardiolipin; BN, blue native; DDM, dodecyl maltoside; Ni-NTA, nickel-nitrilotriacetic acid; CCCP, carbonyl cyanide p-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; MBS, m-maleimidobenzoyl-N-hydroxysuccinimide ester; PG, phosphatidylglycerol; OCR, oxygen consumption rate; TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride.
IV, which, together with Cox2 and Cox3, is involved in the binding of substrate, cytochrome c, to complex IV (23, 24). A close association of the Rcf1 and AAC proteins has also been reported (23). It is currently unknown whether the roles of AAC and the Rcf1/Rcf2 proteins in supporting complex IV levels are related and whether they may involve the lipid CL.

Rcf1 and Rcf2 are both members of the conserved protein family termed hypoxia-induced gene 1 family (Hig1) (also referred to as hypoxia-inducible gene domain (HIGD1) family). The Hig1 protein family can largely be divided into two subgroups of isoforms, termed the Hig1 type 1 and the more universally found type 2 isoforms, and the classification of these isoforms is based largely on the presence or absence of a conserved (Q/I)₃(R/H)X₃(Q/R) motif (termed here the QRRQ motif), which is characteristically found in Hig1 type 2 family members. The Hig1 type 1 proteins characteristically contain a modified version of this motif, e.g. the HIGD1A protein in mammals has (I/V/L)HLIHMR₃Q motif instead. Although the Hig1 type 2 isoforms are universally found in all eukaryotes (and in α-proteobacteria) and appear to represent hypoxia- and stress-induced isoforms (25–27), Rcf1 and Rcf2 are Hig1 type 2 proteins, and S. cerevisiae, like many lower eukaryotes (e.g. fungi and nematodes), does not contain Hig1 type 1 isoforms.

In this study, we have sought to gain further understanding of the significance of Rcf1 involvement with complex IV assembly and its enzymatic properties. We have explored the relevance of the Hig1 type 2 QRRQ motif for the function of yeast Rcf1. As outlined below, our findings indicate that Rcf1 does not act as a subunit of the assembled complex IV enzyme to support its activity, but, rather, we propose that Rcf1 transiently associates with a late-stage assembly intermediate of complex IV to modify it, possibly its lipid composition, and, by doing so, alters its enzymatic properties.

Results

Expression of Rcf1 QRRQ Mutant Derivatives—Rcf1, like the other members of the Hig1 protein family, is an integral inner mitochondrial membrane protein with two predicted transmembrane segments (Fig. 1A). A limited sequence alignment illustrates the conserved nature of the QRRQ motif from diverse species, such as in α-proteobacteria, yeast (both Rcf1 and Rcf2), nematodes, and mammals (Fig. 1B). In the yeast Rcf1 protein, the QRRQ motif corresponds to residues 61–71, i.e. 61Q₃X₃R₃X₃Q₇1 (Fig. 1, A and B).

The functional significance of the conserved QRRQ motif in the yeast Rcf1 protein was investigated by adopting a strategy of alanine site-directed mutagenesis. Two distinct Rcf1 mutant derivatives were initially created. In the first mutant, glutamates 61 and 71 were simultaneously mutated to alanines to create the rcf1/Q₃G₃A₃G₇₁ derivative. In the second mutant, derivative arginine 67 was mutated to alanine (rcf1/Q₃R₃₇A₃A₃). Rcf1 mutant derivatives were expressed in C-terminal His-tagged derivatives in the Rcf1/Rcf2 double-null yeast strain (Δrcf1Δrcf2). Analysis of isolated mitochondria from the resulting strains revealed that the steady-state levels of the rcf1/Q₃R₃₇A₃A₃ and rcf1/Q₃G₃A₃G₇₁ derivatives appeared to be similar to that of the wild-type rcf1/Q₃R₃₇A₃A₃ control, thus indicating that mutation of the QRRQ motif in this manner did not adversely affect the stability of the Rcf1 protein (Fig. 1C). The Δrcf1Δrcf2 yeast strain displays a respiration-based growth defect (23). The expression of rcf1/Q₃R₃₇A₃A₃ and rcf1/Q₃G₃A₃G₇₁ derivatives, like the Rcf1 wild-type protein, largely complemented the Δrcf1Δrcf2 respiratory growth defect (growth on the non-fermentable carbon source, glycerol) (Fig. 1D). This finding indicates that an intact QRRQ motif is not essential for the ability of Rcf1 to support OXPHOS-based yeast growth.

Expression of rcf1/Q₃R₃₇A₃A₃ Alters the Complex IV Assembly State—The Δrcf1Δrcf2 mutant mitochondria examined contained reduced levels of complex IV subunits (23). In contrast, the Δrcf1Δrcf2 mitochondria harboring His-tagged wild-type Rcf1 or the rcf1/Q₃R₃₇A₃A₃ or rcf1/Q₃G₃A₃G₇₁ derivatives appeared to have normal steady-state levels of all complex IV subunits analyzed (Fig. 2A). We therefore conclude that an intact QRRQ motif does not appear to be required by Rcf1 to support the stable accumulation of complex IV subunits.

