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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)X₃(R/H)XRX₃Q, 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 incorporation and/or remodeling of lipids, in particular cardiolipin, into complex IV and, possibly, other mitochondrial proteins such as ADP/ATP carrier proteins.

Mitochondria are specialized organelles that are a nexus for several critical cellular pathways, including the aerobic production of energy through oxidative phosphorylation (OXPHOS)²

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² 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-

(1–4). The enzymes involved in this cellular energy pathway, referred to as complexes I–IV, are embedded within the inner mitochondrial membrane and facilitate the establishment of a proton gradient through the oxidation of reducing equivalents. In the final steps of this process, the electron carrier cytochrome *c* mediates the transfer of electrons from cytochrome *bc*₁ (complex III) to cytochrome *c* oxidase (COX, complex IV), which donates electrons to O₂, forming water (1, 5–7). The electrochemical proton gradient is utilized by the F₁F₀-ATP synthase and provides the energy necessary to generate ATP from ADP and inorganic phosphate (8). Newly synthesized ATP is then exchanged for cytosolic ADP by the ADP/ATP carrier (AAC) proteins, integral membrane proteins of the mitochondrial inner membrane (9).

OXPHOS enzyme complexes require correct assembly and tight regulation to ensure optimal function and integrity. A point of potential regulation is the organization of OXPHOS enzymes, including AAC and complexes III and IV, into structures termed supercomplexes (1–3, 10–15). These higher-ordered supercomplexes, *e.g.* the III-IV supercomplex, have been proposed to facilitate efficient electron channeling and possibly enable the co-regulation of these enzymes. The III-IV supercomplex is stabilized by the signature mitochondrial phospholipid cardiolipin (CL) and can also include the AAC protein (12, 16–21).

Rcf1 and Rcf2 (respiratory complex factor 1 and 2) are two proteins in the yeast *Saccharomyces cerevisiae* that independently associate with subpopulations of the III-IV supercomplex (22–24). Like the AAC proteins, current data indicate that Rcf1 and Rcf2 support the assembly of complex IV and, thus, III-IV supercomplex organization (12, 22–24). The Rcf1 and Rcf2 proteins have been shown to cooperatively play a critical role in respiration-based growth and influence the activity of complex IV (22–24). The growth defect of the $\Delta rcf1;\Delta rcf2$ mutant, at least in part, is caused by a reduction in complex IV enzyme levels in the absence of both Rcf1/Rcf2 proteins (23). Rcf1 physically interacts with the mitochondrially encoded Cox3 subunit during its assembly and has also been shown to modulate the association of Cox12 (Cox6b in mammals) with complex IV enzyme. Cox12 is a peripheral subunit of complex

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)XRX₃Q 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)HLIHMRX₃Q instead. Although the Hig1 type 2 isoforms are universally found in all eukaryotes (and in α -proteobacteria) and appear to represent constitutively expressed isoforms, the Hig1 type 1 subgroup is found in higher eukaryotes and appears 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 type1 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. ⁶¹QX₃RXX₃Q⁷¹ (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, glutamines 61 and 71 were simultaneously mutated to alanines to create the *rcf1*^{Q61A,Q71A} derivative. In the second mutant, derivative arginine 67 was mutated to Ala (*rcf1*^{R67A}).

Rcf1 mutant derivatives were expressed as C-terminal His-tagged derivatives in the Rcf1/Rcf2 double-null yeast strain (Δ *rcf1*; Δ *rcf2*). Analysis of isolated mitochondria from the resulting strains revealed that the steady-state levels of the *rcf1*^{Q61A,Q71A} and *rcf1*^{R67A} derivatives appeared to be sim-

