The N Terminus of the Qcr7 Protein of the Cytochrome bc$_1$ Complex Is Not Essential for Import into Mitochondria in *Saccharomyces cerevisiae* but Is Essential for Assembly of the Complex*

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Subunit 7 of the yeast cytochrome bc$_1$ complex is encoded by the nuclear QCR7 gene and is essential for respiration. This protein does not contain a cleavable N-terminal mitochondrial targeting sequence, and it is not understood how the Qcr7 protein is imported into mitochondria and assembled into the complex. To test the role of the N terminus of the Qcr7 protein in mitochondrial import, assembly of the complex, and proton translocation, we inactivated the endogenous QCR7 gene and expressed mutated qcr7 genes capable of synthesizing proteins truncated by 7, 10, 14, and 20 residues (Qcr7p-D$_7$, Qcr7p-D$_{10}$, Qcr7p-D$_{14}$, and Qcr7p-D$_{20}$, respectively) from the N terminus. In addition, we studied two mutants containing Qcr7 proteins with point mutations in addition to a D$_{7}$ truncation, Qcr7p-D$_{7}$D$_{13}$V and Qcr7p-D$_{7}$D$_{10}$R10K. All the mutant proteins with the exception of Qcr7p-D$_{10}$ were present in the mitochondria at 30 °C, although most at lower steady-state levels than the Qcr7p from the strain overexpressing wild type QCR7. The absence of the Qcr7p-D$_{10}$ may be the result of an unstable protein or a decrease in the efficiency of mitochondrial import due to its compromised amphipathic a-helix and the presence of a negative charge exposed at the N terminus. Cytochrome c reductase activities and the amounts of ATP synthesized were comparable with the wild type in the strain expressing Qcr7p-D$_{7}$. The strain expressing Qcr7p-D$_{7}$(R10K) had an identical phenotype to the one containing the Qcr7p-D$_{7}$, whereas strains expressing the Qcr7p-D$_{10}$, Qcr7p-D$_{14}$, Qcr7p-D$_{20}$, and Qcr7p-D$_{7}$D$_{13}$V were all respiration-deficient. Examination of the steady-state levels of complex III subunits showed that core protein 2, cytochrome c$_1$, the iron-sulfur protein, and the 11-kDa subunit were reduced in respiration-deficient mutant strains. Results from deletion analyses indicate that the N-terminal 20 residues (after Met-1) of the Qcr7p protein are not essential for import into mitochondria and that the N-terminal seven residues (after Met-1) are not involved in proton translocation. The results of this work show, however, that the N terminus of the Qcr7 protein is essential for the biosynthesis of ubiquinol-cytochrome c reductase.

The mitochondrial respiratory chain consists of multisubunit enzyme complexes that are embedded in the inner mitochondrial membrane. Electron transport through the ubiquinol-cytochrome c reductase and cytochrome oxidase complexes in *S. cerevisiae* is coupled to vectorial H$^+$ translocation into the intermembrane space, resulting in the establishment of a H$^+$ gradient and subsequent membrane potential. The energy from this gradient is then used as the driving force for ion translocation, protein import into mitochondria, and ATP synthesis, which is catalyzed by the F$_0$F$_1$ ATPase (1, 2).

Ubiquinol-cytochrome c oxidoreductase consists of 10 subunits in yeast (2, 3). There are the three heme-containing subunits cytochrome b$_6$, cytochrome c$_1$, and the Rieske iron-sulfur protein as well as an additional six so-called supernumerary subunits whose functions remain largely unknown. The homologous complex in some prokaryotes such as *P. denitrificans* consists of only the three catalytic subunits (4) that have been conserved throughout evolution. Thus, it is possible that the supernumerary subunits do not have any functions in the energy transducing activity of the bc$_1$ complex. However, gene inactivation studies of these supernumerary subunits have shown that all are necessary for the integrity and normal functioning of complex III (5–7).

Cytochrome b$_6$ is encoded by the mitochondrial genome, the other polypeptides being nuclear encoded (8). These nuclear encoded subunits are targeted for import into mitochondria after synthesis on cytoplasmic ribosomes. Thus there is a requirement for the coordination of the two separate genetic systems for assembly of the bc$_1$ complex.

