Journal of Bioenergetics and Biomembranes, Vol. 28, No. 1, 1996

Original Article

Subunit 8 of the *Saccharomyces cerevisiae* Cytochrome bc_1 Complex Interacts with Succinate–Ubiquinone Reductase Complex

Christophe Bruel,[1,2] Robert Brasseur,[1,3] and Bernard L. Trumpower[1]

Received July 1, 1995; accepted August 23, 1995

We have investigated the function of subunit 8 of the cytochrome bc_1 complex by generating six site-directed mutants, F46C, R51S, P62V, G64A, R91N, and W69-stop, in the cloned *QCR8* gene and expressing the mutated genes in a *Saccharomyces cerevisiae* strain in which the chromosomal copy of *QCR8* is deleted. The W69-stop mutation impairs assembly of the bc_1 complex and growth of yeast on nonfermentable carbon sources as does deletion of *QCR8* [Maarse, A. C., De Haan, M., Schoppink, P. J., Berden, J. A., and Grivell, L. A. (1988). *Eur. J. Biochem.* **172,** 179–184], implying that the C-terminus of subunit 8 is important for assembly and/or the stability of the bc_1 complex. The F46C, R51S, P62V, G64A, and R91N mutations do not affect the growth of yeast on nonfermentable carbon sources, not do they lower the activity or alter the inhibitor sensitivity of the bc_1 complex. Rather, some of the mutations increase the cytochrome *c* reductase activity of the bc_1 complex by as much as 40%. However, succinate–ubiquinone reductase activity was consistently reduced 40–60% in mitochondrial membranes from these mutants, while NADH–ubiquinone reductase activity was not affected. In addition, the activation of succinate–ubiquinone reductase activity by succinate was diminished by the F46C, R51S, P62V, and G64A mutations. These results indicate that the cytochrome bc_1 complex participates in electron transfer from succinate to ubiquinone *in situ* and also suggest an interaction between succinate–ubiquinone reductase and cytochrome bc_1 complex which involves subunit 8 of the bc_1 complex.

INTRODUCTION

The cytochrome bc_1 complexes catalyze electron transfer from ubiquinol to cytochrome *c* coupled to proton translocation across the membrane in which they are embedded. The electron and proton transfer mechanism is known as the Q-cycle, which postulates two oxido-reduction centers, P and N, located on opposite sides of the membrane (Trumpower, 1990). Three redox proteins are responsible for the bioenergetic activity; a di-heme cytochrome *b*, a 2Fe–2S cluster iron-sulfur protein, and cytochrome c_1. In addition to these three proteins, as many as eight additional subunits are present in the bc_1 complexes of eukaryotes. The functions of these supernumerary subunits are largely unknown.

QCR8, the nuclear gene encoding the 11-kDa subunit 8 of the *Saccharomyces cerevisiae* bc_1 complex, has been cloned and sequenced (Hemrika *et al.*, 1993; Maarse and Grivell, 1987). Grivell and co-workers disrupted *QCR8* and found that the disruption strain

[1] Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755.
[2] Current address: Laboratoire de Bioénergétique et Ingénierie des Protéines, C. N. R. S., 31, Chemin Joseph Aiguier, 134002 Marseille Cedex 20, France.
[3] Current address: Centre de Biophysique Moléculaire Numérique, Faculté des Sciences Agronomiques de Gembloux, Passage des Déportés, 2, 5030 Gembloux, Belgium.

0145-479X/96/0200-0059$09.50/0

did not grow on nonfermentable carbon sources due to impaired assembly of the bc_1 complex (Schoppink *et al.*, 1989). Yu and co-workers (Usui *et al.*, 1990) proposed that the bovine homologue of subunit 8 is a quinone binding protein involved in the catalytic center N of the bc_1 complex on the negative side of the mitochondrial membrane.

In order to better understand the function of subunit 8 in the bc_1 complex we have constructed site-directed mutants of *QCR8* and expressed these from a plasmid in a yeast strain in which the chromosomal copy of *QCR8* is deleted. A W69-stop mutation led to the same loss of fully assembled bc_1 complex as does deletion of *QCR8*, suggesting that the C-terminus of subunit 8 is important for proper assembly or stability of the complex.

Mutations F46C, R51S, P62V, G64A, and R91N did not impair the activity of the bc_1 complex or the growth of yeast on nonfermentable carbon sources but did cause a significant decrease in the succinate–ubiquinone reductase activity of mitochondrial membranes isolated from these mutants. The rate of activation of succinate–ubiquinone reductase by succinate is also extensively reduced in mitochondrial membranes from the F46C, R51S, P62V, and G64A mutants. These results indicate that the cytochrome bc_1 complex interacts with succinate–ubiquinone reductase complex to participate in electron transfer from succinate to ubiquinone, and that subunit 8 of the bc_1 complex is required for this interaction.