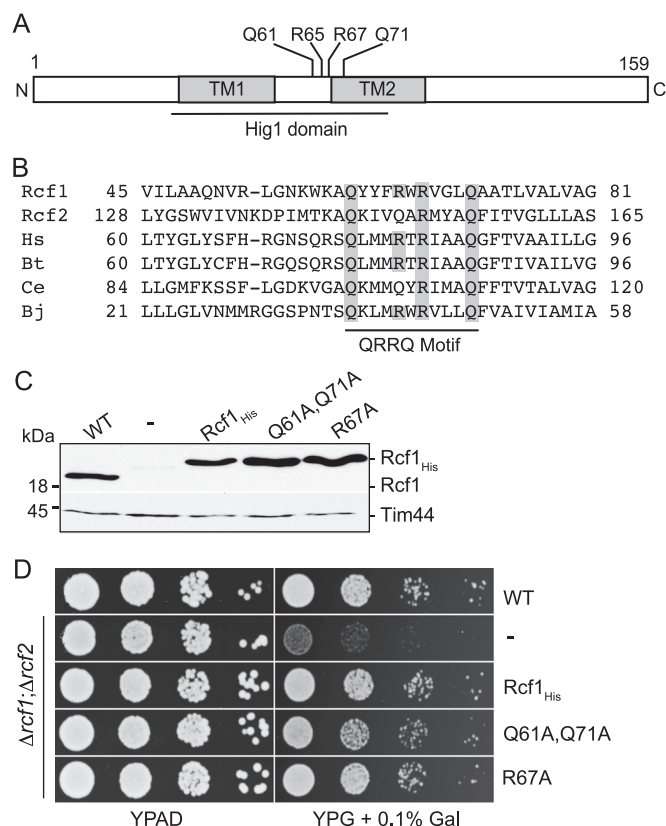


FIGURE 1. Rcf1, an inner membrane protein and member of Hig1 type 2 family and the conserved QRRQ motif. A, the N_{out}-C_{out} orientation of Rcf1 in the inner membrane with N and C tails exposed to the intermembrane space. The location of QRRQ (Gln⁶¹, Arg⁶⁷, Gln⁷¹) motif relative to the two transmembrane 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 Δ *rcf1*; Δ *rcf2* controls (–), or the Δ *rcf1*; Δ *rcf2* strain harboring either His-tagged Rcf1 (Rcf1_{His}), *rcf1*_{His}^{Q61A,Q71A} (Gln⁶¹,Gln⁷¹) or *rcf1*_{His}^{R67A} (R67A) derivatives. Rcf1 and Tim44 (control) levels were analyzed by Western blotting. D, serial 10-fold dilutions of the WT and Δ *rcf1*; Δ *rcf2* expressing Rcf1_{His}, *rcf1*_{His}^{Q61A,Q71A}, or *rcf1*_{His}^{R67A} derivatives or not, as indicated, were spotted on YP plates containing glucose (YPAD) or glycerol supplemented with 0.1% galactose (YPG + 0.1% Gal) and grown at 30 °C.

ilar to that of the wild-type Rcf1_{His} 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 Δ *rcf1*; Δ *rcf2* yeast strain displays a respiration-based growth defect (23). The expression of *rcf1*_{His}^{Q61A,Q71A} and *rcf1*_{His}^{R67A} derivatives, like the Rcf1_{His} wild-type protein, largely complemented the Δ *rcf1*; Δ *rcf2* 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*_{His}^{R67A} Alters the Complex IV Assembly State—The Δ *rcf1*; Δ *rcf2* mutant mitochondria examined contain reduced levels of complex IV subunits (23). In contrast, the Δ *rcf1*; Δ *rcf2* mitochondria harboring His-tagged wild-type Rcf1 or the *rcf1*_{His}^{Q61A,Q71A} or *rcf1*_{His}^{R67A} 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.

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

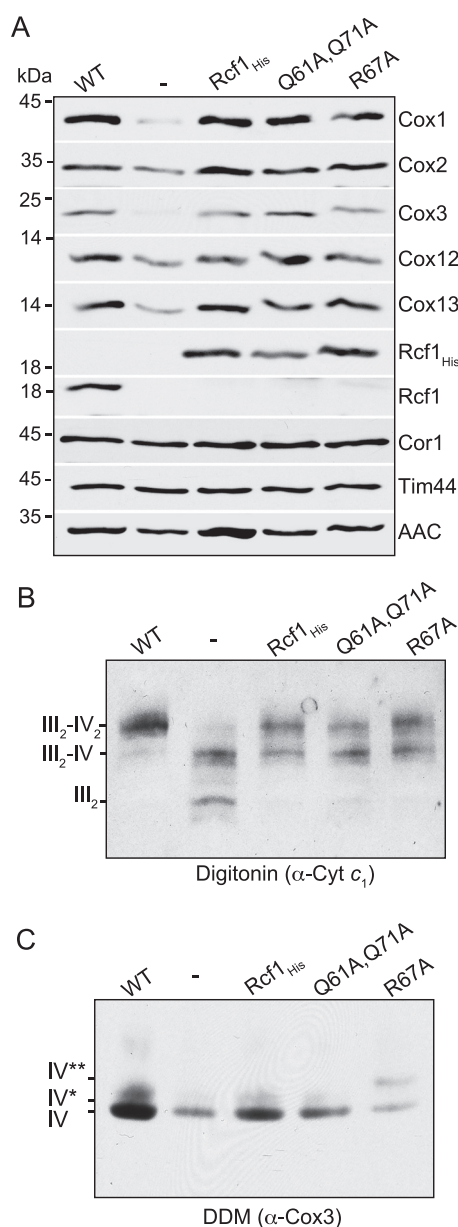


FIGURE 2. Mutation of the QRRQ motif in *Rcf1* affects the stability but not the assembly of complex IV. *A*, steady-state levels of the OXPHOS subunits in mitochondria (50 μ g) isolated from the indicated strains as detailed in Fig. 1C. Tim44 was used as a loading control. *B*, mitochondria isolated from the WT and the indicated $\Delta rcf1$; $\Delta rcf2$ strains were solubilized in digitonin (1%) and subjected to BN-PAGE analysis, Western blotting, and immunodecoration with antibodies against the complex III subunit, cytochrome c_1 (α Cyt c_1). *C*, same as in *B*, except that mitochondria were solubilized with DDM (0.6%) prior to BN-PAGE, and decoration was performed with antibodies against the complex IV subunit Cox3 (α -Cox3).

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 III₂-IV₂ form (observed in the wild-type control mitochondria) to a predominantly III₂-IV form and also to free III₂ complexes (Fig. 2*B*), which has been previously attributed to the limiting levels of complex IV in the absence of *Rcf1*/*Rcf2* (23). The III₂-IV₍₁₋₂₎

supercomplex organizational state was largely restored in $\Delta rcf1$; $\Delta rcf2$ mitochondria harboring wild-type *Rcf1*_{His} (Fig. 2*B*). The *rcf1*_{His}^{Q61A,Q71A} or *rcf1*_{His}^{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. 2*C*). 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 $\Delta rcf1$; $\Delta rcf2$ + *Rcf1*_{His} mitochondria (and also from *rcf1*_{His}^{Q61A,Q71A}) was considerably greater than that observed from the $\Delta rcf1$; $\Delta rcf2$ 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}^{R67A}-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. 2*C*). 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} or *rcf1*_{His}^{Q61A,Q71A} 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 digitonin-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-

