

Topological organization of subunits VII and VIII in the ubiquinol-cytochrome *c* oxidoreductase of *Saccharomyces cerevisiae*

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Abstract To determine the topology of subunit VIII of the yeast ubiquinol-cytochrome *c* oxidoreductase in the mitochondrial inner membrane, an epitope has been introduced in the N-terminal half of this protein. Previous topology studies had shown that at least the C-terminus faces the intermembrane space [Hemrika and Berden (1990) *Eur. J. Biochem.* 192, 761–765]. Based on sensitivity of the protein to proteinase K digestion we now suggest that the N-terminus of subunit VIII is similarly oriented, implying that this subunit does not span the membrane. Despite this, however, subunit VIII cannot be extracted from the membrane even after treatment with 0.1 M Na₂CO₃ at pH 11.5, showing that the protein is integrally embedded in the membrane. A similar behaviour was displayed by another low molecular weight protein of the complex, subunit VII, which faces the matrix side. A model for the topology of these subunits in the membrane is discussed with respect to the structure of the complex and their involvement in quinone binding.

Key words: Membrane topology; Ubiquinol-cytochrome *c* oxidoreductase; Epitope tagging; Integral membrane protein; (*Saccharomyces cerevisiae*)

1. Introduction

The ubiquinol-cytochrome *c* oxidoreductase (complex III, or the *bc*₁ complex) of the yeast *Saccharomyces cerevisiae* consists of 10 subunits and is located in the mitochondrial inner membrane [1,2]. Three subunits, cytochrome *b*, cytochrome *c*₁ and the Rieske Fe-S protein, carry the prosthetic groups and are essential for activity of the enzyme. Much less is known about the function of the other seven subunits which lack a prosthetic group. Subunit VIII, an 11-kDa protein, is thought to be closely associated with cytochrome *b* [3]. In yeast it has been shown that this subunit contributes to the structure of the Q_{out} binding site [4] and the same has been suggested for the bovine heart homologue, a 9.5-kDa protein [5]. In contradiction to these studies are immuno-inhibition experiments performed with the bovine heart 9.5-kDa protein which suggest an involvement in the Q_{in} site [6]. For accurate modelling of ubiquinol binding to the *bc*₁ complex it is important to establish the topology of subunit VIII. Although a low-resolution, three-dimensional structure of the *bc*₁ complex was determined some 15 years ago [7], a high-resolution structure is not yet available. We have therefore used an alternative approach in order to ascertain the topology of subunit VIII with respect to the membrane and to neighbouring subunits in the complex.

Previous topology studies on the *S. cerevisiae* *bc*₁ complex, using antibodies reactive with a C-terminal region of the pro-

tein [8], have shown that at least this part of subunit VIII is exposed to the intermembrane space [9]. In addition, hydrophobicity plots of subunit VIII from *S. cerevisiae*, *K. lactis*, *S. pombe* and the homologous subunit from bovine heart predict the presence of a conserved hydrophobic stretch potentially capable of spanning a lipid bilayer (Fig. 1 and [10]), thus implying that the N-terminus of the protein faces the mitochondrial matrix.

In order to verify the accuracy of these predictions, we have tagged the N-terminal half of the protein with two different epitopes (*c-myc* [11] and HA [12]). As indicated in Fig. 1, these epitopes were not placed at the N-terminus of the protein, since the first 25 amino acids may contain a non-cleaved import signal [13]. Using limited protease treatment we have determined the position of the *c-myc* and the HA epitope with respect to the inner membrane. The outcome of this is combined with carbonate extraction experiments on the entire *bc*₁ complex in order to further model the topology and subunit arrangement of this large respiratory enzyme.

2. Materials and methods

Escherichia coli strain JF 1754 (*lac*, *gal*, *metB*, *leuB*, *hisB*, *hsdR*) was used for DNA manipulations and strain BMH71-18 was used for plasmid transformation after site-directed mutagenesis. *E. coli* transformants were grown in YT medium (1.6% (w/v) bactotryptone, 1% (w/v) yeast extract and 0.5% NaCl) containing 200 µg/ml ampicillin. *S. cerevisiae* strain DLL80 (α , *his3-11,15*, *leu2-3,112*, *ura3-251,328,372*, *qcr8::LEU2*) [8] was used for the transformation of plasmids. Transformation of yeast was performed according to [14]. Transformants were selected on minimal media containing 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) agar supplemented with histidine (20 µg/ml). The respiratory capacity of the transformants was analysed on solid media containing 1% (w/v) yeast extract, 1% (w/v) bacto-peptone, 2% (v/v) glycerol and 2% (v/v) ethanol.

