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The aromatic nature of residue 66 of the 11-kDa subunit of ubiquinol-cytochrome *c* oxidoreductase of the yeast *Saccharomyces cerevisiae* is important for the assembly of a functional enzyme

Wieger Hemrika^a, Gisele Lobo-Hajdu^a, Jan A. Berden^{a,*}, Leslie A. Grivell^b

^a*E.C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands*

^b*Section for Molecular Biology, Department of Molecular Cell Biology, University of Amsterdam, Amsterdam, The Netherlands*

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Abstract

Transformation of multi- and single-copy plasmids carrying a mutated version (*LTN2*, region 66-YWYWW-70 replaced by SASAA) of *QCR8*, the gene encoding the 11-kDa subunit of ubiquinol-cytochrome *c* oxidoreductase of *Saccharomyces cerevisiae*, to a *QCR8*⁰ strain indicated the importance of this aromatic region for the assembly of a functional enzyme. Sequencing of plasmids giving spontaneous restoration of growth to some colonies among the single-copy *LTN2* transformants showed that changing the sequence SASAA into the sequence FASAA could, to a large extent, overcome the observed assembly defect, indicating the importance of the aromatic nature of residue 66.

Key words: Ubiquinol-cytochrome *c* oxidoreductase; Subunit; Mutagenesis; Assembly; *Saccharomyces cerevisiae*

1. Introduction

The 94 aa 11-kDa subunit 8 of the *Saccharomyces cerevisiae* ubiquinol-cytochrome *c* oxidoreductase (complex III or the *bc₁* complex) is encoded by *QCR8*, a single-copy nuclear gene located on chromosome X [1]. Analysis of a *QCR8* disruption mutant showed that the 11-kDa subunit is indispensable for the assembly of a functional complex III. This mutant lacks holo-cytochrome *b* and displays severely reduced levels of apo-cytochrome *b*, the Rieske Fe-S protein and the 14-kDa subunit [1]. Since these pleiotropic effects complicate further analysis of a possible involvement of the 11-kDa subunit in the enzymatic mechanism of complex III, studies were performed in which in vitro mutagenised *QCR8* genes were transformed to a *QCR8*⁰ strain [2, 3].

Schoppink et al. [2] showed that after transformation of the *QCR8*⁰ mutant with a gene encoding the first 66 amino acids of the 11-kDa subunit, fused to the sequence SECEAC (see footnote**) encoded by a STOP-oligonucleotide, assembly of the *bc₁* complex was impaired and

that mitochondria isolated from this yeast mutant had a low complex III activity (5% of that of the wild type) while the turnover number appeared to be only slightly lower than normal. The authors concluded that the C-terminal part of the 11-kDa subunit is involved in the assembly, but not in the catalytic mechanism of complex III.

More recently, Hemrika et al. [3] reported that after transformation of the *QCR8*⁰ strain with a library of hydroxylamine-mutagenised *QCR8* genes a mutant was obtained that was affected in complex III activity while, on Western blots, a protein was detected reacting with antibodies directed against the 11-kDa subunit. This mutant, which appeared not to be a point-, but a deletion mutant (residues 69-WWKNG-73 are replaced by a cysteine), showed an almost normal assembly of the *bc₁* complex, but an increased protease-sensitivity and a turnover number which was approximately half that of the wild type enzyme. Pre-steady state kinetic analysis of this mutant showed that the reaction of the *Q_{out}* semiquinone with the low-potential cytochrome *b* was impaired. It was postulated that the 11-kDa subunit is involved in the formation of the *Q_{out}* reaction domain, possibly contributing to the 'catalytic switch' mechanism as proposed by Brandt and von Jagow [4].

A contribution of the 11-kDa subunit to Q-binding is in agreement with studies on the homologous 9.5-kDa subunit from the bovine-heart complex III [5]. It has been shown that this subunit can be photo-affinity la-

*Corresponding author. Fax: (31) (20) 5255124.