![FIGURE 1. Rcf1, an inner membrane protein and member of Hig1 type 2 family and the conserved QRRQ motif. A, The N-inward orientation of Rcf1 in the inner membrane with N and C tails exposed to the intermembrane space. The location of QRRQ (Gln61, Arg67, Gln71) motif relative to the two transmembrane segments (TM1 and TM2, respectively) is indicated. B, Limited sequence alignment (amino acid residue numbers are indicated) of the QRRQ motif region from a selection of Hig1 type 2 family members. Hs, Homo sapiens (NP_620175); Bt, Bos taurus (NP_001071329); Ce, C. elegans (NP_001254152); Bj, Bradyrhizobium japonicum (WP_014491643). C, mitochondria were isolated from the wild type, the Δrcf1Δrcf2 controls ( ), or the Δrcf1Δrcf2 strain harboring either His-tagged Rcf1 (rcf1/q3A7A), rcf1/q3A7A (Gln61, Gln71) or rcf1/q3A7A (R67A) derivatives. Rcf1 and Tim44 (control) levels were analyzed by Western blotting. D, Serial 10-fold dilutions of the WT and Δrcf1Δrcf2 expressing Rcf1/q3A7A, rcf1/q3A7A, or rcf1/q3A7A derivatives or not, as indicated, were spotted on YP plates containing glucose (YPAD) or glycerol supplemented with 0.1% galactose (YPAD + 0.1% Gal) and grown at 30 °C.](https://asbmb.org/jbc/article-pdf/5217/13/5127/5127_1.pdf)
QRRQ Motif of the Yeast Hig1 Type 2 Protein Rcf1

Through blue native gel electrophoresis (BN-PAGE), we next addressed whether the QRRQ-mutated derivatives of Rcf1 could support the assembly of complex IV and its association with complex III into the III-IV supercomplex, which is altered in the absence of Rcf1 and Rcf2 (23). In \( \Delta rcf1; \Delta rcf2 \) mitochondria, there is a pronounced shift from the \( III_2-IV_2 \) form (observed in the wild-type control mitochondria) to a predominately \( III_2-IV \) form and also to free \( III_2 \) complexes (Fig. 2B), which has been previously attributed to the limiting levels of complex IV in the absence of Rcf1/Rcf2 (23). The \( III_2-IV_{(1-2)} \) supercomplex organizational state was largely restored in \( \Delta rcf1; \Delta rcf2 \) mitochondria harboring wild-type Rcf1\(_{His}\) (Fig. 2B). The Rcf1\(_{His}^{Q61A,Q71A, R67A}\) mutants, analyzed in parallel, displayed a similar capacity as the wild-type Rcf1\(_{His}\) derivative to rescue the organization of the III-IV supercomplex in \( \Delta rcf1; \Delta rcf2 \) mitochondria.

These results indicate that an intact QRRQ motif is not required for the ability of Rcf1 to support the assembly of complex IV and its ability to co-assemble with complex III. Although this conclusion is consistent with the observed steady-state levels of complex IV subunits, which appeared normal in the mitochondria harboring the QRRQ mutant Rcf1 derivatives, we observed a noticeable difference in the behavior of complex IV from Rcf1\(_{His}^{R67A}\) mitochondria when solubilized with the detergent dodecyl maltoside (DDM) prior to the BN-PAGE analysis (Fig. 2C). Solubilization of mitochondria with DDM causes complex IV to become physically separated from the III-IV supercomplex assembly, and released complex IV migrates independently as monomers (IV) on the BN-PAGE (24, 28). In wild-type mitochondria, a small fraction of a slightly larger form of complex IV, termed IV*, was also detected and represents the population of monomeric complex IV, where the peripheral subunits Cox12 and Cox13 remain in association under DDM solubilization conditions (Ref. 24 and data not shown). The level of the DDM-solubilized complex IV from wild-type or \( \Delta rcf1; \Delta rcf2 \) mitochondria was considerably greater than that observed from the Rcf1\(_{His}^{Q61A,Q71A}\) mitochondria (and also from Rcf1\(_{His}^{Q61A,Q71A, R67A}\)) was considerably greater than that observed from the Rcf1\(_{His}^{R67A}\) mitochondria, where complex IV levels (relative to the wild-type control) were reduced by \( ~50\% \), as reported previously (23). In contrast, however, the Rcf1\(_{His}^{Q61A,Q71A}\)-containing mitochondria displayed strongly reduced levels of complex IV monomers following DDM solubilization, with levels that resembled more that of the \( \Delta rcf1; \Delta rcf2 \) mitochondria (Fig. 2C). Furthermore, in addition to the monomeric complex IV population, the Rcf1\(_{His}^{R67A}\) mitochondria contained a novel complex IV subpopulation, termed IV**, that migrated slower on the BN-PAGE gel. The sum of the levels of both complex IV populations (IV + IV**) observed in the Rcf1\(_{His}^{R67A}\) mutant mitochondria following DDM solubilization were significantly lower than those from the mitochondria harboring either the Rcf1\(_{His}^{Q61A,Q71A}\) or Rcf1\(_{His}^{Q61A,Q71A, R67A}\) derivatives. The reduced levels of the complex IV species (IV and IV*) observed in the DDM extracts of Rcf1\(_{His}^{R67A}\) is inconsistent with the apparently normal steady-state levels of complex IV subunits and the regular appearance of the dodecyl-solubilized III-IV supercomplex in these mitochondria. Together with the observed presence of the novel complex IV** species, these results suggest that the assembly state of complex IV is altered and appears more DDM detergent-labile in Rcf1\(_{His}^{R67A}\) mitochondria compared with the wild-type control.

