A pathogenic cytochrome b mutation reveals new interactions between subunits of the mitochondrial bc1 complex

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Running title : Interaction between cytochrome b and Qcr9p

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Keywords :
Yeast / Qcr9p / Rieske protein / cytochrome c1 / myopathy
Interaction between cytochrome b and Qcr9p.

Abstract

Energy transduction in mitochondria involves five oligomeric complexes embedded within the inner membrane. They are composed of catalytic and non-catalytic subunits, the role of these latter proteins often being difficult to assign. One of these complexes, the \( bc1 \) complex, is composed of three catalytic subunits including cytochrome \( b \) and seven or eight non-catalytic subunits. Recently, several mutations in the human cytochrome \( b \) gene have been linked to various diseases. We have studied in detail the effects of a cardiomyopathy generating mutation \( G252D \) in yeast. This mutation disturbs the biogenesis of the \( bc1 \) complex at \( 36^\circ C \) and decreases the steady-state level of the non-catalytic subunit Qcr9p. In addition, the \( G252D \) mutation and the deletion of \( QCR9 \) show synergetic defects that can be partially bypassed by suppressor mutations at position 252 and by a new cytochrome \( b \) mutation \( P174T \). Altogether, our results suggest that the supernumerary subunit Qcr9p enhances or stabilizes the interactions between the catalytic subunits, this role being essential at high temperature.

Introduction

Numerous cellular functions are performed by oligomeric complexes composed of catalytic and non-catalytic subunits and the role of these latter proteins is often difficult to assign. Energy transduction in mitochondria involves five oligomeric complexes that are embedded within the inner membrane. One of these complexes, the \( bc1 \) complex is composed of three catalytic and seven or eight non-catalytic subunits (for review,1). The three catalytic subunits, cytochrome \( b \), cytochrome \( c1 \) and the Rieske iron-sulfur protein (Rieske) are conserved in the bacterial equivalents of this complex. However, several non-catalytic subunits are present in the mitochondrial \( bc1 \) complexes and are often referred to as supernumerary subunits although some are required for the enzymatic activity. In
Saccharomyces cerevisiae the bc1 complex contains seven supernumerary subunits that are conserved in the mammalian enzyme. The mammalian bc1 complex presents an additional subunit that has no equivalent in yeast. The mitochondrial bc1 complexes from bovine, chicken and yeast have been crystallized (2, 3, 4, 5) and an analysis of these structures reveals that the general shape, size and topology of the complexes are similar in the three organisms.

The mitochondrial respiratory chain is required for electron transport and oxidative phosphorylation. The bc1 complex transfers electrons from ubiquinol to cytochrome c and translocates protons into the inter-membrane space, the resulting electrochemical gradient is utilized by the ATP synthase to produce ATP in the mitochondrial matrix. The bc1 complex exhibits two site of ubiquinol binding, the Qo site in the inter-membrane side and Qi site in the matrix side. The oxidation of ubiquinol at the Qo site releases two electrons; one is transferred to Rieske, then to cytochrome c1 and finally to cytochrome c. The second electron is transferred to the low potential heme bL, then to the high potential heme bH and to ubiquinone at the Qi site. Genetic analyses in yeast have shown that the absence of a catalytic subunit always leads to a complete block of electron transfer, while the absence of non-catalytic subunits causes various defects in bc1 complex activity (for review, 6). For example, the absence of the supernumerary subunits Qcr7p or Qcr8p (7, 8) leads to a complete respiratory deficiency at any temperature while Qcr6p or Qcr9p appears to be essential only at 36°C (9, 10). The precise role of these supernumerary subunits and the nature of the functional interactions existing between them and the catalytic subunits still remain to be elucidated.

Cytochrome b plays a crucial role in the activity of the bc1 complex since it harbours the two b hemes and participates in determining the shape of the two ubiquinone fixation sites. This integral membrane protein containing eight trans-membrane segments is the only bc1 subunit encoded by the mitochondrial genome. The sequence alignment between yeast
and bovine cytochrome $b$ shows 51% identity and present also a striking structural conservation between the cytochrome $b$ of the two organisms (5).

Recently several mutations in the human cytochrome $b$ gene have been linked to diseases such as cardiomyopathy, exercise intolerance or Leber’s Hereditary Optic Neuropathy (for review, 11 and references therein, 12, 13, 14, 15). In particular, one mutation that substitutes a glycine by an aspartate residue at position 251 of cytochrome $b$ ($G251D$) is associated with a histiocytoid cardiomyopathy (16, 17). This mutation is located in the inter-membrane space loop connecting the fifth and sixth trans-membrane segments. Analyses on mitochondria purified from patient heart have established a defect in the succinate cytochrome $c$ oxido-reductase activity and in cytochrome $b$ assembly, showing the importance of residue 251 for $bc1$ function.