EXPERIMENTAL PROCEDURES

Materials

Horse heart cytochrome *c*, decyl-ubiquinone, and amino acids were obtained from Sigma. Yeast extract, peptone, and bacto-agar were from Difco. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Oligonucleotides were synthesized and purified by Dr. Tom Ciardelli (Dartmouth Medical School). The Transformer™ Site-Directed Mutagenesis Kit from Clontech was used for mutagenesis and the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit from Applied Biosystem was used for sequencing. Prime-It™ Kit, Nuc-Trap™ columns, and QuickHyb from Stratagene were used for probe labeling, purification, and hybridization. [α-^{32}P] dCTP was from Amersham Corp. Kodak Xar-5 film was used with a DuPont NEN Cronex Lightning Plus intensifying screen for autoradiography. Agarose was from Life Technologies. DNA was isolated from agarose gels using the Geneclean™ Kit from BIO101. Nitrocellulose was from Schleicher & Schuell. Glass beads (0.5 mm) were from Biospec Products.

Yeast Strains and Growth Media

Saccharomyces cerevisiae strains YPH499 (MATa, ura3-52, lys2-801 (amber), ade2-101 (ochre), trp1-Δ63, his3-Δ200, leu2-Δ1) and YPH500 (MATα, ura3-52, lys2-801 (amber), ade2-101 (ochre), trp1-Δ63, his3-Δ200, leu2-Δ1) were from Dr. R. S. Sikorski (Johns Hopkins University). Yeast were grown on YPD,[4] YPEG, YPGal, or a minimal medium containing 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 2% dextrose, and 0.13% "dropout powder" lacking uracil or leucine (Treco and Lundblad, 1989). Plasmids were amplified in *Escherichia coli* strain DH5α (*psi*80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_k^- m_k^+), *sup*E44, *thi*-1, λ-, *gyr*A96, *rel*A1,Δ(*laczya-arg*F)U169, F^-.

Construction of *QCR8* Deletion Strain

A 2.6-kbp *XbaI*/*Kpn*I fragment from plasmid pFL1-285 was subcloned into PRS315 to form PRS315-QCR8 (Frank1). PRS315 is an *E. coli*/*S. cerevisiae* shuttle plasmid carrying the yeast *LEU2* gene. An 800-bp *Hind*III/*Sal*I fragment carrying the *QCR8* reading frame with 275 bp of upstream and 281 bp of downstream sequence was replaced with a 1.28-kbp PvuII/SalI fragment carrying the yeast *URA3* gene that was taken out of the plasmid pJJ244 (Jones and Prakash, 1990). From the resulting deletion plasmid (Frank2) a 3.08-kbp *XbaI*/*Kpn*I fragment was released that carried the *URA3* gene flanked by genomic DNA from the *QCR8* region. This fragment was transformed into a ura3$^-$ isogenic diploid yeast strain (YPH499 $\times$ YPH500), and transformants were selected for uracil prototrophy.

[4] Abbreviations: DBH_2, 2,3-dimethoxy-5-methyl-6-decyl-1, 4 benzoquinol; DCPIP, 2,6-dichlorophenol indophenol; kbp, kilobase pairs; bp, base pairs; PCR, polymerase chain reaction; PMS, phenazine methosulfate; YPD, 1% yeast extract, 2% peptone, 2% dextrose; YPEG, 1% yeast extract, 2% peptone, 4% ethanol, 3% glycerol; YPGal, 1% yeast extract, 2% peptone, 2% galactose.

Insertion of the marker gene at the *QCR8* locus was verified by Southern blots of genomic DNA digests (Southen, 1975). Haploid deletion strains were generated by sporulating the diploids and dissecting tetrads (Sherman and Hicks, 1991). Spores carrying the deletion were identified by their uracil prototrophy.

Site-Directed Mutagenesis

A 27–36-residue oligonucleotide, referred to as the mutagenesis oligonucleotide, was designed for each mutation, and a 20-residue oligonucleotide containing a mutated sequence of the unique *SalI* site of the plasmid pRS315-QCR8 was also synthesized and referred to as the selection oligonucleotide. Each mutagenesis oligonucleotide was integrated together with the selection oligonucleotide in a new pRS315-QCR8 DNA strand synthesized with T4 DNA polymerase using PRS315-QCR8 as template. Nonmutated plasmids were counter-selected by *SalI* digestion and mutated plasmids verified by restriction mapping or sequencing. The mutated plasmids were introduced into the *qcr8* deletion yeast strain by electroporation, and the transformants were selected for leucine prototrophy.

Mitochondrial Preparation

Mitochondria were isolated from the parental strain YPH500, the *qcr8* deletion strain (Δ*QCR8*), and the mutant strains (F46C, R51S, P62V, G64A, W69-stop, and R91N). Cells were inoculated in 5 ml of YPD medium and allowed to grow at 30°C overnight, inoculated into 800 ml of YPGal medium, and incubated for 48 h at 30°C. The cells were harvested, washed by centrifugation, and resuspended in 5 ml of 0.4 M mannitol, 50 mM Tris, 2 mM EDTA, pH 7.4. An equal volume of 0.5 mm diameter glass beads was added to the suspension, and the cells were disrupted using a Vortex mixer at maximum speed for ten 1-min periods, alternating with incubation on ice for 1 min. Mitochondrial membranes were isolated by differential centrifugation, washed, and resuspended in 150 mM KCl, 25 mM K_2HPO_4, pH 7.

