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Ubiquinone accumulates in the mitochondria of yeast mutated in the ubiquinone binding protein, Qcr8p

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Abstract

In *Saccharomyces cerevisiae*, the trans-membrane helix of Qcr8p, the ubiquinone binding protein of complex III, contributes to the Q binding site. In wild-type cells, residue 62 of the helix is non-polar (proline). Substitution of proline 62 with a polar, uncharged residue does not impair the ability of the cells to respire, complex III assembly is unaffected, ubiquinone occupancy of the Q binding site is unchanged, and mitochondrial ubiquinone levels are in the wild-type range. Substitution with a +1 charged residue is associated with partial respiratory competence, impaired complex III assembly, and loss of cytochrome *b*. Although ubiquinone occupancy of the Q binding site is similar to wild-type, total mitochondrial ubiquinone doubled in these mutants. Mutants with a +2 charged substitution at position 62 are unable to respire. These results suggest that the accumulation of ubiquinone in the mitochondria may be a compensatory mechanism for impaired electron transport at cytochrome *b*.

Keywords: Ubiquinone; Complex III; Qcr8p; Ubiquinone binding protein

In *Saccharomyces cerevisiae*, complex III consists of cytochromes c_1 and *b*, a Rieske Fe-S protein, two core proteins, and a group of smaller proteins that contribute to complex III structure. One of these proteins, Qcr8p, is an 11 kDa protein that faces the inter-membrane space [1,2]. The trans-membrane helix of the protein has been identified as a ubiquinone binding region [3]. The trans-membrane helix of Qcr8p also comes into contact with helices G and H of cytochrome *b*. The N-terminus of Qcr8p contributes to one of three β sheets that make up the Rieske protein extrinsic domain, while the C-terminus region is in contact with cytochrome c_1 and Qcr6p [4]. Thus, the trans-membrane domain of Qcr8p is a component of both the Qp and Qn ubiquinone binding sites of complex III [5] and may be critical for respiration.

The effect of mutations in the trans-membrane region of Qcr8p on cellular respiration has been studied by others. In one study [6], complex III assembly was impaired and activity reduced when tyrosine 66 was replaced by serine. Function was restored by phenylalanine, suggesting that an aromatic residue at position 66 is required for complex III function. In another report, substitutions of the residue at positions 62 or 64 did not affect respiratory growth or complex III activity, but a decrease in complex II activity was noted [7]. Others have reported that the replacement of residues 69 to 73 (WWKNG) by a single cysteine residue allowed normal assembly of complex III but specific activity was 45% of control levels and the reduction of cytochrome *b* was impaired [8]. These studies indicate that the trans-membrane ubiquinone binding domain of Qcr8p is required for complex III assembly and respiratory function [5]. However, the effect of mutations in the trans-membrane helix on ubiquinone binding ability has not been investigated. We asked whether point mutations in the trans-membrane region of Qcr8p that impair respiration

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and complex III stability have an effect on the binding of ubiquinone into the Q binding pocket of complex III. In order to address this question, polar substitutions of the non-polar proline residue at position 62 of Qcr8p were identified that were associated with varying abilities of *S. cerevisiae* to respire. As the net charge on the substitution increased, the ability of the cell to respire decreased, as demonstrated by the loss of ability to grow on acetate. The positioning of ubiquinone into the Q binding site was not affected by the mutations; however, a marked increase in mitochondrial ubiquinone in the mutants was observed.

Materials and methods

Creation of mutants. Fig. 1 shows the amino acid sequence of Qcr8p; the trans-membrane domain between residues 56 and 70 is highlighted. PCR primer sets containing the desired point mutations were used to mutagenize the *QCR8* gene sequence using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR amplification products of the Quik-Change procedure were inserted into the plasmid YEp352 at the *Kpn*I/*Sal*I restriction site. Constructs were sequenced (DNA Sequencing Facility, UT Austin) to confirm that the point mutations were correct. In order to create stable Qcr8p mutants, a *QCR8* deletion strain was transformed with the constructs using standard yeast genetic methods [9].