The fact that $S.\ cerevisiae$ is a facultative aerobe, considerably facilitates the study of respiratory deficient mutants. Moreover, the rapid segregation of mitochondrial chromosomes in yeast permits the generation of homoplastic cells containing only the mutated mitochondrial DNA. On the contrary, wild type and mutated mitochondrial DNAs often coexist within the cells of patients carrying a mitochondrial pathology. Thus, yeast mitochondrial mutants can provide invaluable help to fully understand the consequences of a mitochondrial mutation observed in a patient.

In this paper, we have studied in detail the effects of the histiocytoid cardiomyopathy corresponding mutation in yeast ($G252D$). This mutation strongly disturbs the respiratory functions of cells grown at high temperature ($36^\circ C$). We have found that the presence of the aspartate residue at position 252 renders the non-catalytic subunit Qcr9p essential for the activity of the $bc1$ complex at $28^\circ C$ and that this defect can be bypassed by a second mutation in the cytochrome $b$ gene ($P174T$) located in a region close to Rieske. We propose that the supernumerary subunit Qcr9p enhances or stabilizes the interactions between cytochromes $b$, $c1$ and Rieske, particularly at high temperature.
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Experimental Procedures

Strains, media, genetic methods

All strains have the same nuclear background ade2-1 ura3-1 his3-11,-15 trp1-1 leu2-3,-112 can1-100. The construction of the intron-less mitochondrial genome was described in 18 and that of the ∆qcr9 strain in 19. Yeast genetic methods were described in 20 and 21. Non-fermentable ethanol/glycerol medium is 1% yeast extract, 1% casamino acids, 0,05 M sodium potassium phosphate (pH6.25), 3% ethanol, 3% glycerol. Cytoductions experiments were performed by using intermediate strains with the kar1-1 mutation that delays nuclear fusion (22). Rho° derivatives devoid of mitochondrial genome were constructed by growing the cells in complete medium containing ethidium bromide (40 µg/ml). The rho° derivative recipient and the donor strain cells were mixed on complete liquid glucose medium and shaken for 2.5 hours at 28°C. The mixture of cells was harvested by centrifugation and incubated for one hour at 28°C without shaking. Then, the cells were resuspended and shaken for 2.5 hours at 28°C, diluted and plated onto media selective for cytoductants containing the recipient strain nuclear genome and the donor strain mitochondrial genome.

Construction of the intron-less cytochrome b gene with a glycine codon at position 252.

The CYTB gene was site corrected by a two step PCR strategy, using DNA extracted from the strain CW30 carrying the intron-less mitochondrial genome with the G252D mutation as template. The corrected fragment was cloned at the HincII site of the pJM2 plasmid containing the wild type COX2 gene (23). The resulting plasmid (pYS10) was introduced into mitochondria from the rho° strain DFS160 by biolistic transformation (24). Mitochondrial transformants were selected by their ability to rescue the cox2-30 mutation. The cytochrome b corrected 252G gene was then integrated by homologous recombination into the intron-less
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mitochondrial genome of the YST1 strain ($\Delta qcr9\ G252D$) to give the strain YST2 ($\Delta qcr9\ 252G$). The corrected intron-less mitochondrial genome was also introduced by cytoduction into the $rho^o$ derivatives of CW30 and YST6, a $G252D$ strain expressing the c-myc-tagged Qcr9p (19) to give strains CW252 ($252G$) and YST7 ($252G$ strain expressing Qcr9p-c-myc), respectively. The $CYTB$ sequences of all these strains were verified.

**Isolation and genetic characterization of the revertants.**

The $\Delta qcr9\ G252D$ strain (YST1) was subcloned and 10 respiratory deficient subclones were grown in complete liquid medium. 100 $\mu$l of each stationary phase cultures were plated on non-fermentable medium supplemented with 0.1% glucose and incubated at 28°C for 1 week. Nine independent revertants (each from a different liquid cultures) were isolated and called QR1 to QR9. The genetic nature of the suppressor mutations present in the revertants was established according to 21. The revertants and their $rho^o$ derivatives were crossed to a $\Delta qcr9\ G252D$ strain of opposite mating type and the resulting diploids were tested for their ability to grow on glycerol medium at 28°C. Whereas all the diploids resulting from crosses with the $rho^+$ revertants were respiratory competent, all diploids resulting from crosses with $rho^o$ derivatives of the revertants were respiratory deficient, showing that the suppressor mutations had been lost in the $rho^o$ derivatives and thus were located on the mitochondrial genome. The mitochondrial genomes of the revertants QR1, QR2 and QR3 were introduced by cytoduction in the $rho^o$ derivative of CW30 to give WQR1, WQR2 and WQR3 respectively.