Optical Absorption Spectroscopy and Activity Measurements

Optical spectra were recorded on an Aminco DW-2a spectrophotometer in the split-beam mode, and the analog signal was sampled with a MacADIOS II™ Nubus board from GW instruments (Somerville, Massachusetts) installed in an Apple Macintosh IIvx computer connected through a MacADIOS ABO breakout box. Data were synchronized with the monochromator by a signal generated from a stroboscope wheel mounted onto the drive of the monochromator dial; 500 data points were collected every 0.1 nm at a frequency of 144 kHz and averaged to give one point of the spectrum. The 12-bit A/D board was programmed, and data were analyzed using TurboDriver and Superscope II™ software (Version 1.15, GW instruments).

Mitochondrial membranes were suspended at 0.8–1 μM cytochrome *b* in 0.65 M sorbitol, 10 mM KH_2PO_4, 2 mM EDTA, 0.1 M $MgCl_2$, 0.3 % bovine serum albumin, pH 6.5, and the succinate plus antimycin-reduced or dithionite-reduced minus ferricyanide-oxidized spectra were recorded. Electron transfer activities were measured in 150 mM KCl, 25 mM K_2HPO_4, pH 7, containing 9 mM KCN. Ubiquinol–cytochrome *c* reductase, succinate–cytochrome *c* reductase, and NADH–cytochrome *c* reductase were measured with 50 μM cytochrome *c* and 150 μM decyl-ubiquinol, 20 mM succinate, or 0.2 mM NADH, respectively. Succinate-DCPIP reductase and NADH-DCPIP reductase were measured with 20 mM succinate or 0.2 mM NADH, respectively, and 150 μM DCPIP. Activities were recorded in the dual-wavelength mode by following the reduction of cytochrome *c* at 550 versus 540 nm or reduction of DCPIP at 600 nm. Succinate–ubiquinone reductase was activated by preincubating the membranes for 10 min at 30°C in 150 mM KCl, 25 mM K_2HPO_4, pH 7, containing 20 mM succinate and 4 mM KCN.

Computer Analysis of Protein Structure

The mean α-helical hydrophobic moment (μH) and the mean hydrophobicity (H_i) were calculated according to the procedure of Eisenberg *et al* (Eisenberg *et al.*, 1982; Brasseur, 1988). A segment of 11 amino acids was then moved through the protein sequence and the hydrophobicity and mean hydrophobic moment per segment were calculated. μ_H was plotted versus H_i for all possible segments except those containing a proline residue, since this residue has a long helix-breaking capacity (Eisenberg *et al.*, 1982; Brasseur, 1988). These two parameters were also plot-

ted as a function of the midpoint of the amino acid segment along the sequence.

The most stable conformations, corresponding to the lowest energy levels, were calculated for the protein segments through a systematic calculation of the torsional angles ϕ and ψ for the residues of the linker peptides using the stereoalphabet procedure. The conformation with the lowest energy level was subsequently derived using a simplex minimization procedure. The lowest energy structure was calculated as the sum of the London–Van der Waals energy, the Coulomb's electrostatic energy, and the potential energy of rotation of torsional angles. Calculations were performed on an Olivetti CP486 microcomputer equipped with an Intel 80486 coprocessor, using the PC-PROT (Protein Analysis program) and PC-TAMMO (Theoretical Analysis of Molecular Membrane Organization) programs.

RESULTS

Analysis of Subunit 8 Secondary Structure

In order to identify amino acids for site-directed mutagenesis we used a secondary structure prediction algorithm to examine the putative secondary structure of subunit 8 as shown in Fig. 1. This algorithm has previously been used to predict secondary structures for cytochrome *b* of the cytochrome bc_1 complex (Brasseur, 1988), apolipoprotein A-IV (Lins *et al.*, 1993), and apolipoprotein A-I (Brasseur *et al.*, 1990). The algorithm predicts a strongly hydrophobic region in the form of a transmembranous α helix between residues 57–70 and an adjacent amphiphilic α helix, including residues 41–55, which may penetrate the membrane and form an L structure with the transmembranous helix. Finally, two domains likely to be involved in protein–protein interactions were detected between position 48 to 56 (domain 1) and 79 to 94 (domain 2). Previous studies have suggested that subunit 8 spans the membrane, with the C-terminus on the positive side and the N-terminus on the negative side of the membrane (Hemrika *et al.*, 1993; Schoppink *et al.*, 1989).

The sequence of the *QCR8* encoded protein is 22% identical to the bovine 9.5-kDa protein homologue, and if conservative substitutions are allowed, the two proteins are 53% homologous (Hemrika, 1994). Although the sequence identity of the yeast and bovine subunits is only marginally significant, the predicted secondary structures are very similar, as shown in Fig. 1. The predicted structures of both proteins include an amphiphilic helix immediately followed by a transmembranous helix, and the distribution of charges at