The yeast strain deleted in *QCR8* was created using the *HIS3* replacement cassette of *Saccharomyces kluyveri* [10], kindly provided by Dr. M. Longtine (Oklahoma State University). The *QCR8* gene sequence was disrupted with the *HIS3* gene of *S. kluyveri* by PCR amplification. The product was used to transform the wild-type yeast strain Cen.PK2-1C, kindly provided by Dr. C. Clarke (UCLA). Successful integration of the *HIS3* marker into the wild-type *QCR8* gene was confirmed by restoration of histidine autotrophy and PCR amplification of genomic DNA (data not shown).

Growth. Yeast strains (Table 1) were grown on synthetic medium (YNA: 2% acetate, 0.1% glucose, 6% yeast nitrogen base, 0.5% yeast extract, and amino acids). Growth was monitored by optical density readings at 600 nm [9].

Sub-cellular fractionation. Strains were grown 24 h in YNA and cells were pelleted. After incubation with zymolyase (Sigma, St. Louis MO), spheroplasts were isolated, suspended in buffer (0.6 M mannitol, 10 mM Tris, 1 mM PMSF, pH 7.4), and homogenized. Mitochondrial and cytosolic fractions were prepared by sequential centrifugations at 9500*g* [11]. The protein content of fractions was determined using the BCA method (Pierce, Rockford IL).

Cytochrome content. Mitochondrial pellets isolated as described above were suspended in buffer (25 mM potassium phosphate, pH 7.4, 1 mM EDTA, and 1% dodecyl maltoside) and difference spectroscopy was performed [12]. In brief, samples were oxidized with ferricyanide and subsequently reduced with sodium dithionite; spectra were scanned between 420 and 620 nm. Spectra of oxidized samples were subtracted from reduced to obtain difference spectra. Concentrations of cytochrome c_1 were determined at 550 nm using an extinction coefficient of 20.9 mM^{-1} cm^{-1}, and for cytochrome *b*, 560 nm (25.6 mM^{-1} cm^{-1}).

EPR analysis of ubiquinol occupancy of the Q site. Mitochondrial samples were prepared as described above, and EPR run on a Bruker EleXsys E500 equipped with an ER 4116 DM cavity operating at 9.63 GHz and an Oxford instruments ESR 900 helium flow cryostat [13]. EPR spectra of ascorbate-treated mitochondrial preparations were fit using the parameters described by Ding [14]. Spectrometry and fits were performed at the National Biomedical EPR Center (The Medical College of Wisconsin) by Brian Bennett.

Ubiquinone content. Ubiquinone was extracted from growth media and from mitochondrial and cytosolic fractions after 24 h of cell growth and quantified by HPLC as we have described [15]. Lipids were extracted by sequestration in petroleum ether [16] and ubiquinone content analyzed by reversed phase HPLC. Samples were separated on a Zorbax ODS column (Sigma, St. Louis MO) with a mobile phase of hexane:methanol (1:9, v:v) monitored at 275 nm. Coq_7 (Sigma, St. Louis MO) was used as an internal standard. Ubiquinone content is expressed as μg/mg protein.

Table 1
Yeast strains used in this study

CEN.PK2-1C	MAT *a ura3his3leu2trp1*	C. Clarke
QCR8Δ	CEN.PK2-1C, *qcr8::HIS*	This study
P62N	QCR8Δ + YEp352-*QCR8P62N*	This study
P62K	QCR8Δ + YEp352-*QCR8P62K*	This study
P62R	QCR8Δ + YEp352-*QCR8P62R*	This study

Results

Respiratory ability of mutants

Sixteen mutations in Qcr8p were created (Table 2), 14 in the trans-membrane region (residues 56–70) and two control strains containing mutations outside the trans-membrane region. To screen for respiratory ability, strains were grown on acetate (YNA), a non-fermentable carbon

Table 2
Growth of Qcr8p mutants after 24 h in YNA

Mutation	Growth on YNA (24 h)	Proline 62 substitution[a]
CEN.PK2-1C	+	None
V59E	+	None
L60F	+	None
I61K	+	None
P62V	+	Non-polar
P62L	+	Non-polar
P62N	+	Polar, uncharged
P62K	±	Polar, +1 charge
P62R	0	Polar, +2 charge
A63S	+	None
G64R	+	None
G64A	+	None
I65F	+	None
Y66S	+	None
W67R	+	None
R91N	+	None
T25I	+	None

[a] Proline is a non-polar residue.