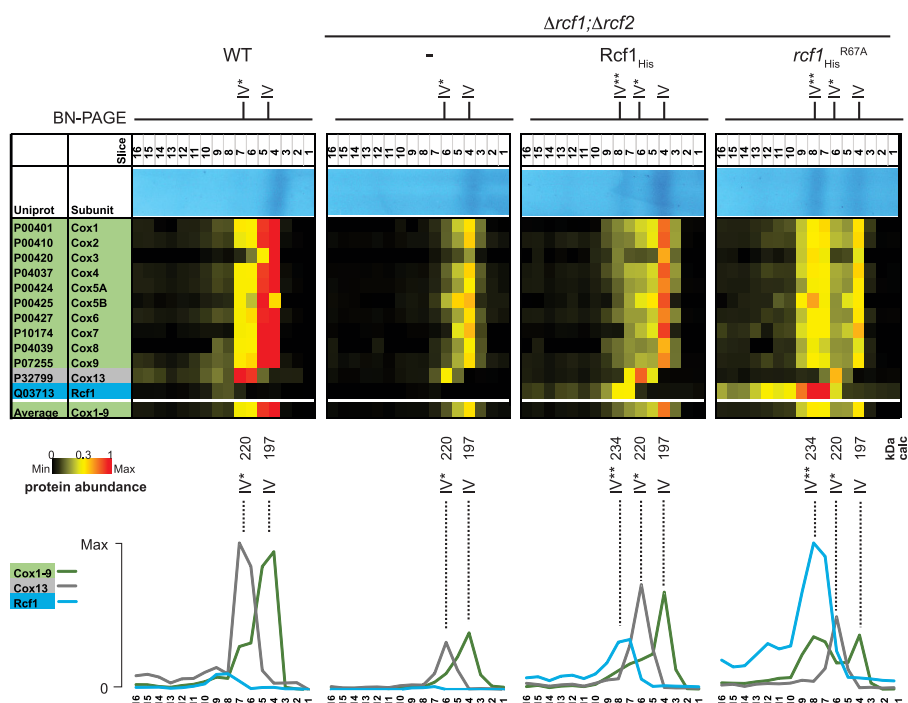


FIGURE 3. Compositional analysis of the complex IV subpopulation.** One-dimensional BN-PAGE analysis of protein complexes in wild-type $\Delta rcf1;\Delta rcf2$ mitochondria and those harboring $Rcf1_{His}$ or $rcf1_{His}^{R67A}$ following solubilization with DDM (0.6%) was performed. The areas of the gel encompassing the complex IV and IV** species (as shown) were fractionated, and proteins were identified and quantified by mass spectrometry. The amounts of identified proteins were quantified using intensity-based absolute quantification value as described under “Experimental Procedures.” Heatmaps and graphs show protein profiles normalized to the maximum abundance of a protein within BN lanes of the wild type and $\Delta rcf1;\Delta rcf2$ mutants.

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 $rcf1_{His}^{R67A}$ 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 $\Delta rcf1;\Delta rcf2$ mitochondria harboring the $Rcf1_{His}$ protein were analyzed, in addition to both IV and IV* populations, small but detectable levels of the IV** subpopulation and some co-migrating $Rcf1_{His}$ species were observed. However, the ratio of the IV** to the IV subpopulation was distinctly increased in $rcf1_{His}^{R67A}$ sample relative to the other mitochondrial types and in agreement with the BN-PAGE analysis shown in Fig. 2C. The total amount of $rcf1_{His}^{R67A}$ protein co-migrating with the IV** species was also considerably greater than that observed with the wild-type $Rcf1_{His}$ 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 $Rcf1_{His}$ (19.9 kDa) with another,

unknown protein of similar size or two copies of $Rcf1_{His}$. 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 $Rcf1_{His}$) 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 $rcf1_{His}^{R67A}$ 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 $rcf1_{His}^{R67A}$ derivative compared with the control $Rcf1_{His}$ protein analyzed in parallel (Fig. 4A). Consistent with the Triton X-100 lysis/purification results, enhanced association of complex IV subunits with the $rcf1_{His}^{R67A}$ derivative relative to the wild-type $Rcf1_{His}$ control was also observed under digitonin conditions (where the III-IV association is preserved) (Fig. 4B).

Co-purification of complex IV subunits with the $rcf1_{His}^{Q61A,Q71A}$ derivative was not observed under Triton X-100 solubilization conditions (Fig. 4A) but was observed (together with complex III subunits) under digitonin solubili-