QCR7, the yeast gene encoding the 14.5-kDa polypeptide, has been cloned and sequenced and was found to be homologous to the 13.4-kDa subunit of the bc$_1$ complex in beef heart mitochondria (9–11). Studies performed on the bc$_1$ complex in beef heart suggest that the N terminus of the 13.4-kDa subunit protrudes into the matrix where it has been postulated to contribute to the H$^+$ conducting pathway(s) from the matrix phase to the primary protolytic redox center (12). Proteolytic cleavage of 7–11 residues from the N terminus of this subunit was associated with decoupling of redox-linked H$^+$ pumping (13).

We have now investigated the involvement of the Qcr7p N terminus with respect to mitochondrial targeting, complex III assembly, and proton conduction. By CD spectroscopy of synthetic peptides we show that the secondary structures of the N termini of the yeast and beef heart proteins strongly resemble one another and are largely a-helical when inserted into the membrane-mimetic environment of SDS micelles. By deletion analysis we found that the N-terminal seven amino acids are not required for conducting protons and that the N-terminal 20 residues are not required for import into mitochondria. How-
ever, the N terminus does contain information required for the assembly of ubiquinol-cytochrome c oxidoreductase.

EXPERIMENTAL PROCEDURES

Materials—Amino acids, uracil, adenine, serum albumin (essentially fatty acid-free), succinate, horse heart cytochrome c, and Total Protein Reagent™ were obtained from Sigma. Yeast extract, peptone, tryptone, and yeast nitrogen base without amino acids were purchased from Difco. Most restriction and modification enzymes were from Pharmacia Biotech Inc. T7 GEN in vitro mutagenesis kit was obtained from U. S. Biochemical Corp. Yeast lytic enzyme, zymolase 100T, and galactose were purchased from ICN Pharmaceuticals. Oligodeoxynucleotides were synthesized by the Department of Clinical Biochemistry at the University of Toronto. Yeast expression vector pG3-14 (14) was a gift from Jacqueline Segall at the University of Toronto. Alkaline phosphatase substrates p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate-toluidine were purchased from Bio-Rad.

Saccharomyces cerevisiae strain W303-1B (mat a, ade 2-1, his 3-11, 15, ura 3-1, leu 2-3, 112, try p-1, can 1-100) was used as the parent strain. Rhö − strain (mat a, 1 r [rco 2321R E514]) was obtained from ATCC 2209 (donated by L. A. Grivell). Transformation of yeast was carried out by the Meso-SE-enhanced whole cell yeast transformation method (15). Transformants were selected on minimal medium containing 0.87% (w/v) yeast nitrogen base, 2% (w/v) glucose, 2% agar, 0.5% chlorate, 0.05% Tween 20, and 0.02 volumes of cold 0.1M CaCl₂ containing 15% (v/v) glycerol. After another 30-min incubation on ice, cells were aliquoted as described in Ref. 15.

Isolation of DNA—Plasmid DNA from E. coli (16) and genomic DNA from yeast (14) were isolated as described.

Competent E. coli and Yeast—E. coli strain JM3D was made competent by growing a culture to an A600 of 0.5, pelleting the cells, and resuspending them in 0.1 volumes of cold 0.1 M CaCl₂ with gentle shaking by hand. Cells were left on ice for 30 min, pelleted, and resuspended in 0.02 volumes of cold 0.1 M CaCl₂ containing 15% (v/v) glycerol. After another 30-min incubation on ice, cells were aliquoted and stored at −70 °C. Yeast were made competent for transformation as described in Ref. 15.

Construction of Plasmids—The original pG3-3 expression plasmid was modified by digestion with ScaI to excise a 1700-base pair fragment in the polylinker region and self-ligated to create the new pG3-3Δ vector. Recombinant plasmids were constructed by digesting pG3-3Δ with Sall and Kpn1 in two steps followed by ligation to an accordingly restricted QCR7 gene generated by polymerase chain reaction mutagenesis. Ligations were carried out overnight at 14 °C.

Construction of the QCR7 Disruption Strain—the coding region of the QCR7 gene was amplified by polymerase chain reaction from yeast genomic DNA and subcloned into the pCR II vector (Invitrogen, San Diego). This construct was linearized with HincII to generate one fragment, disrupted in the middle of the QCR7 gene. The LEU2 gene, isolated in the vector pF1110 by blunt-end cutting enzyme, was subsequently subcloned into this HincII site. The fragment containing the LEU2 gene flanked by the disrupted qcr7 gene was excised from the plasmid and transformed into yeast strain W303-1B. Transformants were selected for on leucine-deficient medium.

