Human Disease-related Mutations in Cytochrome b Studied in Yeast

Several mutations in the mitochondrially encoded cytochrome b have been reported in patients. To characterize their effect, we introduced six "human" mutations, namely G238S, S152P, G252D, Y279C, G291D, and Δ252–259 in the highly similar yeast cytochrome b. G252D showed wild type behavior in standard conditions. However, Asp-252 may interfere with structural lipid and, in consequence, destabilize the enzyme assembly, which could explain the pathogenicity of the mutation. The mutations G33S, S152P, G291D, and Δ252–259 were clearly pathogenic. They caused a severe decrease of the respiratory function and altered the assembly of the iron-sulfur protein in the bc1 complex, as observed by immunodetection. Suppressor mutations that partially restored the respiratory function impaired by S152P or G291D were found in or close to the hinge region of the iron-sulfur protein, suggesting that this region may play a role in the stable binding of the subunit to the bc1 complex. Y279C caused a significant decrease of the bc1 function and perturbed the quinol binding. The EPR spectra showed an altered signal, indicative of a lower occupancy of the Qo site. The effect of human mutation of residue 279 was confirmed by an- other change, Y279A, which had a more severe effect on Qo site properties. Thus by using yeast as a model system, we identified the molecular basis of the respiratory defect caused by the disease mutations in cytochrome b.

The mitochondrial bc1 complex is a membrane-bound enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome c and couples this electron transfer to vectorial proton translocation across the inner mitochondrial membrane. The enzyme exists as a functional dimer, consisting of 10 or 11 polypeptides in the eukaryotic monomeric subunit. One subunit, cytochrome b, is encoded by the mitochondrial genome, whereas the others are nuclearily encoded. Redox prosthetic groups are located within three subunits: cytochrome c1 and the iron-sulfur protein (ISP), which are membrane proteins with large, hydrophilic domains, and cytochrome b, a predominantly hydrophobic protein consisting of eight transmembrane helices that contains two b-type hemes (b1 and b2) and forms the two quinol binding sites: Qo (site of quinol oxidation) and Qi (site of quinol reduction), located on opposite sides of the membrane. The catalytic mechanism of the bc1 complex is essentially described by Mitchell's Q-cycle model. A quinol molecule binds at the Qo-site, is deprotonated, and transfers one electron through the "high potential" electron transfer chain consisting of the [2Fe-2S] cluster of the ISP and the c-type heme of cytochrome c, to the soluble acceptor, cytochrome c. Following a bifurcated pathway, a second electron is transferred across the membrane by the "low potential" pathway formed from hemes b1 and b2 and delivered to quinone bound at the Qo-site, forming a stable semiquinone.

A number of mutations in the human cytochrome b have been linked with diseases. Nonsense or frameshift mutations that result in truncated cytochrome b almost invariably abolish complex assembly. The precise effect of the missense mutations is more difficult to predict, and their characterization is often hampered by the limited amount of tissue available. In this work, we use yeast mutants as models to characterize the deleterious effect of six mutations reported in patients, namely G33S, S152P, G252D, Y279C, G291D, and a short in-frame deletion of eight residues, Δ252–259 (yeast numbering) (Fig. 1).

Two other mutations of residue Tyr-279, Y279A and -W, were also analyzed. Yeast (Saccharomyces cerevisiae) and human cytochrome b share a very high degree of sequence similarity (55%), allowing the study of disease-related mutations in a convenient model system. G33S has been found in a patient with exercise intolerance (1). Gly-33 is located within transmembrane helix A, a hydrophilic environment at the Qo site and close to heme b2. S152P has also been reported in a patient with exercise intolerance (2). Ser-152 is located in a loop connecting helices cd1 and cd2 at the entry of the Qo binding pocket. The mutation G252D and the in-frame deletion of eight amino acids (residues 252–259) have been detected in patients suffering from cardiomyopathy (3) and exercise intolerance (1), respectively. These residues are located in the E-eF loop on the P side of the membrane. The mutation Y279C has been found in a patient with severe exercise intolerance and multisystem disorder (4). It has been also reported in atovaquone-resistant isolates of the parasite Plasmodium yoelii (5). Tyr-279 is close to the highly conserved "PEWY" motif region at the N-terminal region of the ef helix and 3.5 Å from residue 181, a ligand of the ISP (2Fe-2S) cluster. The mutation G291D is also potential cytochrome b heme; EPR, electron paramagnetic resonance; WT, wild type.

