Structure-Function Relationships of the Mitochondrial bc$_1$ Complex in Temperature-sensitive Mutants of the Cytochrome b Gene, Impaired in the Catalytic Center N*

(Received for publication, January 17, 1995, and in revised form, September 21, 1995)

Gael Brasseur†‡§, Jean-Yves Coppée‡, Anne-Marie Colsont‡**, and Paule Brivet-Chevillotte ‡‡

From the 1Laboratoire de Bioenergetique et Ingénierie des protéines, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France and the 1Laboratoire de Génétique Microbienne, Département de Biologie, Place Croix du Sud, 5, Université catholique de Louvain, B1348 Louvain-La-Neuve, Belgium

Seven new structures of cytochrome b have been recently identified by isolating and sequencing revertants from cytochrome b respiratory deficient mutants (Coppée, J. Y., Brasseur, G., Brivet-Chevillotte, P., and Colson, A. M. (1994) J. Biol. Chem. 269, 4221-4226). These mutations are located in the center N domain (QN).

All the revertants exhibited a modified heme $b_{562}$ maximum, confirming that part of the NH$_2$-terminal region is in the vicinity of the extramembranous loop between helices IV-V and heme $b_{562}$. Based on measurements performed on the maximal activities occurring in each segment of the respiratory chain, the decrease observed in the NADH oxidase activities of several revertants was correlated with some bc$_1$ complex activity impairments; this may also explain why a moderate decrease in bc$_1$ complex activity does not limit the succinate oxidase activity. The decrease in the rate of reduction of cytochrome b via the center N pathway is responsible for the impairment of the bc$_1$ complex activity of these revertants.

The three double-mutated revertants (S206L/N208K or -Y, S206L/W30C) are temperature-sensitive in vivo, and their mitochondria like that of the original mutant S206L are thermosensitive in vitro. Isolating the W30C mutation does not yield a thermosensitive phenotype: the replacement of serine 206 by leucine is therefore responsible for the thermoinstability of these strains; this temperature sensitivity is reinforced by additional mutations N208K or N208Y, and not by W30C. These data suggest that serine 206 and asparagine 208 are involved in the thermostability of the protein.

When bc$_1$ complex activity is lost after incubating mitochondria at a nonpermissive temperature (37 °C), heme b is still present, but can no longer be reduced by physiological substrate. The progressive loss of bc$_1$ complex activity seems to be initially linked to a change in the tertiary structure of cytochrome b, which occurs drastically at center N and much more slowly at center P, as shown by kinetic study on the two cytochrome b redox pathways.

The cytochrome bc$_1$ complex is an integral multisubunit membrane enzyme that spans either the inner mitochondrial membrane or the plasma membrane of bacteria. It transfers electrons from ubiquinol to cytochrome c, and this electron transfer is linked to proton translocation across the membrane. This mechanism is best described by the Q-cycle model introduced by Mitchell (1) and recently reviewed by Brandt and Trumpower (2). This mechanism requires two distinct quinone reaction sites, the hydroquinone oxidation ($Q_{	ext{h}}$) and the quinone reduction ($Q_{	ext{n}}$) centers, which are located on opposite sides of the membrane, and linked to cytochrome $b_{562}$ ($b_{1}$) and $b_{562}$ ($b_{2}$) heme, respectively. The bis-heme cytochrome b therefore plays a central role, since it is responsible for the electrogenic electron transfer through the membrane. An eight-transmembrane α helix folding model accounts for the topological organization and heme arrangement (3-5).

Upon comparing about 800 cytochrome b mitochondrial sequences, Degli Esposti et al., (6) found only 9 invariant amino acids, and this number was not affected by adding bacterial sequences. Upon examining the most highly conserved amino acids among 900 sequences, including the four histidine ligands to the hemes, Brandt and Trumpower (2) pointed out that 10 are located at center N and 14 at center P. These well conserved amino acids are natural targets for site-directed mutagenesis, which has been successfully carried out on bacteria (7-14; for review, see Ref. 15). In some cases however, inducing mutation of invariant amino acids did not impair the function (11, 12) and only a few of the evolutionary conserved residues chosen as targets seem to be essential for the functioning of the bc$_1$ complex.

In eucaryotes, cytochrome b is the only subunit of the bc$_1$ complex which is encoded by the mitochondrial genome, while all other subunits (nine in yeast) are of nuclear origin: this means that an eucaryotic organism is not the most suitable for performing site-directed mutagenesis on cytochrome b. This obstacle can be circumvented by selecting respiratory growth-deficient mutants of cytochrome b (16) and numerous intragenic revertants of these mutants (17-21), which has yielded many interesting phenotypes and led to identifying structurally and/or functionally implicated residues without any preconceived notions as to which amino acids will be involved.

Our previous paper (20) has described the isolation of non-native intragenic revertants selected from two cytochrome b respiratory deficient mutants located at the center N domain, namely mutant S206L and frameshift 204. Serine 206 is not an invariant residue, but replacing it by leucine would be lethal in the context of a strictly aerobic organism. These revertants have pseudo-wild type phenotypes, showing discrete but significant differences from the wild type, especially in the case of those which are thermosensitive (they
do not grow at 37 °C with respiratory substrate as carbon source). The aim of this study was to specify the role of the amino acids involved in these modifications and to determine the structure-function relationships of the cytochrome b center N domain, in particular as regards the bc1 complex activity and its thermal stability.