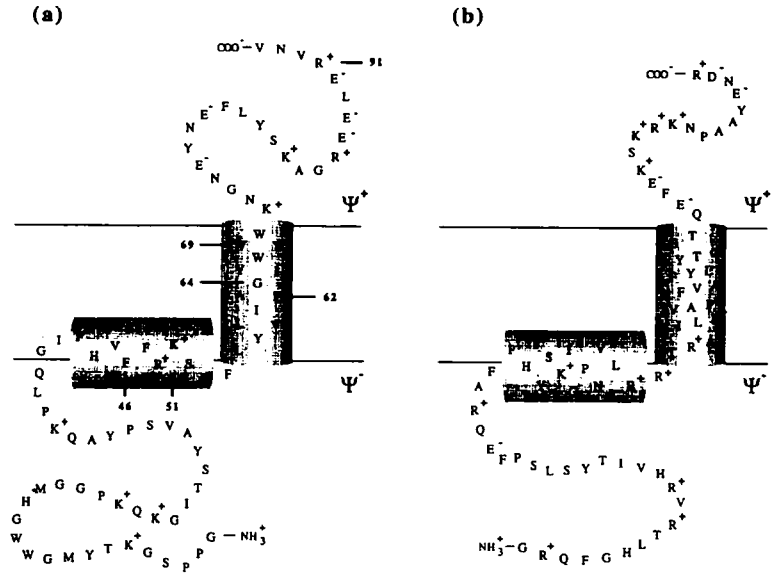

Fig. 1. Predicted secondary structures of yeast subunit 8 (a) and bovine subunit 7 (b). Both proteins are predicted to contain a single transmembranous α-helix and an amphiphilic helix located at the electronegative surface of the membrane. Amino acids that were changed by site-directed mutagenesis are numbered. The bovine subunit 7 structure is based on that proposed by Borchart *et al.* (1986).

the two surfaces of the membrane are also nearly identical. The only difference between the two predicted structures is that the algorithm used by Borchart *et al.* (1986) predicts C and N-terminal helices for the bovine subunit, which are not predicted for the yeast protein.

Since protein interacting domains have been shown to be good mutagenesis targets (Loof *et al.*, 1986; Le *et al.*, 1988), we constructed mutation R91N, which abolishes 50% of domain 2 structure, and R51S, which abolishes 85% of domain 1 structure. Replacement of F46 by a cysteine impairs the hydrophobicity of the N-terminus of the amphiphilic helix and most likely removes the F41HNAVF46 segment from it. The proline residue in the transmembranous helix was also considered as a good mutagenesis target and was changed into valine. Finally, two more mutations were selected: G64A, which should not dramatically modify subunit 8 structure, and W69-stop, which removes the C-terminus of the protein.

Deletion of *QCR8* Results in a Respiratory-Deficient Phenotype

QCR8 was deleted by replacing the genomic *QCR8* gene with the *URA3* gene as a selectable marker. Tetrad spores were plated on fermentable and nonfermentable carbon sources to test for respiratory competence and on a synthetic minimal medium (SD) lacking uracil to follow the segregation of the *QCR8* deletion locus. A total of 25 spores was dissected and the Ura^+/Ura^- spores segregated in a 2:2 fashion. As previously shown by Maarse *et al.* (1988), the haploid *QCR8* deletion strain is unable to grow on nonfermentable carbon sources, and no cytochrome *b* from the bc_1 complex is spectrally detectable in mitochondrial membranes from the deletion strain (results not shown).

Site-directed mutagenesis of the *QCR8* open reading frame was performed to construct the mutants F46C, R51S, P62V, G64A, Trp69, W69-stop, and R91N. The effect of these mutations was tested by expressing the corresponding mutated *QCR8* in the *QCR8* deletion strain.

All of the mutants, except W69-stop, grew with the same characteristics as the parental strain on a nonfermentable carbon source at 30 and 37°C. The W69-stop mutant presented the same phenotype as the *QCR8* deletion strain, no respiratory activity, no spectrally detectable bc_1 complex cytochrome *b*, and no growth on nonfermentable carbon sources.

Optical Properties of the Subunit 8 Mutants

Optical spectra scanned from 500–620 nm of mitochondrial membranes from the F46C, R51S, P62V, G64A, and R91N mutants grown on galactose revealed a slight decrease in cytochromes *b* and c_1 when compared to spectra from the parental strain YPH500, while the cytochrome aa_3 absorption was unchanged (results not shown).

The Subunit 8 Point Mutations Do Not Impair the bc_1 Complex Activity

To investigate the role of subunit 8 in the bc_1 complex, ubiquinol–cytochrome *c* reductase activities were measured on mitochondrial membranes from the F46C, R51S, P62V, G64A, and R91N mutant strains and the parental strain, YPH500. As shown in Fig. 2, none of the mutations impair bc_1 complex activity; rather, when expressed as turnover numbers related to the cytochrome c_1 content of the membranes, the activities of the mutant bc_1 complexes are 110–160% that of the parental strain.

