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Published in:
European Journal of Biochemistry

Citation for published version (APA):
The role of subunit VIII in the structural stability of the bc\textsubscript{1} complex from \textit{Saccharomyces cerevisiae} studied using hybrid complexes

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(Received 22 July/3 September 1997) – EJB 97 1046/6

The \textit{QCR8} genes encoding subunit VIII of the \textit{bc}, complex from \textit{Kluyveromyces lactis} and \textit{Schizosaccharomyces pombe} partially complement the respiratory-deficient phenotype of a \textit{S. cerevisiae} \textit{QCR8}-null mutant. This implies that the heterologous \textit{Qcr8} subunits can be imported by \textit{S. cerevisiae} mitochondria and that they assemble to form a hybrid \textit{bc}, complex that is sufficiently active to support growth. In contrast, the \textit{QCR8} gene from bovine heart, encoding the 9.5-kDa subunit, is not able to restore respiratory function to the \textit{S. cerevisiae} null mutant. This lack of functional complementation is directly attributable to the inability of \textit{S. cerevisiae} mitochondria to import this protein as shown by \textit{in vitro} assays. However, a hybrid gene encoding the N-terminal 26 residues of \textit{S. cerevisiae} subunit VIII and the rest of the 9.5-kDa bovine heart homologue, was able to functionally complement the \textit{QCR8}-null mutant, albeit to a very low extent. Successful import into \textit{S. cerevisiae} mitochondria was confirmed by \textit{in vitro} import experiments. Surprisingly, although assembly of these hybrid complexes is reduced to an extent that is proportional to the evolutionary distance of the homologue to \textit{S. cerevisiae}, the specific activities of the assembled complexes are the same as for the wild-type \textit{bc}, complex. After solubilisation of the mitochondrial membranes with the mild detergent dodecyl maltoside, the wild-type enzyme can be inactivated by incubation at increased temperature, independent of protease activity. The rate of inactivation can be significantly increased by the addition of \textit{o}-phenanthroline [Boumans, H., Grivell, L. A. & Berden, J. A. (1997) \textit{J. Biol. Chem.} 272, 16753–16760]. The hybrid complexes are much more sensitive to both types of treatment. We conclude that substitution of subunit VIII by a homologous counterpart results in a loosening of the structure of the \textit{bc}, complex on the intermembrane space side, resulting in a less stable insertion of the Rieske Fe-S protein \textit{in vivo} and therefore a lower stability of the assembled enzyme under certain \textit{in vitro} conditions, but without an effect on catalytic activity.

\textbf{Keywords:} \textit{bc}, complex; \textit{Saccharomyces cerevisiae}; structural stability; hybrid complex; protein import.

The respiratory chain enzyme ubiquinol−cytochrome-\textit{c} oxidoreductase, or \textit{bc}, complex, transfers electrons from ubiquinol to cytochrome \textit{c} [1]. Coupled to this reaction is the translocation of protons across the inner membrane of mitochondria and across the cytoplasmic membrane in many bacteria. This enzyme complex may consist of up to eleven subunits, as in the bovine heart complex [2], but only three subunits are common to all \textit{bc}, complexes described so far. These subunits are cytochrome \textit{b}, cytochrome \textit{c}, and the Rieske Fe-S protein and this most simple form of the \textit{bc}, complex can be found in prokaryotes such as \textit{Paracoccus denitrificans} [3] and \textit{Rhodospirillum rubrum} [4]. Another prokaryote, \textit{Rhodobacter sphaeroides} [5], has a \textit{bc}, complex containing a fourth subunit that may be involved in quinone binding in addition to its structural role [6]. The number of additional subunits is even larger in eukaryotic organisms. The \textit{bc}, complex from \textit{Saccharomyces cerevisiae} contains at least seven subunits lacking a prosthetic group, giving a total of ten subunits described to date [7, 8]. For the bovine heart complex, an eleventh subunit has been described which has been identified as the presequence of the Rieske Fe-S protein [9]. A similar subunit may be present in \textit{S. cerevisiae}, but since the presequence of the Fe-S protein here is much smaller, it has not been detected so far.

