The aromatic domain ^{66}YWYWW^{70} of subunit VIII of the yeast ubiquinol-cytochrome *c* oxidoreductase is important for both assembly and activity of the enzyme

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Abstract The aromatic character of the region ^{66}YWYWW^{70} of the 11-kDa subunit VIII of ubiquinol-cytochrome c oxidoreductase (bc_1 complex) of the yeast *Saccharomyces cerevisiae* has previously been demonstrated to be important for assembly of a functional complex [Hemrika et al. (1994) FEBS Lett. 344, 15–19]. Especially the aromatic nature of residue 66 appeared to be relevant, as the very low level (5%) of bc_1 complex in the mutant ^{66}SASAA^{70} was restored to nearly 70% of the wild-type level in a phenotypic revertant with the sequence ^{66}FASAA^{70}. In the present study, three other site-directed mutants (^{66}SAYAA^{70}, ^{66}SASAW^{70} and ^{66}SWYWW^{70}) were constructed and analysed. The data indicate that the presence of one aromatic residue is enough for a substantial level of assembly and that its position modulates the level of both assembly and electron transfer activity. The results also confirm the relevance of this region of subunit VIII for the formation of the Q_{out} reaction domain, as reported by Hemrika et al. [(1993) Eur. J. Biochem. 215, 601–609]. It is further shown that the lowered specific activity of the mutant described by these authors is not due to the introduction of a cysteine in the sequence of subunit VIII.

Key words: Ubiquinol-cytochrome *c* oxidoreductase; 11-kDa subunit; Mutagenesis; Aromatic domain; Assembly; Turnover number; *Saccharomyces cerevisiae*

1. Introduction

The 11-kDa subunit VIII of the *Saccharomyces cerevisiae* complex III (bc_1 complex or the ubiquinol-cytochrome *c* oxidoreductase) is encoded by *QCR8*, a single-copy nuclear gene located on chromosome X [1]. Analysis of a *QCR8* disruption mutant showed that this subunit is essential for the assembly of a functional complex. The mutant lacks holo-cytochrome *b* and displays severely reduced levels of apo-cytochrome *b*, the Rieske Fe-S protein and the 14-kDa subunit VII [1].

Since these pleiotropic effects prevent further analysis of the role of the 11-kDa subunit in the mechanism of action of the bc_1 complex, studies were performed in which in vitro-mutagenised QCR8 genes were transformed to a *qcr8* null (DLL80) strain [2–4].

Previous experiments with a truncated version of the 11-kDa subunit VIII revealed that the C-terminal 26 amino acids are dispensable for the enzymatic activity of the protein complex, although the assembly of the complex was markedly affected [2]. This mutant has the original amino acid sequence ending at 66Y fused to residues ^{67}SCSQAC^{72} coming from a stop-oligonucleotide [5]. Note that this fused sequence comprises two cysteine residues, while the original yeast subunit VIII does not contain any.

Recently, a highly aromatic domain at the C-terminal region of subunit VIII was reported to be important for the assembly and catalytic function of the bc_1 complex [3,4]. First, the residues ^{69}WWKNG^{73} were replaced by a cysteine (69C) in a mutant obtained by random mutagenesis [2]. This mutant showed a reduced enzymic activity and alterations in the binding of the Q_{out} inhibitor myxothiazol, implying that this region of the yeast 11-kDa protein contributes to the Q_{out} binding site. Additionally, the region ^{66}YWYWW^{70} in the *S. cerevisiae* subunit VIII was replaced by non-aromatic residues (^{66}SASAA^{70}) by site-directed mutagenesis, resulting in the nearly complete absence of assembled complex III [4]. Based on the occurrence of a spontaneous second mutation, the ^{66}FASAA^{70} mutant, which had a 60–70% recovery of the wild-type activity, it was suggested that the aromatic nature of residue 66 was important for assembly of a functional enzyme.