The presence of the novel larger IV** species in \( \Delta rcf1; \Delta rcf2 \) mitochondria harboring the Rcf1\(_{His}^{R67A}\) derivative was unexpected, and its composition was further investigated (Fig. 3). Mitochondrial proteins were solubilized in DDM, and the complex IV populations were separated by BN-PAGE and subsequently serially analyzed by mass spectrometry. In the wild-type control, the majority of the complex IV subunits detected were present in the IV and IV* species, whereas the Cox13 pro-
tein was detected only in the IV* population. (The small subunit Cox12 was not identified in the datasets.) The absence of detectable levels of Rcf1 in these complex IV or IV* populations is notable and suggests that, in the wild type, Rcf1 is not a component of the fully assembled complex IV/IV* enzyme. A small amount of Rcf1 in the wild-type sample was, however, detected in a higher molecular mass area of the BN-PAGE and co-migrated at a position of the IV** species, a species that was more evident in rcf1His R67A mitochondria. This area of the BN gel was also populated with minor but detectable amounts of complex IV subunits, suggesting that this may correspond to a Rcf1-containing late assembly intermediate of complex IV. When the Δrcf1;Δrcf2 mitochondria harboring the Rcf1His protein were analyzed, in addition to both IV and IV* populations, small but detectable levels of the IV** subpopulation and some co-migrating Rcf1His species were observed. However, the ratio of the IV** to the IV subpopulation was distinctly increased in rcf1HisR67A sample relative to the other mitochondrial types and in agreement with the BN-PAGE analysis shown in Fig. 2C. The total amount of rcf1HisR67A protein co-purifying with the IV** species was also considerably greater than that observed with the wild-type Rcf1His sample (also confirmed by Western blotting analysis, data not shown). Because the relative stoichiometric abundance of the complex IV subunits in the IV** species appeared similar to that of the IV species, we conclude that the novel complex IV** subpopulation is, at least in part, characterized by the stable association of the Rcf1 protein. From its mobility on the BN-PAGE, we estimated the apparent mass of IV** to be 234 kDa. The estimated mass of the IV** species (relative to the IV population, 197 kDa) would indicate that IV** may contain one copy of Rcf1His (19.9 kDa) with another unknown protein of similar size or two copies of Rcf1His. We favor that more than one copy of Rcf1 is present in the IV** subpopulation, as we have observed that the Rcf1 proteins can interact with each other and can at least form dimers (data not shown).

In summary, these findings support the conclusion that, in wild-type (or Rcf1His) mitochondria, the majority of complex IV (i.e. the IV and IV* species) is not present in association with the Rcf1 protein. Furthermore, the data indicate that the rcf1HisR67A derivative may have a tighter association (possibly a higher affinity or less dynamic interaction) with complex IV than its wild-type Rcf1 counterpart and thus results in the accumulation of a larger, novel Rcf1-associated complex IV species, IV**.

The QRRQ Motif Influences the Association of Rcf1 with Complex IV and the Cox3 Subunit—The levels of complex IV associated with the His-tagged Rcf1 derivatives was next investigated through affinity purification via Ni-NTA chromatography following solubilization from mitochondria with Triton X-100 (Fig. 4A). A significantly higher level of complex IV subunits co-purified with the rcf1HisR67A derivative compared with the control Rcf1His protein analyzed in parallel (Fig. 4A). Consistent with the Triton X-100 lysis/purification results, enhanced association of complex IV subunits with the rcf1HisR67A derivative relative to the wild-type Rcf1His control was also observed under digitonin conditions (where the III-IV association is preserved) (Fig. 4B).

Co-purification of complex IV subunits with the rcf1HisQ61A,Q71A derivative was not observed under Triton X-100 solubilization conditions (Fig. 4A) but was observed (together with complex III subunits) under digitonin solubili-
Cox3 subunit. Mutation of Arg67 to alanine causes an enhanced complex IV interaction through an altered interaction with the QRRQ motif has the potential to impact the nature of the Rcf1-D complex IV interaction as evidenced by the elevated levels of the complex IV** species and the increased association of complex IV subunits and radiolabeled Cox3 with the rcf1\textsubscript{His}^R67A derivative.

**Complex IV in Mitochondria Harboring the rcf1\textsubscript{His}^R67A Derivative Displays Altered Enzymatic Properties**—The enzymatic properties of complex IV in rcf1\textsubscript{His}^R67A mitochondria were explored next. The complex IV enzyme solubilized in DDM retains its enzymatic activity, as evidenced by "in-gel" activity assays performed with exogenously added cytochrome c and diaminobenzidine following BN-PAGE analysis (Fig. 5A, center panel). The level of complex IV activity was strongly reduced in the Δrcf1;Δrcf2 mutant compared with both the wild-type control and the null mutant harboring the Rcf1\textsubscript{His} protein. This reduced complex IV enzyme activity can be attributed to the reduction in complex IV protein levels in the Δrcf1;Δrcf2 mutant (Figs. 2A, 2C, and 5A). Despite also having reduced protein content (as judged by parallel Western blotting analysis; Fig. 5A, top panel), the levels of DDM-solubilized complex IV enzyme activity in both the IV and IV** subpopulations in the rcf1\textsubscript{His}^R67A sample appeared similar to, or even higher than, those obtained for the IV population in the Δrcf1;Δrcf2+Rcf1\textsubscript{His} or the rcf1\textsubscript{His}^Q61A,Q71A mitochondria (Fig. 5A, center panel). These results suggest that the complex IV/IV** subpopulations solubilized from rcf1\textsubscript{His}^R67A exhibit an increased level of enzyme-specific activity.