The catalytic mechanism of all \textit{bc}, complexes appears to be largely the same, thus raising the question of the role of the additional subunits present in eukaryotes. One of these subunits in \textit{S. cerevisiae} is an 11-kDa protein, subunit VIII, encoded by the \textit{QCR8} gene [10]. Disruption of \textit{QCR8} results in complete loss of respiratory function, thus showing that subunit VIII is essential for correct assembly of a functional enzyme. Western blot analysis indicated that protein levels of cytochrome \textit{b}, the Rieske Fe-S protein and subunit VII were severely reduced. More detailed studies on subunit VIII, in which C-terminal deletions [11] or amino acid substitutions in a specific region [12, 13] were made, confirmed that this subunit is required for assembly.

The 9.5-kDa subunit in bovine heart, the homologue of subunit VIII, has been identified as a quinone-binding protein by photoaffinity labelling techniques using azido-Q derivatives [14, 15]. In agreement with this is the finding that subunit VIII in
the S. cerevisiae bc, complex contributes to the Q<sub>o</sub> binding site [16]. This became evident from the characterisation of a deletion mutant (residues 69–73 replaced by a cysteine) that showed a reduced electron transfer activity of the mutant enzyme, and from the effect of some mutations in the same region of the protein on both assembly and activity [13]. Amino acid substitutions outside the region 66–70 have as yet not resulted in a complex and the consequences of the heterologous character for the stability of the enzyme, providing additional insight to relevant subunit interactions within the complex. Our localisation experiments [18] are consistent with this idea.

To further study the function of subunit VIII, we have chosen a complementary approach that exploits the evolutionary variation of this protein. By exchanging the S. cerevisiae QCR8 gene with the homologous gene from another organism, a hybrid bc, complex is created in which subunit VIII has been replaced by the homologous protein in vivo. For this approach, we have chosen three organisms varying in evolutionary distance to S. cerevisiae: Kluyveromyces lactis, a yeast species related to S. cerevisiae, Schizosaccharomyces pombe, a fission yeast that has diverged extensively during evolution from the budding yeast S. cerevisiae [19], and the higher eukaryote Bos taurus (see Table 1). The homologous genes from the two yeast species were isolated in our laboratory [20, 21] and the gene encoding the 9.5-kDa subunit from bovine heart [22] was a kind gift from Chang-Allen Yu, Oklahoma, USA.

Here we report on the characterisation of the hybrid bc, complexes and the consequences of the heterologous character for the stability of the enzyme, providing additional insight to subunit interactions involving subunit VIII.

**MATERIALS AND METHODS**

*Strains and media.* Escherichia coli strain JF 1754 (lac, gal, metB, leuB, hisB, hisD) was used for DNA manipulations, strain BMH71-18 was used for plasmid transformation after site-directed mutagenesis and strain JM109 was used for the generation of single-stranded DNA. E. coli transformants were grown in YT medium [1] (mass/vol.) yeast extract, 1% (mass/vol.) bacto-tryptone and 0.5% (mass/vol.) NaCl containing 200 μg/ml ampicillin.

The S. cerevisiae strain DAL273(167) (a, leu2, lys2) (gift from Herman Pel) was grown on minimal media [0.67 (mass/vol.) bactopeptone and 2% (mass/vol.) NaCl] supplemented with lysine, uracil and uracin (20 μg/ml) and containing 0.5 g/l 5-fluoro-otic acid to screen for uru3- cells [23], giving strain DALU273 (a, leu2, lys2, ura3). Disruption of the QCR8 gene was performed as described before [10], giving DALU80 (a, ura3, lys2, qcr8::LEU2).

S. cerevisiae strains DLL80 (a, his3, ura3, qcr8::LEU2) [10] and DALU80 were used for the transformation of plasmids. Transformation of yeast was performed according to [24]. Transformants were selected on minimal media supplemented with histidine or lysine (20 μg/ml). The respiratory capacity of the transformants was checked on solid media containing 1% (mass/vol.) yeast extract, 1% (mass/vol.) bactopeptone and 2% (mass/vol.) glycero1 and 2% (mass/vol.) ethanol (EG medium).

