The properties of the ubiquinol-cytochrome c reductase complex (bc1 complex) have been studied in respiratory defective mutants of Saccharomyces cerevisiae bearing lesions in the core 1 subunit. All the core1 mutants examined have greatly reduced concentrations of mitochondrial cytochrome b and display succinate-cytochrome c reductase activities near the limits of detection. Two mutants (E576 and C7), however, had 5% of wild type activity when the cells were grown at 23 °C, but not at 37 °C. The temperature-sensitive phenotype was determined to result from substitution of either Arg or Glu for Gly in the core 1 subunit.

The respiratory competent revertants E576/R8 and C7/R4 derived from E576 and C7 retain the temperature sensitivity of the original mutants. Both revertants are temperature sensitive in vivo, but only mitochondrial mitochondria isolated from E576/R8 are temperature sensitive in vitro. The bc1 complex of mitochondria isolated from this revertant displays a normal value of the ratio kcat/Km for cytochrome c and four times higher than the wild type for duroquinol. The succinate-cytochrome c reductase activity of E576/R8 is almost completely abolished after incubation at 37 °C for 90 min. It is inferred that the quaternary structure of mitochondrial ubiquinol-cytochrome c reductase complex is more labile at the nonpermissive temperature in the mutant and undergoes an alteration such that cytochrome b is no longer able to receive electrons through either the "o" or the "i" site pathway. The temperature lability and kinetic properties of the mutant enzyme point to a requirement of the core 1 not only for assembly but also for the catalytic activity of the complex.

Ubiquinol-cytochrome c reductase (complex III or bc1 complex) is a component of eukaryotic and prokaryotic respiratory chains (1, 2). Purified preparations of the bacterial and mitochondrial enzymes prome the transfer of electrons from ubiquinol to cytochrome c (3) with a concomitant vectorial translocation across the membrane of one proton/electron (4). Even though the mechanism by which the coupled oxidation of ubiquinol is catalyzed has been conserved during evolution, the bacterial and mitochondrial complexes exhibit quite remarkable differences in their subunit compositions. Bacterial ubiquinol-cytochrome c reductase consists of three catalytic subunits each having the following different redox centers: two cytochromes b associated with a single polypeptide, cytochrome c1, and the Rieske iron-sulfur protein (2, 5).

The same three subunits are present in the mitochondrial complex. The latter, however, contains six to eight other polypeptides with no detectable prosthetic groups (1, 6). This additional set of proteins includes two subunits of approximately 44 and 40 kDa referred to as core 1 and core 2 and an array of smaller proteins identified by their size (1, 6–8).

Notwithstanding the extensive information gathered in recent years about the structure and composition of mammalian and fungal ubiquinol-cytochrome c reductase, the functions of constituents other than those with identified redox centers remain largely unknown. The higher order of structural complexity of the mitochondrial enzymes cannot be correlated with the acquisition of any recognizable new function nor does the presence of the additional subunits appear to alter the kinetic properties of the enzyme in any significant way (1, 2). Their essentiality for the assembly and function of the complex is implied from both genetic and biochemical evidence. Yeast mutants lacking either the core or some of the low molecular weight subunits fail to express the complex (9, 10). Attempts to isolate from either the mammalian or fungal enzyme a catalytic unit composed of the three subunits shared with the bacterial enzyme have been unsuccessful suggesting that the extra subunits are indispensable for function as well.

The two large core proteins can be separated from the rest of the complex as a structural unit (10, 11). A functional role for these proteins is suggested by the recent discovery of a homology of yeast core 1 and core 2 to the two subunits of the matrix protease (12). Even more surprising is the reported identity of the Neurospora crassa core 1 to the regulatory subunit of the protease (12). To better understand the relationship between the complex to the catalytic activity of the mitochondrial bc1 complex, we have begun a study of yeast mutants with temperature-sensitive lesions in the core subunits. Here we report the properties of the complex in strains with mutations in core 1.

**MATERIALS AND METHODS**

**Strains of Yeast and Growth Media**—The sources and genotypes of the strains of S. cerevisiae used in this study are described in Table 1. The pet mutants were obtained by mutagenesis of the respiratory competent haploid yeast S. cerevisiae D273-10B/A1 with either ethylmethane sulfonate or nitrosoguanidine (16). Revertants showing partial restoration of a respiratory function were selected by spreading a lawn of a culture of yeast cells grown in liquidYPD on solid YPEG. Plates were incubated at room temperature for 5–15 days at which time revertants were collected.

