Volume 321, number 2,3, 159–162 FEBS 12369 April 1993

Insight into topological and functional relationships of cytochrome *c* oxidase subunit I of *Saccharomyces cerevisiae* by means of intragenic complementation

Brigitte Meunier, Françoise Coster, Philippe Lemarre and Anne-Marie Colson

Université Catholique de Louvain, Laboratoire de Génétique Microbienne, Place Croix du Sud 4, 1348 Louvain-la-Neuve, Belgium

Received 25 February 1993; revised version received 14 March 1993

In yeast, revertants were selected from four respiratory deficient mutants carrying mutations in the cytochrome *c* oxidase subunit I gene. Intragenic second site mutations revealed amino acids which are functionally complementary to the original mutated position and may be in topological interaction with it. The results provide additional data in favour of the model proposed for the structure of the binuclear centre in proton-motive oxidases.

Cytochrome *c* oxidase; Yeast; Intragenic complementation; Models

1. INTRODUCTION

Cytochrome *c* oxidase, the terminal acceptor of the mitochondrial respiratory chain, catalyses the reduction of oxygen into water. Electron transfer from cytochrome *c* to oxygen catalysed by this enzyme is coupled to translocation of protons across the membrane. The yeast mitochondrial cytochrome oxidase composed of up to 13 subunits [1] and containing two haems and two copper atoms is a member of a large family of proton pumping oxidases [2] which includes oxidases from bacteria, plants and animals. Only three subunits (subunits I, II and III), mitochondrially encoded, appear to be constituents of the minimal catalytic core and are homologous to the subunits of the prokaryote enzymes. Subunit I is very highly conserved and has 12 transmembrane helices as predicted from hydropathy analyses. It contains the ligands for haem *a* and the binuclear centre, haem a_3–Cu_B. Three-dimensional models for the structure of subunit I have been made on the basis of sequence comparisons, biophysical data and the assignment of the histidine ligands to the metal prosthetic groups [3–8]. In a recent model [7], the binuclear centre is located at about the same level as haem *a*, near the outer positive side of the membrane. A proton channel connects the redox centres to the negative aqueous phase. Subunit II, essential for catalytic activity, has two transmembrane spans and a large hydrophilic domain which contains the ligands of the Cu_A and the docking site for the cytochrome *c* [8]. Subunit III does not contain redox active centre and appears not to be essential for the activity [9] although it may have a role in assembly, stability or activity modulation.

Respiratory deficient mutants with optically detectable cytochrome *c* oxidase, considered most interesting in bioenergetics studies, have been isolated in recent studies using *Saccharomyces cerevisiae* ([10,11], and unpublished data). These have identified a large number of mutants in the catalytic subunits I, II and III. The use of a strain lacking all mitochondrial DNA introns to generate mutants [10] and rapid spectrophotometric techniques, which allowed high quality spectra of whole cells grown on appropriate media, have been very effective [11]. Genetic analyses and sequencing have been able to identify the nucleotide changes and the resulting amino acid replacements responsible for the deficiency in several mutants of subunit I [10].

In the present work, four deficient mutants, L13, L29, L45 and L53, with known nucleotide substitution [10] (Fig. 1) in the subunit I encoding gene, were chosen for the reversion analyses. The search for intragenic reversions of deficient mutations is a fruitful approach in order to identify amino acid replacements compatible with the function of the enzyme. Distant reversions allow the detection of pairs of amino acids which are functionally complementary and potentially close in space. Mutant L13 (G253D) with a glycine to aspartate change in position 253 located in the transmembrane-spanning helix 6, in the vicinity of the Cu_B ligand, histidine-241, is likely to carry a limited topological alteration restricted to the binuclear centre environment since it still has optically detectable cytochrome oxidase and shows a significant faster CO recombination kinetics compared to the parental strain [11]. This change, how-

Correspondence address: B. Meunier, Université Catholique de Louvain, Laboratoire de Génétique Microbienne, Place Croix du Sud 4, 1348 Louvain-la-Neuve, Belgium. Fax: (32) (10) 47-3109.