The O2 consumption capacity of mitochondria harboring the Rcf1 derivatives under different bioenergetic states was mea-
levels of Coomassie-stained complex V (F1F0-ATP synthase) are shown in the both wild-type Rcf1His and the QRRQ mutant derivatives (Fig. 5E). A similar effect was observed in Δrcf1;Δrcf2 mitochondria. DCCD binds to Cox3 (Glu99 residue in bovine Cox3, equivalent to Glu98 in yeast) and, in doing so, interferes with the oxygen uptake pathway of complex IV, which involves associated lipids, phosphatidylglycerol (PG1/PG2), and CL molecules within the enzyme (29, 30). We speculate that structural changes in complex IV in rcf1His R67A mitochondria may alter the accessibility of DCCD to its target. Alterations in the lipid composition of complex IV may underlie this and would be consistent with the observed instability of complex IV from rcf1His R67A mitochondria to extraction with the detergent DDM.

**QRRQ Motif of the Yeast Hig1 Type 2 Protein Rcf1**

QRRQ motif in an α-helical motif that arginine help stabilize a complex IV enzyme in the rcf1His R67A mitochondria displayed an increased sensitivity to DCCD relative to those harboring the wild-type Rcf1His (Fig. 5E). A similar effect was observed in Δrcf1;Δrcf2 mitochondria. DCCD binds to Cox3 (Glu99 residue in bovine Cox3, equivalent to Glu98 in yeast) and, in doing so, interferes with the oxygen uptake pathway of complex IV, which involves associated lipids, phosphatidylglycerol (PG1/PG2), and CL molecules within the enzyme (29, 30). We speculate that structural changes in complex IV in rcf1His R67A mitochondria may alter the accessibility of DCCD to its target. Alterations in the lipid composition of complex IV may underlie this and would be consistent with the observed instability of complex IV from rcf1His R67A mitochondria to extraction with the detergent DDM.

Rcf1 May Be Involved in Posttranslational Modification of Complex IV—Analysis of the amino acid sequence region encompassing the QRRQ motif in an α-helical motif that arginine help stabilize a complex IV enzyme in the rcf1His R67A mitochondria displayed an increased sensitivity to DCCD relative to those harboring the wild-type Rcf1His (Fig. 5E). A similar effect was observed in Δrcf1;Δrcf2 mitochondria. DCCD binds to Cox3 (Glu99 residue in bovine Cox3, equivalent to Glu98 in yeast) and, in doing so, interferes with the oxygen uptake pathway of complex IV, which involves associated lipids, phosphatidylglycerol (PG1/PG2), and CL molecules within the enzyme (29, 30). We speculate that structural changes in complex IV in rcf1His R67A mitochondria may alter the accessibility of DCCD to its target. Alterations in the lipid composition of complex IV may underlie this and would be consistent with the observed instability of complex IV from rcf1His R67A mitochondria to extraction with the detergent DDM.

Despite the strongly reduced levels of the rcf1His Q61A,R65A protein, the complex IV subunit levels in the mutant mitochondria were similar to that of the Rcf1His control and significantly higher than those observed in the Δrcf1;Δrcf2 null mitochondria (Fig. 6B). The organization and levels of the III-IV supercomplex in the rcf1His Q61A,R65A mitochondria also resembled those of the Rcf1His control analyzed in parallel (Fig. 6C, top panel). Furthermore, the levels of DDM-solubilized complex IV in the rcf1His Q61A,R65A mitochondria were similar to those of the wild-type Rcf1His control (Fig. 6B). Thus, mutation of either of the Gln/Arg pairs, compared with the wild-type Rcf1His control (Fig. 6B). Thus, mutation of either of the Gln/Arg pairs in this manner strongly impacted the stability and steady-state levels of the Rcf1 protein. Mutation of residue Arg62 alone (i.e. rcf1His R65A) did not compromise the stability of Rcf1 or its ability to support the assembly of a functional complex IV (supplemental Fig. S1). Similar to the rcf1His Q67A derivative (albeit to a lesser extent), an increase in the level of complex III-IV subunits in association with the affinity-purified rcf1His Q61A,R65A derivative was observed (supplemental Fig. S1).

QRRQ motif in an α-helical motif that arginine help stabilize a complex IV enzyme in the rcf1His R67A mitochondria displayed an increased sensitivity to DCCD relative to those harboring the wild-type Rcf1His (Fig. 5E). A similar effect was observed in Δrcf1;Δrcf2 mitochondria. DCCD binds to Cox3 (Glu99 residue in bovine Cox3, equivalent to Glu98 in yeast) and, in doing so, interferes with the oxygen uptake pathway of complex IV, which involves associated lipids, phosphatidylglycerol (PG1/PG2), and CL molecules within the enzyme (29, 30). We speculate that structural changes in complex IV in rcf1His R67A mitochondria may alter the accessibility of DCCD to its target. Alterations in the lipid composition of complex IV may underlie this and would be consistent with the observed instability of complex IV from rcf1His R67A mitochondria to extraction with the detergent DDM.