Presteady-state reduction kinetics of cytochrome b_H were also examined with these membranes, and no difference was seen between the parental and the mutants strains, whether reduction was through center P in the presence of antimycin or through center N in the presence of myxothiazol (results not shown). In

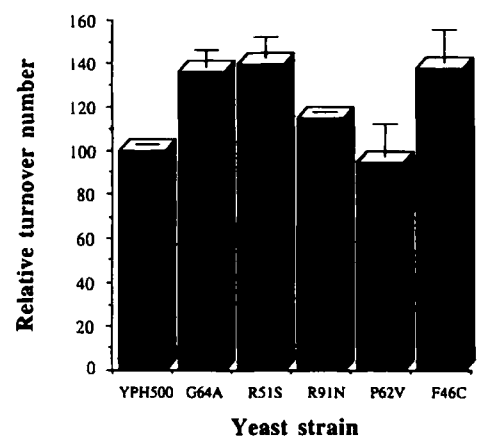

Fig. 2. Ubiquinol–cytochrome *c* reductase activity is increased in mitochondrial membranes of the F46C, R51S, G64A, and R91N subunit 8 mutant strains. Mitochondrial membranes were isolated from yeast strains grown on YPGal at 30°C. The histogram shows the turnover numbers of the bc_1 complex in the F46C, R51S, P62V, G64A, and R91N mutant strains expressed as a percentage of the YPH500 parental strain activity.

view of the increased bc_1 complex turnover numbers in some of the mutants (see above), these rates might be higher in the mutants but not detectable in our experiments due to the relatively slow mixing time in the spectrophotometer cuvette. The titration curves for inhibition of cytochrome *c* reductase activity by antimycin and myxothiazol were also identical in the mutant and parental strains (results not shown). We thus conclude that the structure of the bc_1 complex at centers N and P is unaffected by any of these mutants.

Succinate–Ubiquinone Reductase Activity Is Impaired by Mutations in Subunit 8 of the bc_1 Complex

We tested NADH and succinate–cytochrome *c* reductase activities in the mutant and parental mitochondrial membranes. NADH–cytochrome *c* reductase activity is not impaired by the subunit 8 mutations (results not shown). However, as shown in Fig. 3, there is a 30% decrease in succinate–cytochrome *c* reductase activity. Although the extent of this decrease is small, it occurs consistently with all five of the site-directed mutations in which the bc_1 complex is assembled.

Since the diminished succinate–cytochrome *c* reductase activity could not be attributed to an effect on the bc_1 complex, we examined the activity of succinate–ubiquinone reductase complex by measuring succinate–DCPIP reductase activity of the mutant and parental mitochondrial membranes. We did not include PMS as a mediator in these experiments, so that the activities depend on the activity of the ubiquinone reductase site in the succinate–ubiquinone reductase complex. Also, in these experiments the membranes were preincubated at 30°C in the presence of succinate to fully activate succinate–ubiquinone reductase complex. Under these conditions there is an average 50% decrease in succinate–ubiquinone reductase activity for all of the subunit 8 mutants, as shown in Fig. 4.

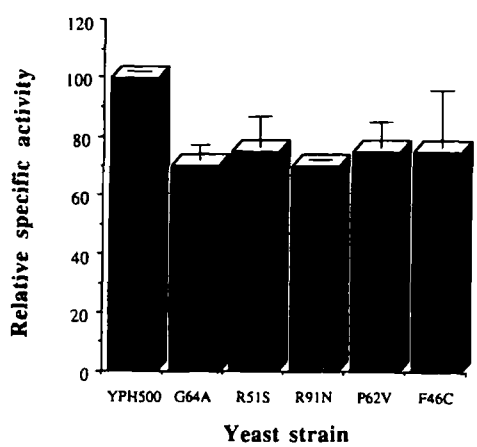

Fig. 3. Succinate–cytochrome *c* reductase activity is decreased in mitochondrial membranes isolated from the subunit 8 mutant strains. The histogram shows the succinate–cytochrome *c* reductase activities of the F46C, R51S, P62V, G64A, and R91N mutant strains relative to that of the parental strain YPH500.

Activation of Succinate–Ubiquinone Reductase Complex Is Slowed by Mutations in Subunit 8 of the bc_1 Complex

Succinate–ubiquinone reductase activity can be activated by preincubation of mitochondrial membranes with succinate (Ackrell *et al.*, 1978). To test the possibility that the differences in cytochrome *c* reductase activities of the mutant and parental membranes might reflect different extents of activation of succinate–ubiquinone reductase, we examined succinate–cytochrome *c* reductase activities of the mutant and parental strains before and after activation of the succinate–ubiquinone reductase complex as shown in Fig. 5. If the membranes are not preincubated with succinate, the rates of cytochrome *c* reduction by the membranes from the YPH500 parental strain and the

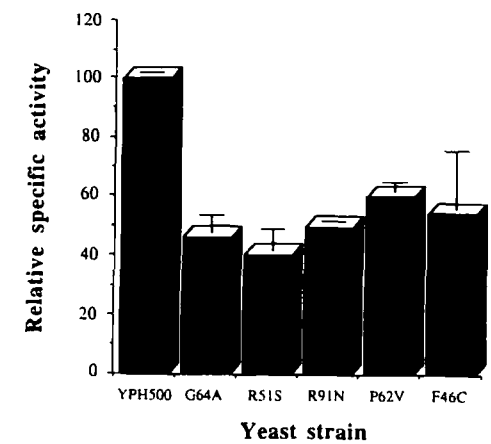

Fig. 4. Succinate–ubiquinone reductase activity is decreased in mitochondrial membranes isolated from the subunit 8 mutant strains. Succinate–ubiquinone reductase activities were measured on mitochondrial membranes isolated from the parental strain YPH500 and the subunit 8 mutant strains F46C, R51S, P62V, G64A, and R91N. The membranes were activated by preincubation with succinate as described under Experimental Procedures. The results are expressed as a percentage of the parental strain activity after normalization to the quantity of protein used for the assay.