Construction of Deletion and Point Mutants—Mutants were constructed using polymerase chain reaction. For the deletion mutants, degenerate oligodeoxynucleotides were synthesized missing the bases corresponding to the N-terminal 2–8, 2–11, 2–15, and 2–21 amino acids, respectively. Point mutants were generated from the Qcr7Δ by using oligonucleotides containing the missense mutations R10K and D13V, respectively.

Sequencing of the QCR7 Gene—Mutant genes generated by polymerase chain reaction were digested with Sall and Kpn1 and subcloned into the respective sites of the pG3-3Δ vector. DNA was sequenced by the dideoxy chain termination method using the T7 Sequencing kit from Pharmacia. The sequencing primers used were in the central portion, 5'-GGGAGTCTTCTTAGGCGG-3', and in the 3' downstream region of the coding sequence, 5'-CCTGGCGACCGGTGGTTGTTGTTG-3'.

Northern Analysis—Total yeast RNA was prepared by growing a 25-ml culture to an A600 of 1.5, harvesting the cells, and resuspending them in 2 ml of AE buffer (50 mM NaAc, pH 4.8, 10 mM EDTA). The suspension was vortexed after the addition of 200 µl of 10% SDS and again after the addition of 450 µl of phenol saturated with AE buffer. The suspension was incubated at 65 °C for 5 min during which it was vortexed three times. The mixture was washed with equal volume of phenol/chloroform, and the RNA was precipitated from the supernatant with 2.5 volumes of ethanol. Northern blotting was performed as described by Fournier et al. (17).

Enzyme Assays and Spectrophotometric Analyses—NADH-cytochrome c reductase and succinate-cytochrome c reductase activities were assayed in 0.1 M potassium P₄ buffer, pH 7.0, containing 94 µM cytochrome c and 1 mM azide. To start the reaction, either 34.2 µM NADH or 10 mM succinate was added, and reduction of cytochrome c was monitored at 550 nm. Cytochrome oxidase activities were assayed in 0.1 M potassium P₄ buffer, pH 7.0. Cytochrome c was reduced with 3.2 molarolate followed by dithionates against 0.1 mM potassium P₄ for 2 days, during which the buffer was changed twice. Reduced cytochrome c (94 µM) was added to start the reaction and oxidation of cytochrome c was followed at 550 nm. For spectral analyses of the cytochromes, mitochondria were resuspended in 0.1 mM potassium P₄, pH 7.4, 0.25 mM succrose, 0.5% cholic acid (10). To obtain a spectrum containing cytochromes c and c₁, cytochrome b, and cytochromes a and a₁, a ferricyanide (grains) oxidized spectrum was deducted from a dithione (grains) reduced spectrum. To obtain a spectrum containing cytochrome b only, dithio- nite reduced minus ascorbate (grains)-N,N,N,N'-tetramethyl-p-phenylendiamine (0.2 mM) reduced samples were run. Spectra were recorded on a DW-2a Aminco spectrophotometer from 520–620 nm.

Western Analyses—SDS-polyacrylamide gel electrophoresis was run according to the published procedure (16). Proteins were transferred for 2 h at 55 V at 4 °C in 1 × running buffer (3.02 g of Tris, 14.4 g of glycerine/liter), 0.1% SDS, and 20% methanol. Blots were subsequently blocked with 2% gelatin in Block A (10 g Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h and then incubated overnight with primary antibody in Blotto containing 1% gelatin. Blots were washed 4 × 30 min in Blotto and then incubated for 1–2 h under the above conditions with a secondary antibody coupled to alkaline phosphatase. Membranes were washed 4 × 15 min in Blotto, and the proteins were visualized using p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate-toluidine as substrates in 0.1 M NaHCO₃-1 mM MgCl₂.

Mitochondrial Isolations— Cultures were grown for 2 days in synthetic medium containing a variety of different carbon sources. Mitochondria were isolated essentially as outlined (14) with the substitution of breeder breaking buffer (0.6 m sucrose, 20 mM HEPES-KOH, pH 6.5, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride) for menthol. Isolated mitochondria were suspended in breaking buffer without phenylmethylsulfonyl fluoride (0.6 m sucrose, 20 mM HEPES-KOH, pH 6.5, 0.1% bovine serum albumin).