**Isolation of mitochondria and respiratory chain activities.**

Mitochondria were purified from cells grown in complete galactose medium (25). The activities of the different respiratory complexes were measured from purified mitochondria at 28°C or 34°C. Ubiquinol cytochrome $c$ oxido-reductase activity was measured as in (26). Antimycin sensitive NADH cytochrome $c$ oxido-reductase activity was detailed in 27,
cytochrome c oxidase activity to 19. Cytochrome spectra were recorded with a Cary 400 spectrophotometer.

**Immunoblotting experiments.**

All steps of Western blotting were performed as in 25. The anti-Rieske protein polyclonal antibody raised against the human Rieske Iron Sulfur protein is from Prof. C. Godinot (CGMC, Lyon, France). The monoclonal antibody anti-porin from yeast was purchased from Molecular Probes. The polyclonal antibody anti-apocytochrome \(c1\) was raised in our lab against a fusion protein ProA-apocytochrome \(c1\) expressed in \(E.\ coligot\) (P. Hamel and C. Lemaire, unpublished). Anti-c-myc is a gift from Dr. J.M. Galan (Institut J. Monod, Paris, France).

**Results**

*The yeast cytochrome b gene encoded by the intron-less mitochondrial genome carries a G252D mutation*

*Saccharomyces cerevisiae* mitochondrial genome contains up to thirteen introns, but an intron-less mitochondrial genome has been constructed (18). We have sequenced the yeast cytochrome b gene encoded by this genome and shown that there is a transition at position 755 of the open reading frame. By comparison to the reference sequence of the yeast intron-containing mitochondrial genome (28), this mutation changes a GGT codon to GAT and leads to the substitution of a glycine by an aspartate at position 252 of the apocytochrome b ORF (G252D). This glycine is conserved in several bacterial cytochromes b (*e. g.* *Rhodobacter capsulatus*) as well as in higher eukaryotes such as mammals (Figure 1). In humans, it has been shown that, the substitution of the homologous glycine 251 by an aspartate leads to an hystiocytoid cardiomyopathy (17). This demonstrates the critical importance of this residue for respiratory function.
The G252D mutation affects the biogenesis of the bc1 complex at high temperature.

Using biolistic transformation, we have corrected the G252D mutation by introducing a glycine codon at position 252 of cytochrome b in the intron-less mitochondrial genome and we have compared the respiratory functions of two isonuclear strains that differ only at codon 252 of the cytochrome b gene.

As shown in Figure 2, both strains grow on non-fermentable medium at 28°C (strains 1 and 2), but the doubling time of the G252D mutant was slightly increased compared to the wild type strain. The respiratory growth of the mutant was severely affected at 36°C, leading to a substantially increased doubling time (5h20 versus 2h40). Mitochondria were purified from cells grown at 28°C or 34°C and the respiratory complex activities were measured at 28 and 36°C for each preparation of mitochondria. No differences in the enzymatic activities were ever observed between mutant and wild type mitochondria purified from cells grown at 28°C (data not shown). However, when mitochondria were purified from cells grown at high temperature, the antimycin-sensitive NADH-cytochrome c oxido-reductase (NADH-cyt$c$) and the ubiquinol-cytochrome c oxido-reductase (bc1) activities were diminished in the mutant by approximately 50% of the corresponding wild type level (Table I). This decrease was independent of the temperature used for the activity measurements. The cytochrome c oxidase activity (Cox) is not affected in the mutant. These results suggest that the respiratory growth defect of the mutant at high temperature is due to a deficiency in the bc1 complex biogenesis rather than activity.

A priori, this could be simply due to a defect in the assembly of the catalytic subunits at 36°C. Since an assembly defect generally leads to the degradation of unassembled subunits, we have looked at the accumulation of the three catalytic subunits: cytochrome b, cytochrome c1 and Rieske in the mutant versus wild type. Absorption spectra of cytochromes reduced by dithionite are identical at both 28°C and 36°C and do not reveal any difference in
the amount of cytochromes $b$ and $c_1$ between the strains (Figure 3A, traces 1 and 2 and data not shown). A Western blot analysis revealed a nearly wild type level of Rieske in the mutant grown at 36°C (data not shown).

In conclusion, the yeast mutant $G252D$ grown at high temperature, is affected in the biogenesis of the $bc_1$ complex leading to a substantial decrease of the enzymatic activity. However, the accumulation of the three catalytic subunits is not significantly modified.