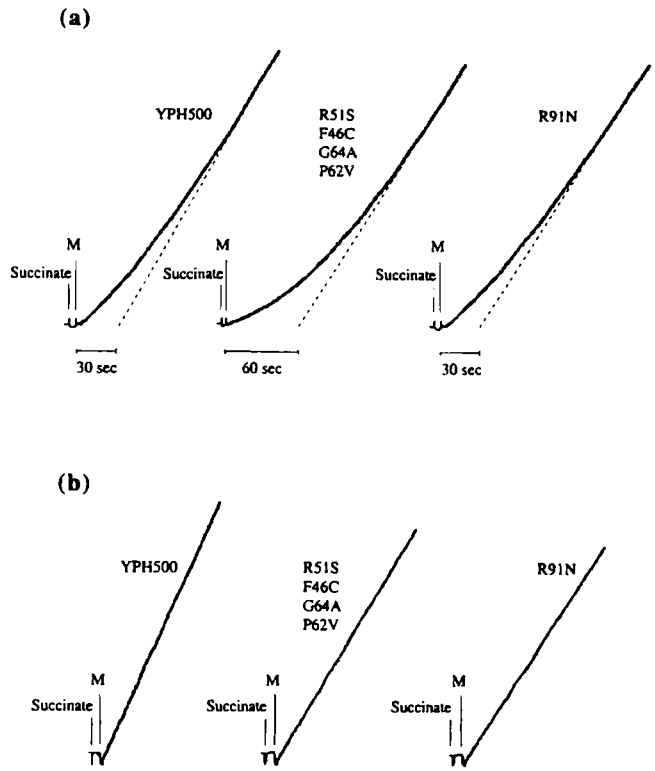

Fig. 5. Activation of succinate–ubiquinone reductase by succinate is slowed by mutations in subunit 8 of the cytochrome bc_1 complex. The traces in (a) show the succinate–cytochrome *c* reductase activities of mitochondrial membranes from the parental strain YPH500 and the subunit 8 mutant strains in which the succinate–ubiquinone reductase complex has not been activated. The dashed lines are extrapolations of the linear portions of the cytochrome *c* reduction rates, showing the times required for activation. The traces in (b) show the succinate–cytochrome *c* reductase activities of the mitochondrial membranes from the same yeast strains after activation with succinate. Reduction of cytochrome *c* was measured at 550–540 nm after addition of succinate and mitochondrial membranes (M).

R91N mutant increase for approximately 30 sec before attaining a constant, linear rate as shown in Fig. 5a. Under these same conditions the membranes from the F46C, R51S, P62V, and G64A mutants exhibit slower initial rates of cytochrome *c* reduction and do not attain their maximum rate for approximately 60 sec (Fig. 5a). This lag phase, which is approximately twice as long in the latter four mutants, corresponds to the time required to activate succinate–ubiquinone reductase complex by the succinate in the assay.

When the complex is activated with succinate before the cytochrome *c* reductase assay, the initial lag phase is eliminated and cytochrome *c* reduction proceeds in a linear manner as soon as the mitochondrial membranes are added to the sample, in both the parental and mutant strains, as shown in Fig. 5b. Under these conditions, where the activity is maximally activated by succinate, there is still a 25–30% decrease in the rates of cytochrome *c* reduction by the mutant membranes. Similarly, the rates of succinate–ubiquinone reductase were 40–50% lower in the mutants after activation of the membranes by preincubation with succinate (results not shown).

DISCUSSION

Subunit 8 of the yeast bc_1 complex shows a low degree of sequence identity but a high degree of secondary structure homology to the 9.5-kDa subunit 7

of the bovine bc_1 complex. A similar low sequence but high structural similarity was found between subunit 10 of yeast bc_1 complex and the homologous bovine subunit 11 (Brandt *et al.*, 1994). Conservation of structure therefore appears to be more important than sequence identity to the function of two of the supernumerary subunits of the bc_1 complex. A notable structural feature of subunit 8 and its bovine counterpart is that both proteins have peripheral domains which are predicted to be involved in protein–protein interactions. Our results suggest that this subunit interacts with one or more proteins of the succinate–ubiquinone reductase complex.

Deletion of subunit 8 results in an absence of bc_1 complex, and it was therefore proposed that this subunit is either required for the assembly of the bc_1 complex or for stabilization of the enzyme once assembly has occurred (Hemrika *et al.*, 1993; Hemrika, 1994). A similar lack of bc_1 complex is observed when some of the other supernumerary subunits are deleted (Trumpower, 1990). The loss of bc_1 complex resulting from deletion or mutation of a subunit does not necessarily indicate that the missing subunit is an assembly factor. Alternatively, it may be that the particular subunit is inserted into the complex early in the assembly pathway, and deletion of the subunit blocks assembly because addition of subsequent subunits requires the presence of the missing subunit. By this reasoning subunits which are inserted early in the assembly pathway would have a more deleterious effect on assembly than would subunits which are inserted late in the pathway.