Liquid and solid growth media had the following composition: YPD (2% glucose, 2% peptone, 1% yeast extract, YPGal (2% galactose, 2% peptone, 1% yeast extract), YPG (3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), W (2% glucose, 0.67% nitrogen base without amino acids (Difco)). Solid media contained 2% agar. Where required media were supplemented with amino acids or other auxotrophic requirements at a final concentration of 20 μg/ml.

**Isolation of Yeast Mitochondria and Enzyme Assays**—Yeast were...
Temperature-sensitive cor1 Mutants

TABLE I
Genotypes and sources of S. cerevisiae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
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<tr>
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<td>Ref. 13</td>
</tr>
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<td>aW303-1A</td>
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<td>R. Rothstein*</td>
</tr>
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</tr>
<tr>
<td>C153</td>
<td>αGirls, met6, cor1</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Department of Human Genetics, Columbia University, New York, NY.

grown aerobically at the permissive temperature (23 °C) in YPGal to early stationary phase. Mitochondria were prepared by the procedure of Faye et al. (17) except that Zymolyase 20,000 instead of Glusulase was used. Mitochondria were converted to submitochondrial particles by sonic irradiation as described previously (18).

Succinate-cytochrome c reductase activity was measured in 100 mM potassium phosphate buffer, pH 7.5, in the presence of 20 mM sodium succinate, 3 x 10^{-7} M KCN and variable amounts of horse heart cytochrome c (Sigma Type III). Reduction of cytochrome c was monitored at 550 nm, using an extinction coefficient \( \Delta \varepsilon_{550} = 19.1 \text{ mM}^{-1} \text{ cm}^{-1} \) (19) for the reduced minus oxidized a-band. Duroquinol-cytochrome c reductase activity was measured in the same buffer in the presence of half-saturating concentrations of cytochrome c and different concentrations of duroquinol (K&K Laboratories, Plainview, NY), dissolved in degassed methanol. The concentration of duroquinol used in the assay was determined using an extinction coefficient \( \varepsilon_{600} = 2.1379 \text{ mM}^{-1} \text{ cm}^{-1} \) for the reduced form in water (20). The rate of the chemical reduction of cytochrome c by duroquinol was determined prior to the addition of the mitochondrial suspension and subtracted from the final rate. For the spectral analysis of cytochromes, a suspension of mitochondria at a protein concentration of 3-8 mg/ml in potassium phosphate buffer, pH 7.5, was clarified by sonic irradiation for 20 s with the micropipet of a Branson sonicator set at 50% of maximum output. Difference spectra were recorded at room temperature in a Cary 14 spectrophotometer after the addition of potassium ferricyanide to the reference cuvette and 15 μM of succinate plus 0.5 μM of potassium cyanide or 10 μg of antimycin A to the sample cuvette. Complete reduction was achieved with sodium dithionite. The concentration of cytochromes b and c + c1 in mitochondria was determined from the absorption maxima of the a-bands relative to the base line 537-570 nm (21) using the following extinction coefficients: cytochromes c + c1 \( \varepsilon_{550\text{baseline}} = 18 \text{ mM}^{-1} \text{ cm}^{-1} \), cytochrome b \( \varepsilon_{600\text{baseline}} = 22.8 \text{ mM}^{-1} \text{ cm}^{-1} \). The concentration of cytochromes a + a2 was determined from the absorbance at 605 nm relative to the base line from 590 to 630 nm using an \( \varepsilon = 14 \text{ mM}^{-1} \text{ cm}^{-1} \) (22). Liquid nitrogen temperature spectra of the cytochromes b and c were obtained in a Perkin-Elmer 557 spectrophotometer as differential scan versus the isosbestic point at 540 nm. Mitochondria suspended in 50 mM Tris-Cl, pH 7.5, at a concentration of 18 mg of protein/ml were used for this analysis.