ever, leads to a total inability of yeast to grow on glycerol. Mutant L29 (T316K), with a threonine to lysine change in position 316 located in the α-helix 8, has optically detectable cytochrome oxidase and is similarly affected in the CO recombination kinetics. In contrast to L13, it grows poorly on glycerol indicating a partial cytochrome oxidase activity. This CO recombination effect may indicate, as suggested by Brown et al. [7,11], that helix 8 and position 316 are also located in the binuclear centre environment or in the channel leading to it from the negative aqueous phase. Mutant L53 (G352V), carrying a glycine to valine mutation in position 352 located in helix 9 has an optically detectable cytochrome oxidase and shows no modification of the CO recombination kinetics [11]. Like L29 (T316K), this mutant has a weak growth on glycerol. Mutant L45 with a replacement of aspartate to asparagine (D369N) in position 369 located in the extramembranous loop between helices 9 and 10, has no optically detectable cytochrome oxidase and is totally unable to grow on glycerol. The mutated position is included in a sequence of seven well conserved amino acids which might be involved in intersubunit electron transfer between Cu_A in subunit II and haem *a* in subunit I [7]. It is also separated by seven amino acids from histidine-376 which is the ligand of haem a_3 in the binuclear centre.

2. MATERIALS AND METHODS

2.1. *Strains and media*

The strains are described in [10]. Revertants were selected from the mutants L13, L29, L45 and L53, after transfer of the mutant mitochondrial genome in the nuclear context of W303–1B. The yeasts were grown in glucose medium (YD: 1% yeast extract, 3% glucose) or glycerol medium (YG: 1% yeast extract, 3% glycerol).

2.2. *Selection of revertants*

The Mit^- mutants are unable to grow on glycerol medium. Spontaneous revertants (respiratory competent clones) can easily be obtained after selection on glycerol medium. For increased frequencies of revertants, treatments using $MnCl_2$, a Mit DNA mutagen were performed. From each Mit^- mutant, 50–100 single colonies grown on glucose medium were plated on glycerol medium to obtain a lawn of about 10^8 cells. A small number of respiratory competent colonies appeared after one to three weeks of incubation at 28°C. One revertant per mutant colony was picked up and subcloned. Mutagenesis was performed on 10^7 cells per ml in glucose medium with 8 mM of $MnCl_2$ during 6 h. After mutagenesis, the cell suspension was spread on glycerol medium. After one to three weeks of incubation at 28°C, revertants appeared and were picked up and subcloned. For large numbers of independent treatments, 96-well microtiter plates were used. Treatments were performed in the same conditions as described above and only one revertant per mutagenesis was picked up and subcloned. The selection of revertants from L29 and L53 was done at 22°C. At this temperature, the mutants did not grow on glycerol whereas at 28°C, they showed a weak growth on glycerol.

3. RESULTS AND DISCUSSION

The revertants derived from the mutant L13 (G253D) were directly sequenced. For the revertants selected from L29 (T316K), L53 (G352V) and L45 (D369N), the mitochondrial heredity of the reversion was determined as described in [12] before sequencing. Mutations were sequenced directly on DNA segments amplified by asymmetrical polymerase chain reaction [10]. The correlation between the deficiency expressed in the mutants and the nature and position of their mutations found in the nucleotide sequence is confirmed by the production of wild type reversions, i.e. the restoration of the wild type nucleotide sequence. Only with mutant L29, wild type reversions were not found. But the location of the deficiency mutation in L29 was confirmed within a region of about 400 nucleotides by genetic mapping using discriminating deletion mutants (petite mutants). This region was entirely sequenced and no other change except in codon 316 was found. Non-wild-type reversions are presented in Table I.