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Although the steady-state levels of the rcf1His R67A,Q71A derivative were marginally higher than that of rcf1His Q61A,R65A protein, the presence of the rcf1His R67A,Q71A protein only partially restored the complex IV subunits to wild-type levels, indicating...
that the ability of this Rcf1 derivative to support complex IV assembly was compromised through mutation of the Arg67/Gln71 pair (Fig. 6B). Consistently, a partial restoration of the III+ - IV- species was observed in the BN-PAGE analysis of the digitonin-solubilized rcf1HisR67A,Q71A mitochondria, paralleling the observation that the levels of the complex IV subunits in these mitochondria were greater than those in the Δrcf1;Δrcf2 control but not equivalent to those of the wild-type control (or of the rcf1HisQ61A,R65A mitochondria) (Fig. 6C, top panel). However, when solubilized with DDM, the levels of free complex IV from rcf1HisR67A,Q71A mitochondria appeared similar to those of the Δrcf1;Δrcf2 null mitochondria and in contrast to those from the rcf1HisQ61A,R65A mitochondria, where the levels of complex IV were similar to the wild-type Rcf1His control (Fig. 6C, bottom panel). These results together suggest that complex IV from the rcf1HisR67A,Q71A mitochondria may exhibit some detergent instability to DDM extraction in a similar manner as observed previously in the rcf1HisR67A mitochondria (Fig. 3C). In contrast to the rcf1HisR67A mitochondria, the presence of a complex IV** form was not detected in the rcf1HisR67A,Q71A mitochondrial sample. This may be attributed to the strongly reduced levels of rcf1HisR67A,Q71A derivative in these mitochondria and, thus, would be limiting to form detectable levels of the complex IV** species, and/or the introduction of the additional Q71A mutation may have compromised the stability of the Rcf1-complex IV association.

Measurement of the maximal O2 consumption rates of complex IV in the rcf1HisQ61A,R65A mitochondria demonstrated them to be fully restored to the levels in wild-type Rcf1His control mitochondria, whereas, in rcf1HisR67A,Q71A mitochondria, although they were significantly higher than those measured in the Δrcf1;Δrcf2 null mitochondria, they were not equivalent to the rcf1HisQ61A,R65A or wild-type Rcf1His mitochondria (Fig. 6D). The increased level of complex IV activity in both of these mutants was sufficient to restore aerobic growth, as expression of both rcf1HisQ61A,R65A and rcf1HisR67A,Q71A derivatives ensured complementation of the growth defect phenotype of the Δrcf1;Δrcf2 strain (Fig. 6E).

In summary, the Gln61/Arg65 and Arg67/Gln71 residue pairs within the QRRQ motif of Rcf1 are independently critical for the stability of the Rcf1 protein. However, despite being strongly reduced in levels, the Gln/Arg pair mutated Rcf1 derivatives could support (fully for the Arg67/Gln71 mutant) the assembly and activity of complex IV.

The Integrity of the Rcf1 QRRQ Motif Alters the Molecular Environment of the Rcf1 Protein—As described previously, wild-type Rcf1His, the most notable being a 45-kDa adduct reported previously to represent cross-linking of Rcf1 to the AAC proteins (23). The rcf1HisQ61A,Q71A derivative maintained the ability to be cross-linked to AAC, whereas the cross-linking between rcf1HisR67A and AAC was significantly reduced (Fig. 7A) despite the normal levels of the AAC protein in these mitochondria (Fig. 2C). The MBS cross-linking profile of the rcf1HisR65A derivative resembled that of the rcf1HisQ61A,R65A or wild-type Rcf1His mitochondria (Fig. 3C). In addition to the loss of the Rcf1-AAC adduct in rcf1HisR67A mitochondria, we observed the gain of a novel adduct near 50 kDa. This rcf1HisR67A adduct was close in size to, but distinct from, a 52-kDa Rcf1 adduct observed in rcf1HisQ61A and rcf1HisQ61A,Q71A mitochondria. As the mass of the 50-kDa Rcf1 adduct was similar to the sum of the His-tagged Rcf1 (20 kDa) and subunit 2 of complex IV, Cox2 (30 kDa), we explored whether this represented an Rcf1-Cox2 adduct. Decoration of a parallel experiment with Cox2-specific-antiseraum demonstrated that a MBS-formed adduct of similar size (50
A linking was performed and analyzed as described in IV is altered in the therefore conclude that the arrangement of Rcf1 with complex shown) and consistent with the Rcf1-antibody decoration. We (epitope antiserum was performed. The positions of the dominant 45-kDa cated. Following SDS-PAGE and Western blotting, decoration with His tag (23). Thus, by binding as an additional subunit to populations of the enzyme (i.e. in stochiometrically equivalent amounts as other complex IV subunits), Rcf1 may exert an influence over substrate binding and the enzymatic properties of complex IV. Indeed, the binding of a Hig1 family member, HIGD1A, to the complex IV enzyme has been indicated to enhance the activity of the enzyme (33), and evidence to indicate altered cytochrome c binding properties to the complex IV enzyme is obtained in mitochondria lacking the Rcf1 protein (15).