Western Blot Analysis—The concentrations of subunits of the bc complex of wild type and mutant mitochondria were determined by Western blot analysis with polyclonal antibodies raised against either the purified subunit (cytochrome b) or against trpE fusion proteins (10). Samples were dissociated in a buffer containing 5% (w/v) sodium dodecyl sulfate, 0.1 M Tris-Cl, pH 6.8, 1% 2-mercaptoethanol, 10% glycerol, 20 μg of phenylnethylsulfonyl fluoride/ml and separated by electrophoresis on a 12% (w/v) polyacrylamide gel. After transfer to nitrocellulose, immunochemically reactive bands were detected by reaction with [125I]Protein A (23).

Cloning of the Mutant cor1 Genes—Nuclear DNAs purified from the revertants E576/R8 and C7/A4 were digested with a combination of BglII and HindIII, separated by electrophoresis on a preparative 1% agarose gel and the region containing fragments ranging from 2.3 to 2.8 kb was excised and the DNA collected by electroelution. The mixture of fragments was ligated into the shuttle vectors YEp352 or YEp351 (24) linearized with HindIII and BamHI. Ampicillin resistant clones of Escherichia coli RLI [supE44 hsdS20(rpsL50) rpsL20 xyl-5 met-1 lacY1 galK2 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 met-1 lacY1 galK2 rpsL20 xyl-5 met-1] transformed with the ligation mixture were screened by colony hybridization (95) with a nick-translated 1.6-kb XbaI-HindIII fragment encompassing the region of CORI coding for the amino-terminal half of the protein (14). Positive clones were purified and assessed by restriction analysis to harbor plasmids with the 2.5-kb BglII fragment coding for the core subunit of ubiquinol-cytochrome c re-
ductase (14). All the cor1 mutants examined lack ubiquinol-cytochrome c reductase activity. This phenotype is due solely to recessive mutations in COR1 since the mutants are complemented by pet strains with lesions in other subunits of ubiquinol-cytochrome c reductase or in nuclear gene products required for the expression of cytochrome b (10). Western analysis of mitochondria indicates that cor1 mutants are either completely devoid of or have severely reduced concentrations of immunochemically detectable core 1 in their mitochondria (Fig. 1). These observations suggest that most mutations in COR1 affect the stability of this subunit thereby blocking assembly of the complex.

A feature common to all cor1 mutants is the almost complete absence of cytochrome b as measured by difference spectra of submitochondrial particles reduced with substrate (succinate) in the presence of antimycin A versus particles oxidized with ferricyanide (Fig. 2, Table II). Similar spectra of chemically reduced submitochondrial particles exhibit a shoulder on the main absorption bands of the c-type cytochromes. This absorption band, partially obscured by cytochromes c and c1, probably corresponds to cytochrome b5 (21).

The concentrations of mitochondrial cytochromes and of succinate-cytochrome c reductase activity in mitochondria of wild type and cor1 mutants are reported in Table II. Mutants grown at 30 °C have less than 2% of the wild type concentration of succinate-reducible cytochrome b. In addition to the near complete absence of ubiquinol-cytochrome c reductase and cytochrome b, cor1 mutants also have less cytochromes a and a6. The decrease in cytochrome oxidase is a pleiotropic effect observed in most pet mutants, regardless of the genetic lesion.

Most of the cor1 mutants in our collection do not grow on the non-fermentable substrate glycerol in the temperature range of 23 to 37 °C. The two exceptions are E576 and C7 which grow slowly on glycerol at 23 °C (approximately 5% of the wild type growth rate) but not at higher temperatures. Consistent with their growth properties, E576 and C7 have higher succinate oxidation rates when grown at 23 than at 30 °C (Table II).

**Phenotype of Temperature-sensitive Revertants of E576 and C7**—The core 1 subunit was found in earlier studies to be required for the assembly of ubiquinol-cytochrome c reductase (10, 14). To determine whether this subunit is also needed for enzymatic activity, temperature-sensitive cor1 mutants were obtained which could be used to study the effect of the non-permissive temperature on the catalytic activity of a complex formed at the permissive temperature. Such conditional mutants were isolated by selecting revertants of cor1 mutants on YEPG and further testing them for growth on this medium at 23 and 37 °C. Of all the cor1 mutants tested only E576 and C7 produced temperature-sensitive revertants whose growth on glycerol is abolished at 37 °C. Two such revertants, E576/R8 and C7/R4, were grown at 30–40% of the wild type rate at the permissive temperature and have approximately 10% of the normal amount of substrate reducible cytochrome b (Fig. 3). Both revertants also have noticeably higher concentrations of core 1 than the original mutants (Fig. 4).