**Interaction between residue 252 of cytochrome $b$ and the supernumerary subunit Qcr9p.**

In addition to the three catalytic subunits, the yeast $bc_1$ complex contains seven other subunits apparently not directly involved in the energy transducing mechanism (for review, 1). The 7.3 kDa subunit Qcr9p is one of these supernumerary subunits. The deletion mutant $\Delta qcr9$ exhibits a temperature-dependent respiratory growth (9, 29 and Figure 2, strain 3). The similarity between the respiratory phenotypes of both $G252D$ and $\Delta qcr9$ mutants have led us to measure the accumulation of Qcr9p in presence of the glycine or aspartate residue at position 252 of cytochrome $b$. As no anti-Qcr9p antibody was available, we have used a Qcr9p tagged at its C-terminus with c-myc epitopes. Whether they carried the wild type or $G252D$ variants of cytochrome $b$, the $QCR9$-$c$-myc strains were both able to grow on a non-fermentable substrate. However, Western blot analysis of mitochondrial proteins showed that the steady-state level of Qcr9p-c-myc was lower in the $G252D$ mutant than in the wild type strain whatever the temperature is (Figure 4 and not shown). To test if this decreased level of Qcr9p was responsible for the phenotype of the $G252D$ mutant at 36°C, we have over-expressed $QCR9$ in the $G252D$ mutant and did not found any improvement of growth. Finally, no major effect on the accumulation of cytochrome $c_1$ and Rieske protein was observed in the presence of the $G252D$ mutation in the Qcr9p-c-myc strain (data not shown). The relationships between the mutant $G252D$ and Qcr9p were further analysed by constructing the
double mutant $\Delta qcr9 \ G252D$. As shown in Figure 2 (strain 4), the respiratory growth of the double mutant is totally blocked at 28°C while each single mutant grows at this temperature. Altogether, these results suggest either a direct or a long-range interaction between Qcr9p and the residue 252 of cytochrome $b$.

Electron transfer between cytochromes $b$ and $c1$ is interrupted in the $\Delta qcr9$ G252D double mutant

We have purified mitochondria from the $\Delta qcr9 \ G252D$ double mutant and both single mutants and measured the activities of the respiratory complexes at 28°C. Only complex $bc1$ activity was affected in the mutants. Table II shows that the antimycin-sensitive, NADH cytochrome $c$ oxido-reductase activity at 28°C was wild type in the $G252D$ mutant, decreased by about 30% in the $\Delta qcr9$ mutant and undetectable in the double mutant. This synergetic defect suggests the existence of a genetic interaction between the two mutants. The ubiquinol cytochrome $c$ oxido-reductase activity (data not shown) was also undetectable in the $\Delta qcr9 \ G252D$ strain showing that there is no $bc1$ complex driven electron transfer in this double mutant.

In order to determine if this lack of $bc1$ activity was due to an assembly defect, we measured the amounts of the catalytic $bc1$ subunits in 28°C grown cells. Cytochrome $b$ and $c1$ were detected by recording cytochrome spectra after full reduction by dithionite. As shown in Figure 3A, there was only a slight decrease in the amounts of cytochrome $b$ and $c1$ in the $\Delta qcr9$ strains and the $G252D$ mutation had no additional effect. Immuno-detection experiments, revealed a threefold decrease of the steady-state level of Rieske in the $\Delta qcr9$ strains, as expected from 9. However, the decrease due to the $\Delta qcr9$ mutation was not dramatically modified by the $G252D$ mutation (Figure 3B and legend). Moreover, and as expected from cytochrome spectra, the accumulation of apo-cytochrome $c1$ (Cyt1p) was not
modified. Altogether, these results suggested that the \textit{bc1} activity defect observed at 28°C in the double mutant was not due to a defect in the accumulation of catalytic subunits.

To determine which step of the electron transfer was blocked in the mutants, the endogenous reduction of the respiratory chain cytochromes was investigated by recording the cytochrome absorption spectra without exogenous reducing agent (Figure 3C, trace 3). All the peaks specific of reduced cytochromes \textit{b}, \textit{c1} and \textit{c} were present in the \textit{Δqcr9} strain, showing that electrons can be transferred through a \textit{bc1} complex lacking Qcr9p. In the \textit{Δqcr9 G252D} double mutant, the \textit{in situ} endogenous reduction of cytochromes \textit{c1} and \textit{c} was strongly decreased, while the reduction of cytochrome \textit{b} was only slightly diminished (Figure 3C, trace 4). This indicates that the combination of both mutations leads to an interruption of electron transfer between cytochromes \textit{b} and \textit{c1}.

Altogether these results suggest that the interaction between the residue 252 of cytochrome \textit{b} and Qcr9p is important for the electron transfer from cytochrome \textit{b} to cytochrome \textit{c1}.