MGPPSGKTYMGWWGHMGGPKQKGITSYAVSPYAQKPLQGIFHNAVFNSFRR
FKSQ**FLY VLI*PAG*IYWYWW**KNGNEYNEFLYSKAGREELERVNV

Fig. 1. The Qcr8p amino acid sequence. The ubiquinone binding site is highlighted and the wild-type proline residue at position 62 is italicized (http://www.yeastgenome.org/).

source. Fourteen of the mutants of Qcr8p grew on YNA similarly to wild-type cells (Table 2). Impaired respiratory ability was observed for some substitutions of the wild-type non-polar proline residue at position 62. Substitutions at position 62 with other non-polar amino acids (valine, P62V, and leucine, P62L) did not affect respiratory ability. Substitution of proline 62 with a polar uncharged residue (asparagine, P62N) also did not affect respiratory ability. However, substitutions with charged polar residues (lysine, P62K, and arginine, P62R) were associated with either diminished (P62K) or complete (P62R) loss of ability to grow on YNA.

Growth of P62 mutants

In Fig. 2, growth on YNA of the mutants P62N, P62K, and P62R is compared to the wild-type strain (CEN.PK2-1C) and the Qcr8p deletion strain (QCR8Δ). P62N grows on YNA comparably to the wild-type strain. P62K is able to grow on YNA, but never achieves the cell densities of P62N or the wild-type strain, suggesting that respiration is impaired in P62K. P62R and QCR8Δ are unable to grow on YNA. The differing abilities of the P62 mutants to respire, as indicated by their growth patterns on YNA, suggest that the proline residue at position 62 is critical for Qcr8p function. Proline is a non-polar amino acid with constrained side chain mobility. Asparagine (N) is a polar uncharged amino acid with a short side chain. Lysine (K) and arginine (R) are polar amino acids with different net charges (+1, and +2, respectively) and long, flexible side chains. The growth data suggest that when a conformationally unrestricted side chain with positive charge is substituted at position 62 of Qcr8p, the ability to respire decreases.

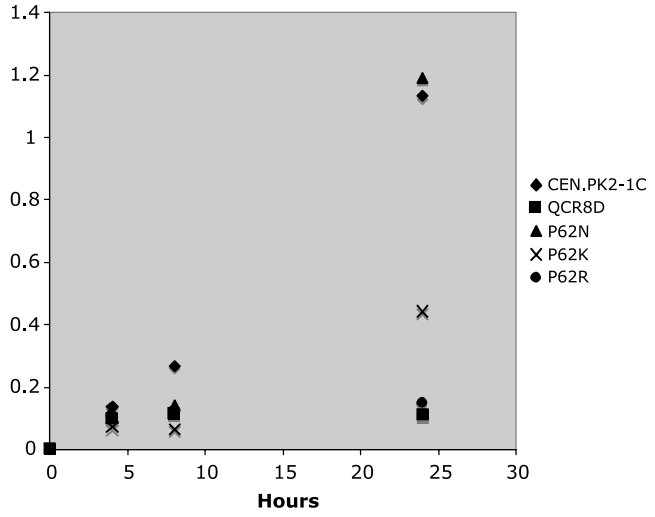

Fig. 2. Growth of strains on non-fermentable carbon. Cells were grown to saturation in complete glucose medium and diluted to equal concentrations at time 0 into YNA (2% acetate 0.1% glucose complete medium). Aliquots were taken at specified time points and absorption read at 600 nm.