**The changed reading frame of the carboxy-terminus of *QCR8* as a result of the extra cytosine found at position 185 [3] also affects the sequence encoded by the STOP-oligo, changing it from PAAKLAD [2] into SECEAC.

Abbreviations: *Q₂H₂*, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol.

belled with a quinone derivative [6] and Usui et al. [7] isolated and identified the labelled peptide. Alignment of the 11-kDa subunit to the 9.5-kDa subunit shows that this labelled peptide is adjacent to the region mutated in the LTN1 mutant described in [3]. Since this region contains a high number of aromatic residues among which five consecutive ones (see Fig. 1) and since the mutation described in [3] affects two of these five aromatic residues we decided to monitor the effect of changing the aromatic nature of this region by replacing the sequence 66-YWYW-70 by the sequence SASAA. Here we report the results of this study.

2. Materials and methods

2.1. Strains and media

Escherichia coli strain JF 1754 (*lac*, *gal*, *metB*, *leuB*, *hisB*, *hsdR*) was used for the propagation of recombinant DNA constructs, 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% w/v yeast extract, 1% 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.

Saccharomyces cerevisiae strain DL1-QCR8⁰ (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 [8]. 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) bacto-peptone and 2% (w/v) glycerol and 2% (w/v) ethanol.

2.2. Plasmids and site-directed mutagenesis

Single-copy and multi-copy shuttle plasmids YCplac33 and YEplac195 [9] were used as carriers of the wild type or the mutated *QCR8* genes.

Plasmid pSelect-H11S was constructed by isolating the 840 bp *HindIII*–*SalI* fragment carrying the *QCR8* gene from plasmid pUC18-H11S [3] ligating this into the pSelect plasmid digested with the same enzymes.

Site-directed mutagenesis of the pSelect-H11S plasmids was performed with an 11-kDa substitution oligonucleotide (5'-CTCGTACCCTTCTTAGCAGCAGAAGCAGAAATCCCGCAGGTAT-3') according to the pSelect manufacturers protocol.

2.3. Isolation of mitochondria and immunoblotting

Mitochondria were isolated as described earlier [10]. Mitochondrial proteins were separated on SDS-polyacrylamide slab gels according to [11] and blotted according to [12]. After incubation of the blots with antibodies directed against specific subunits of complex III [13] the antigen-antibody complexes were made visible with the horseradish peroxidase colour-development assay [14]. The antisera used were raised in rabbits or mice.

2.4. Spectral analysis and complex III assay

Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer model DW2. Concentrations of cytochromes were determined using the following absorbance coefficients and wavelength pairs for the reduced minus oxidized proteins: 24.0 mM⁻¹·cm⁻¹ at 605–630 nm for cytochrome *aa₃* [15] 20.1 mM⁻¹·cm⁻¹ at 550–540 nm for cytochromes *c* + *c₁* [16] and 28.5 mM⁻¹·cm⁻¹ at 562–575 nm for cytochrome *b* [17].

The ubiquinol-cytochrome *c* oxidoreductase assay was performed spectrophotometrically at 25°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 (Q₂H₂). 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 [18].

Spectral and kinetic data were analysed, using Popspec software on an Atari 1040ST computer coupled to the spectrophotometer via the Poptronics 4 channel A/D converter.

2.5. Miscellaneous

Protein concentrations were determined with the Lowry method [19]. Published procedures were used for DNA manipulation and sequencing [20]. 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 the 11-kDa subunit and the position of the aromatic residues (66-YWYW-70) to be substituted. The *QCR8* gene was liberated as an 840 bp *HindIII*–*SalI* 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 oligonucleotide as given in section 2. After mutagenesis, plasmid DNA was isolated from 10 ampicillin-resistant colonies to check for the presence of the 840 bp *HindIII*–*SalI* insert and DNA-sequence analysis was performed on 4 of these plasmids containing the correct insert. The desired substitution was present in 3 of these plasmids. The mutated *QCR8* gene, which was named LTN2, was ligated as a *HindIII*–*SalI* fragment into the single- and multi-copy shuttle vectors YCplac33 and YEplac195 giving, YCp-LTN2 and YEp-LTN2.