ATP Synthesis Assays—Freshly prepared mitochondria were resuspended in 200 µl of breaking buffer without phenylmethylsulfonyl fluoride. To start the reaction, 5 m potassium P₄, pH 7.4, 1 mM ADP, and 5 mM sucinate were freshly added. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped with 80 mM perchloric acid. Proteins were pelleted, and the supernatant was assayed for the amount of ATP synthesized by using a hexokinase/glucose-6-phosphate dehydrogenase coupled assay (18), and the NADPH generated was measured by an Eppendorf fluorimeter.

CD Spectra—Peptides were chosen as follows from the N terminus of the Qcr7 protein in yeast and the homolog in beef heart synthesized by the Alpha Peptide Institute: AGRPVSASSWRL (beef heart, peptide 1), AGRPVSASSWRLGEIRKWWYNAG (beef heart, peptide 2), PQPSTSIAIGIDY (yeast peptide), and PQPSTSIAIGIDYLIK-SPVLSKL (yeast peptide, peptide 4).

For recording CD spectra, peptides were dissolved at 1 mg/ml in either 10 mM NaCl, 10 mM NaH₂PO₄·H₂O, methanol, or SDS (to a 30-fold molar excess diluted in 10 mM NaCl, 10 mM NaH₂PO₄·H₂O). Spectra were recorded on a JASCO J-720A spectropolarimeter and scanned two to three times each from 250 to 190 nm at 25 °C. Base-line spectra for each solvent were subtracted from the peptide spectra.

RESULTS

Characterization of Strain YSM-qcr7Δ Containing the qcr7 Gene Disruption—The yeast strain YSM-qcr7Δ, which contains the chromosomal qcr7ΔLEU2 disruption, lacks a mRNAs transcript for the qcr7 gene (results not shown). The absence of mRNA indicates that a fusion transcript containing the LEU2 gene within the disrupted qcr7 coding region is not synthesized. In a stable transformant, the mRNAs transcript corresponding to the 5' end of the qcr7 gene. A cDNA clone of approximately 450 bases can be seen in YSM-qcr7Δ when complemented with QCR7 on the expression vector pG3-3A (not shown).

Immunoblotting with an antibody recognizing the core proteins, the iron-sulfur protein and the 17-, 14-, and 11-kDa subunits, showed that no Qcr7 protein is present in mitochondria.
drial membranes isolated from YSM-qcr7Δ (Fig. 1, lane 5). A band, however, can be seen in Fig. 1 (lane 6) corresponding to the Qcr7p from the strain YSM-qcr7Δ complemented with the wild type QC7 gene. This indicates that the Qcr7p is being produced from the expression plasmid and imported into mitochondria.

Circular Dichroism Spectra of N-terminal Peptides—Link et al. (11) have previously suggested that the N terminus of the 13.4-kDa subunit of complex III from beef heart mitochondria forms an amphipathic α-helix. To confirm this helicity and to compare the yeast N terminus of the Qcr7 protein with its beef heart homolog, two peptides corresponding to amino acids 2–16 and 2–26, respectively, were probed with monoclonal antibodies raised against each of these subunits. Lane 1, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ14; lane 2, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ20; lane 3, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ10; lane 4, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ7; lane 5, protein from YSM-qcr7Δ; lane 6, protein from YSM-qcr7Δ overexpressing wild type Qcr7 protein, cytc1, cytochrome c1, ISP, iron-sulfur protein; i, intermediate processed forms of ISP or cytochrome c1.

FIG. 1. Composite of Western blot analyses of mitochondrial proteins from YSM-qcr7Δ strains overexpressing wild type and N-terminally truncated proteins Qcr7p-Δ7, Qcr7p-Δ10, Qcr7p-Δ14, and Qcr7p-Δ20. Mitochondrial proteins (100 μg) were dissolved in SDS-polycrylamide gel electrophoresis buffer containing dithiothreitol and heated for 3 min at 95 °C. Samples were run on 16% polycrylamide gels and then transferred to nitrocellulose membranes. The blot containing core proteins 1 and 2 and the blot containing the 17-, the 14-, and the 11-kDa subunits were probed with antibodies detecting all those subunits as well as the iron-sulfur protein. The two blots containing cytochrome c1 and the iron-sulfur protein, respectively, were probed with monoclonal antibodies raised against each of these subunits. Lane 1, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ14; lane 2, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ20; lane 3, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ10; lane 4, protein from YSM-qcr7Δ overexpressing Qcr7p-Δ7; lane 5, protein from YSM-qcr7Δ; lane 6, protein from YSM-qcr7Δ overexpressing wild type Qcr7 protein, cytc1, cytochrome c1, ISP, iron-sulfur protein; i, intermediate processed forms of ISP or cytochrome c1.