On the other hand, it was suggested previously that Rcf1 may play a role in the proper assembly of complex IV rather than acting as a stoichiometric component supporting the III-IV supercomplex arrangement (34). A number of lines of evidence presented here support this proposal. First, the BN-PAGE/proteomic analysis demonstrates that Rcf1/Rcf1His is not found in association with the predominant subpopulations of complex IV, i.e. the IV** species, and is recovered only with the IV* subpopulation. The IV** species represented a minor population of total complex IV in wild-type (or in Δrcf1; Δrcf2+Rcf1His) mitochondria, and the level of IV** was significantly increased through the R67A mutation, which also promoted the Rcf1-Cox3 interaction. Consistently, earlier

FIGURE 7. Mutation of the QRRQ motif or the absence of CL alters the environment of Rcf1. A, chemical cross-linking using MBS was performed on mitochondria isolated from the Δrcf1;Δrcf2 strain harboring either the wild-type Rcf1His, Rcf1HisQ61A,Q71A, or Rcf1HisR67A mutant derivatives as indicated. Following SDS-PAGE and Western blotting, decoration with His tag epitope antiserum was performed. The positions of the dominant 45-kDa (Rcf1-AAC) and 36-kDa (Rcf1-Rcf1) Rcf1-containing adducts are indicated. The position of a less abundant Rcf1 50-kDa adduct detected in Rcf1HisR67A mitochondria is indicated by two asterisks. Note that a slightly larger (52-kDa), still uncharacterized Rcf1 adduct is also observed in the Rcf1His or Rcf1HisQ61A,Q71A mitochondria and is indicated by one asterisk. B, cross-linking was performed and analyzed as described in A. Parallel Western blots were decorated with either Rcf1 (left panel) or Cox2 (right panel) antiserum. Only the area encompassing the 50-kDa Rcf1-Cox2 adduct is shown. C, cross-linking of Rcf1 was performed in mitochondria isolated from the wild type, Δrcf1, Δtam41, and Δaac2,Δaac1 strains, as indicated. Samples were further analyzed as described in A using Rcf1 (top panel) or AAC-specific (bottom panel) antibodies. Only the areas of the gel encompassing both the 45-kDa Rcf1-AAC adduct and the monomeric AAC are shown.

kDa) was detected with both Rcf1 and Cox2 antisera in Δrcf1;Δrcf2 mitochondria harboring the Rcf1HisR67A mutant derivative (Fig. 7B). In contrast, no Cox2-containing adduct was detected in Δrcf1;Δrcf2 mitochondria harboring His-tagged wild-type Rcf1 or the Rcf1HisQ61A,Q71A derivative (data not shown) and consistent with the Rcf1-antibody decoration. We therefore conclude that the arrangement of Rcf1 with complex IV is altered in the Rcf1HisR67A mutant so that a close proximity between Rcf1 and Cox2 is gained and revealed through the cross-linking approach.

Similar to the rcf1HisR67A mitochondria, loss of Rcf1-AAC adduct formation was also observed in mitochondria isolated from mutants displaying defective CL biosynthesis, such as the CL synthase mutant Δcrd1 (where AAC levels are normal) or the Tam41 null mutant Δtam41 (Fig. 7C) (albeit with reduced levels of AAC). Tam41 is a CDP-diacylglycerol synthase required for CL biosynthesis in mitochondria, and thus, like the Δcrd1, Δtam41 mitochondria are deficient in CL (31). Interestingly, despite being reduced in levels, a population of Taz1, an enzyme involved in remodeling of CL, was recovered in association with the affinity-purified rcf1HisR67A protein (Fig. 4B). Taz1 has also been found previously in association with the CL-containing AAC protein (32). Taken together, these results indicate that the molecular environment of Rcf1 is altered through the R67A (and R65A) mutation and in a similar manner as that observed for wild-type Rcf1 in mitochondria deficient in CL.

Discussion

Here we have studied the yeast Hig1 protein Rcf1, a member of the Hig1 type 2 subgroup, and have probed the functional importance of the conserved QRRQ motif of Rcf1, an important distinguishing feature between the type 2 and type 1 subgroups of the Hig1 protein family. Our findings here indicate that the QRRQ motif may form Gln/Arg pairs (Gln61/Arg65 and Arg67/Gln67), which serve to support the stability of the Rcf1 protein within the mitochondrial membrane. Furthermore, our data demonstrate that an intact QRRQ motif supports the ability of Rcf1 to associate with complex IV, in particular with Cox3 and Cox2 subunits, and facilitate the correct maturation and stable assembly of the complex IV enzyme. In particular, our results illustrate the importance of the residue Arg67 in modulating the interaction of Rcf1 with Cox3/complex IV.