*Construction of plasmids.* An 840-bp HindIII–SalI fragment carrying the QCR8 gene was cloned from plasmid pUC18H11S [16] into the multiple cloning site of the centromeric E. coli–S. cerevisiae shuttle vector pRS313 [25] to create pRS/Sch. NcoI restriction sites were created at the respective ATG start codons and ClaI sites directly downstream of the S. cerevisiae QCR8 gene and its homologues from S. pombe and bovine heart (if not already present). By exchanging the NcoI–ClaI fragments, clones were made in which the S. pombe and bovine heart genes were flanked by S. cerevisiae sequences (named pRS/Sp8 and pRS/bh8, respectively). K. lactis QCR8 was cloned into the centromeric vector YCplac 33 [26] as described before (named YCplK8). The S. cerevisiae-bovine heart hybrid gene is constructed using the Ndel site in S. cerevisiae QCR8 at nucleotide position 77 and by introducing an Ndel site in the bovine heart gene at position 47. The Ndel–ClaI fragment from pRS/Sch was subsequently replaced by the Ndel–ClaI fragment from the bovine heart gene giving pRS/Sc-bh8. The HindIII–SalI fragment from pRS/bh8 and pRS/Sc-bh8 were cloned into the multicopy vector YEp195 [26] giving YEp/bh8 and YEp/Sc-bh8, respectively.

*Isolation of mitochondria and immunoblotting.* Mitochondria were isolated as described previously [27]. Protein concentrations were determined using the Lowry method [28]. Mitochondrial proteins were separated on SDS/polyacrylamide slab gels according to [29] and blotted according to [30]. After incubation of the blots with antibodies directed against the bc, complex of bovine heart, the antigen-antibody complexes were made visible with the horseradish peroxidase colour-development assay [31]. The antisera used were raised in rabbits.

*Import of proteins into isolated yeast mitochondria.* After in vitro transcription with T7 RNA polymerase, proteins were synthesised in rabbit reticulocyte lysates in the presence of ["S"methionine or ["S"cysteine. Import into isolated yeast mitochondria was performed according to [32]. Where indicated, the mitochondria were incubated with 100 μg/ml proteinase K for 30 min on ice. The mitochondria were resolated, washed, and the proteins were separated by SDS/PAGE. For dissipation of the membrane potential Δψ, 0.5 μM valinomycin, 8 μM antimycin A, and 20 μM oligomycin were included [33].

*Spectral analysis and bc, complex assay.* Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer model DW2000. Concentrations of cytochromes were determined using the following absorption coefficients and wavelength pairs for the reduced minus oxidised proteins: 24.0 M<sup>–1</sup> cm<sup>–1</sup> at 603–625 nm for cytochrome aa<sub>3</sub>, [34], 20.1 M<sup>–1</sup> cm<sup>–1</sup> at 550–540 nm for cytochromes c<sub>c</sub> and c<sub>c</sub>, [35], and 28.5 M<sup>–1</sup> cm<sup>–1</sup> at 562–575 nm for cytochrome b [35]. The ubiquinol–cytochrome-c oxidoreductase (or bc, complex) assay was performed spectrophotometrically at 30°C by measuring the reduction of 18 μM horse-heart ferricytochrome c at 550–540 nm by 25 μM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (reduced Q-2). The buffer used contained 2 mM EDTA, 0.5 mM potassium cyanide, and 20 mM potassium phosphate, pH 7.4, in order to obtain maximal activity with horse-heart cytochrome c as acceptor [36].

**Chemicals.** 1,10-Phenanthroline and 1,10-phenanthroline–ferrous sulphate complex (electron motive force +1.08 V; specific extinction E<sub>1%1cm</sub> = 180 – 200; pH 7; 510 nm) were from BDH Chemicals. The complete protease inhibitor cocktail was from Boehringer.

**RESULTS**

**Growth properties of the mutants.** The respiratory deficiency of the QCR8-disruption strain (DLL80) can be complemented by transforming these cells with a plasmid carrying the QCR8 gene [10]. This set-up was used to introduce the genes encoding
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the subunit VIII homologues of K. lactis, S. pombe, and bovine heart into S. cerevisiae, thereby allowing the construction of hybrid bc1 complexes. To rule out different levels of protein synthesis due to the presence of the genes on a plasmid rather than on a chromosome, we considered the disruption strain complemented with the S. cerevisiae QCR8 gene as the wild type in all experiments.