The N Terminal of the Qcr7 Protein of the Cytochrome bc1 Complex—When examining the levels of the other subunits in the complex (Fig. 1), it can be seen that core protein 1 is

lipid bilayer (21–23).

The Qcr7 protein does not contain a cleavable N-terminal mitochondrial targeting sequence. However, given the α-helical nature of the wild type Qcr7p N terminus and its potential for forming an amphipathic helix (Fig. 3), we decided to investigate whether this region contains any information for the mitochondrial localization of the protein. Accordingly, we constructed four deletion mutants in which residues 2–8, 2–11, 2–15, and 2–21 (Qcr7p-Δ7, Qcr7p-Δ10, Qcr7p-Δ14, and Qcr7p-Δ20, respectively) were deleted from the N terminus (Fig. 3). In each case the initial Met (residue 1) was retained; however, a number of residues typical for import sequences were deleted. When expressing the Qcr7p-Δ7, the Qcr7p-Δ14, and the Qcr7p-Δ20 in the strain YSM-qcr7Δ at 30 °C, the respective, truncated Qcr7 proteins are synthesized and transported into mitochondria, as can be seen from the Western analysis in Fig. 1 (lanes 1, 2, and 4). However, it is evident that the amount of protein present in the mitochondria is lower in yeast transformed with the deletion mutants than in yeast transformed with the wild type gene. It is also obvious that in yeast transformed with the gene encoding Qcr7p-Δ10, the protein is not present in the mitochondria (Fig. 1, lane 3). This protein may be unstable and degraded in the cytoplasm or in the mitochondria.

Characterization of Deletion Mutants by Growth—Growth studies were performed at 30 °C on two types of solid media to determine whether any of the mutants are respiration-deficient. Fig. 4 (top) shows that when grown on synthetic deficient medium with ethanol/glycerol containing 0.1% glucose, the yeast Qcr7p-Δ7 is comparable in size with the wild type, whereas YSM-qcr7Δ as well as yeast strains containing Qcr7p-Δ10, Δ14, and Δ20 are pet mutants, indicative of a respiratory chain defect. Similarly, when grown on synthetic deficient medium containing glucose as the sole carbon source (Fig. 4, bottom), the deficient deletion mutants remain white. The wild type strain has a red phenotype due to the ade2 mutation, which causes a pigment to accumulate (24). In respiration incompetent mutants, this pigment is not formed, and the cells retain a white phenotype. Growth characteristics of the strains containing the point mutants (not shown) were in agreement with the results from the enzyme activities. The strain expressing Qcr7p-Δ7(R10K) was comparable with the wild type, whereas that containing Qcr7p-Δ7(D13V) resembled the profile of the respiration-deficient deletion mutants.

Activities of Respiratory Chain Complexes—Having established that the majority of mutant proteins are present in the mitochondria, it was of interest to find out whether they assemble into a functional enzyme complex. Mitochondrial membranes from the yeast YSM-qcr7Δ were devoid of NADH-cytochrome c reductase (Fig. 5) and succinate-cytochrome c reductase activities. Cytochrome c oxidase activity was diminished by about 35% when compared with the wild type (not shown).

Strains carrying Qcr7p-Δ7 and the Qcr7p-Δ7(R10K) were assayed for respiratory chain complex activities and found to have wild type activities for NADH-cytochrome c reductase (Fig. 5) as well as succinate-cytochrome c reductase and cytochrome c oxidase (not shown). Expression of qcr7 genes encoding Qcr7p-Δ7(D13V), Qcr7p-Δ14, and Qcr7p-Δ20, which produce stable but truncated protein products that are located into mitochondria, did not restore NADH-cytochrome c reductase (Fig. 5) or succinate-cytochrome c reductase activities. Cytochrome c oxidase activities were lowered in these strains by 35% similarly as in YSM-qcr7Δ.
present at comparable levels in all the mutant strains and the wild type. Intermediate iron-sulfur and cytochrome c$_1$ proteins are present in high amounts in the wild type and in somewhat lower amounts in the strain expressing the Qcr7p-D$_7$. Strains carrying Qcr7p-D$_{14}$ and Qcr7p-D$_{20}$, however, only contain trace amounts of these intermediates, and they are not detectable in the strain with Qcr7p-D$_{10}$ and the YSM-qcr7p.