Materials and Methods

Strains—The wild type strain and the original mutants were from the Gif collection. Parental strain (wild type box1): the diploid KM91 was obtained by crossing 777-3A α adel op1 rho- mit+ with KL14-4A a his1 trp2 OP1 rho-. The original ρ- mit- respiratory deficient mutants ρ- mit- strains 777-3A/M1G1988 and 777-3A/M472 were isolated from the haploid strain 777-3A α adel op1 rho- mit+ (22, 23). These haploid strains were crossed with KL14-4A a his1 trp2 OP1 rho2 to give frameshift 204 and S206L, respectively. Their mutations were localized by deletion mapping in exon B4 (22), and their mtDNA sequence was determined (16).

The respiratory competent revertants were selected from the respiratory deficient mutants S206L and frameshift 204 (20) as described previously by de Rago et al. (17). The experiments were carried out with the haploid strains, which are all isogenic except for the cytochrome b mutations.

Media, Growth Conditions, Isolation of Mitochondria, and Phosphorylation Efficiency in Vivo—These were as described previously (24, 25).

Cells were grown with galactose medium (2% yeast extract, 1% bactopeptone, 2% galactose, 0.1% glucose, 1.2 g/liter of ammonium sulfate). Mitochondria were extracted from cells in the early stationary phase of growth. Yield and specific growth rate with ethanol medium were determined as described by Coppe et al. (20). In vitro RC and phosphate to oxygen ratios, as defined by Chance and Williams (26), were determined polarographically with a Gilson oxygraph. The following buffers were used: MRb buffer (0.05 M sorbitol, 10 mM KH2PO4, 2 mM EDTA, 0.1 mM MgCl2, 0.3% bovine serum albumin, pH 6.5) and phosphate buffer (50 mM potassium phosphate, 50 μM EDTA, pH 7.4).

Spectral Analysis—Low temperature spectra were recorded at liquid nitrogen temperature (77 K); heme b562 was obtained by difference spectra of succinate-reduced minus ascorbate-reduced mitochondria. Heme b562 was obtained by difference spectra of succinate-reduced mitochondria in the presence of antimycin (after anaerobiosis was reached) minus succinate-reduced mitochondria (16).

Activities of the Whole Respiratory Chain and of Its Various Segments—Respiratory activity measurements were carried out as described by Meunier-Lemesle et al. (24). DBH2-cytochrome c reductase activity was determined spectrophotometrically as described (27) except that a synthetic analog of ubiquinol, decylubiquinol (DBH2-100 μM), was used as electron donor. The activity of the segment I of the respiratory chain (NADH-decyubiquinone-reductase activity) was measured by NADH oxidation monitored at the 340/425-nm wavelength pair with DB as quinone electron acceptor. Complex II activity (succinate-decyubiquinone-reductase activity) was measured by DBH2 reduction at 280/325 nm with succinate as electron donor. Complex IV activity (cytochrome c-oxidase) was measured as described previously, with a Gilson oxygraph (27).

Kinetics of Center P and Center N Cytochrome b Reduction Pathways—Reactions were followed at 562/575 nm with the dual wavelength spectrophotometer DW3A Aminco Chance using the rapidly stirred reaction cuvette procedure; NADH was used as electron donor, because it is a better substrate for mitochondrial cytochrome b reduction, especially through center N, than DBH2. Antimycin (20 μM) and myxothiazol (40 μM) were used at saturating level in order to observe the center P and center N kinetic reduction pathways, respectively.

Kinetics of Cytochrome oxidase by the Center N Pathway—Reactions were also monitored with the rapidly stirred reaction cuvette procedure at 562/575 nm. DB was used as electron acceptor after heme b562 has been entirely reduced with endogenous substrate in the presence of KCN. Myxothiazol (40 μM) was added at saturating level to prevent reduction by the center P pathway.

Electrophoresis and Western Blot Analysis—SDS-PAGE (polyacrylamide gel electrophoresis) was performed using 10–14% acrylamide gels as in the Laemmli’s method (28) modified with Tricine running buffer (100 mM Tris, 100 mM Tricine, 1% SDS, pH 8.3). Immunoblotting was carried out as described previously (29) using a polyclonal antibody raised against purified wild type bc1 complex. This antibody mainly cross-reacted with the two core proteins, the iron sulfur protein and the subunit 8 of the bc1 complex. The purified cytochrome bc1 complex was prepared as described by Chevillotte-Brivet et al. (27).

Results

General Characteristics of Mutants and Revertants—Non-native intragenic revertants have been selected (20) from two respiratory deficient mutants of cytochrome b gene which are unable to grow on respiratory substrates (ethanol or glycerol). As recently described (16), the missense mutant S206L is well assembled, synthesizes reducible cytochrome b, and retains a partial electron transfer activity. The frameshift 204 mutant synthesizes a truncated apocytochrome b without any heme. Immunoblot analysis of mitochondria isolated from this mutant and from the wild type strain with polyclonal antibodies raised against purified wild type bc1 complex showed that the assembly of the complex is affected with the frameshift mutant; in particular, a loss of core protein I was observed together with that of the Rieske iron-sulfur protein (Fig. 1). Together with the complete absence of the mature FeS protein in this mutant, a band of higher molecular weight is detected (Fig. 1, lane 2) which is not present with the bc1 complex and the wild type mitochondria and might be the premature FeS protein; this would mean that the Rieske iron-sulfur protein is synthesized and imported into the mitochondria with the frameshift mutant, but due to the cytochrome b mutation leading to an absence of apoprotein and heme of this cytochrome, the FeS protein is not matured and not assembled into the bc1 complex.