The meiotic progeny issued from crosses of E576/R8 and C7/R4 to a respiratory competent haploid strain, 5–10% had the original mutant phenotype indicating that neither suppressor is linked to the original cor1 alleles. Backcrosses of E576/R8 and C7/R4 and of their ρ0 (cytoplasmic petite mutant of yeast lacking mitochondrial DNA) derivatives to the original mutants further showed both extragenic suppressors to be partially recessive and to be in nuclear DNA.

**Temperature-sensitive Respiration in E576/R8 Mitochondria**—To determine whether core 1 is essential for catalytic activity, mitochondria were isolated from E576/R8, C7/R4, and from wild type yeast grown at 23 °C and their succinate-cytochrome c reductase was measured as a function of time of incubation at the non-permissive temperature (37 °C). Even though C7/R4 does not grow on glycerol at 37 °C, the in vitro stability of succinate-cytochrome c reductase of this strain is identical to that of wild type (Fig. 5). In contrast, mitochondria of E576/R8 are much more susceptible to inactivation at the high temperature. Virtually all activity is destroyed after exposure of mitochondria from this strain at 37 °C for 90 min (Fig. 5).

The concentrations of some subunits of the complex such as cytochrome b, the iron-sulfur protein, cores 1 and 2, and the 11-kDa polypeptide are lower in E576/R8 than in wild
Table II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cytochrome content</th>
<th>Activity</th>
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<tr>
<td></td>
<td>c + c, b, a + a0</td>
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</tr>
<tr>
<td></td>
<td>nmol/mg-protein</td>
<td>Cells grown</td>
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<tr>
<td></td>
<td></td>
<td>temperature at 30°C</td>
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<tr>
<td>D273-10B/A1</td>
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<td>400</td>
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<td>W3095COR1</td>
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<tr>
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<td>0.55 0.016 0.25</td>
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<td>10 0.9</td>
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<td>C7/R4</td>
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</table>

Fig. 3. Spectra of submitochondrial particles from E576 and E576/R8. Difference spectra of oxidized versus reduced submitochondrial particles suspended in 10 mM Tris-HCl, pH 7.5, at a protein concentration of 6.2 mg/ml (E576) and 5.3 mg/ml (E576/R8) were recorded at room temperature as in Fig. 2. The particles were reduced either with sodium dithionite (Na2S2O4) or with succinate plus antimycin A (succ). The original mutant E576 and the revertant E576/R8 were both grown at 23°C, the permissive temperature.

Fig. 4. Western blot analysis of core 1 in mitochondria of temperature sensitive cor1 mutants. The respiratory competent strain D273-10B/A1 (WT), and the cor1 mutants C7, E576, C7/R4, and E576/R8 were grown at 23°C. Total mitochondrial protein (50 µg) from each strain were separated on a 12% polyacrylamide gel and tested with antibody against a trpE/COR1 fusion protein as described in the legend to Fig. 1.

Fig. 5. Temperature sensitivity of succinate-cytochrome c reductase activity in isolated mitochondria of wild type yeast and of cor1 mutants. The respiratory competent haploid strain D273-10B/A1 (C) and the two cor1 revertants C7/R4 (C3) and E576/R8 (Δ) were grown at 23°C in liquid YPGal medium. Cells were harvested in early stationary phase and mitochondria prepared. Mitochondria suspended in 10 mM Tris-HCl, pH 7.5, at a protein concentration of 12-14 mg/ml were incubated at 37°C for the indicated periods of time and were then assayed for succinate-cytochrome c reductase activity at 23°C. The specific activities of the mitochondria at time 0 are reported in Table II.