Mature iron-sulfur and cytochrome c$_1$ proteins are present in all strains, although in varying amounts. Of the strains examined in Fig. 1, yeast expressing wild type and Δ7 Qcr7 proteins contain comparable levels, whereas the remaining mutants have lower amounts. A similar pattern is seen for the 11-kDa subunit, with the highest levels in the mutants expressing wild type and Qcr7p-Δ7, and the lowest levels in the strain expressing the Qcr7p-Δ10 and the YSM-qcr7Δ.

To determine more precisely what the function of the N terminus may be, we constructed two point mutants in the context of a D$_7$ deletion. The mutations targeted two charged residues, Arg-10 (changed to Lys) and Asp-13 (mutated to Val). From examining the subunit composition of the strains containing these mutant proteins (Fig. 6), it is evident that the mutant Qcr7 proteins are all located in the mitochondria. Interestingly, in the strain expressing Qcr7p-Δ7(D13V) the level of 14-kDa subunit is comparable with the wild type, whereas in

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**FIG. 2. Circular dichroism spectra.** Peptides corresponding to the N-terminal 25 amino acids of the beef heart 13.4-kDa subunit and the N-terminal 23 amino acids of the yeast 14.5-kDa subunit were assayed for secondary structure by CD. Peptides were dissolved at a concentration of 1 mg/ml in buffer (10 mM NaCl, 10 mM NaH$_2$PO$_4$, H$_2$O), methanol, or SDS to a 30-fold molar excess (diluted in above buffer). Spectra were recorded in duplicates at 25 °C on a Jasco J-720A spectropolarimeter. *Top panels*, beef heart peptide 2; *bottom panels*, yeast peptide 4.

**FIG. 3. Helical wheel plots and Qcr7 amino acid sequence.** The N-terminal 18 residues (starting from residue 2) are plotted (top). WT, wild type. Qcr7 protein was truncated by residues 2–8 from the N terminus (Δ7). A helical wheel projection of the wild type shows that all the charged and most of the polar residues are located on one face of the helix, whereas the majority of the hydrophobic residues are located on the opposing face. A helical wheel projection of the Qcr7p-Δ7 shows that charged residues are located on one face of the helix, whereas hydrophobic and polar residues are interspersed throughout.
the strain with Qcr7p-Δ7(R10K) the level is similar to that of the yeast containing the Qcr7p-Δ7, Qcr7p-Δ14, and Qcr7p-Δ20. All the other subunits are present at comparable amounts in the yeast containing Qcr7p-Δ7(R10K) as in the yeast expressing the wild type QCR7 gene. As for the strain expressing the Qcr7p-Δ7(D13V), its subunit composition is similar to that of the Δ14 and Δ20 deletion mutants; unchanged amounts of core protein 1, however, lower amounts of mature cytochrome c₁ and the iron-sulfur protein. Furthermore, the 11-kDa subunit as well as intermediate cytochrome c₁ and iron-sulfur proteins are nearly undetectable in this strain. From the spectral analyses it is evident that the strain carrying the conservative substitution R10K displays near wild type levels of cytochrome b, whereas the strain expressing proteins with the D13V mutation contains low and undetectable amounts of cytochrome b, respectively (spectra not shown). Taken together with the results from the enzyme activities, the varying levels of complex III subunits implicate the Qcr7p N terminus in assembly.

Deletion of Seven Amino Acids from the N Terminus Does Not Impair H⁺ Pumping—Because truncation of the N-terminal seven amino acids does not impair electron transport, we were interested in determining whether this segment contributes to the proton conducting pathways from the matrix phase to the primary protolytic redox center. Studies have been performed on the homologous subunit in beef heart where cleavage of 7–11 residues from the N terminus was correlated with decoupling of redox-linked proton pumping (13). To establish a proton gradient, electron transport has to be intact; hence, of the strains tested, only Qcr7p-Δ7 produced ATP in the assay system. With
succinate as substrate, the integrity of the proton gradient that is established by complexes III and III+IV was measured. When ferricyanide was used as electron acceptor and cytochrome oxidase was inhibited by azide, the ATP produced was solely due to the action of complex III. In both cases, the yield of ATP in the mutant with Qcr7p-Δ7 was comparable with the wild type (not shown). This indicates that in yeast, unlike in beef heart, the N-terminal seven amino acids are unlikely to be involved in proton translocation.