Both LTN2 plasmids were used to transform DL1-QCR8⁰ to uracil prototrophy and 5 uracil prototrophs from either transformation were subsequently transferred to ethanol/glycerol plates to monitor their respiratory capacity.

Fig. 2 shows that after one week of growth on the ethanol/glycerol plates the YCp-LTN2 transformant shows hardly any growth while also the growth of the YEp-LTN2 transformant is reduced compared to that of the wild type. Interestingly, colonies of the YCp-LTN2 transformants, growing on ethanol/glycerol plates, gave rise to phenotypic revertants with growth rates approximately equal to that of the wild type cells (Fig. 2). Plasmid DNA, isolated from the fastest growing colony conferred the same phenotype on transformation to the DL1-QCR8⁰ showing that this restoration of growth derives from a change in the plasmid itself, rather than from a change in a nuclear gene from the host. Sequence analysis of the isolated plasmid DNA indicated that the phenotypic reversion was the result of a C to T transition at nucleotide position 197 of the LTN2 gene, which changes the serine at position 66 into a phenylalanine. The SASAA motif of the LTN2 gene thus becomes

MGPPSGKTYMGWGWGHMGGPKQKQKIGITSYAVSPYAQKPLQGIHFHNAVFNFRFRFKSQFLYV
 LIPAGI**YWYWW**KNGNEYNEFLYSKAGREELERVNV (11-kDa subunit)
SASAA (LTN2)
FASAA (LTN3)

Fig. 1. Amino acid sequence of the 11-kDa subunit and the residues replaced by the *LTN2* and *LTN3* encoded proteins. Fig. 1 shows the sequence of the 11-kDa subunit of *Saccharomyces cerevisiae*. The residues (66–70) which are replaced in the mutants are given in bold face. The replaced sequences encoded by the *LTN2* and *LTN3* genes are given below the wild type sequence.

FASAA. This mutant gene was named *LTN3*. Two fast growing revertants subsequently analysed showed the same genotype.

3.2. Properties of the mutants and the phenotypic revertant

To rule out any differences due to the presence of the *QCR8* alleles on the plasmids YEplac-195 and YCplac-33 in the mutants, as compared to the *QCR8* gene in its chromosomal context, we decided to use D11-*QCR8*⁰ transformed with a wild type copy of the *QCR8* gene on YCplac33, and not D11 itself, as the wild type in the following experiments.

Fig. 3 shows the results of a Western blot analysis of mitochondria from ethanol/glycerol-grown cells of the wild type, both *LTN2* and the YCp-*LTN3* transformants. Equal amounts of mitochondrial protein were separated on a SDS-polyacrylamide gel and blotted to nitrocellulose paper to determine the steady-state levels of the complex III subunits. It can be seen that the mutated 11-kDa subunits run faster on the SDS-polyacrylamide gel than the wild type 11-kDa subunit. Moreover, as, lanes 1 and 2 indicate, assembly of complex III is severely impaired in both single- and multi-copy *LTN2* transformants, as judged from the low levels of apo-cytochrome *b*, the Rieske-FeS protein and the 14-kDa subunit and overexpression of the *LTN2* gene does not detectably increase the levels of these subunits (Fig. 3, lane 2).

The partial restoration of the aromatic nature of the mutated region in the YCp-*LTN3* transformant apparently overcomes the assembly defect observed in the *LTN2* transformants since the steady-state levels of all the complex III subunits are approximately equal to those of wild type cells (Fig. 3, lanes 3 and 4).