The effect of the non-permissive temperature on the complex was further assessed by estimating the amount of substrate-reducible cytochrome b. As indicated above, E576/R8 mitochondria possess 10% of the wild-type level of functional cytochrome b as defined by its substrate reducibility in the presence of antimycin A (Fig. 3, Table II). Incubation of mitochondria at 37°C leads to a progressive loss in the ability of cytochrome b to accept electrons from ubiquinol. This is illustrated in Fig. 7 by the spectra of mitochondria incubated at 23 and 37°C for 90 min and then reduced by the addition of succinate in the presence of antimycin A. The variable amount of cytochrome c and c1 reduced by succinate is due to a partial depletion of oxygen between the time of addition of the substrate and freezing of the sample. Since most of cytochrome oxidase is still oxidized, however, there is sufficient dissolved oxygen left in this experiment to elicit the antimycin-dependent extra reduction of cytochrome b (29, 30). Only 26% of the cytochrome b heme was lost at the non-permissive temperature as determined from the differential spectrum of the dithionite reduced minus the ascorbate-reduced samples (data not shown).

The temperature-induced decrease in functional cytochrome b is seen not only with the "o" site assay described above but also using the alternative entry of electrons at the "i" site (31). In the latter assay, reduction of cytochrome b by substrate is measured in the presence of myxothiazol. Spectra of mitochondria incubated at the non-permissive temperature...
Temperature-sensitive corl Mutants

The respiratory competent strain D273-10B/A1 and the corl mutant E576/R8 were grown at 23 °C in YPGal. Mitochondria were prepared from cells harvested in early stationary phase. The mutant mitochondria were incubated either at 23 or 37 °C for the indicated periods of time, and 40 µg of protein were separated on a 12% polyacrylamide gel. Wild type mitochondria without prior incubation served as a control. The proteins were transferred to nitrocellulose by electrophoresis and reacted with antibodies against core 1, core 2, cytochrome c (cyt. c), cytochrome b (cyt. b), iron-sulfur protein (FeS), and the 14- and 11-kDa subunits of the complex.

The loss of functional cytochrome b at the non-permissive temperature is unlikely to be due to an alteration in the iron-sulfur center since transfer of electrons to cytochrome b in the presence of myxothiazol does not require a functional Rieske protein (32).

Kinetic Properties of the bc Complex of E576/R8—The effect of the temperature-sensitive mutation in the core 1 subunit on the function of the bc complex was further tested by determining the kinetic constants of the wild type, the mutant E576, and the revertant E576/R8 enzymes with respect to cytochrome c and duroquinol, an analog of ubiquinol. The Lineweaver-Burk plots obtained for the two different substrates are presented in Figs. 8 and 9 and the constants derived from those data are summarized in Tables III and IV. The values of $k_{cat}$ were normalized to the amount of functional cytochrome b detected in mitochondria by the o-site assay.

Since the mitochondria used for the assays are totally uncoupled, the reduction of exogenous cytochrome c occurs in an essentially irreversible mode and the $k_{cat}/K_m$ ratios can be taken as indices of the minimal association rate constants at the cytochrome c and duroquinol sites. To ascertain that the bc complex is freely accessible to cytochrome c under the conditions of the assay, the kinetic constants for cytochrome c were also determined in the presence of 0.1% potassium cholate. The addition of the detergent decreases by 50% the turnover numbers of E576 and E576/R8 complexes are very similar. However, the total amount of functional cytochrome b is much higher in E576/F# suggesting that the suppressor mutation increases the stability of the complex without altering its kinetic properties.

The effects of the 37 °C incubation on the kinetic constants of the E576/R8 enzyme with respect to cytochrome c are shown in Fig. 9 and Table IV. The loss of enzyme activity observed as a result of the heat treatment is due mainly to a decrease in the total amount of functional bc complex. Thus, the turnover of the enzyme is not significantly different after 30 and 60 min of incubation at 37 °C. However, the apparent increase in $K_m$ for cytochrome c at 30 and 60 min suggests that the loss of activity is preceded by an alteration of the quaternary structure.