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Nicholas Fisher‡, C. Kate Castleden‡, Ingrid Bourges‡, Gael Brasseur‡, Genevieve Dujardin‡, and Brigitte Meunier‡

From the ¶Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, United Kingdom, the Laboratoire de Bioenergetique et Ingenierie des Proteines, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France, and the ¶Centre de Gene´tique Mole´culaire, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

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transmembrane helix F1, in close vicinity of the Q$_o$ site. These human mutations were introduced into yeast cytochrome $b$, and their effects on the assembly and activity of the mutant $b_{c1}$ complexes were studied. The mutations G33S, S152P, H900252–259, and G291D were highly deleterious. Y279C had a less severe effect on the respiratory function. G252D has no effect in standard conditions. Respiratory competent clones were selected from mutants S152P and G291D. Compensatory mutations were identified in cytochrome $b$ and in the hinge region of the ISP.

**EXPERIMENTAL PROCEDURES**

**Media and Chemicals**—The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone, 3% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and transformation medium (0.7% yeast nitrogen base, 3% glucose, 2% agar, 1 M sorbitol, and 0.8 g/liter of a complete supplement mixture minus uracil, supplied by Anachem). Decylubiquinone and myxothiazol were purchased from Sigma. Stigmatellin was purchased from Fluka.

**Generation of the Mutant Strains**—The plasmid pBM5 carrying the wild type intronless sequence of the CYTB gene has been constructed by blunt end cloning of a PCR product of CYTB into the pCRscript vector (Stratagene). The mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. After verification of the sequence, the plasmids carrying the mutated genes were used for biolistic transformation. The mitochondrial transformation by microprojectile bombardment was adapted from Ref. 7 and described in Refs. 8 and 9.

**Isolation and Genetic Analysis of the Revertants**—Diploid strains respiratory growth deficient generated by biolistic transformation were used to select revertants. The mutants were subcloned. Several subclones were grown in YPD, and then incubated on respiratory medium (YPG). Respiratory competent clones appeared after a 1- or 2-week incubation. Four to 15 independent revertants (each from different subclones) were then sporulated. Respiratory competent haploid clones were analyzed as described in Ref. 10 to determine the mitochondrial or nuclear heredity of the reversion mutation. For the mitochondrial reversion, the cytochrome $b$ gene of the revertant was sequenced to identify the secondary change. The nuclear reversions were identified by directly sequencing the most probable candidate gene, which was the nuclearly encoded gene RIP, coding for the ISP. The cytochrome $b$ gene

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**Fig. 1. Location of the mutations in the bc$_1$ structure.** The figure was prepared using the coordinates of the yeast enzyme (Protein Data Bank accession code 1KYO) with VMD (24, 25). The cytochrome $b$ polypeptide backbone is represented in orange, the ISP in cyan. Cytochrome $b$ residues involved in disease-associated point mutations are shown in green, and the location of the Δ252–259 deletion is indicated in white. The location of the compensatory mutations in the ISP and cytochrome $b$ are shown in yellow and purple, respectively. Stigmatellin bound at the Q$_o$ site is shown in pink, and the position of residue Ala-144 in blue.
was also sequenced to confirm the presence of the primary mutation. For characterization on the effect of nuclear reversions in ISP, strains combining the mutations in ISP with either the WT or mutant cytochrome b were constructed by cotransduction (11), by transferring the mitochondridal genome from a donor strain into the rho' derivative of the recipient strain harboring the ISP mutation.

Preparation of Decylubiquinol—10 mg of 2,3-dimethoxy-5-methyl-n-decyl-1,4-benzoquinone (decylubiquinone), an analogue of ubiquinone (Sigma), was dissolved in 400 μl of nitrogen-saturated hexane. An equal volume of aqueous 1.15 M sodium dithionite was added, and the mixture was shaken vigorously until colorless. The upper, organic phase was collected, and the decylubiquinol recovered by evaporating off the hexane under nitrogen. The decylubiquinol was dissolved in 100 μl of 96% EtOH (acidified with 10 μl of HCl) and stored in aliquots at −80 °C. Decylubiquinol concentration was determined spectrophotometrically from absolute spectra, using ε_{280-292} = 4.44 mm M cm⁻¹.