Difference spectra and cytochrome content

Difference spectra were obtained for CEN.PK2-1C, P62N and P62K (Fig. 3). Cytochrome c_1 and b concentrations were calculated from the difference spectra. Cytochrome c_1 concentrations in all strains were similar (3.9, 4.0, and 3.5 nmol/mg, for CEN.PK2-1C, P62N, and P62K, respectively). Although cytochrome b concentrations were similar for CEN.PK2-1C and P62N (4.2 and 3.8 nmol/mg, respectively), cytochrome b content was markedly reduced in P62K (1.9 nmol/mg). The decrease in cytochrome b content in P62K is in agreement with its impaired ability to respire. It was not possible to characterize P62R because it grows so poorly on YNA.

Ubiquinol occupancy of the Q site

To determine whether Q site occupancy differed in the mutant strains compared to wild-type, Q binding site

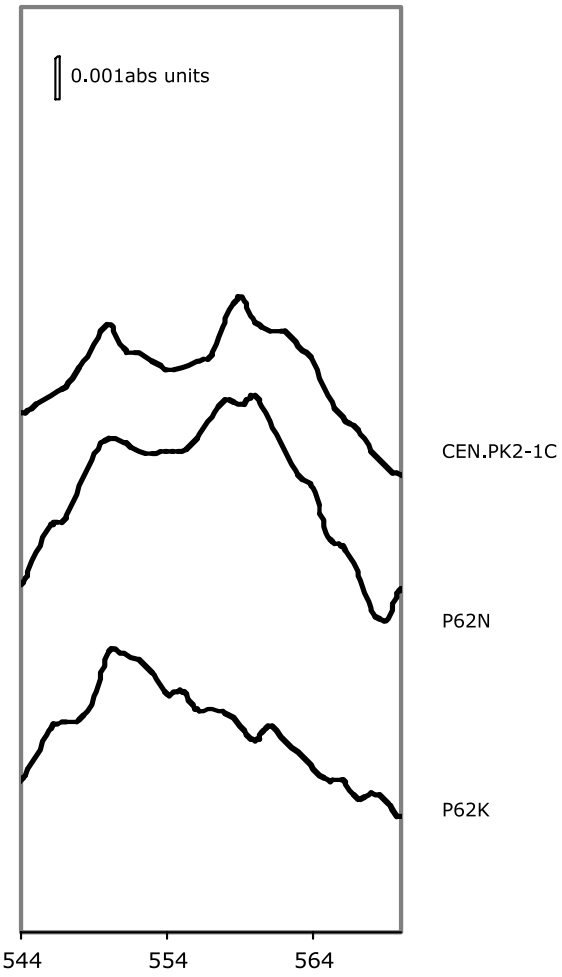

Fig. 3. Representative difference spectra of CEN.PK2-1C, P62N, and P62K. Cells were grown in YNA (2% acetate 0.1% glucose complete medium) for 24 h and mitochondria were prepared. Samples were oxidized with ferricyanide and subsequently reduced with sodium dithionite; spectra were scanned between 420 and 620 nm. Spectra of oxidized samples were subtracted from reduced to obtain difference spectra.

occupancy was determined by EPR. The $g(x)$ signal centered at $g = 1.79$ is typically shifted over 1% upfield in organisms with defective ubiquinol binding at site Qp [17–19]. In our mutants, the $g(x)$ signals for both P62N and P62K (1.785 and 1.787, respectively) were within 0.4% of the $g(x)$ signal for the wild-type (1.792). These data suggest that substitutions of proline 62 in the trans-membrane domain of Qcr8p do not affect the ability of ubiquinone to bind at the Q binding site.

Ubiquinone content of cells and media

After 24 h of growth on YNA, ubiquinone was extracted from mitochondrial preparations. Twice as much ubiquinone was isolated from P62K (25.0 μg/mg protein) as from wild-type or P62N (12.2 and 14.1 μg/mg protein, respectively). Cytosolic fractions of all strains contained trace amounts of ubiquinone, from 5 to 10% of mitochondrial content. No ubiquinone was detected in media samples after 24 h of growth.