Characterization of the corl Mutations Responsible for the Temperature-sensitive Phenotypes of C7/R4 and E576/R8—The mutant corl genes were cloned in two different ways. The first involved colony hybridization screens of mutant plasmid libraries constructed from the nuclear DNA of E576/R8 and C7/R4 (see "Materials and Methods"). In the second method the 1.8-kb XbaI-HindIII fragment coding for the
Temperature-sensitive cor1 Mutants

The mutations were identified from the sequences of the mutant genes cloned by colony hybridization. Only the 1.8-kb XbaI-HindIII regions of the genes were sequenced. The nucleotide sequences of this region revealed that the same amino acid (Gly6') is mutated in the core 1 protein of the two mutants although each has a different substitution. In E576/R8 Gly68 is replaced by an arginine (codon change, GGG → AGG) and in C7/R4 by a glutamic acid (codon change, GGG → GAG). The replacement of the same residue is consistent with the temperature-sensitive growth phenotype of the mutants. The difference in the in vitro heat inactivation of enzyme activity in the two revertants could be a consequence of the different charges introduced by the mutations or a function of the suppressors in the two strains.

**DISCUSSION**

The essentiality of the core 1 subunit for the synthesis of ubiquinol-cytochrome c reductase is supported by the phenotype of cor1 mutants of yeast (9, 10). That this protein may be equally important for catalytic activity is suggested by earlier studies of chemical modification (34, 35) as well as reconstitution of enzymatically active complex from its constituents (11). Even though both experimental approaches point to the importance of the core 1/core 2 subcomplex in both electron transfer and proton translocation, they have some shortcomings. Chemical modifiers are only partially selective making the attribution of the observed effects to any single subunit problematic. The reconstitution experiments are also subject to some criticism. The use of detergents and chaotrope reagents for the separation of the subunit components can affect the tertiary structure of the components being analyzed and the final reconstituted activities are generally low compared with the native enzyme.

In the present study we have resorted to an alternative more genetically oriented approach to probe possible roles of the core 1 subunit in the structure and function of the bc, complex. Two mutations in the COR1 gene have been identified which confer a temperature-sensitive growth phenotype when glycerol, a non-fermentable substrate, is used as the carbon source. The slow growth of the two mutants C7 and E576 on this substrate, even at the permissive temperature, and the low ubiquinol-cytochrome c reductase activity measured in mitochondria, however, made these strains difficult to analyze biochemically. Derivative mutants retaining the temperature conditional phenotype but manifesting better growth properties on the non-fermentable substrate were therefore isolated as partial revertants of C7 and E576. The improved ubiquinol-cytochrome c reductase activity in both
revertants was ascertained genetically to be due to extragenic
nuclear suppressor mutations.

The availability of mutants with a heat labile form of core 1
attained an opportunity to test whether the requirement for
the native subunit persists after assembly of the complex
is allowed to occur at the permissive temperature. Mitochondria
of E576/R8 grown at 23 °C have approximately 25% of the
wild-type succinate-cytochrome c reductase activity. A com-
parison of the effect of incubation of isolated mitochondria at
the non-permissive temperature also point
to a subtle alteration of the quaternary and/or tertiary struc-
ture of the mutant complex.

Two other properties of the bc1 complex in E576/R8, both
related to its catalytic activity, were examined. Ubiquinol is
known to donate its electrons to cytochrome b at the o site in
the presence of antimycin A and at the i site in the presence of
myxothiazol (32). These respiratory inhibitors can therefore
be used to assay the catalytic competence of the two sites.

The results of temperature shift experiments indicate that
the amount of reducible cytochrome b decreases to the same
extent during the heat treatment, independent of whether
electrons are introduced at the o or the i site. These results
suggest that the ability of cytochrome b to interact with its
substrate is critically dependent on core 1. A reasonable
interpretation is that core 1 in conjunction with core 2 and
perhaps some of the low molecular weight subunits stabilizes
the quaternary structure of the complex. The lower Kc, for
duroquinol and the increase in the Kc, for cytochrome c during
the incubation at the non-permissive temperature also point
to a subtle alteration of the quaternary and/or tertiary struc-
ture of the mutant complex.

The higher kcat/Km ratio for duroquinol corresponds to an
increment of Gibbs free energy for the transfer of this sub-
strate from the enzyme to the aqueous phase of 3.4 kJ/mol
(0.81 kcal/mol) (36). This value is consistent with the incre-
mental binding energy provided by a methyl group relative to
a hydrogen atom (37-39). A possible interpretation of this
result is that in E576/R8 one additional methyl of duroquinol
is involved in the binding to the bc1 complex.