Preparation of Crude Mitochondrial Membranes and Measurement of Cytochrome c Reductase Activity—Wild type and mutant yeast strains were grown to stationary phase (48 h) in 200 ml of YPD cultures at 28 °C. The cells (∼2 g wet weight per culture) were harvested by centrifugation at 4000 × g for 10 min. Cell pellets were washed by resuspension in 40 ml of 50 mM potassium phosphate, 2 mM EDTA (pH 7.5) and centrifuged as before. The harvested cells were resuspended in 10 ml of 50 mM potassium phosphate, 2 mM EDTA (pH 7.5) supplemented with 0.2 mM phenylmethylsulfonyl fluoride and 0.08% (w/v) bovine serum albumin prior to disruption in a Retsch MM300 glass bead mill operating at 30 Hz for 10 min at 4 °C. Membranes were separated from cell debris by centrifugation at 10,000 × g for 20 min. The supernatant was centrifuged at 100,000 × g for 90 min and the pellet membranes were suspended in a minimal volume of 50 mM potassium phosphate (pH 7.5), 2 mM EDTA containing 10% (v/v) glycerol. Resuspended membranes (2 μM bc1) were stored in 100-μl aliquots at −80 °C.

Cytochrome c reductase activity measurements were assayed in 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 10 mM KCl, 0.025% (w/v) lauryl maltoside, and 30 μM equine cytochrome c at room temperature. Membranes were diluted to 2.5 μM cytochrome bc complex (determined from the dithionite reduced minus ferricyanide-oxidized difference spectra, using ε_{280-292} = 25.5 mM M cm⁻¹ (12). Cytochrome c reductase activity was initiated by the addition of decylubiquinol (5–100 μM). Reduction of cytochrome c was monitored in a Cary 4000 spectrophotometer at 550 versus 542 nm. Initial rates (computer-fitted as zero-order kinetics) were measured as a function of decylubiquinol concentration, and V_m and K_m values were derived from Eadie-Hofstee (v versus v/S) plots. All rate measurements were performed in triplicate.

Spectroscopic Analysis of Cytochromes in Whole Cells—Spectra were generated by scanning of cell suspensions with a single beam spectrophotometer built in-house operating at room and cryogenic temperatures (77 K). The cells, grown on YPD plates for 48 h, were re-suspended at a concentration of around 200 mg (room temperature spectra) or around 400 mg (77 K spectra) of cells/ml and reduced by dithionite. Quadratic baseline compensation was carried out on the data as described (13) to remove the distortion of the baseline. Spectral resolution was 0.2 nm.

Western Blotting Analysis—Immunodetection analyses were performed on crude mitochondrial membranes. Loading solution of 62.5 mM Tris-HCl (pH 6.8), 0.01% (w/v) bromphenol blue, 25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol was added to each sample (1.2, v/v). The samples were heated for 5 min at 95 °C. The analyses were performed as described in Ref. 10. The mitochondrial membrane preparations (40 mg of total protein/sample) were electrophoresed on SDS-polyacrylamide gels (4–20% linear gradient polyacrylamide gel) prior to transfer to polyvinylidene difluoride membrane by semi-dry electroblotting. “Precision Plus Protein Dual Color” standards (Bio-Rad) (10–250 μg) were used for sizing. The polyclonal antisera against subunits Cox V1a and Cox VI were kindly provided by Dr. J. W. Taa- mazian of Dartmouth Medical School. Longs Peak Biotechnology of Longmont, CO provided antisera against cytochrome C2, iron-sulfur protein, and QCR7p were generously provided by Prof. B. L. Trumpower (Dartmouth Medical School, Hanover, NH).