The homologue of the yeast 11-kDa subunit VIII in bovine heart is the 9.5-kDa subunit VII [6], shown to be photo-affinity labelled with a quinone derivative [7,8]. This so-called 'small ubiquinol-binding protein of cytochrome c reductase (QPc)' most likely forms part of one of the two ubiquinol/ubiquinone reaction sites: the oxidation (center o) or the reduction site (center i). The Q-binding domain is located close to the interface between the inner membrane and the intermembrane space (residues 48–57 of the bovine sequence) and is adjacent to the conserved aromatic region (see Fig. 1). Recently, the cloning, sequencing and expression of QPc (9.5-kDa subunit) were reported [9].

One modification of the originally reported amino acid sequence [6] was noted: residue 61 is tryptophan instead of cysteine [9], increasing the aromatic nature of this domain.

The sequence conservation amongst the protein family that contains the yeast 11-kDa subunit VIII, the bovine 9.5-kDa subunit VII and other homologous proteins, referred to here as the 11-kDa protein family, is quite low [10]. However, all these proteins have a strikingly similar secondary structure [5,11], including a hydrophobic area close to the C-terminal domain. This region is just long enough to span the membrane and comprises 3–5 aromatic residues present in all sequences reported so far. The subunit IV from *R. sphaeroides*, which is a ubiquinol-binding protein [12], thought to fulfil the same role as the bovine 9.5-kDa subunit, also has a large hydrophobic area at the C-terminal domain comprising 3 aromatic residues located in the segment labeled by azido-Q [13].

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This might indicate a role of the aromatic domain in the structure of the Q-pocket or even in Q-binding.

Here we report the results of a further study of the aromatic domain of *S. cerevisiae* subunit VIII, using three other site-directed mutants with scattered aromatic residues. We also investigated the fate of the bc_1 complex when a cysteine residue is introduced into the sequence of the 11-kDa subunit VIII without any further deletion.

2. Materials and methods

Escherichia coli strain DH5a (recA, (rk^-, mk^+) l^-) was used for the propagation of recombinant DNA constructs, strain BMH71-18 was employed 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% (w/v) yeast extract, 1.6% (w/v) bactotryptone and 0.5% (w/v) NaCl) containing 100 μg/ml ampicillin for normal transformations or 100 μg/ml tetracycline for the transformation of the pSelect plasmids.

S. cerevisiae strain DLL80 (α, *his3*, *ura3*, LEU2::*qcr8*) [2] was used for the transformation of plasmids carrying the wild-type or mutated QCR8 genes (see below for further details). 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 checked on solid media containing 1% (w/v) yeast extract, 1% (w/v) bactopeptone, 2% (w/v) glycerol and 2% (w/v) ethanol.

Plasmid pSelect-H11S was constructed by isolating the 840 bp *Hin*dIII-*Sal*I fragment carrying the QCR8 gene from plasmid pUC18-H11S [3] and ligating this into the pSelect plasmid digested with the same enzymes.

Site-directed mutagenesis of the pSelect-H11S plasmids was performed with the following 11-kDa substitution oligonucleotides: 66SAYAA70 (5′-CTCGTTACCGTTCTTAGCAGCATAAGCAGAAATTCCCGCAGGTAT-3′); 66SASAW70 (5′-CTCGTTACCGTTCTTCCAAGCAGAAGCAGAAATTCCCGCAGGTAT-3′); 66SWYWW70 (5′-GTTCTTCCACCAGTACCAAGAAATTCCCGCAGG-3′); 66YWYWC70 (5′-CTCGTTACCGTTCTTGCACCAGTACCAATAAATTCC-3′) according to the pSelect manufacturer's protocol (Altered Sites in vitro Mutagenesis System/Promega).

Single-copy and multi-copy shuttle plasmids YCplac33 and YEplac195 [15] and the centromeric *E. coli*/*S. cerevisiae* shuttle vector pRS316 [16] were used as carriers of the wild-type or the mutated QCR8 genes.