\textbf{Suppressor mutations restore respiratory function to the \textit{Δqcr9 G252D} double mutant.}

To further our understanding of the nature of the interaction between the residue 252 of cytochrome \textit{b} and Qcr9p, we have searched by-pass suppressor mutations able to alleviate the respiratory deficiency of the \textit{Δqcr9 G252D} double mutant at 28°C (Figure 2). Nine independent spontaneous revertant strains were selected and the genetic analysis concluded that all the suppressor mutations were located on the mitochondrial chromosome (see Materials and Methods). As cytochrome \textit{b} is the only \textit{bc1} complex subunit encoded by the mitochondrial genome, we have PCR-amplified and sequenced the gene from the nine revertant strains. Interestingly enough, all the suppressor mutations are located in two regions of the cytochrome \textit{b} gene. Seven revertants carried a mutation at codon 252 : three changed
the aspartate to an asparagine (D252N) and four to a tyrosine (D252Y). The two last suppressors exhibited a C to T transition in codon 174, leading to the substitution of a proline by a threonine (P174T). As shown in Table II, the doubling time of the revertant strains in non-fermentable medium varied from five to six hours at 28°C, the P174T mutation being the least efficient suppressor mutation. All revertants failed to grow on non-fermentable medium at 36°C (Figure 2).

The bc1 complex activity and assembly were analysed at 28°C in the three classes of revertant strains. The antimycin-sensitive, NADH cytochrome c oxydo-reductase activity was partially restored in the three revertants, the bc1 activity in the D252N suppressor strain is restored almost to Δqcr9 single mutant level (Table II). As shown in Figure 5, cytochrome absorption spectra and Western blot analysis showed a slight diminution of the steady-state level of cytochromes b, c1 and Rieske in the revertant strains (Figure 5A, traces 5 and 7 and figure 5B), similar to the decrease described above for the single mutant Δqcr9 (Figure 3, trace 3 and lane 3). This suggests that the suppressor mutation does not compensate for the mild assembly defect due to the absence of Qcr9p.

In order to examine the respiratory phenotype of the three suppressor mutations in presence of Qcr9p, we have introduced the mitochondrial genomes of the revertants in a wild type QCR9 context by cytoduction. The D252N and D252Y suppressor mutations had no effect on the respiratory phenotype as assessed by cell doubling time, assembly or activity of the bc1 complex compared to the wild type at 28° or 36°C (Table II; Figure 5A, trace 8 and data not shown). The P174T G252D strain which carries two substitutions in cytochrome b, has a slightly longer doubling time, this phenotype being nearly undetectable on plates, and no significant difference in the bc1 complex activity was detected when compared to the wild type (Table II).

Thus, the location of the two suppressor mutations D252N and D252Y stresses the importance of the residue 252 of cytochrome b for the bc1 complex activity in absence of
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Although, the \( P174T \) mutation is located in the intermembrane space domain of cytochrome \( b \) like the \( G252D \) mutation, the two mutations appear rather distant according to the crystal structure (5). This speaks in favour of a long range interaction between these two residues of cytochrome \( b \).

**Discussion**

In this study, we have shown that the intron-less mitochondrial chromosome from the yeast *S. cerevisiae* carries a mutation leading to the substitution of a glycine residue by an aspartate at position 252 of cytochrome \( b \). The same mutation has been recently described in a patient with a cardiomyopathy (17) associated with a defect in succinate cytochrome \( c \) oxido-reductase activity (16). This \( G252D \) mutation has no major effect on respiratory function of yeast cells grown at 28°C, the appropriate growth temperature of yeast but leads to a 50% decrease of the \( bc1 \) complex activity at 36°C, which is close to the temperature of the human body.

A growing number of mutations in the human mitochondrial DNA were shown to be responsible for numerous pathologies (for review, 30). Among a dozen mutations mapped to the human cytochrome \( b \) gene, only two have been investigated in the yeast system (11). The \( G34S \) mutation has been observed in a patient suffering from exercise intolerance (31) and the substitution of the corresponding glycine by aspartate in yeast leads to a total defect of the \( bc1 \) complex activity at 28°C (32). The \( G339E \) mutation is responsible for a human myopathy and the same mutation totally abolishes the \( bc1 \) complex assembly in yeast at 28°C (33, 343). Thus, \( G252D \) is the first cytochrome \( b \) mutation corresponding to a human pathology that leads to a thermosensitive \( bc1 \) activity. This stresses the interest of testing different growth temperatures when using yeast as a model to study mutations implicated in human pathology.