How do Rcf1 and the Hig1 family members support complex IV assembly/activity and how is this related to their QRRQ motif? One possibility is that Rcf1 represents a physical component (i.e. a stoichiometric subunit) of complex IV and, through Cox3 (and possibly Cox2), may serve to enhance the structure and activity of this enzyme in a manner that influences the association of both Cox12 and the substrate cytochrome c (23). Thus, by binding as an additional subunit to subpopulations of the enzyme (i.e. in stochiometrically equivalent amounts as other complex IV subunits), Rcf1 may exert an influence over substrate binding and the enzymatic properties of complex IV. Indeed, the binding of a Hig1 family member, HIGD1A, to the complex IV enzyme has been indicated to enhance the activity of the enzyme (33), and evidence to indicate altered cytochrome c binding properties to the complex IV enzyme is obtained in mitochondria lacking the Rcf1 protein (15).
QRRQ Motif of the Yeast Hig1 Type 2 Protein Rcf1

BN-PAGE and affinity purification approaches have shown that the majority of Rcf1 is not together with complex IV, and only a minor percentage of complex IV is recovered together with Rcf1 (23). Second, an enhanced specific activity of both complex IV and the novel IV** species was observed through in-gel complex IV activity analysis in the extracts of the rcf1<sub>His</sub><sup>R67A</sup> mitochondria, yet the rcf1<sub>His</sub><sup>R67A</sup> protein was present in association with only the IV** species. Third, complex IV activity (and steady-state levels) were fully restored in the Δrcf1;Δrcf2 mutant harboring the rcf1<sub>His</sub><sup>G61A,R65A</sup> protein (and partially in those containing the rcf1<sub>His</sub><sup>R67A,Q71A</sup> protein) despite the strongly reduced levels of these mutated Rcf1 derivatives. These findings indicate that Rcf1 does not need to be present at its wild-type levels to fully support the activity of complex IV and, thus, argue against Rcf1 exerting its influence on complex IV activity as a stoichiometric equivalent component of the enzyme.

Taking these findings together, we propose here that Rcf1 functions to support the assembly and activity of complex IV by dynamic and transient associations with it, possibly during the assembly process to modify the enzyme composition and, thereby, its stability and catalytic properties. Thus, when analyzed, only a small fraction of complex IV is found in association with Rcf1, and this may reflect a late-stage Rcf1-complex IV assembly intermediate or a small population of the assembled complex IV being modified in an Rcf1-dependent fashion. Moreover, we suggest that this modification of complex IV, i.e., Rcf1’s “fingerprint” on it, may involve non-protein elements of complex IV, such as associated lipids. The association of Rcf1 with Cox3-containing assembly intermediates may be important to secure the correct maturation (or regulatory modification) of this subunit. We speculate that an otherwise transient interaction of Rcf1 and Cox3 protein is stalled upon mutation of the key Arg<sup>67</sup> residue, and thus, more of complex IV (IV**) remains in association with the rcf1<sub>His</sub><sup>R67A</sup> species, and this may have interfered with the normal maturation and stability of the enzyme. A number of results reported here support the speculation that the function of the fingerprint of Rcf1 may be related to the incorporation or remodeling of the CL or other lipid species associated with the complex IV enzyme. Both CL and PG lipids are associated with complex IV, in particular with the Cox3 subunit, and together are proposed to form an integral part of the O<sub>2</sub> translocation channel to the active site of the enzyme (30). The observed instability of the complex IV enzyme to DDM detergent extraction and the increased sensitivity to DCCD inhibition in rcf1<sub>His</sub><sup>R67A</sup> mitochondria may suggest an altered CL and/or PG arrangement within the complex IV enzyme.

A possible role for Rcf1 in the lipid maturation of mitochondrial enzyme complexes may not be limited to complex IV, as we also demonstrate that the rcf1<sub>His</sub><sup>R67A</sup> mutant displays a decreased ability to cross-link to AAC proteins, a result that was mirrored with the wild-type Rcf1 protein in the CL-deficient mitochondria Δcrd1 and Δtam41. Rcf1 has been shown to exist in a close physical relationship with the Cox3, Cox12, AAC proteins (Ref. 23 and our results here), and also with cytochrome c<sub>1</sub> of complex III (22), and all are proteins known to be intimately associated with CL molecules. Lipid profile analysis of Δrcf1;Δrcf2 mutant mitochondria has not indicated a major alteration in the content of CL or PG in the absence of Rcf1/ Rcf2 proteins. The recovery of a population of Taq1 with the rcf1<sub>His</sub><sup>R67A</sup> mutant may also add further support to a possible involvement of Rcf1 in CL maturation or remodeling within the mitochondrial membrane complexes.

Finally, as our data highlight the importance of the QRRQ motif for the function of Rcf1, a Hig1 type 2 family member, it is important to note that this motif is noticeably different in the Hig1 type 1, stress-induced isoforms, where the QRRQ motif is replaced with (I/V/L)HLHMRX<sub>QQ</sub>. It is possible that the differences in these motifs between the type 2 and type 1 Hig1 family members reflect the need for differential lipid modifications of complex IV and other enzymes, designed to fine-tune the respiratory chain to operate under stress conditions such as hypoxia.

Experimental Procedures

Yeast Strains and Growth Conditions—All S. cerevisiae strains used in this study were in the haploid W303–1A genetic background (W303-1A, mat a leu2, trp1, ura3, his3, ade2) and include the WT, Δrcf1;Δrcf2 (RCF1::HIS3, RCF2::KAN) (23), Δaac2 (AAC2::KAN) (35), Δtam41 (MMP37::KAN) (36), and Δcrd1 (CRD1::KAN). Yeast strains were maintained and cultured at 30 °C on YP (yeast extract, peptone) medium supplemented with 2% glucose and 20 mg/liter adenine hemisulfate (YPAD) following standard protocols. All cultures were grown in YP medium containing 0.5% lactate and supplemented with 2% galactose.