When grown at 28 °C, the revertants isolated from the two cytochrome b respiratory deficient mutants S206L and frameshift 204 exhibited phenotypes which were almost similar to that of the parental strain (box1). In particular, these revertants had identical growth yields on galactose and ethanol as substrates, while the two original mutants did not grow at all on respiratory substrate (20). From these data, the phosphorylation efficiencies in vivo were deduced with the revertants grown on ethanol, which were found to be identical to that of the wild type (±5%), as summarized in Table I. This identity was confirmed by directly measuring the coupling characteristics using the polarographic method (with ethanol as substrate) on the mitochondria isolated from these strains. The phosphate to oxygen ratios (moles of ATP synthesized from added ADP/
atom of consumed oxygen) were between 1 and 2 (Table I), as expected with the yeast S. cerevisiae, which has only two phosphorylation sites (30).

At this growth temperature of 28 °C, only differences in the doubling time of some revertants were observed when cells were grown on respiratory substrates such as ethanol (Table I). Moreover, three revertants exhibiting these significantly modified doubling times also turned out to be thermosensitive strains, because they did not grow on ethanol at 37 °C (Table I). The increase in the doubling time of the three double-mutated revertants might reflect some changes in the kinetic parameters of these mutants, which were tentatively investigated in this study.

Activities in the Four Segments of the Respiratory Chain and in the Whole Chain—NADH and succinate oxidase activities have been measured in the various strains, and the results are given in Table II. The NADH oxidase activity decreased in the three double-mutated revertants isolated from the original mutant S206L, while the succinate oxidase activity remained similar to that of the wild type in all the revertant strains. With the two respiratory deficient mutants, these two activities either decreased considerably (S206L) or were practically abolished (frameshift 204).

With a view to explaining the discrepancy between the decrease in the NADH oxidase and the stability of succinate oxidase activities with some revertants, the maximal activities of each of the four segments of the respiratory chain were investigated in the wild type strain, the revertants, and the two original mutants which exhibited nil specific growth rate on ethanol (20). In each trial, the cytochrome b and a,a3 contents of the strain were determined as previously (20) and used to express the change in the specific activity at the complex III and IV level (turnover). As shown in Table II, point modifications in cytochrome b gene in the respiratory deficient mutants lead to a drastic decrease not only in complex III activity but also in complex II and IV activities in the case of S206L and frameshift 204 mutants. The situation is quite different with the revertants isolated from these two box mutants (Table II), since complex II shows no change as compared with the wild type value. In all the cases studied, NADH-DB activity (segment I) remained stable (+10%), as did the complex IV activity. With the three thermosensitive double mutants isolated from S206L, the only significant variation was that observed at the complex III level, which decreased by 30-40% in comparison with the wild type value (box an), while S206T and S206V revertants recovered activities similar to that of the wild type strain (Table II). These variations observed at the complex III level were correlated with, and were probably mainly responsible for, those recorded in the NADH oxidase activity.

The maximum activity values measured in each segment of the respiratory chain (Table II) provide a means of accounting for the differences observed between the unmodified succinate and the decrease in the NADH oxidase activities of the revertants exhibiting a decrease in complex bc, activity. Assuming that the excess ubiquinone is a mobile carrier linking dehydrogenases with cytochrome chains, Kröger and Klingenberg (31) described the overall oxidase activity (V) as follows.

\[
V = \frac{V_1 \times V_2}{V_1 + V_2} \quad \text{(Eq. 1)}
\]

\(V_1\) (or \(V_{\text{red}}\)) was defined as the maximum velocity of the ubiquinone-reducing system and \(V_2\) (or \(V_{\text{ox}}\)) as the maximum velocity of the ubiquinol-oxidizing system. A similar formulation has been described based on the enzymatic kinetic formalism, taking into account the measured concentration of endogenous quinones in the chain (in excess as in the above Q-pool hypothesis) and the presence of excess oxidant (32).

In Equation 1, \(V_1\) is proportional to the maximum velocity of dehydrogenases, and \(V_2\) might be expressed as the maximum rate of the cytochrome chain (bc complex if taking V to stand for succinate or NADH-cytochrome c reductase activity, the whole cytochrome chain (C1I + CIV) in the presence of oxygen, taking V to stand for succinate or NADH oxidase activity). If \(V_2^\prime\) is the modified bc complex activity with the revertant and \(V^\prime\) the resulting succinate or NADH-cytochrome c reductase activity, then the relative rate of the revertant (referred to that of the wild type \(V^\ast\)) can be expressed from Equation 1, as follows.

\[
\frac{V^\prime}{V^\ast} = \frac{1 + \frac{V_1}{V_1^\prime}}{\frac{V_2}{V_2^\prime} + \frac{V_2^\prime}{V_2}} \quad \text{(Eq. 2)}
\]

If only complex III activity is modified in revertants, this relative rate will vary hyperbolically with the relative activity \(V_2^\prime/V_2\) of complex III, and the concavity of the curve will depend on the value of \(V_1/V_2\) where \(V_1\) is the succinate or NADH-
ubiquinone reductase maximum activity and \( V_2 \), the complex III activity in the wild type strain. As it can be deduced from the legend of Table II, this ratio \( V_1/V_2 \) is lower with succinate than with NADH as substrate. Hence, Equation 2 clearly illustrates the fact that partial loss of bc1 complex activity affects NADH-cytochrome c reductase activity to a greater extend than succinate-cytochrome c reductase activity: when the bc1 complex activity is reduced to 50% of the wild type value, the NADH-cytochrome c reductase activity declines to about 60%, while the succinate-cytochrome c reductase activity only declines to 85% of the respective wild type values.