Whereas the presence of subunit 8 is essential to stable assembly of the bc_1 complex, none of the site-directed mutations we constructed inhibits activity of the complex; rather several of the mutations resulted in increased ubiquinol–cytochrome *c* reductase activity. A possible explanation for this effect is discussed below. We also performed random mutagenesis of the cloned *QCR8* by low-stringency PCR but were unable to isolate any mutants which grew slowly on nonfermentable carbon sources. Hemrika (1994) similarly reported that no missense mutations resulting in a single amino acid change and affecting bc_1 complex activity were obtained after chemical mutagenesis of the cloned gene. These negative results suggest that subunit 8 is not directly involved in ubiquinol–cytochrome *c* reductase activity.

Our results indicate that mutations in a subunit of the cytochrome bc_1 complex inhibit succinate–ubiquinone reductase activity. The fact that partial inhibition of succinate–ubiquinone reductase activity has no detectable effect on growth of yeast on nonfermentable carbon sources suggests that this activity is not limiting for respiration or that the capacity of the respiratory chain is not limiting for nonfermentative growth. The observed inhibition of succinate–ubiquinone reductase activity may indicate that subunit 8 of the cytochrome bc_1 complex is required in some manner for assembly or stability of succinate–ubiquinone reductase complex, or it may indicate that the cytochrome bc_1 complex participates in electron transfer from succinate to ubiquinone *in situ*.

That mutations in the cytochrome bc_1 complex might inhibit electron transfer from succinate to ubiquinone is consistent with previous observations that succinate–duroquinone reductase activity is inhibited by antimycin (Chen *et al.*, 1986; Yu and Yu, 1982), which binds specifically to the bc_1 complex. In addition, our finding that the mutations retard the activation of succinate–ubiquinone reductase activity by preincubation with succinate supports the possibility that the mutations in subunit 8 of the bc_1 complex alter the activity of existing succinate–ubiquinone reductase molecules, rather than affecting assembly thereof. Since all of the site-directed mutants lowered succinate–cytochrome *c* reductase activity to a similar extent, but none abolished the activity, it appears that the interaction between cytochrome bc_1 complex and succinate–ubiquinone reductase complex facilitates, but is not essential for, electron transfer from succinate to ubiquinone.

The effects of the subunit 8 mutations cannot be attributed to a lower amount of succinate–ubiquinone reductase, since this would not account for the diminished rate of activation of the succinate–ubiquinone reductase and cytochrome *c* reductase activities by succinate. It is also unlikely that subunit 8 reversibly dissociates from the bc_1 complex and thus interacts with succinate–ubiquinone reductase complex, since subunit 8 is an integral membranous subunit which remains tightly associated with cytochrome *b* of the bc_1 complex, even when the complex is dissociated into subcomplexes (Schägger *et al.*, 1986). We thus conclude that the cytochrome bc_1 complex physically interacts with succinate-ubiquinone reductase complex and that subunit 8 of the bc_1 complex is required for this interaction.

The activation, as opposed to an expected inhibition, of ubiquinol-reductase activity by the subunit 8 mutations is also consistent with an interaction between the cytochrome bc_1 complex and succinate–

ubiquinone reductase complex. The ubiquinol–cytochrome *c* reductase assay measures the oxidation of the ubiquinol pool by the bc_1 complex. If the bc_1 complex interacts with succinate–ubiquinone reductase, it would not be surprising if this interaction favored oxidation of the endogenous, bound ubiquinol at the expense of oxidation of the ubiquinol pool. Thus, mutations in the bc_1 complex which prevent interaction between the two complexes might be expected to enhance oxidation of the ubiquinol pool. Consistent with this explanation is the fact that the increases in ubiquinol–cytochrome *c* reductase activities are quantitatively reciprocal to the decreases in succinate–ubiquinone reductase activities.

There have been numerous reports suggesting an interaction between succinate–ubiquinone reductase and cytochrome bc_1 complex. As noted above, succinate–duroquinone reductase activity is inhibited by antimycin (Chen *et al.*, 1986; Yu, and Yu, 1982), and this same inhibitor stabilizes the ubisemiquinone radical which is bound to the succinate-ubiquinone reductase complex (Ohnishi and Trumpower, 1980). The increased rotational correlation time of spin-labeled cytochrome bc_1 complex when it is mixed with succinate–ubiquinone reductase complex (Thomas *et al.*, 1976) and the less than additive exothermic enthalpy change of thermodenaturation of protein-phospholipid vesicles containing both succinate–ubiquinone reductase and ubiquinol–cytochrome *c* reductase (Gwak *et al.*, 1986) also indicate a direct physical interaction between these two complexes.

It has been proposed that activation of succinate–ubiquinone reductase involves a conformational change in the enzyme which facilitates removal of oxaloacetate from the active site of the dehydrogenase and alters the electronic environment around iron-sulfur center S-2 (Ackrell *et al.*, 1978). The F46C and R51S mutations, which are predicted to be in an amphiphilic helix at the negative side of the membrane, and the P62V and G64A mutations, which are predicted to be in the transmembrane helix, decrease the rate of activation of succinate–ubiquinone reductase activity by succinate. These results suggest that these regions of subunit 8 interact with the succinate–ubiquinone reductase complex and retard the conformational change involved in the activation. The R91N mutation, which is predicted to be on the positive side of the membrane, does not affect the activation of succinate–ubiquinone reductase.