Discussion

Our study suggests that Qcr8p-cytochrome *b* interactions are important to the assembly of complex III of the yeast respiratory chain. We identified a mutant of Qcr8p that is impaired in respiration and found that cytochrome *b* levels are diminished in that mutant. Although some studies have suggested that the trans-membrane helix of Qcr8p participates in ubiquinone binding [5], we found that ubiquinone occupancy of the Q binding site is not affected by Qcr8p mutations that impair respiration. However, the amount of mitochondrial ubiquinone in the mutant is twice that of wild-type cells.

Substitution of the non-polar proline at position 62 of the Qcr8p trans-membrane domain with a polar uncharged residue such as asparagine (N) does not impair respiratory ability or complex III assembly. Substitution with a +1 charged polar amino acid (K) partially impairs respiration, apparently by interfering with the incorporation of cytochrome *b* into the complex. Increasing the charge at position 62 to +2 (R) is associated with a complete inability to grow on non-fermentable carbon. Residue 62 of Qcr8p is adjacent to residue F324 of cytochrome *b* [4]. Structural manipulations of complex III (pdb 1ezv) using the mutation tool in DeepView (http://swissmodel.expasy.org//SWISS-MODEL.html) demonstrate that replacing proline 62 with a polar uncharged amino acid with a short side chain, such as asparagine (N), does not introduce unfavorable interactions with cytochrome *b*. Substituting lysine (K,+1) for proline generates a somewhat unfavorable interaction with cytochrome *b*. Arginine (R,+2) penetrates deeply into the adjacent region of cytochrome *b*, with unfavorable interactions in all predicted rotamers. Thus, as charge and chain length are increased at residue 62, unfavorable interactions between Qcr8p and cytochrome *b* increase. The reduction in cytochrome *b* content that we noted in P62K is consistent with impaired complex III assembly due to unfavorable interactions between cytochrome *b* and the mutant Qcr8p [20]. Therefore, we conclude that the defect in respiration observed in P62K is due to the inability of complex III to fully incorporate cytochrome *b*.

We observed a substantial increase in mitochondrial ubiquinone content in P62K. It is unknown whether this difference is due to increased ubiquinone synthesis or to a decreased rate of ubiquinone degradation. It has been reported that complex III is unstable in strains in which ubiquinone synthesis is blocked [21,22]. In those strains, stability can be restored by the addition of exogenous ubiquinone. Thus, it is possible that the function of the increased ubiquinone levels noted in P62K may be to improve stability of the complex III under conditions leading to unfavorable interactions between cytochrome *b* and Qcr8p.

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References

[1] M. Iwata, J. Abramson, B. Byrne, S. Iwata, Structure and function of quinone binding membrane proteins, Adv. Protein Chem. 62 (2003) 151–176.
[2] W. Hemrika, J.A. Berden, Membrane topography of the subunits of ubiquinol-cytochrome *c* oxidoreductase of *Saccharomyces cerevisiae*, Eur. J. Biochem. 192 (1990) 761–765.
[3] L. Yu, K.-P. Deng, C.-A. Yu, Cloning, gene sequencing, and expression of the small molecular mass ubiquinone-binding protein of mitochondrial ubiquinol-cytochrome *c* reductase, J. Biol. Chem. 270 (1995) 25634–25638.
[4] C. Hunte, J. Koepke, C. Lange, T. Rossmanith, H. Michel, Structure at 2.3 Å resolution of the cytochrome bc1 complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment, Structure 8 (2000) 669–684.
[5] S. Usui, L. Yu, C.-A. Yu, The small molecular mass ubiquinone-binding protein (Qpc-9.5 kDa) in mitochondrial ubiquinol-cytochrome *c* reductase, Biochemistry 29 (1990) 4618–4626.
[6] W. Hemrika, G. Lobo-Hajdu, J.A. Berden, L.A. Grivell, The aromatic nature of residue 66 of the 11-kDa subunit of ubiquinol-cytochrome *c* oxidoreductase of the yeast *Saccharomyces cerevisiae* is important for the assembly of a functional enzyme, FEBS Letts. 344 (1994) 15–19.
[7] C. Bruel, R. Brasseur, B.L. Trumpower, Subunit 8 of the *Saccharomyces cerevisiae* cytochrome bc1 complex interacts with succinate-ubiquinone reductase complex, J. Bioenerg. Biomembr. 28 (1996) 59–68.
[8] W. Hemrika, J.A. Berden, L.A. Grivell, A region of the C-terminal part of the 11-kDa subunit of ubiquinol-cytochrome-*c* oxidoreductase of the yeast *Saccharomyces cerevisiae* contributes to the structure of the Q out domain, Eur. J. Biochem. 215 (1993) 601–609.
[9] F. Sherman, Getting started with yeast, Methods Enzymol. 194 (1991) 3–21.