The fact that a decrease occurred in the NADH oxidase activity and not in the succinate oxidase activity can therefore be explained in terms of a decrease at the complex III level and the restricted flux occurring in complex II in comparison with those recorded in complexes I and III; complex III is rate-limiting as regards the NADH oxidase but not the succinate-oxidase activity.

Spectral Analysis of the Two Cytochrome b Hemes in the Various Strains—The respiratory deficient cytochrome b mutant S206L exhibits a specific red shift of heme \( b_{562} \) absorption maximum of 1.5 nm which can be observed in the \( \alpha \) and \( \gamma \) bands, at room temperature as well as at liquid nitrogen temperature (16). This shift toward the red (bathochromic effect) may correspond to a decrease in the energy transition barrier of this heme. The frameshift cytochrome b mutant exhibited no cytochrome b heme. Low temperature spectral analysis of the revertants, having a new cytochrome b configuration at center N, has been carried out and the data on the \( \alpha \) band maximum of the two hemes are summarized in Table III. This study showed that the heme \( b_{562} \) maximum has intermediate positions between that of the original mutant S206L (561 nm) and that of the wild type strain (559.5 nm) (Table III). Reversions S206T and S206V in the same position 206 than the original mutation (S206L) and mutations in positions 204 and 205 (H204Y and H204S/G205C) induce a heme \( b_{562} \) absorption maximum (between 560.1 and 560.6 nm, Table III) as compared with the reversion in position 208 (S206L/N208K, S206L/N208Y) which induce a heme \( b_{562} \) absorption maximum at 559.8 nm. It thus emerges that the heme \( b_{562} \) characteristics might be affected by changes in environmental amino acids other than the pair of histidines 96 and 197 which coordinate the heme, whether the cytochrome b resulting from the new conformation is functional (revertants) or practically not (mutant S206L). This also confirms the proximity of position 30 to heme \( b_{562} \) in the tertiary cytochrome b structure. The heme \( b_{562} \) was not modified in any of the strains tested, which is in agreement with the localization of this heme on the positive side of the inner mitochondrial membrane (center P).

In Vivo Thermosensitivity of the Center N-mutated Strains—The growth of three double-mutated revertants on respiratory substrates such as ethanol or glycerol is abolished at 37 °C (Table I): these thermosensitive strains have the original mutation S206L together with the proximal mutation N208K or N208Y or with the distal mutation W30C located on the NH\(_2\)-terminal part of the protein.

In order to test the phenotype arising from the reverse mutation alone in the revertant S206L/W30C, a recombinate strain carrying the wild type serine in position 206 and the mutated codon in position 30 was obtained (20). This strain carrying only the mutation W30C grows at 37 °C on nonfermentable substrate such as the parental strain. Similarly, the other two revertants, S206V and S206T, as well as the revertants from the original frameshift cytochrome b-deficient mutant in position 204, namely H204Y and H204S/G205C, are also nonthermosensitive strains. It therefore seems likely that the replacement of serine 206 by leucine might confer thermosensitivity on the cells. However, since mutant S206L is respiratory growth-deficient and is unable to grow on non fermentable carbon sources at any temperature, it is impossible to determine whether or not it may be thermosensitive in vivo.

In Vitro Thermosensitivity of Cytochrome bc\(_2\) Complex Activity with Mitochondria of the Center N Mutated Strains—Mitochondria were isolated from the parental strain (box\(^+\)) and the original mutant S206L and its revertants grown at permissive temperature (28 °C) on galactose medium. Under these conditions, the revertants synthesized cytochrome b contents in proportion quasi-identical to that of the wild type strain (about 90%) and exhibited an appreciable level of bc\(_2\) complex activity: the respiratory growth-deficient mutant S206L was found to synthesize less cytochrome b (44%) and to still show a low bc\(_2\) complex activity (Table II).

To determine whether the activity of the bc\(_2\) complex is affected by the temperature when the cells are grown at the permissive temperature (28 °C), under the conditions where the complex is well folded and assembled, the DBH\(_2\)-cytochrome c reductase activity was measured as a function of the incubation time at nonpermissive temperature (37 °C). As shown in Fig. 2, the in vitro bc\(_2\) complex activity of the wild

### Table II

| Strains | NADH oxidase | Succinate oxidase | Segment I (NADH-DB activity) | Complex II (succinate-DB activity) | Complex III (DBH-cyt. c (turnover)) | Complex IV (cyt. c oxidase activity) | Complex IV (turnover) |
|---------|--------------|------------------|-----------------------------|-----------------------------------|-------------------------------------|--------------------------------------|----------------------|
| K191 (box\(^+\)) | 100           | 100              | 100                         | 100                               | 100                                 | 100                                  | 100                  |
| S206L (original mutant) | 5             | 19               | 104                         | 20                                | 10                                  | 25                                   | 73                   |
| S206V | 106           | 100              | ND                          | 81                                | 95                                  | 111                                  | 97                   |
| S206L/N208Y | 50           | 90               | 110                         | 88                                | 62                                  | 67                                   | 110                  |
| S206L/N208K | 40           | 90               | 95                          | 88                                | 62                                  | 63                                   | 95                   |
| S206L/W30C | 50           | 90               | 105                         | 88                                | 62                                  | 75                                   | 109                  |
| Frameshift 204 (original mutant) | 3             | 3                | 80                          | 13                                | 17                                  | 17                                   | 100                  |
| H204Y | 80            | 96               | 111                         | 90                                | 76                                  | 92                                   | 96                   |
| H204S/G205C | 89           | 92               | 114                         | 84                                | 82                                  | 96                                   | 94                   |