Interaction of succinate–ubiquinone reductase complex with the cytochrome bc_1 complex would allow ubiquinol or ubisemiquinone that is formed in the ubiquinone reductase complex to directly reduce ubiquinone or ubisemiquinone in the cytochrome bc_1 complex, without obligatory oxidation–reduction of the ubiquinone pool in the membrane. Mitochondrial succinate–ubiquinone reductase complex contains four subunits. A 13-kDa subunit and a 15-kDa subunit are embedded in the inner membrane and form the ubiquinone reductase, or QPs, domain, and a 27-kDa subunit and a 70-kDa subunit are located in the matrix and form the succinate dehydrogenase, or SDH, domain (Yu and Yun, 1981).

Our results suggest that subunit 8 of the cytochrome bc_1 complex interacts with one or both of the QPs subunits of succinate–ubiquinone reductase complex and is proximal to the site of ubiquinone reduction in the cytochrome bc_1 complex. Support for the latter suggestion comes from the finding that the bovine homologue of subunit 8 reacts with a photoaffinity–labeled analog of ubiquinone and is thought to constitute the ubiquinone reductase site in the bc_1 complex on the negative side of the mitochondrial membrane (center N), along with cytochrome *b* of the bc_1 complex (Yu and Yu, 1982; Ohnishi and Trumpower, 1980).

ACKNOWLEDGMENT

This work was supported by NIH Grant GM 20379.

REFERENCES

Ackrell, B. A. C., Kearney, E. B., and Singer, T. P. (1978). *Methods Enzymol:* **LIII part D**, 466–495.

Borchart, U., Machleidt, W., Schägger, H., Link, T. A., and von Jagow, G. (1986). *FEBS Lett.* **200**, 81–86.

Brandt, U., Uribe, S., Schägger, H., and Trumpower, B. L. (1994). *J. Biol. Chem.* **269**, 12947–12953.

Brasseur, R. (1988). *J. Biol. Chem.* **263**, 12571–12575.

Brasseur, R., Meutter, J. D., Vanloo, B., Goormaghtigh, E., Ruysschaert, J. M., and Rosseneu, M. (1990). *Biochim. Biophys. Acta* **1043**, 245–252.

Chen, M., Liu, B.-L., Gu, L.-Q., and Zhu, Q.-S. (1986). *Biochim. Biophys. Acta* **851**, 469–474.

Eisenberg, D. (1984). *Annu. Rev. Biochem.* **53**, 595–623.

Eisenberg, D., Weiss, R. M., and Terwilliger, T. C. (1982). *Nature* **299**, 371–374.

Gwak, S.-H., Yu, L., and Yu, C.-A. (1986). *Biochemistry* **25**, 7675–7682.

Hemrika, W. (1994). *Yeast Ubiquinol–Cytochrome c Oxidoreductase: Studies on the Location and the Function of the 14-kDa and the 11-kDa Subunits.* Ph.D. thesis, University of Amsterdam, The Netherlands.

Hemrika, W., Berden, J. A., and Grivell, L. A. (1993). *Eur. J. Biochem.* **215**, 601–609.

Jones, J. S., and Prakash, L. (1990). *Yeast* **6**, 363–366.

Le, L., Brasseur, R., Wemers, C., Meulemans, G., and Burny, A. (1988). *Virus Genes* **1**, 333–350.
Lins, L., Brasseur, R., Rosseneu, M., Vanloo, B. and Ruysschaert, J.-M. (1993). *Biochim. Biophys. Acta* **1149**, 267–277.
Loof, H. D., Rosseneu, M., Brasseur, R., and Ruysschaert, J. M. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 2295–2299.
Maarse, A. C., and Grivell, L. A. (1987). *Eur. J. Biochem* **165**, 419–425.
Maarse, A. C., Haan, M. D., Schoppink, P. J., Berden, J. A., and Grivell, L. A. (1988). *Eur. J. Biochem.* **172**, 179–184.
Ohnishi, T., and Trumpower, B. L. (1980). *J. Biol. Chem.* **255**, 3278–3284.
Schägger, H., Link, T. A., Engel, W. D., and Von Jagow, G. (1986). *Meth. Enzymol.* **126**, 224–237.
Schoppink, P. J., Jong, M. D., Berden, J. A. and Grivell, L. A. (1989). *Eur. J. Biochem.* **181**, 681–687.
Sherman, F., and Hicks, J. (1991). *Meth. Enzymol.* **194**, 21–37.
Southern, E. M. (1975). *J. Mol. Biol.* **98**, 503–517.
Thomas, D. D., Dalton, L. R., and Hyde, J. S. (1976). *J. Chem. Phys.* **65**, 3006–3024.
Treco, D. A., and Lundblad, V. (1989). In *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.) Wiley, New York, pp. 13.1.1–13.1.4.
Trumpower, B. L. (1990). *Microbiol. Rev.* **54**, 102–129.
Usui, S., Yu, L., and Yu, C.-A. (1990). *Biochemistry* **29**, 4618–4626.
Yu, C.-A., and Yun, L. (1981). *Biochim. Biophys. Acta* **639**, 99–128.
Yu, L., and Yu, C.-A. (1982). *J. Biol. Chem.* **257**, 10215–10221.