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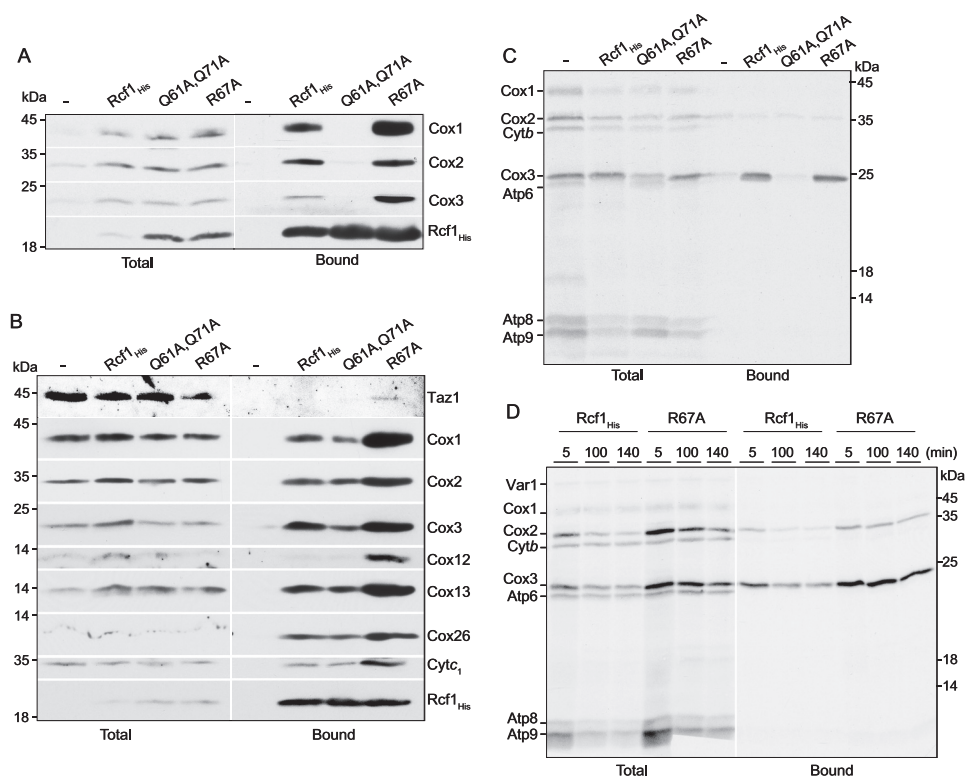


FIGURE 4. Mutation of the QRRQ motif affects the association of Rcf1 with Cox3 and complex IV. *A*, affinity purification of Rcf1_{His}, Rcf1_{His}^{Q61A,Q71A}, Rcf1_{His}^{R67A} derivatives following solubilization with Triton X-100 detergent was performed, followed by SDS-PAGE, Western blotting, and immunodecoration as indicated. *Total*, 5% of solubilized material; *Bound*, 100% of affinity-purified material on the Ni-NTA beads. *B*, the same as *A*, except solubilization was performed with 1% digitonin. *C*, *in organello* labeling in the presence of [³⁵S]methionine was performed for 20 min in isolated mitochondria prior to solubilization with Triton X-100. Affinity purification of the His-tagged Rcf1 derivatives was performed as described in *A*, but, following SDS-PAGE, the gels were subjected to autoradiography. *D*, as in *C*, except, following the pulse of translation (20 min), translation was stopped through addition of puromycin and cold methionine and further chased for the time periods indicated prior to solubilization of the mitochondria with Triton X-100 and affinity purification of the Rcf1 derivatives.

zation conditions (Fig. 4*B*). Thus, mutation of the Gln⁶¹/Gln⁷¹ residues in the QRRQ motif disturbs the integrity of the Rcf1-complex IV interaction so that it does not remain stable in the presence of Triton X-100 but can be preserved when the milder detergent (digitonin) solubilization conditions are applied. Complex IV substrate cytochrome *c* was not recovered with affinity-purified wild-type or QRRQ mutant Rcf1_{His} proteins under either detergent conditions (data not shown).

Association of the Rcf1 protein with complex IV, at least in part, involves the mitochondrially encoded subunit 3, Cox3, of complex IV, which can physically associate with Rcf1 prior to its assembly into final complex IV (23). *In organello* synthesized, radiolabeled Cox3 co-purified with both wild-type Rcf1_{His} and the Rcf1_{His}^{R67A} derivatives. Association of radiolabeled Cox3 with the Rcf1_{His}^{Q61A,Q71A} derivative under these Triton X-100 solubilization conditions, however, was not observed (Fig. 4*C*). Furthermore, a pulse-chase kinetic analysis indicated that the association of radiolabeled Cox3 with the Rcf1_{His}^{R67A} derivative appeared to be more prolonged than with the wild-type Rcf1_{His} control, suggesting that mutation of the Arg⁶⁷ residue to alanine causes enhanced association with Cox3, which may protect Cox3 from subsequent proteolytic turnover (Fig. 4*D*).

Taken together, these results indicate that mutation of the QRRQ motif has the potential to impact the nature of the Rcf1-complex IV interaction through an altered interaction with the Cox3 subunit. Mutation of Arg⁶⁷ to alanine causes an enhanced 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_{His}^{R67A} derivative.

Complex IV in Mitochondria Harboring the Rcf1_{His}^{R67A} Derivative Displays Altered Enzymatic Properties—The enzymatic properties of complex IV in Rcf1_{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. 5*A*, center panel). The level of complex IV activity was strongly reduced in the Δ Rcf1; Δ Rcf2 mutant compared with both the wild-type control and the null mutant harboring the Rcf1_{His} protein. This reduced complex IV enzyme activity can be attributed to the reduction in complex IV protein levels in the Δ Rcf1; Δ Rcf2 mutant (Figs. 2*C* and 5*A*). Despite also having reduced protein content (as judged by parallel Western blotting analysis; Fig. 5*A*, top panel), the levels of DDM-solubilized complex IV enzyme activity in both the IV and IV** subpopulations in the Rcf1_{His}^{R67A} sample appeared similar to, or even higher than, those obtained for the IV population in the Δ Rcf1; Δ Rcf2+Rcf1_{His} or the Rcf1_{His}^{Q61A,Q71A} mitochondria (Fig. 5*A*, center panel). These results suggest that the complex IV/IV** subpopulations solubilized from Rcf1_{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-