**EPR Analysis**—The analysis was performed as described in Ref. 14.

### Results

#### Generation of the Yeast Mutants and Respiratory Growth Competence—The yeast mutants harboring the mutations in the mitochondrially encoded cytochrome b gene were generated by the biologic methods as described in Refs. 8–10. It is interesting to note that all the strains constructed were homoplasmic and contained only one population of mitochondrial DNA. The mutants G33S, S152P, G291D, and A252–259 were not able to grow on respiratory medium. Y279C had a less severe impairment of the respiratory growth. The growth rate (doubling time) in respiratory medium (YPG) at 28 °C was ∼12 h, compared with 4 h for the wild type (WT) cells. G252D showed no respiratory growth deficiency, as observed previously (10). The respiratory growth rate was 4 h, identical to WT.

**Effect of the Mutations on Cytochrome b and ISP Content**—The effects of the mutations on the level of cytochrome b in whole cells were monitored (Table I). The cytochrome b content (based on the dithionite reduced spectra in the visible region, as exemplified in Fig. 2) was decreased by 25 to 50% of the wild type level, whereas the cytochrome c content was increased as observed previously for other respiratory deficient mutants (15). G252D showed the WT level of cytochrome b, as expected. It is interesting to note that in the respiratory deficient mutants, as shown for A252–259 in Fig. 2, the aerobic spectrum (dotted line) showed a peak at 562 nm, corresponding to reduced cytochrome b, presumably b_65, and a small peak at 575 nm, corresponding to oxylflavohemoprotein (16). Upon addition of dithionite (solid line), cytochromes c, c_1, and oxidase (aa_3) were reduced and the signal of oxylflavohemoprotein disappeared. This behavior was observed for all the mutants studied here, except for G252D. WT and G252D exhibited fast O_2 consumption. The cell suspensions became anaerobic immediately, and the cytochromes became fully reduced.

To analyze further the content in bc_1 complex subunits, mitochondrial membranes were prepared and the level of ISP, cytochrome c_1, and QCR7p monitored by Western blotting as described under “Experimental Procedures.” As shown in Fig. 3, a severe decrease in ISP content was observed for mutants G33S, S152P, A252–259, Y279W, and G291D, whereas Y279C and -A contained the WT level of this subunit. The amount of QCR7p was also decreased in these mutants, but to a lesser degree, whereas the cytochrome c_1 content was not affected. Mutant S152P seemed the most affected as ISP was only detected after a longer exposure. The decreased level of the ISP in the G33S mutant was unexpected because this residue is located at the Q, site, far from the domains of contact between cytochrome b and the ISP. However, 77 K spectra of whole cell suspensions (Fig. 4) showed that the peak position of cytochrome b was red-shifted from 561 to 562.5 nm. This is sug-
gestive of distortion of the local environment around heme $b_h$. Total loss of heme $b_h$ in the G33S mutant could be excluded as the $bc_1$ complex retained a low level of catalytic activity (Table I). The resulting alteration in the structure of the cytochrome $b$ polypeptide is apparently responsible for the destabilization and loss of the ISP from the complex.

Effect of the Mutations on the $bc_1$ Activity—The cytochrome $c$ reductase activity was monitored spectrophotometrically as a function of decylubiquinol (QH$_2$) concentration, as described under “Experimental Procedures.” The apparent $V_m$ and $K_m$ for QH$_2$ were calculated from initial rate measurements using derived Eadie-Hofstee plots (Table I). No $bc_1$ activity could be detected in mitochondrial membranes prepared from mutants Δ252–259 and Y279W. It seemed likely therefore that the small population of ISP-containing enzyme observed in Fig. 3 was non-functional. G33S, S152P, and G291D showed a severely decreased catalytic activity: $V_m$ 10, 5, and 9 s$^{-1}$, respectively, compared with 80 s$^{-1}$ for the WT. The decreased level of ISP in the mutants could account for the low turnover rate observed. Because the mutated residues are located in the catalytic domain of the enzyme, it is likely that they would also hinder the functioning of a fully assembled complex. Y279A and -C exhibited a different behavior because the enzyme was assembled at near WT level, as judged by the amount of ISP. However, the catalytic activity of these two mutants was diminished compared with WT ($V_m$ values: 21 and 59% of the WT rates for Y279A and -C, respectively, under the assay conditions). The mutation Y279C increased the $K_m$ for quinol from 18 to 23 μM, which is suggestive of a less efficient binding interaction at Qo. The mutation Y279A decreased the $K_m$ for quinol 2-fold, indicating that the Qo site was becoming saturated at lower concentrations of quinol than the WT, and may also be indicative of a decreased quinol “on” rate. It should be noted that the $k_{min}$ (an apparent second-order rate constant equal to $V_m/K_m$) values for the Y279A and -C mutants are very similar at 1.89 and 2.04 μM$^{-1}$ s$^{-1}$ (cf. 4.44 μM$^{-1}$ s$^{-1}$ for the WT). EPR analyses were performed to obtain more information on quinol binding in these two mutants.