The S. pombe and bovine heart genes were cloned under the control of the S. cerevisiae QCR8 promoter to provide levels of transcription comparable with that of the S. cerevisiae gene (see Materials and Methods section). The K. lactis QCR8 gene has been expressed in the S. cerevisiae strain DLL80 before and the properties of the hybrid enzyme were found to be comparable to the wild type [37]. Therefore, we decided not to perform any additional cloning steps with this gene.

First, the single-copy constructs carrying the genes from S. cerevisiae, K. lactis, S. pombe, and bovine heart were transformed into DLL80 and cells were screened for growth on EG medium. All but the pRS/bh8 transformant were able to at least partially complement respiratory deficiency. Subsequent transformation of the multicopy construct YEplbh8 into DLL80 also failed to restore growth on non-fermentable carbon sources.

The S. cerevisiae-bovine heart hybrid protein. To examine which domain of subunit VIII is essential for a functional bc1 complex, we constructed a hybrid gene encoding a protein consisting of the N-terminal part of S. cerevisiae subunit VIII (residues 1–26) fused to residues 16–81 of the bovine heart protein (see Materials and Methods section for details). It should be noted that the fusion results in substitution of Ser17 by glycine. Both the single-copy (pRS/bh8) and multi-copy constructs (YEplbh8) were transformed into DLL80, but only the YEplSc-bh8 transformant showed measurable growth on EG medium. Western blot analysis of cytosolic and mitochondrial fractions of YEplbh8 and YEplSc-bh8 transformants (Fig. 1) showed the presence of the Qcr8 protein only in the case of YEplSc-bh8 transformants.

Subsequently, in vitro protein import assays were performed to clarify the absence of the bovine heart 9.5-kDa protein in yeast mitochondria after expression in yeast. Fig. 2 shows that in contrast to the yeast protein, this protein is not imported into yeast mitochondria in the presence of a membrane potential. The hybrid protein is imported, as could be expected from its capacity to partly complement the respiratory-negative phenotype of the QCR8-disruption strain.

Characterisation of hybrid complexes. Mitochondria were isolated from the mutants with a hybrid bc1 complex in which subunit VIII has been replaced by the homologue of K. lactis (named bc1/K18), S. pombe (bc1/Sp8) and an S. cerevisiae-bovine heart hybrid protein (bc1/Sc-bh8), respectively. Growth of cells containing the S. cerevisiae-bovine heart hybrid prior to the isolation of mitochondria gave rise to large colonies when grown for five days on plates containing non-fermentable media. These colonies were analysed (as described in [39]) and shown to contain secondary nuclear mutations. Cells containing only a secondary mutation displayed only minor differences in growth rate compared with the wild type on EG medium. We have not characterised these cells further.

Strain DALU80 was subsequently transformed with the YEplSc-bh8 construct and grown on galactose medium for isolation of mitochondria to avoid problems of slow growth rates and accumulation of suppressor mutations.

Table 1 presents results of measurements of electron transfer activity and cytochrome content. Surprisingly, the turnover number, calculated as electron transfer activity relative to cytochrome b content, was found to be the same for all the complexes when compared to the corresponding wild-type strain. It should be noted, however, that because of the very low value of cytochrome b content of the bc1/Sc-bh8 complex, a large uncertainty is introduced in the calculated turnover number. The $K_m$ for reduced Q-2 was the same for the bc1/Sp8 with respect to the wild-type complex (data not shown).

Thus, the steady-state level of bc1 complex is reduced in the mutants and from the reduced level of cytochrome b one may speculate (see below for further discussion) that this is due to a reduced level of assembly of the enzyme. The enzyme proper-
ties of the mutants are not changed, although this cannot completely be excluded for the bc/Sc-bh8 complex. However, since this mutant contains very small amounts of bc, complex, this could not be studied further.