The glycine 252 of yeast cytochrome \( b \) is not only conserved in mammals and plants but is also present in several bacteria such as *Rhodobacter capsulatus*. The \( G252D \) mutation
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leads to the substitution of a small amino acid by a large and negatively-charged one. Thus, it is reasonable to postulate that the respiratory defect is due to steric and/or electrostatic conflicts within cytochrome $b$ or between cytochrome $b$ and another subunit. The glycine 252 is located in the E-ef loop at the positive side of the membrane, close to the ubiquinol oxidation site, Qo according to the crystal structure (5). It was previously proposed (17) that the presence of D instead of G in human cytochrome $b$ should cause charge repulsion with the glutamate 271 of the Qo site (E272 in yeast) and would impair hydroquinone binding. The fact that both the mutations D252N and D252Y, in an otherwise wild type background, totally restore the respiratory function at 36°C suggests that the defect observed in the G252D mutant is rather due to the negative charge brought by the aspartate residue rather than the increased size. If this charge inhibits the Qo site function, cytochrome $b$ can be only reduced at the Qi site. However, the cytochrome spectra of the ∆qcr9 G252D double mutant recorded with antimycin that blocks the Qi site, revealed the presence of reduced cytochrome $b$ (data not shown). This suggests that the cytochrome $b$ hemes can be reduced by ubiquinol at the Qo site in the G252D mutant.

Our results suggest the existence of an interaction between cytochrome $b$ residue 252 and Qcr9p, the mutation G252D leading to a decrease in the steady-state level of Qcr9p. However, according to (5), the residue 252 is far from Qcr9p and cannot directly interact with Qcr9p while cytochrome $c1$ is close to the residue 252 (Figure 6). Thus, it is tempting to propose that the interaction between the residue 252 and Qcr9p would be a long range interaction via cytochrome $c1$. Since, the closest amino acid in cytochrome $c1$ is the lysine 182, the G252D mutation could create an illegitimate electrostatic interaction with this positively-charged amino acid. This illegitimate interaction would affect cytochrome $c1$ conformation and partially destabilize Qcr9p or affect the insertion of Qcr9p within the membrane. This would lead to a decrease of the bc1 complex activity at high temperature, as in the ∆qcr9 mutant. Previous studies have shown that Qcr9p interacts with Rieske and cytochrome $c1$ (5, 9, 35),
therefore we propose that Qcr9p could enhance or stabilize the interactions between these two catalytic subunits. It was previously shown that in the total absence of Qcr9p, the conformation of Rieske and its iron-sulfur cluster insertion is altered (9). Thus, in the $\Delta qcr9 G252D$ double mutant, the conformation of both cytochrome $c1$ and Rieske would be affected and this would completely prevent the electron transfer between Rieske and cytochrome $c1$ even at 28°C. This hypothesis is consistent with our results showing a block in electron transfer between cytochrome $b$ and cytochrome $c1$. However, a direct evidence of this hypothetical illegitimate interaction could be provided by introducing a compensatory mutation in the partner residue, e.g. the proposed candidate lysine 182 of cytochrome $c1$.

The suppressor mutations able to restore the respiratory growth of the $\Delta qcr9 G252D$ double mutant mapped either at codon 252 or at codon 174 of the cytochrome $b$ gene. All partially compensate for the respiratory defect at 28°C but not at 36°C, suggesting that they restore the electron transfer between cytochrome $b$ and cytochrome $c1$ but do not compensate for the absence of Qcr9p. This suggests that the cytochrome $b$ substitutions, that we have isolated, cannot strengthen the interactions between Rieske and cytochrome $c1$ at high temperature and thus cannot functionally substitute for Qcr9p. While suppressor mutations at residue 252 could lower the illegitimate interactions occurring at this position in the original mutant, the suppression mechanism by proline 174 ought to be different. Both the glycine 252 and the proline 174 are located in the inter-membrane space but are quite distant (Figure 6), the proline 174 is at the vicinity of the tether domain of Rieske, that is necessary for the Rieske movement and the electron transfer between the iron-sulfur cluster and the heme of cytochrome $c1$ (2, 36, 37). Substituting a proline by a threonine in this domain could improve the interaction between Rieske and cytochrome $c1$ that is deficient in the $\Delta qcr9 G252D$ double mutant, and allow partial electron transfer at 28°C.
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Altogether, these results show that the residue 252 plays an important role in the biogenesis of the $bc_1$ complex at 36°C and is probably involved in long range interactions within the $bc_1$ complex and between the supernumerary subunit Qcr9p and the two other catalytic subunits, cytochrome $c_1$ and Rieske. It has recently been shown that protein-phospholipid interactions also play a role in the structure of the $bc_1$ complex (38) and it is well known that phospholipids are highly sensitive to temperature. Thus, elucidating the relative roles of non-catalytic subunits and phospholipids constitutes the next challenge to fully understand respiratory complex assembly at high temperature.