An 840 bp *HindIII*-*SalI* fragment carrying the *QCR8* gene was cloned from plasmid pUC18-H11S [4] into the multiple cloning site of the pSelect plasmid (Promega). Site-directed mutagenesis was performed with a subunit VIII substitution oligonucleotide (5'-GGAAA-ATACCTTGTAATGAATTCTGAGCATATGGAG-3') according to the pSelect manufacturers protocol (Promega). This introduced an *EcoRI* restriction site at base 103 in the *QCR8* gene. Two oligonucleotides (5'-AATTCGGAACAAAACCTTATTCTGAAGAA-GATCTG-3' and 5'-AATTCAGATCTTCTTCAGAAATAAGTTT-TTGTTCCG-3') were annealed together to form double-stranded DNA encoding the *c-myc* epitope (EQKLISEEDL) flanked by 5' cohesive ends compatible with an *EcoRI* site. This DNA construct was cloned into the *EcoRI* site in *QCR8* creating the *QCR8/c-myc* fusion gene. The same approach has been undertaken to create the *QCR8/HA* fusion gene (oligonucleotides 5'-AATTCTTACCCATAC-GACGTCCCAGACTACGCTTGG-3' and 5'-AATTCCAAAGCG-TAGTCTGGGACGTCGTATGGGTAAG-3', epitope YPYDVP-DYA). These fusion genes were subsequently cloned as an 876 bp *HindIII*-*SalI* fragment into the multi-copy vector YEplac195 [15] yielding pQCR8c-myc and pQCR8HA, respectively.

Mitochondria were isolated as described previously [16]. Mitochon-

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drial proteins were separated on SDS-polyacrylamide slab gels according to [17] and blotted according to [18]. After incubation of the blots with antibodies the antigen-antibody complexes were made visible with the horseradish peroxidase colour-development assay [19]. Antibodies against the *c-myc* epitope were obtained from Cambridge Research Biochemicals and antibodies against the HA epitope from Boehringer-Mannheim. The antisera used to detect subunits VII and VIII are described in [9]. Mitoplasts were obtained by diluting the mitochondrial suspension 3-fold in 20 mM Tris-HCl pH 7.4, incubating the suspension 15 min on ice, sedimenting the mitoplasts by centrifugation and resuspending them in 0.6 M sorbitol, 20 mM KPi pH 7.5, 1 mM EDTA, 1 mM MgCl₂. Treatment with Proteinase K (250 µg/ml) was performed at 37°C for 30 min, membranes were solubilised in 0.5% Triton X-100. Carbonate treatment [20] was performed by pelleting wild-type mitochondria, resuspension in 0.1 M Na₂CO₃ (pH 11.5) at 1 mg mitochondrial protein/ml and incubation for 45 min on ice followed by centrifugation at 150 000×g.

Spectral measurements and the ubiquinol-cytochrome *c* oxidoreductase assay were performed as described previously [4].

3. Results

3.1. Complementation of DLL80 with the *c-myc*- or HA-tagged subunit VIII

In a first attempt to test the functionality of the *c-myc*-tagged subunit VIII, the QCR8⁰ strain DLL80 was transformed with a single-copy vector (pRS316 [22]) carrying the *QCR8/c-myc* hybrid gene. This resulted in partial complementation of the respiratory-negative phenotype of the *QCR8*-disruption strain, indicated by slow growth on ethanol/glycerol medium, which was estimated to be around 30% of the wild-type strain. Therefore the multi-copy vector YEplac195 was used as described in Section 2. Transformation with this construct resulted in a growth rate on non-fermentable carbon source after transformation of DLL80 of around 75% of that

of the wild type. Electron transfer activity of the *bc*₁ complex in this mutant was determined to be 60% of that of the wild-type enzyme. Similar values were obtained when the cytochrome *b* content was measured by spectral analysis, which means that the turnover number of the enzyme has not changed (data not shown). This suggests that insertion of the epitope has an effect on the assembly efficiency of the complex.