Fig. 4A,B and Table 1 show the results of the spectral and kinetic analysis of the different yeast strains. The difference spectra of Fig. 4A show that in the presence of succinate and antimycin almost no holo-cytochrome *b* can be detected in the YCp-*LTN2* transformant while also in the YEplac-*LTN2* transformant its amount is severely diminished. The serine to phenylalanine conversion in the *LTN3* gene increases the holo-cytochrome *b* level of the YCp-*LTN3* transformant to 73% of that of the wild type. Calculation of the turnover number based on the amount of holo-cytochrome *b* and the specific

complex III activity in mitochondria isolated from the different yeast strains (see Table 1) shows that this is approximately the same in the wild type and the YCp-*LTN3* transformants while it is slightly lower in both *LTN2* transformants.

Fig. 4B shows that the cytochrome *c+c₁* levels are lowered in both *LTN2* transformants as compared to these levels in the wild type and the YCp-*LTN3* transformants, while for the cytochromes *a+a₃*, the situation is reversed. Since it was shown that in the *QCR8*⁰ mutants the levels of these cytochromes are comparable to those of the wild type [1,2] and since these levels tended to vary between different mitochondrial preparations of the two *LTN2* transformants, we prefer not to speculate on the significance of this observation.

No differences were observed in the affinities for myxothiazol and antimycin between the wild type and the mutant *bc₁* complexes (results not shown).

4. Discussion

To study the importance of the aromatic region (residues 66-YWYWW-70) of the C-terminal part of the 11-kDa subunit of the yeast *bc₁* complex, we constructed, via site-directed mutagenesis, a gene (*LTN2*) in which this aromatic region was replaced by the sequence SASAA.

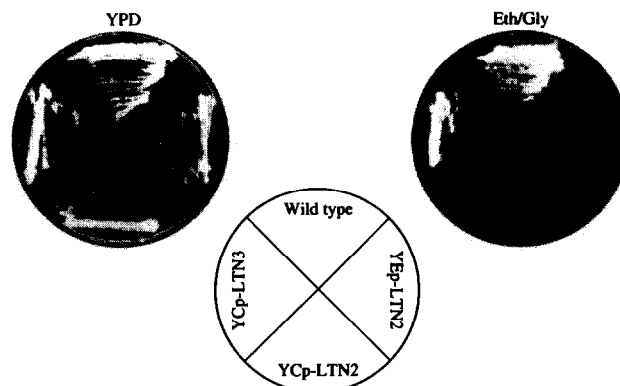


Fig. 2. Analysis of the growth characteristics of the wild type, the YCp-*LTN2*, the YEplac-*LTN2* and the YCp-*LTN3* transformants. Cells of the different yeast strains were streaked on plates containing rich glucose medium or rich ethanol/glycerol medium. See section 2 for details on the composition of the media.

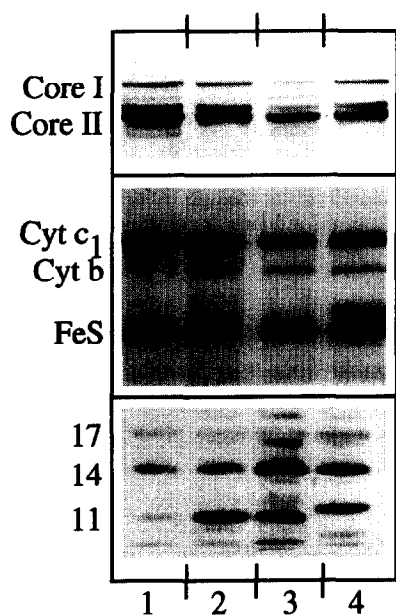


Fig. 3. Western blot analysis of mitochondrial protein from the wild type, the YCp-LTN2, the YEp-LTN2 and the YCp-LTN3 transformants. 30 μ g of mitochondrial protein of the wild type the YCp-LTN2, the YEp-LTN2 and the YCp-LTN3 transformants were separated on a 15% SDS-polyacrylamide gel and blotted to nitrocellulose paper. The blots were incubated with polyclonal antibodies directed against the core II subunit, the Rieske Fe-S protein, the 17-kDa, 14-kDa and the 11-kDa subunits and against cytochrome *c*₁, cytochrome *b* and the Rieske Fe-S protein. Lane 1, mitochondrial protein isolated from the YCp-LTN2 transformant. Lane 2, mitochondrial protein isolated from the YEp-LTN2 transformant. Lane 3, mitochondrial protein isolated from the YCp-LTN3 transformant. Lane 4, mitochondrial protein isolated from the wild type transformant.