The hybrid complex bc/Sc-bh8 is unstable after solubilisation. The mutant bc complexes we have constructed show a decreased level of assembly efficiency that parallels the evolutionary distance from S. cerevisiae of the organism supplying the heterologous subunit VIII. The evolutionary distance is reflected by the percentages of sequence identity shown in Table 1. However, once assembled all complexes show wild-type electron transfer activity. To examine whether subunit VIII, in addition to its function in the assembly of the complex, also has a role in the stability of the complex after complete assembly, we solubilized the mitochondrial membranes using dodecyl maltoside. After addition of detergent during incubation on ice, electron transfer activity was measured at various time intervals. bc/Sc-bh8 appeared to be inactivated in a time-dependent manner, inactivation being complete within 15 min. All other complexes were found to be fully stable, even after prolonged incubation (data not shown).

Temperature-dependent inactivation of the hybrid bc complexes. Although the bc/K18 and bc/Sp8 complexes are fully stable on ice upon solubilisation using the detergent lauryl maltoside, we found that these hybrid complexes are less stable than the wild-type enzyme in response to increased temperature. The stability of the different complexes can be described in terms of the turnover time at which the activity is reduced to 50%.

Fig. 3 shows the inactivation of wild-type and hybrid bc complexes. Electron transfer activities of all complexes were first normalised to 100%. Each sample was incubated for 10 min at the temperature indicated. Inactivation was quantitated in terms of a 10-min incubation period resulting in inactivation of 50% of the enzyme. Fig. 3 shows that half of the wild-type enzyme is inactivated after 10 min at 40°C, whereas the same is achieved for bc/K18 at 37°C and at 30°C for bc/Sp8. The stability of the hybrid complexes thus correlates approximately with the evolutionary distance of subunit VIII to the respective homologue.

Although protease inhibitors are added during the isolation of mitochondria, solubilisation of the membranes in the mitochondrial isolate may liberate additional proteases. Repeating the experiment in the presence of 2 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors, however, did not change the stability of the enzymes, indicating that the decrease of activity can be entirely assigned to thermal inactivation.

In a previous section, it was shown that the mutants containing a hybrid bc complex exhibit reduced steady-state levels of this enzyme, which is generally explained in terms of a reduced assembly efficiency, either due to decreased rate of assembly or to a decreased lifetime of the limiting subunit. However, it may be possible that the decreased stability of these complexes under certain in vitro conditions has an effect on the steady-state level of the enzyme in vivo. To examine this, isolated mitochondria from the wild-type strain and DLL80/Sp8 were incubated at 30°C and 37°C for up to 5 h. Only at 37°C and after incubation for at least 4 h could a small decrease in enzyme activity be observed. Since no significant difference was found between the wild type and the hybrid complex and since the generation time of these cells is within the same order as the incubation time, we can conclude that the relative instability of the hybrid bc complex found in vitro does not contribute to the stability of the complex in vivo once fully assembled.

Table 1. Enzyme activity and spectral analysis of wild-type and heterologous bc complexes determined with isolated mitochondria. Note that the complex containing the Sc-bh hybrid subunit VIII (hyb) can only be compared with the corresponding wild-type DAL273. n.a., not applicable; n.d., not determined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence identity</th>
<th>Specific activity</th>
<th>Specific activity relative to wild-type</th>
<th>Cytochrome b content</th>
<th>Turnover number</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>nmol · mg⁻¹ · s⁻¹</td>
<td>%</td>
<td>nmol · mg⁻¹ · s⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (DL1)</td>
<td>100</td>
<td>40.2</td>
<td>100</td>
<td>0.16</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>K18</td>
<td>70</td>
<td>37.1</td>
<td>92</td>
<td>0.14</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td>Sp8</td>
<td>51</td>
<td>17.5</td>
<td>44</td>
<td>0.08</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>bh8</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Wild type (DAL273)</td>
<td>n.a.</td>
<td>27.1</td>
<td>100</td>
<td>0.26</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>hyb</td>
<td>n.a.</td>
<td>1.0</td>
<td>4</td>
<td>0.01</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Inactivation using o-phenanthroline. Recently, we have shown that 1,10-phenanthroline slowly inhibits the electron transfer activity of the bc complex in solubilised wild-type yeast mitochondria, by binding at the IMS side of the complex, presumably by hydrogen bonding with one of the histidine residue ligands of the Rieske Fe-S cluster [40]. Shortly after inactivation, 1,10-phenanthroline chelates an iron atom derived from the Rieske Fe-S cluster, thereby distorting the structure of the Rieske protein, while leaving the iron atoms of the cytochromes of the complex unaffected [40]. The rate of inactivation by 1,10-phenanthroline may be used as a measure of the structural stability of the enzyme complex in the region of the Qₚₚ site. Fig. 4 shows the inactivation of wild-type bc and bc/K18 by 1,10-phenanthroline versus time at 25°C. The hybrid complex bc/K18 is omitted since it differs only slightly from the wild type.
anthroline (1 pg/mg protein) at 25°C and electron transfer activity was monitored versus time. Electron transfer activities of both complexes were first normalised to 100%.