The fact that the *c-myc* epitope contains three glutamate residues may be a factor contributing to reduced assembly efficiency. It has been reported that glutamate residues in nuclear encoded mitochondrial inner membrane proteins occur 10 times more often on the IMS side than on the matrix side, suggesting a role of this residue in directing the protein topology [23]. We therefore transformed DLL80 also with the single-copy vector pRS316 and with the multi-copy vector YEplac195 both carrying the *QCR8* gene tagged with the HA epitope [12], which lacks glutamate residues. The level of complementation by the first construct was very low, comparable with that of the *QCR8/c-myc* fusion gene on the single-copy vector. The latter construct resulted in a high level of complementation and the activity of the *bc*₁ complex from this mutant was found to be 75% of that of the wild-type enzyme. The difference with the *c-myc* tagged equivalent is far too small to be explained by a difference in membrane topology for the two tagged proteins.

Van Loon et al. [24] have reported that overexpression of subunit VIII on a multi-copy vector results in a 5–10-fold increase of this protein present in mitochondria, together with increased turnover. Fig. 2a shows a quantitation of the HA-tagged subunit VIII after expression on the multi-copy vector YEplac195 in comparison to the wild-type protein

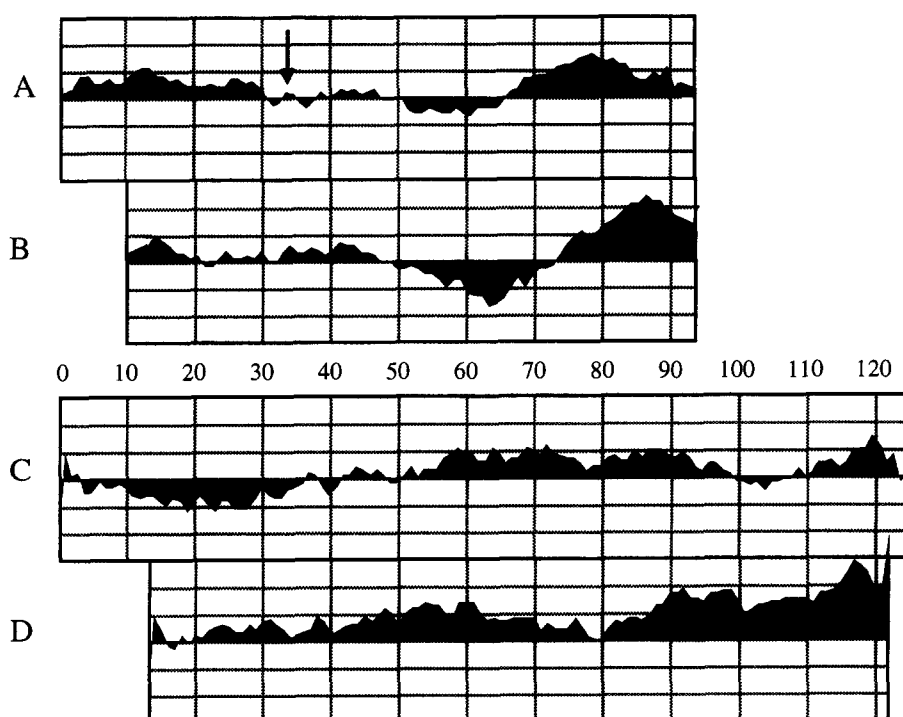


Fig. 1. Hydrophilicity plots of subunits VIII (A) and VII (C) from *S. cerevisiae* and their respective bovine homologues (B,D). Hydrophilicity plots were produced according to the Kyte-Doolittle scale [21]. A window size of 19 was used. Negative values predict hydrophobic regions. The position where the *c-myc* epitope is inserted is indicated by an arrow. Numbers correspond to amino acid positions of the *S. cerevisiae* proteins.