The Mutant Containing Qcr7p-Δ7 Is Temperature-sensitive—The growth of the yeast strain expressing the Qcr7p-Δ7 displays a different profile at 37 °C when compared with the phenotype at 30 °C. At 37 °C this strain is a "pet" mutant with undetectable NADH-cytochrome c reductase activity. In this case the immunoblotting (Fig. 7) showed that the Δ7 protein was not present in the mitochondria (lane 1) in contrast to the wild type (lane 2). This result suggests that the Qcr7p-Δ7 is not sufficiently stable at 37 °C to be imported into mitochondria and therefore degraded in the cytoplasm or, alternatively, that it is imported and rapidly degraded in the mitochondria.

DISCUSSION

Previous studies of the QCR7 gene have shown that this subunit is an essential component of ubiquinol-cytochrome c oxidoreductase (10) and that the C terminus may be involved in the assembly of a functional enzyme complex (25). In the current work, we have investigated the role of the N terminus of the Qcr7 protein with respect to proton translocation, because previous studies have postulated that this subunit faces the matrix (13, 26) and is involved in the uptake of protons from the matrix (13). We were also interested in determining the importance of this region with respect to assembly of the bc1 complex and mitochondrial targeting, because the Qcr7 protein does not contain a cleavable N-terminal signal sequence but displays features characteristic such sequences. We have approached the above issues by expressing a number of point and deletion mutants in the strain YSM-qcr7Δ in which we inactivated the chromosomal QCR7 gene and through investigation of the secondary structure(s) of selected N-terminal peptides by CD spectroscopy and comparison with their beef heart homologs.

We have confirmed earlier findings of Schoppink et al. (10) that inactivating the QCR7 gene gives rise to a respiration-deficient strain. Complementation of this strain with the gene encoding Qcr7p-Δ7 restored cytochrome c reductase activities to wild type levels at 30 °C (Fig. 5) and resulted in a strain that was no longer respiration-deficient (Fig. 4). This result indicated that electron transport had been restored, and we proceeded to examine whether these N-terminal amino acids play a role in proton transfer by assaying the amount of ATP synthesized in coupled mitochondria. Efficiency of ATP synthesis in the mutant containing Qcr7p-Δ7, however, was observed to be equivalent to the wild type, and it was concluded that the involvement of the N-terminal seven amino acids in proton translocation and ATP synthesis is unlikely to be critical.

When complementing YSM-qcr7Δ with the gene encoding Qcr7p-Δ10, a substantially different profile is seen. The Qcr7p-Δ10 is not present in the mitochondria (Fig. 1), and as a result, complex III is inactive and the mutant strain is respiration-deficient. Truncation of 10 amino acids from the N terminus of the Qcr7p results in the exposure of the negative charge of Asp-13 at the front end (now residue 2 of mature Qcr7p) of the protein. It is conceivable that the exposure of this negative charge is detrimental to the import process. Mitochondrial signal sequences rarely contain negatively charged residues; however, depending on their location and orientation they may not always be as disruptive. In addition, it is not clear whether this or any of the other truncated proteins can form amphiphilic α-helices because the charged residues are more widely interspersed with hydrophobic residues and thus result in sequences uncharacteristic for amphiphilic helices. Alternatively, this result may simply imply that the protein is unstable and degraded in the cytoplasm or the mitochondria.

The phenotype of the strains containing the Qcr7p-Δ14 and Qcr7p-Δ20 is significantly different from mutants with Qcr7p-Δ7 and Qcr7p-Δ10. Strains expressing these truncated proteins form stable products that are imported into mitochondria (Fig. 1), albeit to a lesser degree than the Qcr7p from the wild type strain in which QCR7 is overexpressed. Significantly, NADH-cytochrome c reductase activities are absent, and these mutants display a pet- phenotype (Fig. 4), despite the fact that their truncated proteins are present in the mitochondria at levels comparable with those of the respiration-competent mutant containing Qcr7p-Δ7. Examination of the steady-state levels of the 11-kDa subunit, as well as the iron-sulfur protein and cytochrome c1, shows that these subunits are significantly reduced in strains with Qcr7p-Δ14 and Qcr7p-Δ20 when compared with the wild type strain.