Doubling times were determined by inoculating 50 ml lactate medium (0.5% yeast extract, 0.2% (w/v) magnesium sulphate, 0.6% (w/v) ammonium phosphate, 2% sodium lactate (70% w/v) and 1.3% lactic acid (75% w/v) pH 4.5) with yeast cells from an overnight culture in the same medium to give a starting A_{600} of about 0.2. Growth proceeded at 28°C to stationary phase. At different time points samples were taken to measure the optical density at 600 nm on a Zeiss spectrophotometer.

Mitochondria were isolated as described earlier [17]. Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer model DW2000. Concentrations of cytochromes were determined using the following absorbance coefficients and wavelength pairs for the reduced minus oxidized proteins: 21.3 mM^{-1} cm^{-1} at 605–625 nm for cytochrome aa_3 [18], 20.1 mM^{-1} cm^{-1} at 550–540 nm for cytochromes $c+c_1$ [19] and 28.5 mM^{-1} cm^{-1} at 562–575 nm for cytochrome *b* [20].

The ubiquinol-cytochrome *c* oxidoreductase 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 (Q_2H_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

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S.tuberosum  .........  .........  .......MGK  QPVKLKAVV.
  O.sativa   .........  .........  .......MGK  TPVRMKAVV.
A.thaliana   .........  .........  .......MGK  QPVKXKAVV.
  B.tauris   .........  .........  ...GRQFGHL  TRVRH..VIT
 H.sapiens   .........  .........  ..MGREFGNL  TRMAM..XIS
  K.lactis   .........  .MGGPHAK..  AYMGW.WGSI  GSPAQKGITT
S.cerevisiae .........  .MGPPSGK..  TYMGW.WGHM  GGPKQKGITS
  S.pombe    .........  MGGAAGGK..  TYLGW.WGHL  GGPKQKGIIT
  N.crassa   MRPTQTMLGG  GGGAPIGKHN  HYLGG.WGNF  GGMKQRGIIS
  Consensus  ----------  ----------  ---G---G--  -----K----

S.tuberosum  YAISPFQQKI  MPGLWKDLPG  KIHHKVSENW  ISATLLLGPL
  O.sativa   YALSPFQQKV  MPGLWKDITT  KIHHKVSENW  ISATLLLAPI
A.thaliana   YALPPFQQXI  XTGLWKXLPE  KIHHKVSXNW  XSATLLVTPV
  B.tauris   YSLSPFEQRA  FPHYFSKGIP  NVLRRTRACI  L...RVAPPF
 H.sapiens   YSLSPFEQRP  YPHVFTKGIP  NVLRRIRESF  F...RVVPQF
  K.lactis   YTVSPYAQKP  LNNIFHNAVF  NTFRRVKSQI  L...YMALPA
S.cerevisiae YAVSPYAQKP  LQGIFHNAVF  NSFRRFKSQF  L...YVLIPA
  S.pombe    YSLSPFQQRP  MAGFFKTSTQ  NMFRRVMTEG  L...YVAIPF
  N.crassa   YGISPNRQNP  LAGTAHDAVF  NTFRRVSSQF  L...YWAPSL
  Consensus  Y--SPF-Q--  --G-------  N--RRV----  --------P-

S.tuberosum  VGTYSYVQHF  LEKEKLEHRY  ..........  .
  O.sativa   VGTYEYAMYY  KEQEKLSHRY  ..........  .
A.thaliana   VGTYWYAQYF  KEQEKLLHRF  ..........  .
  B.tauris   VAFYLVYTWG  TQ..EFEKSK  RKNPAAYEND  R
 H.sapiens   VVFYLIYTWG  TE..EFERSK  RKNPAAYEND  K
  K.lactis   ALYWAWWVNC  RDYNAYLYTK  AGREELERVN  V
S.cerevisiae GIYWYWWKNG  NEYNEFLYSK  AGREELERVN  V
  S.pombe    GIAYYIYCWG  KERNEFLNSK  HGRHLVEE..  .
  N.crassa   VAGYYIMNWA  IERNHYLNSK  AGRAEFAGQE  E
  Consensus  V--Y------  -E-------K  ----------  -
R.sphaeroides VWKYRYRLGG[87]
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Fig. 1. Multiple sequence alignment of the 11-kDa protein family. Sequences were aligned using the GCG Pile Up program (gap weight, 3.00; gap length, 0.10) [34]. Amino acids appearing in at least 75% of the sequences are listed in the consensus line. *S. tuberosum* (potato) [11], *O. sativa* (rice) [10], *A. thaliana* (plant) [10], *B. tauris* (bovine) [9], *H. sapiens* (human) [GenBank accession no. T47406], *K. lactis* (yeast) [35], *S. cerevisiae* (yeast) [1], *S. pombe* (yeast) [5], *N. crassa* (fungus) [36], *R. sphaeroides* (bacterium) [37]. The residues underlined in the bovine sequence correspond to the ones labelled by azido-Q [8].