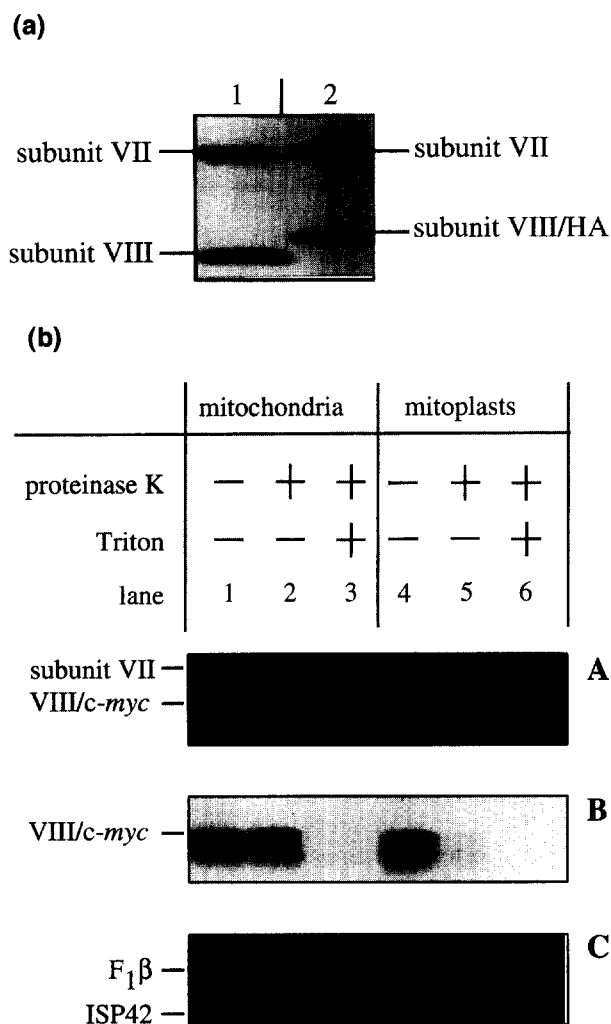


Fig. 2. Topology of epitope-tagged subunit VIII. (a) Quantitation of the HA-tagged subunit VIII when expressed on a multi-copy vector. Samples were run on 16.5% T, 3% C SDS-PAGE, blotted and stained with antibodies against subunits VII and VIII. In lane 1 mitochondria were run from the wild-type strain and in lane 2 from the strain containing the HA-tagged subunit VIII. Subunit VII serves as a control for the amount of layered material. (b) Proteinase K treatment of mitochondria and mitoplasts (see Section 2). Samples were run on 20% SDS-PAGE and subsequently analysed by Western blotting using antibodies directed against: (A) subunits VII and VIII, (B) *c-myc*, (C) F₁β and ISP42.

which was expressed on a single-copy vector. It can be seen that there is no increase in the amount of subunit VIII relative to subunit VII which serves as a reference. This means that turnover of the tagged subunit VIII is even further increased relative to the wild-type protein. This might also explain why the two constructs derived from the single-copy vector could complement DLL80 to only a very low extent.

The quantitation of the signal in combination with the enzyme activity shows that almost all of the HA tagged as well as the *c-myc* tagged subunit VIII that is visible on the Western blots, is present in an assembled and functional complex and therefore in its native topology.

3.2. Localisation of the N-terminus of subunit VIII

Mitochondria were isolated and a fraction of the preparation was converted to mitoplasts (see Section 2). Both mito-

chondria and mitoplasts were incubated with proteinase K as depicted in Fig. 2b. Lanes 3 and 6 serve as a control for the protease activity. Triton X-100 solubilizes all membranes and therefore makes all proteins accessible for proteinase K. Panel C shows results obtained for mitochondrial proteins facing either the matrix (F₁β) or the IMS (ISP42). Panel A shows the localisation of subunits VII and VIII as was determined before [9]. Resistance to proteinase K of the 14-kDa subunit VII (lane 5) shows that this protein is not exposed to the IMS, consistent with earlier findings which indicated that this protein faces the mitochondrial matrix [9]. The VIII/*c-myc* fusion protein, as recognised by subunit VIII antibodies which only recognize the C-terminus, is degraded showing that this part of subunit VIII is exposed to the IMS. Panel B shows the same protein recognised by *c-myc* antibodies. Since the fusion protein is sensitive to proteolytic activity and no lower molecular weight bands were generated, we conclude that the N-terminus is also exposed to the IMS. This implies that subunit VIII does not span the membrane. In order to exclude the appearance of very small peptides after proteinase K incubation, digestion products were analysed by electrophoresis on a 16.5% T, 3% C Tricine acrylamide gel [25]. This system is able to separate proteins as small as 1 kDa. However, also under these conditions no lower molecular weight proteins could be detected.

To exclude the possibility of an aberrant topology of the VIII/*c-myc* fusion protein due to the presence of the *c-myc* tag, we performed identical experiments with the VIII/HA fusion protein (data not shown). Since this fusion protein is also degraded in mitoplasts similar to the VIII/*c-myc* fusion protein, we can conclude that insertion of the *c-myc* tag does not alter the membrane topology of subunit VIII and that the results obtained here also count for the wild-type subunit VIII protein.

3.3. Subunit VIII is an integral membrane protein

The membrane association of subunit VIII, being a non-transmembrane protein, still needed to be elucidated. To achieve this, mitochondria were treated with sodium carbonate as described in Section 2. AAC, an integral membrane protein, could not be solubilized by this procedure, while F₁β, a peripheral membrane protein was recovered in the soluble supernatant fraction (Fig. 3). Subunit VIII co-fractionates with AAC, thus behaving as an integral membrane protein. We conclude from this that the hydrophobic stretch in this protein (see Fig. 1) anchors the protein firmly in the lipid bilayer, where it may interact with cytochrome *b* as has previously been suggested [26].