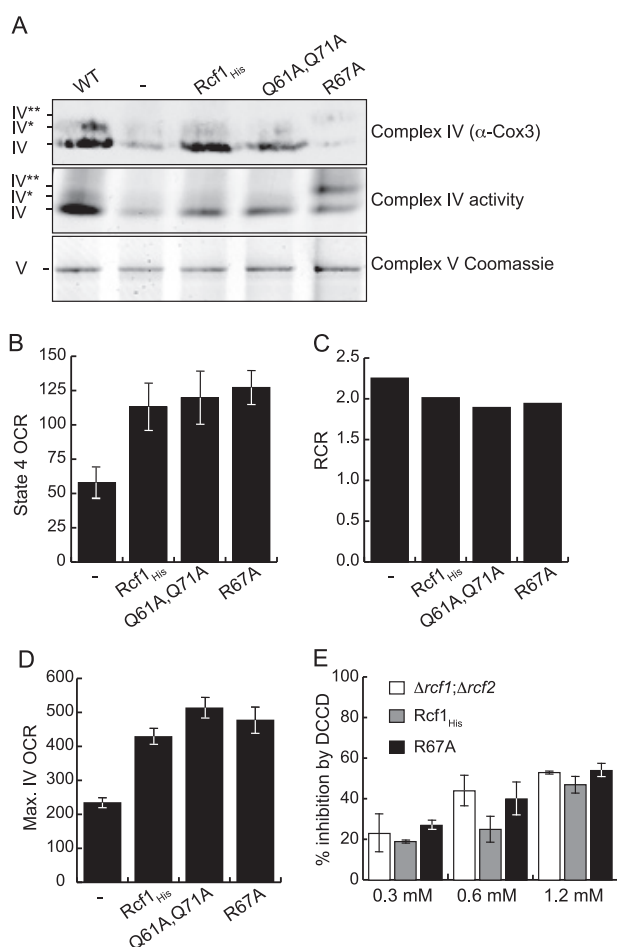


FIGURE 5. Complex IV in $rcf1_{His}^{R67A}$ mitochondria displays altered catalytic properties. A, mitochondria isolated from WT or the $\Delta rcf1;\Delta rcf2$ strain harboring the Rcf1_{His}, $rcf1_{His}^{Q61A,Q71A}$, $rcf1_{His}^{R67A}$ derivatives, as indicated, were solubilized with DDM (0.6%) and subjected to duplicate BN-PAGE analysis. The complex IV enzyme activity levels were analyzed in one set of samples by performing an in-gel enzyme activity assay (center panel), and in a parallel sample, the complex IV protein levels were determining following Western blotting and decoration with Cox3 antibody (top panel), as indicated. The levels of Coomassie-stained complex V (F_1F_0 -ATP synthase) are shown in the bottom panel as a loading control. B and C, the OCRs of the NADH-driven state 4 and respiratory control ratio (the ratio of state 3 (NADH/ADP) to state 4 (NADH)) were measured in the indicated isolated mitochondria ($n = 3$). D, the maximal capacity of complex IV was determined by measuring the OCR driven by ascorbate/TMPD in the presence of CCCP to dissipate the membrane potential ($n = 6$). E, isolated $\Delta rcf1;\Delta rcf2$ mitochondria and $\Delta rcf1;\Delta rcf2$ harboring the Rcf1_{His} or $rcf1_{His}^{R67A}$ derivatives were incubated with DCCD at the indicated concentrations for 90 min at room temperature prior to measuring the maximal complex IV OCR in the presence of ascorbate/TMPD and CCCP. The percent of inhibition caused by the DCCD treatment relative to the control (no DCCD) is indicated.

sured. The rates of O₂ consumption following addition of the substrate NADH, i.e. basal or “state 4” respiration, were similar in all mitochondrial types, i.e. harboring either the Rcf1_{His}, $rcf1_{His}^{Q61A,Q71A}$ or $rcf1_{His}^{R67A}$ proteins. The respiratory control rate (state 3 respiration [NADH+ADP]/state 4) in QRRQ mutant mitochondria was also similar to the other mitochondria measured, indicating the coupled state of these mitochondria (Fig. 5C). The maximum oxygen consumption capacity of complex IV (measured in the presence of ascorbate/TMPD/CCCP) was also found to be similar in mitochondria harboring both wild-type Rcf1_{His} and the QRRQ mutant derivatives (Fig. 5D). Therefore, we conclude that both the $rcf1_{His}^{Q61A,Q71A}$ and

$rcf1_{His}^{R67A}$ mutant derivatives displayed the capacity to fully support maximal complex IV activity when expressed in the $\Delta rcf1;\Delta rcf2$ strain even when the enzyme is working to its maximum capacity.

Finally, the observed elevated in-gel enzyme activity assay suggests that the complex IV enzyme in the $rcf1_{His}^{R67A}$ mitochondria may display differential enzymatic properties than when the wild-type Rcf1 control protein is present. In support of this, we also observed that the complex IV enzyme in $rcf1_{His}^{R67A}$ mitochondria displayed an increased sensitivity to DCCD relative to those harboring the wild-type Rcf1_{His} (Fig. 5E). A similar effect was observed in $\Delta rcf1;\Delta rcf2$ mitochondria. DCCD binds to Cox3 (Glu⁹⁰ 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 $rcf1_{His}^{R67A}$ 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 $rcf1_{His}^{R67A}$ 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 plot indicated that the Arg⁶⁷ residue co-aligned with the Gln⁷¹ residue, where the Gln⁶¹ residue neighbored the Arg⁶⁵ residue, suggesting that they may form Gln/Arg pairs (Fig. 6A). We further explored the QRRQ motif in the Rcf1 protein by making double mutants, mutating one or other of the Gln/Arg pairs, i.e. $rcf1_{His}^{Q61A,R65A}$ and $rcf1_{His}^{R67A,Q71A}$, and then expressed them in the $\Delta rcf1;\Delta rcf2$ null mutant. Steady-state analysis of proteins isolated from the resulting strains indicated that the levels of both mutant Rcf1 derivatives were strongly reduced, in particular the $rcf1_{His}^{Q61A,R65A}$ derivative, compared with the wild-type Rcf1_{His} 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 Arg⁶⁵ alone (i.e. $rcf1_{His}^{R65A}$) 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 $rcf1_{His}^{R67A}$ derivative (albeit to a lesser extent), an increase in the level of complex III-IV subunits in association with the affinity-purified $rcf1_{His}^{R65A}$ derivative was observed (supplemental Fig. S1).