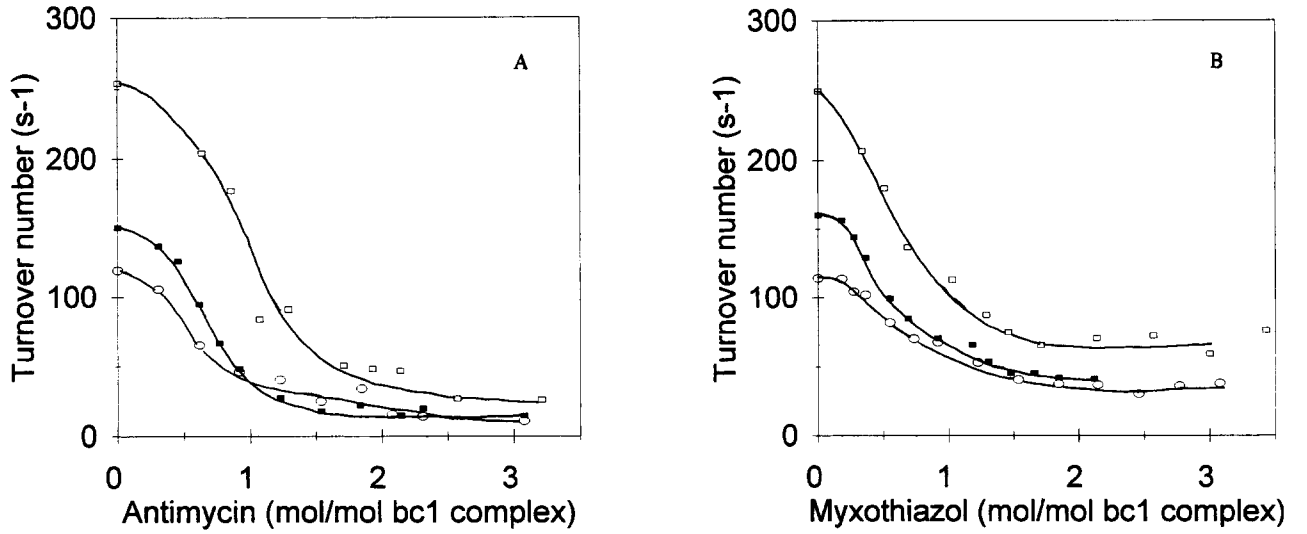

Fig. 2. Inhibitor titration of wild-type and mutant mitochondria: 101 μg of wild-type (□-□), 148 μg of SASAW (○-○) and 146 μg of SWYWW mitochondria (■-■) were incubated for 5 min in 2.0 ml of the bc_1 complex assay buffer, with different concentrations of the specific inhibitors. Thereafter, first KCN (0.5 mM) and cytochrome *c* (18 μM) were added and after the subsequent addition of 25 μM Q_2H_2 the reduction of cytochrome *c* was measured at 550–540 nm. The values given on the *x*-axis are calculated from the spectrally determined concentrations of antimycin, myxothiazol and cytochrome *b*, assuming 2 mol heme *b*/mol bc_1 complex. (A) Titrations with antimycin. (B) Titrations with myxothiazol.

with horse-heart cytochrome *c* as acceptor [21]. Spectral and kinetic data were analysed, using the DW2000 software.