Temperature-sensitive Center N Cytochrome b Mutants

To determine whether or not it may be thermosensitive in vivo.
Temperature-sensitive Center N Cytochrome b Mutants

TABLE III

Values of the α band maxima of heme b$_{562}$ (b$_{1}$) and heme b$_{559}$ (b$_{2}$) observed in low temperature spectral analysis with mitochondria isolated from the wild type strain (box*), respiratory-deficient mutant S206L, and its revertants, and the two revertants of the frameshift 204 mutant. The heme b$_{559}$ maximum was obtained from difference spectra of succinate-reduced sample minus ascorbate-reduced reference; the heme b$_{562}$ maximum was obtained from difference spectra of succinate + antimycin reduced sample minus succinate-reduced reference. ND = not determined.

| Strains          | box* (±0.1 nm) | S206L/ N208K | S206L/ N208Y | S206L/ W30C | S206T | S206V | S206L | H204Y | H204S/ G205C |
|------------------|---------------|--------------|--------------|-------------|--------|-------|--------|-------|--------------|
| b$_{559}$ (+0.1 nm) | 559.5         | 559.8        | 559.8        | 560.2       | 560.6  | 560.1 | 561    | 560.1 | 560.2        |
| b$_{562}$ (+0.2 nm) | 562.2         | 562.2        | 562.2        | 562.2       | 562.2  | 562.2 | 562.2  | ND    | ND           |

Fig. 2. Temperature sensitivity of the ubiquinol-cytochrome c reductase activity in the wild type strain and various mutants. Mitochondria were incubated at 37 °C for the indicated period of time in phosphate buffer (50 mM potassium phosphate, 50 μM EDTA, pH 7.4), in the presence of 2 mM KCN. Cells were grown at permissive temperature (28 °C) with galactose medium and harvested in the stationary phase. Mitochondria were isolated from the wild type strain ( ), one nonthermosensitive revertant S206V ( ), the nonthermosensitive recombinant strain S206L/W30C ( ), the original mutant S206L ( ), and the three thermosensitive revertants (two proximal revertants, S206L/N208K ( ) and S206L/N208Y ( ), and the distal revertant, S206L/W30C ( + )). DBH$_{2}$ cytochrome c reductase activity was measured at 25 °C as described under “Materials and Methods”; the specific activities of mitochondria isolated from the strains were also carried on in the presence of protease inhibitor phenylmethylsulfonyl fluoride, and similar results were obtained which suggests that this thermoinactivation of bc$_{1}$ complex activity may not be due to proteolysis of some subunits of the complex (results not shown). This was confirmed by SDS-PAGE and Western blot analysis using polyclonal antibodies against whole bc$_{1}$ complex, which did not show any decrease in the subunits after mitochondria had been incubated for several hours at 37 °C with or without protease inhibitors such as phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (data not shown).

In order to determine what happens with the cytochrome b heme when the cytochrome bc$_{1}$ complex activity decreases or is abolished, the reducibility of cytochrome b$_{1}$ was checked using a physiological substrate, succinate, and a nonphysiological one, dithionite, after exposure of mitochondria for 7 h at 37 °C. The results obtained with the wild type and two revertants are given in Fig. 3 (A–C): with all the strains, the heme was still present after incubating the mitochondria at the nonpermissive temperature (upper trace, dithionite-reduced minus oxidized mitochondria), even with the revertant S206L/N208K, which had completely lost its activity; however cytochrome b is not reducible in that strain (Fig. 3B) neither with succinate alone (middle trace) nor with succinate in the presence of antimycin (bottom trace, which corresponds to the reduction of cytochrome b via the center P pathway). The distal revertant S206L/W30C is an intermediate case between the parental strain (box*) and revertant S206L/N208K: cytochrome b is partially reduced by succinate alone, and through the center P pathway, which is in agreement with the fact that this mutant is still partially active after being exposed for 7 h at 37 °C (Fig. 2).

Control experiments were carried out in which mitochondria isolated from thermosensitive and nonthermosensitive strains were incubated at 0 °C and at the permissive temperature 25 °C for several hours: no significant decrease in the bc$_{1}$ complex activity was observed.

With all the revertants, the temperature sensitivity in vitro at the bc$_{1}$ complex level was found to be correlated with that observed in vivo. This thermoinactivation of bc$_{1}$ complex activity is an irreversible phenomenon: the decrease in activity cannot be restored by incubating the mitochondria at permissive (25 °C) nor at freezing temperature whatever the incubation time may be (data not shown). This cytochrome b conformational change is not an elastic process and reflects some irreversible structural modification.

Experiments such as that described in Fig. 2 involving the thermoinactivation of bc$_{1}$ complex activity of thermosensitive strains were also carried on in the presence of protease inhibitor phenylmethylsulfonyl fluoride, and similar results were obtained which suggests that this thermoinactivation of bc$_{1}$ complex activity may not be due to proteolysis of some subunits of the complex (results not shown). This was confirmed by SDS-PAGE and Western blot analysis using polyclonal antibodies against whole bc$_{1}$ complex, which did not show any decrease in the subunits after mitochondria had been incubated for several hours at 37 °C with or without protease inhibitors such as phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (data not shown).