Other subunits that could not be extracted are cytochrome *b*, which is assumed to span the membrane eight times [27], cytochrome *c*₁, also known to be an integral membrane protein, and subunit VII. Core I and core II can be extracted from the membrane, which is consistent with the fact that the subcomplex that contains these two proteins was found to be water-soluble [28]. Other subunits that are extracted under these conditions are the Rieske FeS protein and the 17-kDa subunit VI, a very hydrophilic and acidic protein which is involved in the interaction between the *bc*₁ complex and cytochrome *c* [29]. Subunits IX and X are not shown in Fig. 3.

This experiment was repeated with mitochondria isolated from the mutant strain which contains the *c-myc* tagged sub-

unit VIII. This fusion protein, similar to subunit VIII, could not be extracted from the membrane, indicating identical membrane association.

4. Discussion

This paper reports a detailed study of the membrane topology of subunit VIII of the yeast *bc*₁ complex. In an earlier report it was shown that subunit VIII is exposed to the IMS [9]. However, the fact that the antibodies used in this earlier study recognize only the C-terminus has prompted us to re-investigate this issue. Since structure predictions indicate a hydrophobic region in the protein long enough to span a lipid bilayer (see Fig. 1A, amino acids 47–65), it became important to establish the location of the N-terminus. In this report we show that an epitope tag inserted into the N-terminal half of subunit VIII is accessible to the action of externally added proteases, thus strongly suggesting that this part of the protein also faces the IMS.

If subunit VIII is a transmembrane protein, only the C-terminus should be accessible to and subsequently degraded by proteinase K, thereby producing a C-terminally truncated protein of approx. 9.5 kDa. This product, which should be visualizable by the *c-myc* antibodies, is not observed. Although the lack of detection of this product could be due to its intrinsic instability, we consider this unlikely: a similar protein of a C-terminal deletion mutant, containing only the first 66 residues, is sufficiently stable to permit assembly of a partially functional *bc*₁ complex [8].

The resistance of subunit VIII to extraction from the membrane by sodium carbonate at alkaline pH shows that this protein is an integral membrane protein and thus by definition interacts with the hydrocarbon region of the bilayer. Combining the results we propose a model for the topology of subunit VIII in the inner membrane in which the hydrophobic domain of the protein makes a loop in the membrane (Fig. 4). This hydrophobic domain contains a conserved proline residue approximately in the middle which might be involved in 'kinking' the α -helix. However, it should be mentioned that prolines occur rather frequently in membrane-spanning α -helices [30]. The position of the hydrophobic loop is highly relevant in relation to the function of subunit VIII in the formation of the *Q*_{out} binding site. Deletion analysis defined the region that is involved herein, indicated in Fig. 4 by the grey region [4]. It is generally accepted that the *Q*_{out} site is located near the surface of the membrane at the IMS side [31]. For cytochrome *b* a large number of residues have been identified exhibiting inhibitor resistance for the *Q*_{out} site or the *Q*_{in} site ([32] and references therein). This suggests that these amino acids are at or near the respective quinone binding site. Based on these data the quinone binding domains were mapped using a helical wheel diagram which positions the transmembrane helices with respect to each other [32]. Since subunit VIII contributes to the *Q*_{out} site it is now possible to localize subunit VIII relative to cytochrome *b*. We propose that the hydrophobic stretch in subunit VIII penetrates the membrane in the vicinity of helices C and F of cytochrome *b*, or may be embedded in the membrane in this region similar to the helix between C and D.

Like subunit VIII, subunit VII cannot be solubilized by Na-carbonate treatment. This subunit was shown to be located at the matrix side of the inner membrane [9]. In yeast this pro-