Protein concentrations were determined according to Lowry et al. [22]. Published procedures were used for DNA manipulation and sequencing [23]. Restriction and other enzymes used in DNA manipulation were purchased from Boehringer, Biolabs and Sigma and used as recommended by the manufacturers. Radioactive chemicals were obtained from Amersham. All other chemicals were of the highest purity available.

3. Results

3.1. Production of the mutants

Fig. 1 shows the amino acid sequence of all known members of the 11-kDa protein family, displaying in bold the position of the aromatic residues in the aromatic domain. The part of the bovine sequence labelled with azido-Q [8] is underlined. In *S. cerevisiae* the residues ^{66}YWYWW^{70} correspond to the ones substituted in this study. The QCR8 gene was liberated as an 840 bp *Hin*dIII-*Sal*I fragment from plasmid pUC18-H11S [3] and ligated into the pSelect plasmid digested with the same enzymes. Site-directed mutagenesis was performed on single-stranded DNA with the substitution oligonucleotides as given in Section 2. After mutagenesis, plasmid DNA was isolated from 12 ampicillin-resistant colonies from each mutant to check for the presence of the 840 bp *Hin*dIII-*Sal*I insert and DNA-sequence analysis was performed on 10 of these plasmids containing the correct insert. The desired substitution was present in 50–75% of these plasmids depending on the mutant. The mutated QCR8 genes were ligated as a *Hin*dIII-*Sal*I fragment into the single-copy shuttle vectors YCplac33 or pRS316, and the 66SASAA70/LTN2 mutant also into the multi-copy vector YEplac195 [4].

The plasmids containing the different mutants were used to transform DLL80 to uracil prototrophy and the uracil prototrophs from either transformation were subsequently replica plated to ethanol/glycerol (EG) plates to monitor their respiratory capacity. Table 1 lists the different mutants.

3.2. Properties of the mutants

Table 2 shows the results of the spectral and kinetic analysis of the different yeast mutants. Calculation of the turnover number based on the amount of holo-cytochrome *b* and the specific bc_1 complex activity in mitochondria isolated from the different yeast mutants shows that this is approximately the same in the wild-type and the LTN3/^{66}FASAA^{70} transformant; it is slightly lower in the LTN2/^{66}SASAA^{70}, ^{66}SAYAA^{70} and ^{66}YWYWC^{70} transformants and is 50–60% reduced in the ^{66}SASAW^{70}, ^{66}SWYWW^{70} and LTN1 transformants.

The cytochrome $c+c_1$ and cytochrome $a+a_3$ levels are also different (lower or higher) in most of the transformants as compared to the levels in the wild type. Since it was shown that also in the *qcr*8 null mutant the levels of these cytochromes are comparable to those of the wild type [1,2] and these levels tended to vary slightly between different mitochondrial preparations of the transformants, we prefer not to speculate on the significance of the minor differences observed.

The presence of a wild-type level of complex III, and therefore full assembly, in the mutants SASAW and SWYWW was additionally shown by titrations of isolated mitochondria with antimycin (Q_{in}-site inhibitor) and myxothiazol (Q_{out}-site in-

Table 1
List of the different mutants

Mutant name and vector	Amino acid sequence	Reference
Wild-type/pRS316	^{66}YWYWW^{70}	this study
LTN2/YCplac33	^{66}SASAA^{70}	[4]
LTN2/YEplac195	^{66}SASAA^{70}	[4]
LTN3/YCplac33	^{66}FASAA^{70}	[4]
SAYAA/pRS316	^{66}SAYAA^{70}	this study
SASAW/pRS316	^{66}SASAW^{70}	this study
SWYWW/pRS316	^{66}SWYWW^{70}	this study
YWYWC/pRS316	^{66}YWYWC^{70}	this study
LTN1/YCplac33	^{69}WWKNG^{73}/69C	[3]