Acknowledgments

We are particularly grateful to N. Lachacinsky for technical assistance. We thank Drs C. Godinot, A. Lombes and J.M. Galan for the gift of antisera. We also thank C. Lemaire, O. Groudinsky, C.J. Herbert and P. Hamel for the critical reading of the manuscript. This work was supported by a grant from the Association Française contre les Myopathies.

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Legends to figures and tables

**Figure 1: Sequence comparison of the E-ef loop of cytochrome b from various organisms.**

The E-ef loop represents the N terminal part of the inter-membrane loop located between the fifth (E) and sixth (F) trans-membrane segments of cytochrome b (5). E-ef loops from *S. cerevisiae* and various other organisms were aligned using the ClustalW program (39). *S. cerevisiae*: Saccharomyces cerevisiae; *R. capsulatus*: Rhodobacter capsulatus; *G. galus*: Galus galus; *B. taurus*: Bos Taurus; *N. crassa*: Neurospora crassa; *A. thaliana*: Arabidopsis thaliana; *D. melanogaster*: Drosophila melanogaster; *H. sapiens*: Homo sapiens. The upper line represents the *S. cerevisiae* reference sequence (28), the second line the mutant sequence present in the intron-less mitochondrial genome. The residue 252 is in a black background. The bold characters represent the strictly conserved residues in all the sequences. The conserved residues PEWY are involved in the quinone deprotonation (1).

**Figure 2: Effect of cytochrome b mutations on the respiratory growth.**

1: CW252; 2: CW30; 3: YST2; 4: YST1; 5: QR1; 6: QR2; 7: QR3. All strain constructions were described in Materials and Methods. Serial dilutions were made and spotted onto glucose (G) or glycerol/ethanol (E) plates and incubated for 5 days at 28°C or 36°C.
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$+$ : $QCR9$ wild type allele ; $\Delta$ : $qcr9::URA3$ allele

**Figure 3 : Effect of the G252D mutation on respiratory complex assembly.**

1: CW252; 2: CW30; 3: YST2; 4: YST1. Panels A and C: Low temperature cytochrome absorption spectra of cells grown on galactose at 28°C were recorded with (A) or without (C) dithionite. The absorption maxima for the alpha bands of cytochromes $c$, $c1$, $b$ and $aa$, are 546 nm, 552 nm 558nm and 602 nm respectively. Strain genotypes are indicated in panel B. The arrow represents the wavelength of the $\cdot$ band of cytochrome $c1$. Strain genotypes are indicated in panel B. Panel B: 60 micrograms of mitochondrial proteins from cells grown at 28°C were separated on a 12 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The blot was probed with antibodies raised against apocytochrome $c1$, Rieske or porin. Rieske amount was quantified using porin as an internal control. There is a 15% decrease in the $G252D$ mutant (lane 2), a 60% decrease in the $\Delta qcr9 G252$ (lane 3) and a 70% decrease in the $\Delta qcr9p G252D$ (lane 4) as compared to $QCR9 G252$ wild type strain (lane 1).

**Figure 4 : Effects of the G252D mutation on the Qcr9p accumulation**

1: CW252; 2: CW30; 1’: YST7; 2’: YST6. Western blotting was performed as in Figure 3 except that 30 micrograms of mitochondrial proteins from cells grown at 34°C were loaded on the gel and probed with antibodies raised against either the c-myc epitope or porin.

**Figure 5 : Respiratory complex assembly in the revertant strains**

1: CW252; 5: QR1; 7: QR3; 8: WQR1; 9: WQR3. Panel A: Low temperature cytochrome absorption spectra of cells grown at 28°C recorded with dithionite. Strain genotypes are given in panel B and the legend is as in Figure 4B. Panel B: Western blot analysis with antibodies raised against apocytochrome $c1$, Rieske or porin.
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**Figure 6 : Schematic representation of the three catalytic subunits and Qcr9p within the bc1 complex.**

Qcr9p, cytochrome $c1$ (Cyt1p), cytochrome $b$ (Cytbp) and Rieske of an active monomeric $bc1$ complex are schematically represented according to the structure of the yeast $bc1$ complex (5). Grey circles represent the glycine 252 (G252), the glutamate 272 (E272) of the Q$_0$ site and proline 174 (P174) of cytochrome $b$ as well as the lysine 182 (K182) of cytochrome $c1$.