[10] M.S. Longtine, A. McKenzie, D.J. Demarini, Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*, Yeast 14 (1998) 953–961.
[11] E. Zinser, G. Daum, Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*, Yeast 11 (1995) 493–536.
[12] R.A. Hagerman, P.J. Trotter, A mutation in the yeast mitochondrial ribosomal protein Rml2p is associated with a defect in catalase gene expression, Mol. Cell Bio. Res. Commun. 4 (2001) 299–306.
[13] N. Fisher, I. Bourges, P. Hill, G. Brasseur, B. Meunier, Disruption of the interaction between the Rieske iron–sulfur protein and cytochrome *b* in the yeast bc1 complex owing to a human disease-associated mutation within cytochrome *b*, Eur. J. Biochem. 271 (2004) 1292–1298.
[14] H. Ding, D.E. Robertson, F. Daldal, P.L. Dutton, Cytochrome bc1 complex [2Fe-2S] cluster and its interaction with ubiquinone and ubihydroquinone at the Q0 site, Biochemistry 31 (1992) 3144–3158.
[15] R.A. Hagerman, M.J. Anthony, R.A. Willis, Solid-phase extraction of lipid from *Saccharomyces cerevisiae* followed by high-performance liquid chromatography analysis of coenzyme Q content, Anal. Biochem. 296 (2001) 141–143.
[16] A. Kroger, Determination of contents and redox states of ubiquinone and menaquinone, Methods Enzymol. 52 (1978) 579–591.
[17] A.S. Sariba, H. Ding, P.L. Dutton, F. Daldal, Tyrosine 147 of cytochrome *b* is required for efficient electron transfer at the ubihydroquinone oxidase site (Q sub(o)) of the cytochrome bc sub(1) complex, Biochemistry 34 (1995) 16004–16012.
[18] G. Brasseur, J.-P. Di Rago, P.P. Slonimski, D. Lemesle-Meunier, Analysis of suppressor mutation reveals long distance interactions in the bc1 complex of *Saccharomyces cerevisiae*, J. Biol. Chem. 1506 (2001) 89–102.
[19] G. Brasseur, D. Lemesle-Meunier, F. Reinaud, B. Meunier, QO site deficiency can be compensated by extragenic mutations in the hinge region of the iron–sulfur protein in the bc1 complex of *Saccharomyces cerevisiae*, J. Biol. Chem. 279 (2004) 24203–24211.
[20] H. Boumans, J.A. Berden, L.A. Grivell, The role of subunit VIII in the structural stability of the bc1 complex from *Saccharomyces cerevisiae* studied using hybrid complexes, Eur. J. Biochem. 249 (1997) 762–769.
[21] C. Santos-Ocana, T.Q. Do, S. Padilla, P. Navas, C.F. Clarke, Uptake of exogenous coenzyme Q and transport to mitochondria is required for bc1 complex stability in yeast coq mutants, J. Biol. Chem. 277 (2002) 10973–10981.
[22] T.Q. Do, A.Y. Hsu, T. Jonassen, P.T. Lee, C.F. Clarke, A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in *Saccharomyces cerevisiae* abc1 mutants, J. Biol. Chem. 276 (2001) 18161–18168.