Changes in the Kinetic Characteristics of Cytochrome b Reduction and Oxidation with the Incubation Time at Nonpermissive Temperature in Mitochondria Isolated from Thermosensitive Revertants—The three thermosensitive revertants isolated from mutant S206L showed a decrease in bc$_{1}$ complex activity depending on the incubation time at 37 °C (Fig. 2). The progressive effect of the nonpermissive temperature was further assessed by observing the kinetics of cytochrome b reduction and oxidation, in order to investigate which step is modi-
Fig. 3. Physiological and nonphysiological reducibility of cytochrome b in the wild type strain and thermosensitive revertants isolated from the respiratory deficient cytochrome b mutant S206L, after incubating mitochondria at the permissive (25°C) or nonpermissive (37°C) temperature. Cells were grown at 28°C and spectra recorded at 25°C. A, mitochondria of the wild type strain (box) incubated for 7 h at 37°C at the concentration of 0.32 nmol of cytochrome b/ml; B, mitochondria of the thermosensitive revertant S206L/N208K incubated for 7 h at 37°C at 0.29 nmol of cytochrome b/ml; C, mitochondria of the thermosensitive revertant S206L/W30C incubated for 7 h at 37°C at 0.41 nmol of cytochrome b/ml; D, mitochondria of the thermosensitive revertant S206L/N208K incubated for 7 h at permissive temperature (25°C) at 0.33 nmol of cytochrome b/ml. Upper trace, difference spectra of dithionite-reduced mitochondria in the sample cuvette versus potassium ferricyanide-oxidized mitochondria in the reference cuvette: this is the nonphysiological reduction. Middle trace, difference spectra of succinate-reduced versus potassium ferricyanide-oxidized mitochondria: this is the physiological reduction. Bottom trace, difference spectra of succinate-reduced mitochondria in the presence of antimycin (20 μM) versus potassium ferricyanide-oxidized mitochondria.

fied in the overall steady-state electron transfer from quinol to cytochrome c (DBH2-cytochrome c reductase activity). This mechanism, described by the Q cycle model, postulates the existence of two oxidoreduction centers on opposite sides of the membrane, which are inhibited by specific inhibitors. Cytochrome b might be reduced either via center P (in the presence of antimycin, as in Fig. 3, bottom trace) or via center N (in the presence of myxothiazol).

The reduction kinetics of cytochrome b with NADH as electron donor were studied via these two pathways and the results are given in Fig. 4. The data obtained before any incubation at the nonpermissive temperature (at t = 0) correspond to the kinetic characteristics of the strains grown with galactose medium at permissive temperature (28°C). While the kinetic behavior via center P showed no apparent change with the two revertants in comparison with the data on the wild type strain, the reduction of cytochrome b via center N was drastically slowed down (Fig. 4B). The kinetics of cytochrome b reduction with revertant S206L/W308K at 28°C were apparently identical to those of the wild type strain (data not shown). The impairment of bc complex activity was therefore correlated with the observed defect in the reduction pathway of heme bS562 at the center N level. This defect is responsible for the decrease in NADH oxidase activity and probably for that in the specific growth rate observed with respiratory substrate as carbon source.

With the thermosensitive revertant S206L/N208K (the activity of which was abolished after incubating mitochondria for about 4 h at 37°C), the kinetics of cytochrome b reduction by the reverse electron transfer from quinol to cytochrome bS562 through the center N pathway were drastically affected by the temperature, as regards both the apparent reduction rate and the amount of substrate reducible cytochrome b (Fig. 4B). The cytochrome b reduction kinetics through center P were also affected by the temperature in this revertant, as shown in Fig. 4A, but to a lesser extent than through center N: after incubating the mitochondria for 3 h, these kinetics showed only moderate difference with that of the wild type, while electron transfer through center N reduction pathway was nil. Another less thermosensitive revertant which has a ts50 of 4 h 10 min (Fig. 2), namely S206L/W30C, was also studied in detail and it emerged that cytochrome b reduction by center P pathway shows almost no change during thermoinactivation up to 7 h, in comparison with the wild type strain (Fig. 4A). This is in accordance with the spectrum in Fig. 3C. On the other hand, under similar conditions, the reduction kinetics decreased strongly by the center N pathway (Fig. 4B). Hence, while both strains are severely affected through the center N pathway, mutations affecting the more thermosensitive revertant S206L/N208K, has in addition long distance repercussions on the reduction kinetics by the center P pathway when mitochondria were incubated at nonpermissive temperature; this resulted in more drastic effects.

The characteristics of cytochrome b oxidation by decylubiquinone via the center N pathway (in
in the presence of antimycin (20 μM) (A) and via center N in the presence of myxothiazol (30 μM) (B). NADH (2 mM) was used as substrate (S), and the same dithionite-reduced cytochrome b content was present in all the experiments. The kinetic experiments were performed at 25°C in a rapidly stirred reaction cuvette with mitochondria extracted from cells grown at 28°C with galactose medium and incubated in MR1 buffer at 37°C during 0, 1 h, 40 min, 3 h, 15 min, and 7 h. The reactions were followed at 562/575 nm.