Table 2
Doubling time, enzymatic activity and spectral analysis of wild-type and mutant cells and mitochondria

Mutant	Doubling time (min)	Specific activity (nmol/mg per s)	$c+c_1$ (nmol/mg)	b (nmol/mg)	aa_3 (nmol/mg)	Turnover number (s^{-1})
Wild-type	281 (3)	12.7 (2)	0.20	0.14	0.04	181
YCpLTN2	–	1.7 (2)	0.14	0.02	0.05	170
YEpLTN2	–	4.7 (1)	0.24	0.06	0.06	157
LTN3	514 (1)	6.4 (1)	0.19	0.07	0.04	183
SAYAA	700 (1)	5.1 (1)	0.07	0.06	0.04	170
SASAW	624 (1)	5.6 (2)	0.25	0.13	0.05	86
SWYWW	470 (2)	7.3 (2)	0.19	0.13	0.07	112
YWYWC	312 (2)	11.0 (2)	0.21	0.14	0.07	157
LTN1	676 (1)	6.0 (1)	0.10	0.11	0.06	109

The number of measurements on individual isolates is given in parentheses. See Section 2 for experimental conditions.

hibitor) (see Fig. 2). The lowered turnover of the assembled enzyme in these mutants was confirmed. The titration curves show that the affinity for myxothiazol is unaffected in both mutants, despite the lowered turnover, but that the affinity for antimycin is lowered in the SASAW mutant (see Fig. 2). The LTN1 mutant, on the other hand, displayed a decreased affinity for myxothiazol [3], while for the LTN2/^{66}SASAA^{70} and LTN3/^{66}FASAA^{70} no differences in the affinities for either inhibitor was observed [4]. The affinities of the ^{66}SAYAA^{70} and ^{66}YWYWC^{70} mutants for either myxothiazol or antimycin were not changed (not shown).

4. Discussion

As deduced from mutant studies [24,25], the assembly of the yeast bc_1 complex involves the formation of a subcomplex between cytochrome *b*, the 14-kDa subunit VII and the 11-kDa subunit VIII. Both the 14-kDa and 11-kDa subunits are closely associated with cytochrome *b* and the mitochondrial inner membrane [26].

The bovine heart 9.5-kDa subunit is assumed to span the inner membrane [6,9]. This protein and the subunit IV from *R. sphaeroides* show no significant amino acid sequence similarity, although their structural features, such as hydrophobicity and topological arrangements of the quinone binding domains with respect to the membrane look alike. Both have only one transmembrane helix and their supposed quinone binding domains, as derived from azido-quinone binding data, are located in the transmembrane helix near the surface of the membrane [6,8,9,27].

Also the yeast 11-kDa subunit VIII shows little sequence similarity with the bovine 9.5-kDa subunit VII, but shares the same structural features [5]. It has been shown recently [28] that this polypeptide, although not extractable with carbonate, is not transmembranous. We may assume that this also holds for the homologous bovine 9.5-kDa subunit, although in this polypeptide the hydrophobic domain shows a larger hydrophobic moment [5]. The association of such a protein with the membrane will be achieved via interaction of the hydrophobic region of this protein with phospholipids and probably with hydrophobic residues of cytochrome *b*. The domain ^{66}YWYWWKNG^{73}, including 5 consecutive aromatic residues, is just on the interface between the inner membrane and the intermembrane space. The results obtained in the study of several in vitro mutants [3,4] show that this region is crucial both for stable interaction (via aromatic stacking?) with other subunits of the complex, supposedly cytochrome *b*, and for the electron transfer activity of center o.