**Table I : Effect of the G252D mutation on enzymatic activities.**

1 : CW252; 2 : CW30. Enzymatic activities were measured at 28°C and 36°C on mitochondria purified from cells grown on galactose medium at 34°C because mitochondria were difficult to prepare at 36°C even for wild type cells. The enzymatic activities are expressed as a percentage of the corresponding wild type activity (CW252). Two independent experiments were performed. The antimycin sensitive, NADH cytochrome $c$ oxido-reductase (NADH-cyt $c$) activity of CW252 was 2253 ± 342 nmoles of reduced cytochrome $c$/minute/mg at 28°C and 2216 ± 82 nmoles of reduced cytochrome $c$/minute/mg of protein at 36°C. The ubiquinol cytochrome $c$ oxido-reductase ($bc1$) activity of CW252 was 1895 ± 239 nmoles of reduced cytochrome $c$/minute/mg at 28°C and 937 ± 36 nmoles of reduced cytochrome $c$/minute/mg of protein at 36°C. The cytochrome $c$ oxidase activity (Cox) of CW252 was 1887 ± 158 nmoles of reduced cytochrome $c$/minute/mg at 28°C and 2263 ± 70 nmoles of reduced cytochrome $c$/minute/mg of protein at 36°C.

**Table II: Generation time and bc1 complex activity in the Δqcr9 G252D double mutant and revertant strains.**

1: CW252; 3: YST2; 4: YST1; 5: QR1; 6: QR2; 7: QR3; 8: WQR1; 9: WQR3 . To determine the doubling time, cells were grown in non-fermentable medium at 28°C. For the NADH-cyt$c$ oxido-reductase activity measurement, mitochondria were purified from cells grown on...
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galactose medium at 28°C. The activities measured at 28°C are expressed as a percentage of the wild type strain activity (CW252): 1958 ± 272 nmoles of reduced cytochrome $c$/minute/mg.
Interaction between cytochrome $b$ and Qcr9p.

**Table I**

| Strain | Genotype     | NADH-cytc 28°C | NADH-cytc 36°C | bcl 28°C | bcl 36°C | Cox 28°C | Cox 36°C |
|--------|--------------|----------------|----------------|----------|----------|----------|----------|
| 1      | CYTB-G252    | 100            | 100            | 100      | 100      | 100      | 100      |
| 2      | CYTB-G252D   | 51             | 57             | 49       | 57       | 95       | 92       |

**Table II**

| Strain | Genotype     | Doubling time | NADH-cytc (%) |
|--------|--------------|---------------|---------------|
| 1      | QCR9 CYTB-G252 | 2h00          | 100           |
| 3      | ∆qcr9 CYTB-G252 | 4h30          | 67            |
| 4      | ∆qcr9 CYTB-G252D | -            | 0             |
| 5      | ∆qcr9 CYTB-D252N | 5h00          | 63            |
| 6      | ∆qcr9 CYTB-D252Y | 5h30          | 39            |
| 7      | ∆qcr9 CYTB-G252D,-P174T | 6h00          | 21            |
| 8      | QCR9 CYTB-D252N | 2h00          | 108           |
| 9      | QCR9 CYTB-G252D,-P174T | 3h20          | 100           |
| Organism               | Sequence                      |
|-----------------------|-------------------------------|
| *S. cerevisiae G252*  | PNTLGHPDNYIPGNPLVTAPASIVPEWY |
| *S. cerevisiae G252D* | PNTLDHPDNYPGNPLVTAPASIVPEWY |
| *R. capsulatus*       | PNYLGHPDNYVQANPLSTPAHIVPEWY  |
| *G. galus*            | PNLLGDPENFTPAANPLVTPPHIKPEWY |
| *B. Taurus*           | PDLLGDPDNYTPANPLNTPPHIKPEWY  |
| *N. crassa*           | PNVLGDSENYIMANPMQTTPAIVPEWY  |
| *A. thaliana*         | PNVLGHPDNYIAPANPMSSTPHIVPEWY |
| *D. melanogaster*     | PNLLGDPDNFIAPANPLVTPAHIQPEWY |
| *H. sapiens*          | PDLLGDPDNYTLANPLNTPPHIKPEWY  |
| Strain | QCR9 | CYTB   | G 28°C | E 28°C | E 36°C |
|--------|------|--------|--------|--------|--------|
| 1      | +    | G252   | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 2      | +    | G252D  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 3      | Δ    | G252   | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 4      | Δ    | G252D  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 5      | Δ    | D252N  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 6      | Δ    | D252Y  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
| 7      | Δ    | P174T  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
|        |      | G252D  | ![Image](G28C.png) | ![Image](E28C.png) | ![Image](E36C.png) |
A pathogenic cytochrome b mutation reveals new interactions between subunits of the mitochondrial bc1 complex
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J. Biol. Chem. published online October 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207219200

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