It is generally believed that the continuous oxidation of
duroquinol by the complex requires the presence of endoge-
nous quinone (40, 41). However, duroquinol can also be ox-
idized directly at the o center without the involvement of the
dependent quinone (42, 45). Duroquinol appears to react
preferentially at this site in the yeast enzyme (44). Although
the high kcat/Km for duroquinol need not neces-
sarily indicate a similar change in the interaction of the
complex with the endogenous quinones, an increase in the
second order rate constant for the oxidation of ubiquinol
could explain the higher values of Kc, and Vmax observed for
cytochrome c. The bc1 complex has been proposed to behave
kinetically as a ping-pong system (45). One of the character-
istics of such systems is an increase in the Kc, and Vmax values
at one site with higher saturation of the other site (46).

The two mutations in core 1 identified in this study appear to
exert an effect on both assembly, as evidenced by the
reduced steady-state concentrations of the complex in cells
grown at the permissive temperature, and the catalytic prop-
erties discussed above. Both are consequences of mutations

---

### Table III

**Kinetic properties of the bc1 complex in the cor1 mutants in relation to different substrates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Km</th>
<th>Vmax</th>
<th>Cytochrome b</th>
<th>kcat/Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>nmol cyt/c min/mg protein</td>
<td>nmol/mg protein</td>
<td>s^-1</td>
<td>10^6 x s^-1 M^-1</td>
</tr>
<tr>
<td>D273-10B/A1</td>
<td>19.5</td>
<td>563</td>
<td>0.42</td>
<td>45</td>
<td>2.3</td>
</tr>
<tr>
<td>E576</td>
<td>55.5</td>
<td>66</td>
<td>0.016</td>
<td>137</td>
<td>2.5</td>
</tr>
<tr>
<td>E576/R8</td>
<td>59.0</td>
<td>195</td>
<td>0.050</td>
<td>132</td>
<td>2.2</td>
</tr>
</tbody>
</table>

### Table IV

**Effect of the non-permissive temperature (37 °C) on the kinetic properties of the cytochrome c reduction site in the bc1 complex of the revertant E576/R8**

The amount of functional cytochrome b was determined at each time from the differential spectra recorded in
condition of "oxidant induced" extra reduction (succinate + antimycin A versus ferricyanide). The concentration
of active complex was considered equal to half of that of cytochrome b.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Km</th>
<th>Vmax</th>
<th>Cytochrome b</th>
<th>kcat/Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>nmol cyt/c min/mg protein</td>
<td>nmol/mg protein</td>
<td>s^-1</td>
<td>10^6 x s^-1 M^-1</td>
</tr>
<tr>
<td>0</td>
<td>59.7</td>
<td>195</td>
<td>0.006</td>
<td>132</td>
<td>2.2</td>
</tr>
<tr>
<td>30</td>
<td>93.7</td>
<td>148</td>
<td>0.031</td>
<td>161</td>
<td>1.7</td>
</tr>
<tr>
<td>60</td>
<td>87.8</td>
<td>67</td>
<td>0.018</td>
<td>127</td>
<td>1.5</td>
</tr>
</tbody>
</table>
at Gly\(^{6}\) of core 1. In C7 this residue is substituted by a glutamic acid and in E876 by an arginine. In general, mutations that affect the stability at the non-permissive temperature of an already folded protein map preferentially to sites with lower than average crystallographic thermal factors and low solvent accessibility (47). The two replacements at Gly\(^{6}\) appear to belong to this class of temperature-sensitive mutations suggesting that this residue is probably located in a charged residue such as arginine or glutamic acid probably weakens local hydrophobic interactions thereby destabilizing the tertiary structure of the protein. This would account for the increased susceptibility of the mutant protein to proteolysis as reflected by its lower concentration even when cells are grown at the permissive temperature. The protein surviving degradation is incorporated into a complex with the somewhat modified catalytic properties described above. In E576/R8 the catalytically competent structure of the complex is lost by disruption of the tertiary structure of core 1 at the non-permissive temperature. The enhancement of respiratory activity in the partial revertants is probably due to the further stabilization of the mutant core 1 proteins by some other component of the mitochondrial inner membrane. At present we have no information regarding the identity of the suppressors.

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REFERENCES

Structure and function of the mitochondrial bc1 complex. Properties of the complex in temperature-sensitive cor1 mutants.
D L Gatti and A Tzagoloff


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