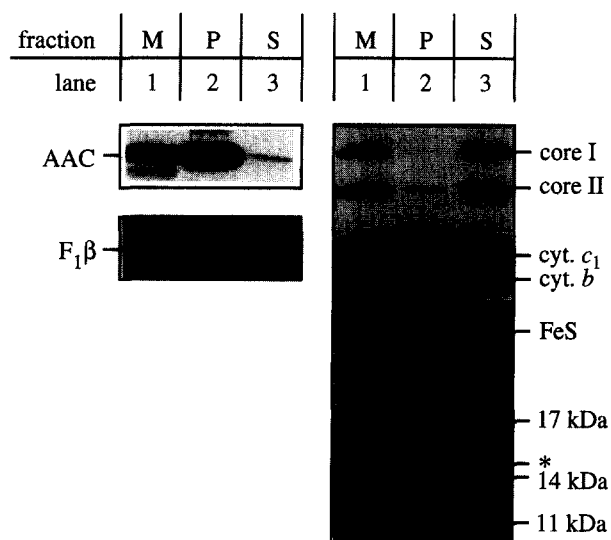


Fig. 3. Sodium carbonate treatment of mitochondrial membranes. Mitochondria were treated as described in Section 2. Samples were run on 15% SDS-PAGE, blotted and stained with antibodies directed against the components of the *bc*₁ complex. Lanes: 1, mitochondria (M); 2, pellet fraction (P); 3, supernatant fraction (S). The asterisk marks an unknown protein which is also present in the subunit VII disruption strain.

tein contains a hydrophobic stretch near the N-terminus (see Fig. 1C). Other structure predictions show that this region is highly likely to form an amphiphilic, surface-seeking α -helix [13], contacting the membrane as is shown in the model in Fig. 4. However, the bovine heart homologue does not contain this hydrophobic stretch (Fig. 1D). Controlled proteolytic digestion indicated that the N-terminus of this subunit in the bovine heart *bc*₁ complex is involved in proton translocation [33]. These authors then concluded that the N-terminus must be located at the border of the complex penetrating into the matrix. It remains to be established whether the bovine heart subunit can be extracted from the membrane using carbonate.

Subunit VII is also thought to be involved in quinone binding [34,35], but there is controversy as to which site it contributes. Since this subunit is located at the matrix side and must be partly embedded in the membrane (see Fig. 4), we consider a contribution to the *Q*_{out} site to be unlikely.

The model in Fig. 4 only shows three subunits of the *bc*₁ complex. These three subunits are proposed to form a sub-complex prior to assembly of the entire complex [3,36], which constitutes the centre of the assembled complex. Interaction with other subunits of the complex is (at least in part) established by the C-termini of subunits VII and VIII. These regions are very hydrophilic, containing many charged residues, and it was proposed that they interact with hydrophilic parts of other subunits [8,37].

Our finding that subunit VIII does not span the membrane suggests that topology and immuno-inhibition studies performed on the homologous 9.5-kDa subunit from the bovine heart *bc*₁ complex [6] may need to be re-evaluated. The position of the azido-Q-labeled peptide [6], according to the alignment with yeast subunit VIII [4], is indicated in Fig. 4 by the hatched region. Assuming evolutionary conservation of structure and function in subunit VIII and its homologues, we consider a contribution to the *Q*_{in} site unlikely.

Taken together these structural data should facilitate the

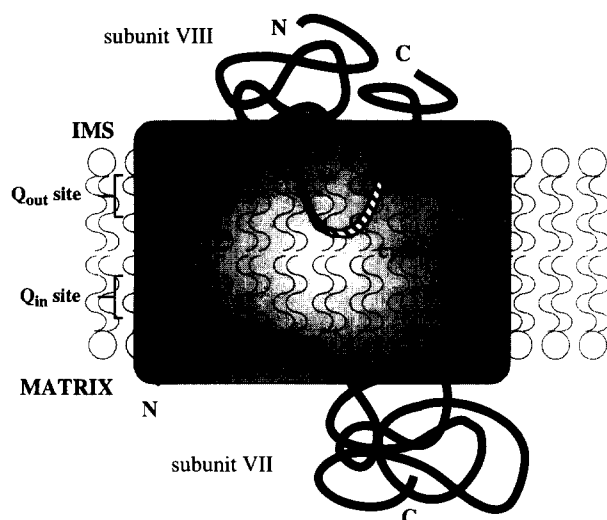


Fig. 4. Model for the topology of subunits VII and VIII in the membrane and with respect to cytochrome *b*. The hatched region in subunit VIII corresponds to the peptide that was azido-Q-labeled in the bovine heart homologue [6]. The grey region indicates the position of the stretch of five amino acids in yeast that was proposed to be involved in the Q_{out} site [4]. The N-terminus of subunit VII is predicted to form an amphiphilic, surface-seeking helix [13]. No secondary structures, like α -helices and β -sheets, are shown for subunits VII and VIII, but at least those regions interacting with the membrane are proposed to be α -helical based on structure predictions.

characterization of the role of both subunits VII and VIII in quinone binding, as well as their interactions with other subunits of the complex.

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