Complementation of a QCR8 disruption strain with a single-copy plasmid carrying this *LTN2* gene showed that the region 66–70 is of great importance for the assembly of the enzyme since in the *LTN2* transformants both complex III activity and holo-cytochrome *b* content are lowered to approximately 6% of that of the wild type transformant, while on Western blots it is shown that also the levels of apo-cytochrome *b*, the Rieske Fe-S protein and the 14-kDa subunit are severely diminished. Overproduction of the *LTN2* gene on a multi-copy plasmid does not increase the levels of these subunits on Western blots but the amount of holo-cytochrome *b* and the complex III activity are increased to 40% and 36% of that of the wild type, respectively. The increase amount of holo-cytochrome *b* without a detectable increase in the level of apo-cytochrome *b* may be indicative for a role of the 11-kDa subunit in the maturation of cytochrome *b*.

The obtained results indicate the importance of the mutated region for the assembly of complex III, but no large effect is found in the turnover number of the enzyme. The effect of the previously described mutation [3] is therefore probably not due to the removal of the aromatic residues 69–70 (WW) as such, but may be the

result of distortion of the conformation of the Q_{out} reaction domain due to the shortening of the 11-kDa subunit and/or the introduction of the cysteine residue, thereby impairing the reaction of the Q_{out} semi-quinone with the low-potential cytochrome *b*.

Sequencing of plasmid DNA responsible for conferring almost wild type growth on QCR8⁰ transformants revealed that the sequence SASAA of the *LTN2* gene had been mutated into the sequence FASAA. This mutation thus largely overcomes the assembly defect. In the YCp-LTN3 transformant the steady-state levels of the complex III subunits on Western blots were comparable to those of the wild type while complex III activity and holo-cytochrome *b* level were restored to 76% and 73% of that of the wild type, respectively.

According to the model of Berden et al. [21] (see also Crivellone et al. [22]), assembly of complex III involves three subcomplexes and the Rieske Fe-S subunit. One of these subcomplexes is formed between cytochrome *b*, the 14-kDa and the 11-kDa subunit. Both the 14-kDa and the 11-kDa subunit are strongly associated with cytochrome *b* and the mitochondrial inner membrane [23] without possessing hydrophobic stretches long enough to be predicted as membrane spanning. It is therefore likely that the association with the membrane is accomplished via interaction of hydrophobic patches of the 14-kDa and the 11-kDa subunits with the hydrophobic residues of cytochrome *b*. Residues 66–70 are, according to the Kyte and Doolittle algorithm, just on the interface between the hydrophobic (residues 55–68) and the more hydrophilic environment (residues 69–74). Since the results obtained with the *LTN3* gene show that especially the aromatic nature of residue 66 is of great importance we believe that at least this residue makes contact (via aromatic stacking) with cytochrome *b*. It is not excluded that also residue 67 can fulfil such a role since this study would not reveal such a possibility because alanine cannot be converted into an aromatic residue by one single base substitution.

We recently proposed that the charged C-termini of the 14-kDa and the 11-kDa subunits are involved in the association of the two hydrophilic subcomplexes to the

Table 1
Enzymic activity and spectral analysis of wild type transformant and mutant mitochondria

Strain	Specific activity of complex III (nmol/mg/s)	Cytochrome content (nmol/mg)			Turnover number (s ⁻¹) Complex III
		Cyt <i>c</i> ₁ + <i>c</i> ₁	Cyt <i>b</i>	Cyt <i>aa3</i>	
D11	30.1 (4)	0.16	0.15	0.06	401
YCp-LTN3	22.8 (4)	0.17	0.11	0.07	415
YEp-LTN2	10.7 (3)	0.11	0.06	0.09	357
YCp-LTN2	1.8 (3)	0.10	0.01	0.09	360

The number of measurements on individual isolates are given between brackets. See section 2 for experimental conditions.