bound by 1,10-phenanthroline to form the 1,10-phenanthroline-ferrous bc,SpR solubilised.

related to the sulphate complex (called ferroin).

hybrid bc,SpS complex using 1,10-phenanthroline.

and solubilised.

deduced from Fig. 3. The nearly complete inactivation of the hybrid bc,SpS complex results in loss of respiratory function due to incomplete assembly of the complex [10]. More detailed studies on subunit VIII have confirmed a role in assembly of a functional complex [11, 12] and have revealed an involvement in the catalytic activity of the enzyme, as part of the Qox binding pocket [13, 16]. Single mutations outside the 66-70 region of the polypeptide, however, did not result in defects in assembly or activity ([17] and Hemrika, W., Lobo-Hajdu, G. and Berden, J. A., unpublished results), but caused a deficiency in the succinate:cytochrome c oxidoreductase activity, indicative of a distortion of the interaction between the bc, complex and succinate:Q oxidoreductase [17]. These data suggest that a large part of subunit VIII is not involved in intersubunit interactions, but in intercomplex interactions, in agreement with both our localisation experiments (no transmembrane helix [18]) and our conclusions about the organisation of the respiratory chain of S. cerevisiae in respiratory units, containing interacting respiratory complexes (unpublished results). Only the region that was detected as being involved in the formation of the Qox reaction centre (around amino acids 66-70) seems to be relevant for assembly and activity of the bc, complex.

To resolve further the role of subunit VIII in these two distinct processes, the homologous genes from two other yeast species and a higher eukaryote were introduced into S. cerevisiae. The corresponding proteins can be considered as natural mutants for S. cerevisiae in which a large number of amino acids (up to 80% for the bovine heart homologue) have been substituted simultaneously. Introduction of these genes results in in vivo assembly of hybrid bc, complexes in which subunit VIII is replaced by the corresponding protein. Despite the relatively large number of changes in primary structure, the introduction of a heterologous subunit VIII did not result in a change in specific activity of the complex: the electron transfer reactions are not

wild-type enzyme with respect to stability. For the wild-type enzyme, 50% inactivation is achieved after 35 min and for bc,Sp8 already after 4 min. As a control, both enzyme complexes were also incubated at 25°C without 1,10-phenanthroline. As shown in Fig. 4, no inactivation within 60 min could be observed for both enzymes. Solubilisation of the mitochondria did not itself result in a measurable inactivation after 10 min at 25°C, either for the wild type, or for the bc,Sp8 mitochondria, as can be deduced from Fig. 5. The nearly complete inactivation of the solubilised bc,Sp8 complex after 10 min, as shown in Fig. 4, is therefore completely due to the presence of 1,10-phenanthroline. Spectral analysis of the bc,Sp8 complex (Fig. 5C) shows that the cytochromes of this complex keep their iron after treatment with 1,10-phenanthroline, in agreement with our previous conclusion that the inactivator binds to the Qox site and the Rieske Fe-S cluster. After heat treatment, the cytochromes remain intact (data not shown).

In solubilized wild-type mitochondria the two stages of the effect of 1,10-phenanthroline, i.e. first binding, which results in inactivation, followed by chelation of an iron from the Rieske Fe-S cluster, could only be separated by using pre-steady-state kinetics of the cytochromes and showing that the inhibition of cytochrome b reduction lags behind the inactivation of the enzyme. With the bc,Sp8 hybrid complex, however, the two processes can be visualised separately using spectral analysis, due to the rapid inactivation of the enzyme by 1,10-phenanthroline. Fig. 5A shows a spectrum of the hybrid complex reduced using succinate after complete inactivation using 1,10-phenanthroline. Cytochrome b is still enzymically reducible, which, according to our earlier findings [40], occurs via the Qox site. After an additional 15 min of incubation with 1,10-phenanthroline, this pathway is also affected and cytochrome b cannot be further reduced enzymically (Fig. 5B).