In Vivo and In Vitro Effects on the Respiratory Chain of Mutant S206L and Its Thermosensitive Revertants Grown at 37°C with Galactose Medium—All these strains have lost the ability to grow at 37°C on respiratory substrate because they are either respiratory growth-deficient, as in the case of mutant S206L, or thermosensitive, as in the case of the three double-mutated revertants isolated from this mutant. Nevertheless, they can grow at 37°C with fermentative substrates such as galactose, and their characteristics were determined after growth under these conditions. The cytochrome content and the activities measured in each segment and in the whole respira-
tory spectra that cytochrome cb is also absent in this strain (data not shown), while cytochrome c1 is still present (Table IV).

DISCUSSION

Among the seven non-native respiratory-competent revertants, derived from two respiratory growth-deficient mutants as described in our previous paper (20), three are affected both in their doubling time at the cellular level and in their NADH oxidase activity at the mitochondrial level (Tables I and II); this study shows clearly that the only significant modification in these revertants is located at the complex III level; more specifically, this moderate decrease in bc1 complex activity was found to be closely related to a slowing down in the kinetics of heme b562 reduction through center N pathway. On the other hand, the mutant S206L is strongly affected in both the reduction and oxidation of heme b562 at center N (36); in addition the two original mutants, S206L and frameshift 204, exhibit lower activities in the three complexes I, II, and III, as previously observed in several missense cytochrome b respiratory deficient mutants (37, 33). This observation is rather surprising and might be related to either a great sensitivity of succinodihydrogenase assembly to the absence of respiration as already...
Cytochrome contents, maximum activities in the four different segments of the respiratory chain, and respiratory activities of mitochondria isolated from cells grown at 37°C with galactose medium, of the wild type strain, box mutant S206L, and two thermosensitive revertants

| Strains                  | Cytochrome content | Respiratory chain segments activities | Respiratory activities |
|-------------------------|--------------------|--------------------------------------|------------------------|
|                         | aa3    | b        | c3     | I     | II    | III    | IV     | Ethanol | NADH | Succinate |
| KM91 (box^*)            | 100    | 100      | 100    | 100   | 100   | 100    | 100    | 100     | 100   | 100       |
| S206L (original mutant) | 3      | 47       | 70     | 99    | 4     | 10 (18)| 1      | 15      | 10    | 2         |
| S206L/N208K              | 5      | 10       | 38 (only c) | 76    | 0     | 0 (0)  | 4      | 10      | 8     | 1         |
| S206L/W30C               | 22     | 38       | 46     | ND    | 9     | 12 (33)| 15     | 16      | 10    | 13        |

**Fig. 5.** Immunoblot analysis of some complex III subunits in mitochondria isolated from cells grown at 37°C in the nonthermosensitive recombinant strain S206L/W30C, the thermosensitive revertant S206L/N208K and in the wild type strain. Cells were grown at 37°C with galactose medium; total mitochondrial protein (in the presence of protease inhibitor 4-(2-aminoethyl)benzenesulfonfyl fluoride and purified wild type bc1 complex were separated on 12% SDS-PAGE and transferred to nitrocellulose. Mitochondrial proteins were probed with polyclonal antibodies against wild type purified bc1 complex; as seen with the purified bc1 complex (lane 1), the antibody strongly reacted with the Core protein I (CP I) and II (CP II). Rieske iron sulfur protein (FeS), and subunit 8 (11 kDa). Lane 1, wild type purified bc1 complex (20 μg); lane 2, nonthermosensitive recombinant strain S206L/W30C mitochondria (75 μg); lane 3, thermosensitive revertant S206L/N208K mitochondria (75 μg); lane 4, wild type mitochondria (75 μg).

noticed by de Kok et al. (38) or to a direct interaction between respiratory complexes, in particular between complexes II and III as previously suggested by Gwak et al. (39), or to both phenomena.

When only complex III is modified by a mutation which decreases its activity (like with some revertants), the resulting succinate cytochrome c reductase or succinate oxidase activity is not very greatly modified, at least up to a threshold in complex III decrease, while NADH-cytochrome c reductase or NADH oxidase may amplify the variation. This is in complete agreement with the finding by Taylor et al. (40) that in patients with respiratory chain abnormalities, a partially defective complex III cannot be reliably detected by measuring succinate cytochrome c reductase activity.

The spectral shift toward the red observed with all the revertants might reflect some modification in the electronic surroundings of heme b_{562}, due to mutations (including the distal W30C) which map at the center N: this confirms the location of Trp-30 in the vicinity of this heme.

The revertants isolated from mutant S206L with an additional mutation in position 208 or 30 have been identified as thermosensitive strains. Thermosensitive mutants have been described previously, which affected the yeast bc1 complex, either as Rieske iron-sulfur mutants (41) or as Core protein I mutants (42). The thermosensitive cytochrome b mutants show progressive loss in the ability for cytochrome b to accept electrons from ubiquinol when mitochondria isolated from cells grown at 28°C were incubated at 37°C; this appears to have resulted mainly from a change in the tertiary structure of cytochrome b, since the assembly of the bc1 complex and cytochrome b hemes did not seem to be modified, which suggests that the quaternary structure of the complex is not greatly perturbed. With all thermosensitive revertants, the kinetics of cytochrome b reduction through center N were drastically and quickly altered; long distance repercussions may also have occurred however with the most thermosensitive revertant S206L/N208K (Fig. 4).