Recent studies suggest that membrane-integrated segments do have specific features, one of these being a clustering of aromatic residues at the polar/non-polar interface, observed in the reaction centers, porins, prostaglandin H synthase and in the peptide ionophore gramidicin A [29]. In this latter case, the four boundary tryptophans may form hydrogen-bonds with the lipid headgroups or water and are likely to play a role in anchoring the peptide in a correct orientation within the bilayer [30]. Tryptophan is a large, aromatic, and polar but neutral residue. Depending on the environment, it can either contribute to the hydrophobicity of a protein region or act as a hydrogen bond donor through its nitrogen free doublet. Studies with model compounds mimicking the side chain of a tryptophan show that the indole ring partitions spontaneously to the lipid headgroup region of a bilayer [31]. The driving force of this process has a substantial enthalpic component, implying that factors other than the hydrophobic effect, for example van der Waals interactions or hydrogen bonding, are also involved in stabilizing the interfacial location of tryptophans [31]. Inspection of the structure of reaction centers shows that many tryptophans participate in hydrogen bonds with distant main chain carbonyls, which may contribute to the stability of the tertiary structure [32]. Site-directed mutagenesis studies with bacteriorhodopsin and with another protein of the rhodopsin family indicate that tryptophans in transmembranous segments, while not essential for function, participate in ligand binding and also contribute to the stability of the protein [33].

The previously described LTN1 mutation [3] showed both a decreased level of assembly and a decreased turnover number, the latter being caused by a decreased rate of reduction of the low-potential cytochrome *b*, implying that this region of the yeast 11-kDa protein contributes to the Q_{out} reaction center. It is tempting to speculate that not only a site of interaction between the 11-kDa subunit VIII and cytochrome *b* resides at the aromatic domain, resulting in a decreased level of assembly in the LTN1 mutant, but that this domain is also part of the reaction site of the Q_{out} semi-quinone with the low-potential cytochrome *b*. While the SASAA mutant showed only very little assembly, a substantial, but still decreased level of assembly, without an effect on the turnover number, is found in FASAA and SAYAA, so an aromatic residue at a position between 66 and 68 improves the assembly drastically, but for full assembly it seems that the presence of a tryptophan at positions 69 or 70 is required, as is the case in the SASAW and SWYWW mutants. A decreased level of assembly was also observed in the LTN1 mutant, in which the two tryptophans at positions 69 and 70 are absent.

The C-terminal deletion mutant described by Schoppink et al. [2], in which the 66Y was followed by a tag of six residues encoded by a fused stop-oligonucleotide (with sequence ^{67}SCSQAC^{72}), can be considered as a variant of the ^{66}FASAA^{70} mutant, in which assembly is further diminished as consequence of the absence of the C-terminus. The introduction of a cysteine residue in the LTN1 mutant had probably less effect than the shortening in length and the removal of the two tryptophans, as demonstrated by the analysis of the ^{66}YWYWC^{70} mutant, which has a similar turnover number and cytochrome *b* concentration as the wild-type enzyme.

The data obtained from the investigation of the phenotype of the SAYAA, SASAW and SWYWW mutants indicate the importance of the aromatic region not only for the assembly of the bc_1 complex, but also for the turnover and stability of the enzyme. The mutant ^{66}SASAW^{70} presents, based on the amount of holo-cytochrome *b* and the specific bc_1 complex activity, a turnover number that is reduced to 48% of the wild-type level. The ^{66}SWYWW^{70} mutant has an intermediate phenotype. The lowered turnover numbers of these mutants indicate that the Q_{out} site is affected, just like in the LTN1 mutant [3]. Based on these data we can speculate about an involvement of subunit VIII in the stabilization of the semiquinone, but no specific residue(s) can be assigned for this role as yet. The final picture that arises from the combined data of all these mutants points to an involvement of the aromatic residues in stabilizing the tertiary structure of the protein. The finding that the SASAW mutant displays a decreased affinity for antimycin cannot be structurally interpreted as yet.

Assuming that the consensus sequence is required for the functionality of an assembled complex, we may postulate that, in addition to the overall structure, the sequence of the regions of interaction with the other subunits of the complex determines the level of assembly. These sequences have evolved together with the sequences of the interacting proteins, so that assembly correlates with evolutionary distance. Here we propose that the conserved aromatic region of the 11-kDa protein family is one of those regions of interaction with cytochrome *b*, the central subunit of the bc_1 complex, and that this site of interaction is part of the center o.

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