Despite the strongly reduced levels of the $rcf1_{His}^{Q61A,R65A}$ protein, the complex IV subunit levels in the mutant mitochondria were similar to that of the Rcf1_{His} control and significantly higher than those observed in the $\Delta rcf1;\Delta rcf2$ null mitochondria (Fig. 6B). The organization and levels of the III-IV supercomplex in the $rcf1_{His}^{Q61A,R65A}$ mitochondria also resembled those of the Rcf1_{His} control analyzed in parallel (Fig. 6C, top panel). Furthermore, the levels of DDM-solubilized complex IV in the $rcf1_{His}^{Q61A,R65A}$ mitochondria were similar to those of the wild-type Rcf1_{His} control (Fig. 6C, bottom panel).

Although the steady-state levels of the $rcf1_{His}^{R67A,Q71A}$ derivative were marginally higher than that of $rcf1_{His}^{Q61A,R65A}$ protein, the presence of the $rcf1_{His}^{R67A,Q71A}$ protein only partially restored the complex IV subunits to wild-type levels, indicating

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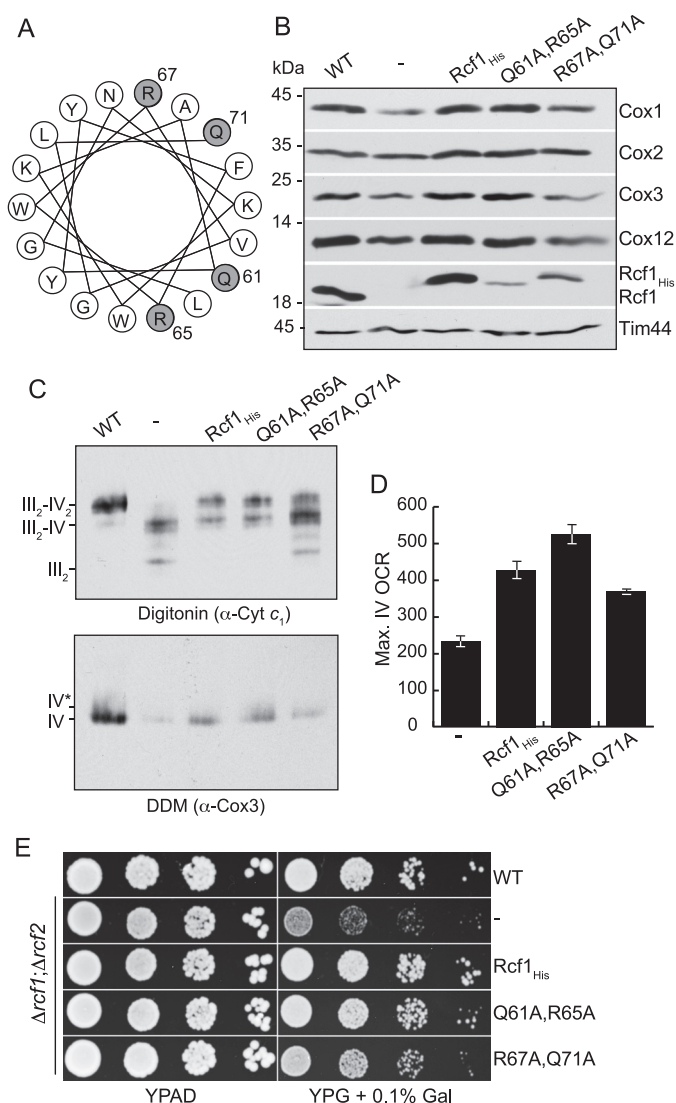


FIGURE 6. Mutation of the Glu/Arg pairs in the QRRQ motif in *Rcf1* affects the stability of *Rcf1*, but complex IV assembles. *A*, an α -helical plot of residues Leu⁵⁴ through Gln⁷¹ of *Rcf1* depicting the co-localization of the Gln⁶¹/Arg⁶⁵ residue pair and the Arg⁶⁷/Gln⁷¹ residue pair on the helix. *B*, steady-state levels of the OXPHOS subunits in the mitochondria (50 μ g of protein) isolated from the indicated strains. Tim44 was used as a loading control. *C*, mitochondria isolated from the WT and the indicated $\Delta rcf1; \Delta rcf2$ strains were solubilized in digitonin (1%, *top panel*) or DDM (0.6%, *bottom panel*) and subjected to BN-PAGE analysis, Western blotting, and immunodecoration with antibodies against the complex III subunit, cytochrome *c*₁ (α -Cyt *c*₁, *top panel*) and the complex IV subunit, Cox3 (*bottom panel*). *D*, maximal OCR of bioenergetically isolated complex IV was measured in mitochondria isolated from the $\Delta rcf1; \Delta rcf2$ strain and the $\Delta rcf1; \Delta rcf2$ strain harboring the *rcf1*_{His}^{Q61A,R65A} or *rcf1*_{His}^{R67A,Q71A} derivatives following addition of ascorbate/TMPD and CCCP. *E*, serial 10-fold dilutions of WT, and $\Delta rcf1; \Delta rcf2$ expressing *Rcf1*_{His}, *rcf1*_{His}^{Q61A,R65A}, *rcf1*_{His}^{R67A,Q71A} derivatives or not, as indicated, were spotted on YP plates containing glucose (YPAD) or glycerol supplemented with 0.1% galactose (YPG + 0.1% Gal) and grown at 30 °C.