With this proximal revertant, the decrease observed in both the rates of cytochrome b oxidation and reduction at the center N level during incubation at 37°C suggests that the oxidoreduction potential of the couples Q/QO and O2/O2^* may have changed in an opposite manner, as it was assumed in the case of the two mutants S206L and M221K (36); this would result in a larger difference between the potentials of these two couples and the semiquinone concentration would hence have diminished (1, 2). The stability of the anionic semiquinone might therefore have undergone a decrease during thermoinactivation at 37°C as known to occur with cytochrome b mutants of Rhodobacter capsulatus (43). Incubation at nonpermissive temperature might initially favor a local distortion of the tertiary structure of cytochrome b, probably due to an increase in the distance inside and/or between the helices. This might thermodynamically destabilize the center N domain, so that the electron transfer rate is blocked (since the variation in the electron transfer rate as a function of the intercenter distance is an exponential law (44)) and/or the quinone binding site is affected, and both oxidation and reduction of cytochrome b through the center N pathway are slowed down.

Whereas the mutant S206L also exhibited sensitivity to heat treatment (Fig. 2), the strain constructed with the isolated mutation W30C did not, on the contrary, lead to a thermosensitive phenotype. These data therefore suggest that the replacement of serine 206 by leucine is responsible for the thermal sensitivity of the original mutant and revertants.

Matsumura et al. (45) have pointed out that mutations affecting protein stability generally have a cumulative effect. Like the thermal stabilizing effects of two substitutions (46), the destabilizing effects might also be additive: mutation W30C in addition to S206L did not increase the thermal instability, while proximal reversions, especially N208K, in addition to S206L, did (Fig. 2). These results were strengthened by the phenotypes of these strains grown on galactose medium at their nonpermissive temperature (37°C); the phenotypes of...
mutant S206L and revertant S206L/W30C are almost similar and show some of the activity of an assembled complex, while that of the revertant S206L/N208K is more drastically perturbed and shows a nonassembled bc\(_2\) complex with a complete loss of activity.

Argos et al. (47) mentioned that the most significant changes leading to thermal stability in proteins were the increase in the internal hydrophobicity, the helix forming ability of amino acids in helices and the increase in the sheet-forming tendency of residues in \(\beta\)-sheets. In particular, these authors reported that the Ser \(\rightarrow\) Thr change inside a \(\beta\)-sheet region had the ability to increase the thermal stability; this can be compared with the present finding that replacing serine 206 by threonine (or valine) resulted in a quite thermostable bc\(_2\) complex (Fig. 2). On the basis of computer analysis, Robert Brassere (Université Libre de Bruxelles) proposed that Ser-206 and Asn-208 are located in a cytochrome b region showing a high probability of \(\beta\)-sheet conformation.\(^2\) This is in agreement with the implication of these two amino acids in the thermostability of the protein, since mutations affecting the stability are assumed to map preferentially in folded regions (\(\alpha\)-helice or \(\beta\)-sheet).

In addition to its probable involvement in the thermostability of cytochrome b, asparagine 208 was found to be involved in funiculosin binding (48). Moreover, replacing asparagine 208 by lysine in this revertant keeping the original mutation S206L leads, when mitochondria are incubated at nonpermissive temperature, to concomitant loss of bc\(_2\) complex activity and funiculosin binding, while the antimycin binding is conserved; these data suggest close proximity between the funiculosin binding site and the catalytic center N domain (Q\(_N\)) (49). Furthermore, the three thermosensitive revertants exhibited a qualitative change in the shift induced by the funiculosin binding; this inhibitor causes a hypsochromic effect (blue-shift) on the spectrum of cytochrome bc\(_{2}\) heme in the wild type strain as well as in the mutant S206L, but it causes a bathochromic effect (red-shift) in the three double-mutated strains. This spectral change was attributed to the interaction between the second mutation and S206L. These data suggest that at least part of the funiculosin binding site is very close to the bc\(_{2}\) heme (49), which is in agreement with the first location of a funiculosin resistance mutation in yeast, L198F, close to histidine 197 linking the bc\(_{2}\) heme (50).

In conclusion, it emerges that the replacement of serine 206 by leucine in the original mutant is involved in the drastic decrease in bc\(_2\) complex activity, in the shift of the spectral maximum of the bc\(_{2}\) heme toward the red and in the thermostability of bc\(_2\) complex activity; whereas, the replacement of leucine 206 by threonine or valine is compatible with the thermostability of the protein and leads to a quasi-wild type phenotype; all temperatures (except for the heme bc\(_{2}\) spectral shift). The three additional mutations, in position 208 or 30, interact with leucine 206, resulting in restoration at the permissive temperature of a pseudo-wild type phenotype, with some decrease in complex III activity; two of these mutations, in position 208, strengthen the thermostability of the strain (N208K, N208Y). The mutations were found to mainly affect the center N domain where they were mapped, which is consistent with the eight transmembrane helix folding model of cytochrome b. These data as a whole indicate that the three amino acids, serine 206, asparagine 208, and tryptophan 30, are involved in the catalytic center N; two of them, serine 206 and asparagine 208, contribute to the thermostability of cytochrome b and are probably located in a folded region (\(\beta\)-sheet); asparagine 208 is also strongly involved in funiculosin binding.

---

\(^2\) R. Brassere, personal communication.
Structure-Function Relationships of the Mitochondrial bc1 Complex in Temperature-sensitive Mutants of the Cytochrome b Gene, Impaired in the Catalytic Center N

Gaël Brasseur, Jean-Yves Coppée, Anne-Marie Colson and Paule Brivet-Chevillotte

J. Biol. Chem. 1995, 270:29356-29364.
doi: 10.1074/jbc.270.49.29356

Access the most updated version of this article at http://www.jbc.org/content/270/49/29356

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 10 of which can be accessed free at http://www.jbc.org/content/270/49/29356.full.html#ref-list-1