EPR Analysis of Y279C and -A—Residue Tyr-279 is located near the docking site of the ISP [2Fe-2S] center and is likely to be involved in the stabilization and/or positioning of the quinol in the active site (17). It was therefore interesting to monitor the Qo site occupancy by EPR using the characteristic interaction between the [2Fe-2S] cluster of the ISP and the quinone/quinol binding at the Qo site. It is well known that a $g_y$ signal centered at $g = 1.80$ is observed when the Q pool is fully oxidized and that this signal shifts to a lower $g_y$ value of around 1.77 when the Q pool is fully reduced (18, 19). When the Q site is empty, because of either a mutation or extraction of quinones, the value of the $g_y$ signal shifts to a lower value of around 1.76. The EPR spectrum of the WT sample reduced with ascorbate showed a $g_y$ peak at $g = 1.90$ and a $g_x$ trough centered at $g = 1.80$, which is characteristic of a Qo site fully occupied with...
oxidized quinone (Fig. 5A). In Y279A and -C, the total level of [2Fe-2S] cluster as indicated by the $g_x$ signal was identical to that of the WT. However, the $g_y$ trough was less pronounced and shifted to a lower value of $g_x = 1.76-1.77$, thus indicating a partially empty Qo site. This result is in agreement with the lower catalytic efficiency for quinol ($k_{\text{cat}}$) observed in the mutants. Addition of stigmatellin fixes the ISP [2Fe-2S] cluster in a position close to cytochrome $b$ (Fig. 5C). The mutants were still able to bind the inhibitor as shown by the $g_y$ trough. However, Y279C exhibited a shifted $g_x$ signal ($g_y = 1.80$) in comparison with that of the WT ($g_y = 1.775$). This indicates a modified binding of stigmatellin within the Qo site or an altered interaction between ISP and stigmatellin.

**Reversion Analysis of Mutations**—From the data obtained here, it appeared that several mutations in the Qo domain affected the assembly of the ISP. We addressed then the question whether other modifications could restore the assembly of the ISP and the enzyme activity. That may highlight domains of the enzyme important for the assembly of the ISP and its interaction with cytochrome $b$. To this end, revertants (respiratory growth competent clones) were selected on respiratory medium from the respiratory deficient mutants S152P and G291D. The revertants were analyzed as described under “Experimental Procedures.” The reversion mutations were identified by sequencing. For S152P, two compensatory mutations were observed: a mutation at the same codon in cytochrome $b$ restoring Ser-152; and a mutation in the ISP, A90D, which was found in three independent revertants. For G291D, several secondary mutations were found: two mutations in cytochrome $b$, D287H located in the vicinity of the primary mutation, and H53D, more than 20 Å from the primary site; five changes in the ISP, V88A/G/D, A90T/D, located in the hinge region of this subunit (Fig. 1). A90D was found in 6 independent revertants from G291D, whereas the other reversions were observed only in one or two revertants. The change A90D was thus the most frequent reversion and suppressed both S152P and G291D mutations. The non-native reversions compensate partially the respiratory defect induced by the primary mutations. Their doubling time was between 10 and 12 h (4 h in WT). Further analyses were performed on the compensatory mutation A90D in the ISP. Membranes were prepared from three strains that combined the mutation in ISP A90D with the WT cytochrome $b$, the mutation S152P, or the mutation G291D. The strains were constructed as described under “Experimental Procedures.”