We may conclude, therefore, that the introduction of a heterologous subunit VIII results in loosening of the structure of the bc, complex at the Qox binding region, thereby facilitating binding of 1,10-phenanthroline to the complex.

**DISCUSSION**

Deletion of the QCR8 gene encoding subunit VIII of the bc, complex results in loss of respiratory function due to incomplete assembly of the complex [10]. More detailed studies on subunit VIII have confirmed a role in assembly of a functional complex [11, 12] and have revealed an involvement in the catalytic activity of the enzyme, as part of the Qox binding pocket [13, 16]. Single mutations outside the 66-70 region of the polypeptide, however, did not result in defects in assembly or activity ([17] and Hemrika, W., Lobo-Hajdu, G. and Berden, J. A., unpublished results), but caused a deficiency in the succinate:cytochrome c oxidoreductase activity, indicative of a distortion of the interaction between the bc, complex and succinate:Q oxidoreductase [17]. These data suggest that a large part of subunit VIII is not involved in intersubunit interactions, but in intercomplex interactions, in agreement with both our localisation experiments (no transmembrane helix [18]) and our conclusions about the organisation of the respiratory chain of S. cerevisiae in respiratory units, containing interacting respiratory complexes (unpublished results). Only the region that was detected as being involved in the formation of the Qox reaction centre (around amino acids 66-70) seems to be relevant for assembly and activity of the bc, complex.

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affected in the mutant complexes and only a diminished assembly is observed. This may seem to conflict with the findings that subunit VIII plays a role in electron transfer, as indicated by a decrease in affinity for myxothiazol and an impaired reduction of cytochrome \( b \) via the \( Q_{\text{int}} \) site in certain mutants [13, 16]. Comparison of the subunit VIII homologues has shown that, despite only small overall sequence conservation, their predicted secondary structures are largely the same and the proteins contain similar structural elements, including a central hydrophobic segment [21]. In other words, the amino acid changes that have occurred in the course of evolution have not affected the overall structure of the protein. Combining this with the data presented here, i.e. no change in electron transfer activity and affinity for ubiquinol, it may be speculated that subunit VIII is not involved in binding of quinone, quinol, or semiquinone itself, but is located in close vicinity of the binding region (which is in cytochrome \( b \)). The conformation of this protein would then indirectly influence the binding pocket. Replacing subunit VIII by a heterologous protein would not change this conformation, while the deletion mutants and mutants in the aromatic region \( 66-70 \), which showed the altered characteristics for the \( Q_{\text{int}} \) site, would. Studies on the bovine heart homologue complicate this matter by showing that this protein can be labelled with azido-Q [14, 15]. Furthermore, recent experiments showed that this protein itself is able to bind quinone [41]. For the yeast homologue such a Q binding has not been shown. Questions that remain on this matter can only be answered by further analysing the region of the protein that binds azido-Q using site-directed mutagenesis.

The stability of the hybrid \( bc \) complexes was examined by solubilising the mitochondrial membranes using dodecyl maltoside. The same treatment is also used as the first step in the isolation of this enzyme complex [42]. Being a mild, neutral detergent, dodecyl maltoside does not negatively affect the catalytic activity of the (wild type) enzyme. However, the hybrid complex in which subunit VIII has been replaced by the \( S. \text{cerevisiae} \)-bovine heart hybrid protein is unstable and electron transfer activity is completely lost after incubation in dodecyl maltoside for 15 min at 0°C. When increasing temperatures are used, the other hybrid complexes also show a reduced stability compared to the wild-type enzyme. The relative instability of the complexes is inversely proportional to the extent of sequence identity between the heterologous subunit and subunit VIII of \( S. \text{cerevisiae} \). Furthermore, the relative stability of the complexes correlates with the steady-state levels found in vivo. It thus seems that the extent of instability found in the in vitro experiments mimics the role of subunit VIII in the stability of the enzyme in vivo. Since no effect on the stability of the hybrid complexes could be detected when present in intact mitochondria, it has to be concluded that subunit VIII has no or little role in the stability of the complex once it is completely assembled. Solubilisation of the mitochondrial membranes, however, decreases the structural stability of the \( bc \) complex, thereby making the relative contribution of subunit VIII to complex stability more important.