To determine more precisely the function of the N terminus of the Qcr7p, we mutated the two charged residues Arg-10 and Asp-13 in the context of a Δ7 deletion. The strain complemented with the Qcr7p-Δ7(R10K), which contains a conservative substitution, has a phenotype comparable with that carrying the Qcr7p-Δ7 and the wild type at 30 °C. On the other hand, the strain expressing Qcr7p-Δ7(D13V) is completely devoid of NADH-cytochrome c reductase activities and displays a pet- phenotype. Furthermore, when examining the levels of complex III subunits in this mutant, it can be seen (Fig. 6) that the levels of cytochrome c1, iron-sulfur protein, and 11-kDa subunit are reduced, with the level of 11-kDa subunit being lower than in mutants with Qcr7p-Δ14 and Qcr7p-Δ20. This stands in contrast to the level of Qcr7p, which is higher in this mutant than in the others and compares with the wild type (Fig. 6). Cytochrome b spectra of strains with the Qcr7p-Δ7(R10K) and Qcr7p-Δ7(D13V) correlate with the results from the NADH-cytochrome c reductase activities; the R10K mutation resulted in a strain with nearly wild type levels of cytochrome b, whereas cytochrome b was not detectable in the strain with Qcr7p-Δ7(D13V).

Although the N-terminal 23 amino acids spontaneously assume a largely α-helical conformation (Fig. 2) when inserted into the membrane-mimetic environment of SDS micelles and there is a potential for the wild type N terminus to form an amphiphilic α-helix (Fig. 3), in strains expressing Qcr7p-Δ7 and Qcr7p-Δ7(R10K) the complex functions just as in the wild type at 30 °C. This finding suggests that the N-terminal seven amino acids are not required for the functioning of the complex.
However, in strains complemented with the Qcr7pΔ14 and Qcr7pΔ20, as well as with Qcr7pΔ7(D13V), the truncated Qcr7 protein products are imported into mitochondria at 30 °C to approximately the same level as Qcr7pΔ7, but the bc₁ complex is not functional.

All the mutants with the exception of the strain expressing the Qcr7pΔ7(D13V) contain lower than wild type levels of Qcr7p in the mitochondria. This fact may point toward a function in mitochondrial import for the N terminus, especially because Qcr7pΔ7(D13V) is present at wild type levels. One could argue that the elimination of the negative charge, which is an uncommon feature for signal sequences, causes the N terminus to assume a more typical character for transit sequences. This may in turn compensate for the truncation of the seven residues and restore mitochondrial protein levels back to wild type levels as seen in the mutant with Qcr7pΔ7(D13V). If the Qcr7 protein were to follow the same import pathway as proteins that have a cleavable N-terminal signal sequence, then deletion of the N terminus could conceivably cause a number of problems that result in decreased import. Truncated proteins may not interact with cytosolic chaperones as efficiently, or alternatively they may not bind as tightly to the outer mitochondrial membrane receptors. The absence of the Qcr7pΔ7 at 37 °C could thus be due to a higher degradation rate at that temperature, resulting from impaired binding to cytosolic chaperones, for example. Roise et al. (27) have suggested that part of the mechanism of import is the perturbation of the phospholipid bilayer by the surface active amphiphilic helix of the presequence. This ability of the presequence to cause a local defect in the membranes would then create a route for the rest of the protein to follow. It is thus conceivable that the deletion mutants in our study cannot enter the membrane as efficiently, because the amphiphilic character (and inherent lytic properties) has been decreased by shortening the presequence. Hence, this again may reduce the efficiency of import.

In summary, the N terminus of the Qcr7p of the bc₁ complex may have some characteristic features of mitochondrial targeting sequences and may indeed facilitate import. Nevertheless, this region is not essential for the localization of the Qcr7 protein into mitochondria. This notion is reinforced by Western blot analyses of cytosolic protein fractions, which do not show an accumulation of nonimported Qcr7 proteins (result not shown). Because the steady-state levels of Qcr7pΔ14, Qcr7pΔ20, and Qcr7pΔ7(D13V) are comparable with or higher than the level of Qcr7pΔ7, the pet character of the deficient mutants cannot be attributed to the decreased levels of Qcr7p. We therefore conclude that the respiratory chain defect of these mutants is the result of the lowered levels of complex III subunits. This implicates the N terminus of the Qcr7p in assembly of the bc₁ complex. More precisely, this region may bind cytochrome b and/or the 11-kDa subunit. Hence, substitution of critical residues in the N terminus of the Qcr7 protein may prevent the formation of the subcomplex consisting of cytochrome b and the 11-kDa and the 14-kDa subunits. This would then lead to the observed lowered steady-state levels of complex III subunits with the concurrent loss of complex III-linked enzyme activities.

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