The QH$_2$-cytochrome $c$ reductase activities were assayed as described under “Experimental Procedures.” Introduction of the ISP A90D mutation into the WT cytochrome $b$ background had no effect on the $bc_1$ complex activity (not shown). Thus despite its high conservation in sequences from various organisms, residue Ala-90 can be replaced by a larger and charged residue without loss of function. Introduction of the ISP A90D mutation into the S152P and G291D cytochrome $b$ mutants resulted in enzymatic activity 2.5- and 3.3-fold higher than the rate observed for the cytochrome $b$ mutant background alone (assayed at 70 μM QH$_2$). This was sufficient to support a slow respiratory rate. Fig. 6 shows the steady-state level of ISP in the strains combining ISP A90D with WT cytochrome $b$ (lane 1), S152P (lane 2), or G291D (lane 3). The level of ISP in the revertants was similar to that of the control strain combining ISP A90D with the WT cytochrome $b$. It appears therefore that the change in A90D stabilizes the binding of ISP to the $bc_1$ complex. The low activity of the S152P and G291D revertants suggests that the cytochrome $b$ mutations alone cause a severe disruption of the Qo site activity in the ISP-containing complex.

Several mutations in the hinge region of the ISP have been previously observed as suppressors of the cytochrome $b$ mutation A144F. The mutation A144F affects the binding of quinol, presumably by altering the local structure of the Qo site. The reported suppressor mutations were likely to correct the positioning of the ISP head group on the mutant cytochrome $b$. It was therefore interesting to see whether these suppressor mutations could compensate the defect caused by S152P and G291D. To this end, we constructed double mutants that combined the ISP mutations with cytochrome $b$ mutations A144F, S152P, or G291D. The respiratory growth competence of the double mutants was then monitored. As shown in Fig. 7, the compensatory effect of the ISP mutations seems specific for cytochrome $b$ mutations. The ISP mutations at residues 85, 92, and 93 compensated A144F only, whereas mutations at residues 88, 89, and 90 corrected partially A144F and G291D. S152P was compensated by the ISP mutation A90D, and very

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Fig. 4. Low temperature (77 K) visible absorption spectra. 77 K visible absorption spectra of cell suspensions were obtained as described under “Experimental Procedures.” Spectral resolution was 0.2 nm. The spectra were normalized for the cytochrome oxidase signal at 606 nm.

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*B. Meunier and G. Brasseur, unpublished data.*
DISCUSSION

In this work, we used yeast mutants to characterize the deleterious effect of six mutations reported in patients, namely G33S, S152P, G252D, Y279C, G291D, and a short in-frame deletion of eight residues, H9262–H9269 (yeast notation).