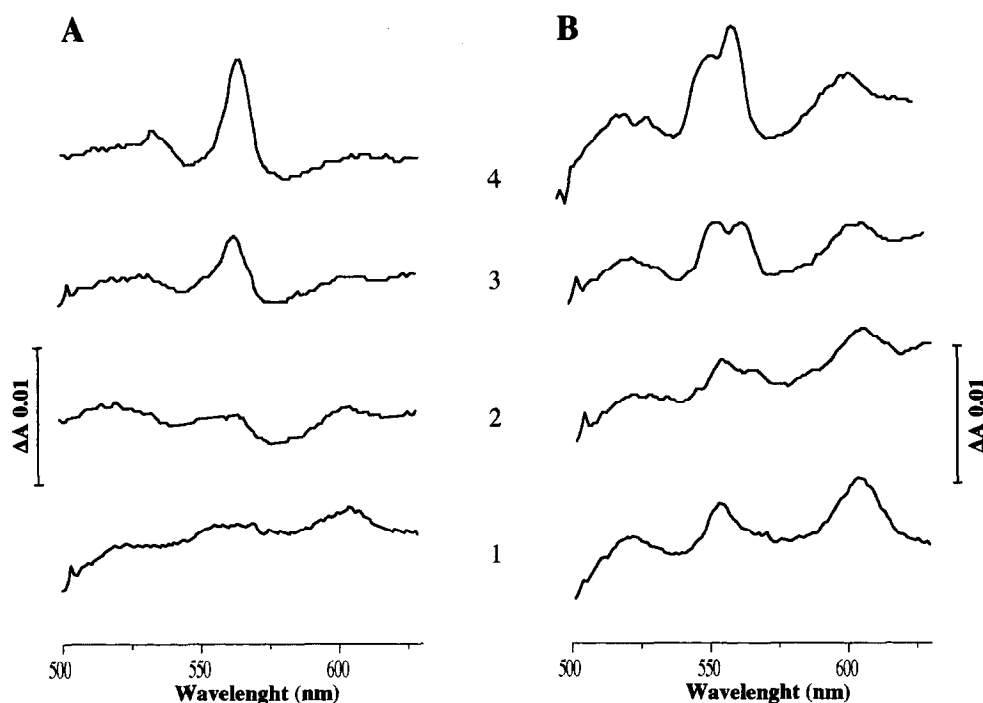


Fig. 4. Spectral analysis of the cytochrome content of mitochondria isolated from the wild type-, the YCp-LTN2 the YEp-LTN2 and the YCp-LTN3 transformant. Mitochondrial protein was isolated from cells grown on ethanol/glycerol medium. The difference spectra were recorded at room temperature. Protein concentrations were 1.7 mg/ml for the wild type and the YCp-LTN3 transformants, respectively, and 1.4 mg/ml and 2.4 mg/ml for the YEp-LTN2 and YCp-LTN2 transformants, respectively. Panel A, difference spectra of succinate-reduced mitochondria in the presence of antimycin, minus ferricyanide-oxidized mitochondria. Panel B, ascorbate+KCN were added to the sample cuvettes of traces 1; the reference cuvettes remained the same. Traces 1, mitochondrial protein isolated from the YCp-LTN2 transformant. Traces 2, mitochondrial protein isolated from the YEp-LTN2 transformant. Traces 3, mitochondrial protein isolated from the YCp-LTN3 transformant. Traces 4, mitochondrial protein isolated from the wild type transformant.

cytochrome *b* subcomplex. Here we propose that residue 66 is involved in the interaction with cytochrome *b*.

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