Generation of His-tagged Rcf1 QRRQ Mutant Derivatives—The Yip351-LEU2 vector containing the open reading frame encoding the Rcf1 protein as a C-terminal His<sub>6</sub>-tagged protein downstream of the galactose-inducible GAL10 promoter (23) was used as a template for PCR-based mutagenesis. Mutations in the QRRQ motif were generated using a PCR site-directed mutagenesis strategy. The resulting plasmids were integrated into the leu2 locus of the Δrcf1;Δrcf2 strain, LEU<sup>+</sup> transformants were selected, and expression of Rcf1<sub>His</sub> derivatives was verified.

Affinity Purification of His-tagged Proteins—Isolated mitochondria (200 μg of protein) harboring the His-tagged Rcf1 derivatives were solubilized in lysis buffer (100 mM KCl, 20 mM HEPES-KOH, 10 mM MgCl<sub>2</sub>, and 0.5 mM PMSF (pH 7.4)) containing either 0.25% Triton X-100, 0.6% DDM, or 1% digitonin (as indicated) for 30 min on ice. Following a clarifying spin, the resulting plasmids were integrated into the leu2 locus of the Δrcf1;Δrcf2 strain, LEU<sup>+</sup> transformants were selected, and expression of Rcf1<sub>His</sub> derivatives was verified.

BN-PAGE Analysis—BN-PAGE analysis of digitonin-solubilized (1%) or DDM-solubilized (0.6%) mitochondrial extracts (30 μg of protein) was performed using Invitrogen NuPAGE gradient (4–12%) gels according to the protocol of the manufacturer, followed by Western blotting and immunodecoration with subunit-specific antisera as indicated. For in-gel complex

3 J. Garlich and R. A. Stuart, unpublished results.
4 S. Claypool, personal communication.
IV activity following BN-PAGE (0.6% DDM lysis), the gel was incubated in activity buffer (50 mM phosphate buffer (pH 7.4), 1 mg/ml 3,3’-diaminobenzidine, 1 mM catalase, 1 mg/ml cytochrome c, and 75 mg/ml sucrose) for 90 min at room temperature, fixed for 1 h in 45% methanol and 10% acetic acid, and destained overnight in 10% methanol and 10% acetic acid.

Quantitative Mass Spectrometry—Protein abundance profiles of subunits within complexes were analyzed by a combination of BN-PAGE and quantitative MS (38). Briefly, the area of the complex IV species in the BN-PAGE gel (linear 3–18% acrylamide gradient) was cut into 16 even slices, digested with trypsin as described previously (38), and further analyzed as described previously (28). MS data were analyzed by MaxQuant (v1.5.3.30) (39). Proteins were identified using the yeast reference proteome database UniProtKB with 6721 entries, released in March 2016, supplemented with the Rcf1-His derivatives as described previously (28). Intensity-based absolute quantification values were recorded and normalized to maximum appearance within native lanes comparing wild-type, Δrcf1Δrcf2, Δrcf1Δrcf2Δrcf1_1His and Δrcf1Δrcf2Δrcf1_1His K67A. To compare these mini abundance profiles, each sample was normalized to the median of protein abundance of the wild type (28). Normalization to the maximum appearance of each protein within the analyzed samples was used to display the results in heatmaps and profile plots. Complexes III2 (473 kDa), ATP synthase (572 kDa), IV (sum of all matured subunits, two copies of Cox8, without Cox12 and Cox13, 197 kDa) were used for native mass calibration (data not shown).

O2 Consumption Assays—Oxygen consumption rates (OCRs) were measured with a Clark-type oxygen electrode (Rank Brothers, digital model 10) using isolated mitochondria (80 μg of protein) in an isosmotic buffer (10 mM potassium P4, 20 mM HEPES-KOH, 2 mM EDTA, 1 mg/ml BSA, and 0.6 M mannitol (pH 7.2)). State 4 respiration was measured following addition of NAH (0.5 mM), and state 3 was then achieved by subsequent addition of ADP (0.2 mM). The bioenergetically isolated complex IV OCR was attained by addition of TMPD/ascorbate (0.4 mM/1.6 mM) to directly reduce cytochrome c, and maximal bioenergetically isolated complex IV activity was achieved by subsequent addition of a protonophore (CCCP, 0.2 mM). DCCD inhibition of bioenergetically isolated complex IV was measured as above following 90-min, 25 °C incubation of mitochondria in MES-Tris buffer (pH 7.3) containing 10% methanol and 0.3, 0.6, or 1.2 mM DCCD (in methanol) or control (methanol alone).

Miscellaneous—In organello translation with [35S]methionine labeling was performed as described previously (40). Chemical cross-linking with N,N-maleimidoazobenzoyl-N-hydroxy-succinimide ester (MBS) was performed as described previously (23). Mitochondrial isolation, protein determination, and SDS-PAGE were performed according to published methods (21, 41, 42). The Cox1 and Cox3 antibodies used in this study were commercially obtained (Cox1: Molecular Probes, anticyo Cox1, mouse monoclonal 11D8-B7, lot 6251–1; Cox3: Invitrogen/Novex anti-Cox3 monoclonal, 459300, lot H3578). All other antibodies used were rabbit polyclonal against the respective purified yeast proteins and generated either in the Stuart laboratory or received as gifts.

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