The stable integration of the ISP into the bc1 complex was defective in mutants G33S, S152P, Δ252–259, and G291D as observed by immunodetection. Δ252–259 and G33S are located far from the region of contact with ISP. An indirect long distance effect needs to be invoked to explain the loss of the ISP. The deletion of eight amino acids (Δ252–259) may reasonably be expected to severely disrupt the assembly of the complex. The mutant bc1 complex was still partially assembled, as judged by the optical signal for cytochrome b, but the level of ISP was dramatically decreased. This mutation is located in a surface loop connecting helices E and ef on the P side of the membrane, and is not directly in contact with the ISP. However, the alteration of the folding of cytochrome b caused by the shortening of this loop is likely to disrupt the stable binding of the ISP to the bc1 complex. The small population of ISP-containing enzyme was inactive. This could be explained by the major alteration of the catalytic site caused by the deletion. Surprisingly, the Q1 site mutation G33S altered the binding of the ISP. Two mutations of that residue have been characterized in yeast. G33D impairs the assembly of holo-cytochrome b, as judged by the loss of cytochrome b signal, whereas G33A restores the respiratory function (20). Gly-33 is found within a hydrophobic environment at the Q1 site, 4.0 Å from the porphyrin...
Introducing the bulkier and potentially anionic aspartyl side chain at this position would be sterically and thermodynamically unfavorable, explaining the failure of the complex to assemble in the G33D yeast mutant. The reversion G33A re-introduces a smaller group that would not disrupt side chain packing or interactions around the heme (21). The serine side chain of the human mutation G33S is non-ionic and less bulky than aspartate, but could still perturb the local environment of heme $b_h$. It was previously reported that a similar mutation, G131S, located near heme $b_i$, decreased the reduction of cytochrome $c_1$. This inhibited yeast growth or $b_c$ activity in crude membrane preparations under standard conditions. It has been reported that the yeast G252D mutant was thermostensitive, as the mutant $b_c$ complex was found to be partly inhibited when the cells were grown at higher temperature (36 °C) (10). The mutation when combined with loss of the subunit Qcr9p severely decreased the complex activity. We have also noted that the isolated mutant enzyme was unstable, with activity lost on purification.2 Examination of the HHDBT-inhibited yeast $b_c$ crystal structure (Protein Data Bank code 1P84 (17)) reveals a tightly bound phospholipid molecule (probably phosphatidylcholine) at the P-side interface between cytochromes $b$ and $c_1$. The lipid head group is stabilized by polar interactions with the side chains of His-185 of cytochrome $c_1$, and Ser-268 of cytochrome $b$, with the fatty acyl chains in hydrophobic contact with Trp-273. An additional hydrogen bond is provided by the imidazole ring of His-253 (cytochrome $b$) to an acyl ester oxygen atom of the bound lipid. Mutation of Gly-252 to aspartate may weaken or disrupt this latter hydrogen bonding association, or otherwise distort the local fold at the lipid:protein interface, facilitating delipidation (and hence inactivation) during purification. The thermosensitivity of the G252D mutant may also be facilitated by an altered interaction with structural lipids. This may, in part, explain the pathogenicity of the G252D mutation in human tissue. In human cardiac muscle, the G252D mutation may alter the enzyme conformation and function in a similar manner to that observed in yeast grown at higher temperature or (but to a lesser extent) after delipidation. Additionally, as noted above, the mutation G252D leads to a decrease in the steady-state level of the Qcr9p subunit of the yeast $b_c$ complex (10). It was proposed that a long range interaction between Gly-252 and Qcr9p was mediated by residue Lys-182 of cytochrome $c_1$, and that the mutation G252D created an illegitimate electrostatic interaction with this lysyl side chain. It is interesting to note that Lys-182 is replaced by arginine in human cytochrome $c_1$, and thus it is plausible to suggest that this switch may increase the severity of the $b_c$ deficiency in the human instance.

The mutation Y279C affects the quinol binding. The position of ubiquinol at the $Q_o$ site has been suggested to be influenced by hydrogen bonding interactions with the side chain hydroxyl group of Tyr-279 (17). In Rhodobacter sphaeroides, mutation to phenylalanine has no effect on the activity of the complex, whereas mutations to leucine, glycine, or glu-

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3 E. Berry, personal communication.
tamine decreased the enzyme activity 3-, 40-, and 50-fold, respectively (23). In addition to the Y279C mutant, we also introduced two other mutations at position 279: Y279A and Y279W. The latter two mutations are not associated with disease in man, but were introduced to further investigate the sensitivity of this site to alteration. The replacement of Tyr-279 by tryptophan was very deleterious, abolishing the activity of the complex and causing the loss of the ISP (Fig. 3), presumably because of distortion of the structure at the Qo site. The mutations Y279A and -C had no major effect on the assembly of the enzyme but affected its catalytic properties. The introduction of a cysteine increased the $K_m$ for quinol, whereas the introduction of a tryptophan was very deleterious, abolishing the activity of the WT because of poor electron transfer, or may reflect a inability because of distortion of the structure at the Qo site. The complex and causing the loss of the ISP (Fig. 3), presumably because of the differences in structure between the double-ring stigmatellin binding, whereas stigmatellin binding appears to be altered in the Y279C mutant (Fig. 5). This is likely to be because of the differences in structure between the double-ring pharmacophore of stigmatellin, and the single ring head group structure of quinol, and the interaction of these groups with the residue at position 279.

In conclusion, using yeast as a model, we have determined the molecular basis of respiratory dysfunction caused by disease mutations in human cytochrome $b_6$. G33S, S152P, G291D, and Δ252–259 were highly deleterious and affected the assembly of the ISP to the $bc_1$ complex. Y279C, which exhibited a clear, albeit less severe impairment of the respiratory function, affected quinol binding. Further disease-associated mutations will be introduced into the yeast $bc_6$ complex and their mode of action studied as new clinical data become available.

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