that the ability of this *Rcf1* derivative to support complex IV assembly was compromised through mutation of the Arg⁶⁷/Gln⁷¹ pair (Fig. 6*B*). Consistently, a partial restoration of the III₂-IV₁₋₂ species was observed in the BN-PAGE analysis of the digitonin-solubilized *rcf1*_{His}^{R67A,Q71A} mitochondria, paralleling the observation that the levels of the complex IV subunits in these mitochondria were greater than those in the $\Delta rcf1; \Delta rcf2$ control but not equivalent to those of the wild-type control (or

of the *rcf1*_{His}^{Q61A,R65A} mitochondria) (Fig. 6*C*, *top panel*). However, when solubilized with DDM, the levels of free complex IV from *rcf1*_{His}^{R67A,Q71A} mitochondria appeared similar to those of the $\Delta rcf1; \Delta rcf2$ null mitochondria and in contrast to those from the *rcf1*_{His}^{Q61A,R65A} mitochondria, where the levels of complex IV were similar to the wild-type *Rcf1*_{His} control (Fig. 6*C*, *bottom panel*). These results together suggest that complex IV from the *rcf1*_{His}^{R67A,Q71A} mitochondria may exhibit some detergent instability to DDM extraction in a similar manner as observed previously in the *rcf1*_{His}^{R67A} mitochondria (Fig. 3*C*). In contrast to the *rcf1*_{His}^{R67A} mitochondria, the presence of a complex IV^{**} form was not detected in the *rcf1*_{His}^{R67A,Q71A} mitochondrial sample. This may be attributed to the strongly reduced levels of *rcf1*_{His}^{R67A,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 *rcf1*_{His}^{Q61A,R65A} mitochondria demonstrated them to be fully restored to the levels in wild-type *Rcf1*_{His} control mitochondria, whereas, in *rcf1*_{His}^{R67A,Q71A} mitochondria, although they were significantly higher than those measured in the $\Delta rcf1; \Delta rcf2$ null mitochondria, they were not equivalent to the *rcf1*_{His}^{Q61A,R65A} or wild-type *Rcf1*_{His} mitochondria (Fig. 6*D*). The increased level of complex IV activity in both of these mutants was sufficient to restore aerobic growth, as expression of both *rcf1*_{His}^{Q61A,R65A} and *rcf1*_{His}^{R67A,Q71A} derivatives ensured complementation of the growth defect phenotype of the $\Delta rcf1; \Delta rcf2$ strain (Fig. 6*E*).

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 Gln⁶¹/Arg⁶⁵ mutant and partially 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 *Rcf1*_{His} forms a number of adducts with the cross-linking reagent MBS, the most notable being a 45-kDa adduct reported previously to represent cross-linking of *Rcf1* to the AAC proteins (23). The *rcf1*_{His}^{Q61A,Q71A} derivative maintained the ability to be cross-linked to AAC, whereas the cross-linking between *rcf1*_{His}^{R67A} and AAC was significantly reduced (Fig. 7*A*) despite the normal levels of the AAC protein in these mitochondria (Fig. 2*C*). The MBS cross-linking profile of the *rcf1*_{His}^{R65A} derivative resembled that of the *rcf1*_{His}^{R67A} protein (supplemental Fig. S1). In addition to the loss of the *Rcf1*-AAC adduct in *rcf1*_{His}^{R67A} mitochondria, we observed the gain of a novel adduct near 50 kDa. This *rcf1*_{His}^{R67A} adduct was close in size to, but distinct from, a 52-kDa *Rcf1* adduct observed in *Rcf1*_{His} and *rcf1*_{His}^{Q61A,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 a *Rcf1*-Cox2 adduct. Decarboxylation of a parallel experiment with Cox2-specific-antiserum demonstrated that a MBS-formed adduct of similar size (~50