If indeed the relative instability correlates with the steady-state level of the complex found in vivo, the heterologous nature of subunit VIII should affect stability at a stage before complex assembly is completed. Models have been proposed for the assembly of the \( bc \) complex in which subunit VIII is involved in assembly of cytochrome \( b \) into the complex by forming a protease-sensitive subcomplex together with cytochrome \( b \) and subunit VII [43, 44]. It is known from many studies that subunits of mitochondrial inner membrane complexes in yeast are rapidly degraded when not assembled. (For a recent review on the role of protein degradation in mitochondrial function and biogenesis see [45].) The same occurs with some of the subcomplexes formed in the early stages of assembly of the \( bc \) complex, including the cytochrome \( b \) subcomplex. The balance between assembly and degradation can be described in terms of quality control: proteins that fail to assemble quickly and stably are presumably more likely to be recognised by and acted on by the degradative machinery. The introduction of a heterologous subunit VIII may reduce stability of the cytochrome \( b \) subcomplex by shifting activity of the quality control machinery more towards degradation. Our finding that in the heterologous complexes the region around the Rieske Fe-S cluster is destabilised, which is only detectable after solubilisation, may be interpreted as indicating relevant intersubunit interactions only in this region of the enzyme, thereby supporting our model for the localisation of subunit VIII [18]. The homologous bovine heart subunit VIII was found to be transmembrane in crystals of the bovine heart enzyme [46], in agreement with the higher hydrophobicity of the hydrophobic domain of the protein. A different localisation of the N-terminal and middle part of the protein in yeast and mammals may explain why the yeast/bovine heart hybrid poly- peptide does not show an appreciable level of assembly.

The final step in the assembly of the \( bc \) complex is the incorporation of the Rieske Fe-S protein, after the remainder of the complex is assembled. Disruption of the gene encoding the Rieske Fe-S protein results in reduced levels of cytochrome \( b \), presumably because partially assembled \( bc \) complex lacking the Fe-S protein is subject to increased rates of degradation [47]. We show here that introduction of a heterologous subunit VIII weakens interaction of the \( bc \) complex with the Rieske Fe-S protein, thus reducing its stabilising effect. The results obtained by use of heterologous subunits therefore further support the suggestions from mutational studies that subunit VIII is only indirectly involved in electron transfer activity by its interactions with other subunits, especially the Rieske Fe-S protein, in the region of the Q reaction centre. The main role of this subunit in yeast may be the interaction on the IMS side of the membrane with other complexes in a respiratory unit, while the analogous bovine subunit is transmembrane and possibly more directly involved in both intersubunit interactions and Q-binding.

In conclusion, the reduced steady-state level of \( bc \) complex found in the mutants described here is the result of a summation of processes effected by the expression of a subunit VIII homologue in \( S. \text{cerevisiae} \). First, reduced stability of both the heterologous \( OQR8 \) transcript and protein in the cytosol may result in less protein to be targeted to the mitochondria. Secondly, the import apparatus of \( S. \text{cerevisiae} \) may not import the heterologous protein as efficiently as the yeast counterpart. Third, the stability of the cytochrome \( b \) subcomplex may be affected due to the heterologous nature of subunit VIII thereby reducing the assembly efficiency. Furthermore, the stabilising effect of the Rieske Fe-S protein is reduced due to a loosening of the complex on the IMS side. Finally, the reduced steady-state level of the enzyme may be influenced by a decreased interaction of the \( bc \) complex with other respiratory complexes, resulting in increased breakdown in vivo. These five processes thus all affect the biogenesis of the mutant \( bc \) complexes.

We thank Aloysius F. Hartog for the synthesis of ubiquinone-2, Sandra van Wilpe for performing in vitro mitochondrial import assays and Prof. Chang-An Yu for the gift of the gene encoding the bovine heart 9.5 kDa subunit. This work was supported in part by grants from the Netherlands Organisation for the Advancement of Pure Research (N. W. O.) under the auspices of the Netherlands Foundation for Chemical Research (SON).

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