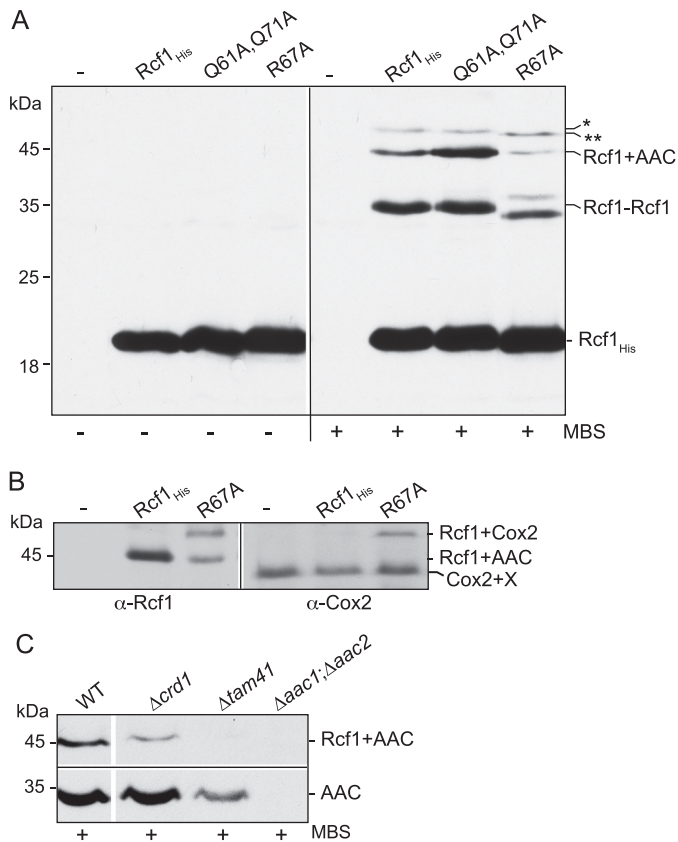


FIGURE 7. Mutation of the QRRQ motif or the absence of CL alters the environment of Rcf1. A, chemical cross-linking using MBS was performed on mitochondria isolated from the $\Delta rcf1$; $\Delta rcf2$ strain harboring either the wild-type Rcf1_{His}, Rcf1_{His}^{Q61A,Q71A}, or Rcf1_{His}^{R67A} mutant 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 Rcf1_{His}^{R67A} mitochondria is indicated by two asterisks. Note that a slightly larger (52-kDa), still uncharacterized Rcf1 adduct is also observed in the Rcf1_{His} or Rcf1_{His}^{Q61A,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, $\Delta rcf1$, $\Delta tam41$, and $\Delta aac2$, $\Delta aac1$ 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 $\Delta rcf1$; $\Delta rcf2$ mitochondria harboring the Rcf1_{His}^{R67A} mutant derivative (Fig. 7B). In contrast, no Cox2-containing adduct was detected in $\Delta rcf1$; $\Delta rcf2$ mitochondria harboring His-tagged wild-type Rcf1 or the Rcf1_{His}^{Q61A,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 Rcf1_{His}^{R67A} mutant so that a close proximity between Rcf1 and Cox2 is gained and revealed through the cross-linking approach.

Similar to the Rcf1_{His}^{R67A} 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 $\Delta rcd1$ (where AAC levels are normal) or the Tam41 null mutant $\Delta tam41$ (Fig. 7C) (albeit with reduced

levels of AAC). Tam41 is a CDP-diacylglycerol synthase required for CL biosynthesis in mitochondria, and thus, like the $\Delta rcd1$, $\Delta tam41$ 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 Rcf1_{His}^{R67A} 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/Gln⁷¹), 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 stoichiometrically 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/Rcf1_{His} is not found in association with the predominant subpopulations of complex IV, i.e. the IV/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 $\Delta rcf1$; $\Delta rcf2$ +Rcf1_{His}) mitochondria, and the level of IV** was significantly increased through the R67A mutation, which also promoted the Rcf1-Cox3 interaction. Consistently, earlier

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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*_{His}^{R67A} mitochondria, yet the *rcf1*_{His}^{R67A} protein was present in association with only the IV** species. Third, complex IV activity (and steady-state levels) were fully restored in the Δ *rcf1*; Δ *rcf2* mutant harboring the *rcf1*_{His}^{Q61A,R65A} protein (and partially in those containing the *rcf1*_{His}^{R67A,Q71A} 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 Arg⁶⁷ residue, and thus, more of complex IV (IV**) remains in association with the *rcf1*_{His}^{R67A} 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 O₂ 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*_{His}^{R67A} 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*_{His}^{R67A} 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 Δ *crd1* and Δ *tam41*. 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 cyto-

chrome *c*₁ of complex III (22),³ and all are proteins known to be intimately associated with CL molecules. Lipid profile analysis of Δ *rcf1*; Δ *rcf2* mutant mitochondria has not indicated a major alteration in the content of CL or PG in the absence of Rcf1/Rcf2 proteins.⁴ The recovery of a population of Taz1 with the *rcf1*_{His}^{R67A} 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)HLIHMRX₃Q. 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, Δ *rcf1*; Δ *rcf2* (*RCF1*::*HIS3*, *RCF2*::*KAN*) (23), Δ *aac2* (*AAC2*::*KAN*) (35), Δ *tam41* (*MMP37*::*KAN*) (36), and Δ *crd1* (*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 His₁₂-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 Δ *rcf1*; Δ *rcf2* strain, LEU⁺ transformants were selected, and expression of Rcf1_{His} derivatives was verified.

Affinity Purification of His-tagged Proteins—Isolated mitochondria (200 μ g of protein) harboring the His-tagged Rcf1 derivatives were solubilized in lysis buffer (100 mM KCl, 20 mM HEPES-KOH, 10 mM MgCl₂, 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, Ni-NTA purification of His-tagged proteins was performed as described previously (37).

BN-PAGE Analysis—BN-PAGE analysis of digitonin-solubilized (1%) or DDM-solubilized (0.6%) mitochondrial extracts (30 μ g 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 nM 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, $\Delta rcf1;\Delta rcf2$, $\Delta rcf1;\Delta rcf2+rcf1_{His}$, and $\Delta rcf1;\Delta rcf2+rcf1_{His}^{R67A}$. 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 P_i, 20 mM HEPES-KOH, 2 mM MgCl₂, 1 mM EDTA, 1 mg/ml BSA, and 0.6 M mannitol (pH 7.2)). State 4 respiration was measured following addition of NADH (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 *m*-maleimidobenzoyl-*N*-hydroxysuccinimide 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, anti-yeast 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.

Author Contributions—J. G. and R. A. S. conceived and coordinated the study and wrote the paper. J. G. designed, performed, and analyzed all the experiments shown together with R. A. S., except those shown in Fig. 3. V. S. and I. W. performed and analyzed the experiments shown in Fig. 3 and prepared the figure. J. G. prepared all other figures. All authors reviewed the results and approved the final version of the manuscript.

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