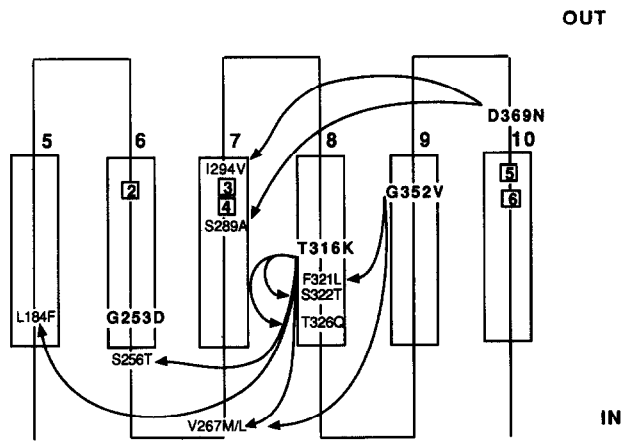

Fig. 1. Localisation of the amino acid substitutions in the folding model of the cytochrome oxidase subunit I. Only the helices 5 to 10 were presented. The histidine ligands of the haems *a* and a_3 and of the Cu_B are represented by the squares. Histidines 2, 3 and 4 are thought to be the ligands of Cu_B; histidine 5, the ligand of haem a_3; histidine 6, one of the haem *a* ligands. The deficiency mutations G253D (L13), D369N (L45), T316K (L29) and G352V (L53) are in bold letters. Their distant reversions are indicated by arrows.

The same second site amino acid replacements were observed in genetically independent revertants (Table I) and the sequencing data were confirmed by the genetic localisation of the reversions by petite mapping [12] and by analysis of the genetic recombination frequencies in diploid progeny issued from the crosses between the revertants and the wild type strain (data not shown). Therefore, the observed distant mutations were considered as responsible for the restoration of the respiratory competence.

No distant reversions have been found so far for the mutant L13 (G253D). All the reversions affect the same codon in position 253: in 16 revertants, the aspartate was replaced by an asparagine (G > D > N) and in 2 revertants, by an alanine (G > D > A). The replacement of aspartate to asparagine leads to a charge change and the amino acid remains significantly larger than the wild type glycine whereas the reversion to alanine reduces the size of the amino acid.

Only distant reversions of L29 mutation (T316K) were found among the 21 revertants. Three changes were located in the loop between helices 6 and 7: in position 267, a valine was replaced by a methionine or by a leucine (V267M and V267L); in position 256, a serine was replaced by a threonine (S256T). These results add data in favour of functional and structural relations between helices 6, 7 and 8 as proposed by the model of Brown et al. [7]. Other reversions include position 184, at the bottom of helix 5, where a leucine was changed in a phenylalanine (L184F). Two changes were found in helix 8: 6 amino acids below the deficient mutation, a serine was changed in a threonine (S322T), and 10 amino acids below the deficient mutation, a threonine was replaced by a glutamine (T326Q) (Fig. 1). Reverse mutations in helix 8 (S322T and T326Q) are located on the same side on the helical wheel (Fig. 2). According to Brown et al. [7], this face of the helix 8 may be oriented towards Cu_B and form part of the proton channel into the binuclear centre.

It will be of interest to investigate the revertants for their CO recombination kinetics. The question may be asked if the second site amino acid replacements, particularly those located in helix 8, restore normal CO recombination kinetics or if the respiratory deficiency can be compensated even if the CO recombination kinetics remain modified. The nature and position of the reversions present interesting properties: all changes led to an increase in the size of the compensatory amino acid without charge modification. The amino acids replacement responsible for the deficiency is located in the middle of the membrane whereas the compensatory amino acid changes are located near or at the inner side of the membrane. Four helices are involved in the compensation of the deficiency: reversions occur in helices 5 and 8 which do not carry any binuclear centre ligands, and in the loop between helices 6 and 7, which carry the three Cu_B ligands.

In one revertant derived from the L53 mutant, the mutated amino acid (G352V) was replaced by an alanine (G > V > A). This replacement with a smaller amino acid was found to be able to restore the respiratory competence, suggesting that a minute steric difference between alanine and valine is critical for the function. Two long distance reversions were found: in the loop between helices 6 and 7, in position 267, a valine was replaced by a leucine (V256L) and in position 321, a phenylananine was changed into a leucine (F321L). The compensatory mutation F321L is found to be located on the opposite side of the helix 8 helical wheel com-

Table I

Codon and amino acid replacements in COX1 mutants and their revertants

Strains	Initial mutation locus		Distant reversion locus		Number of revertants and type of selection		
	Codon	Amino acid	Codon	Amino acid	A	B	C
Wild type	GGT	G253					
L13	GAT	G253D					
Revertant	AAT	G253N			16		
Revertant	GCT	G253A			2		
Wild type	ACA	T316					
L29	AAA	T316K					
Revertant	AAA	T316K	GTA > ATA	V267M	1	1	2
Revertant	AAA	T316K	GTA > TTA	V267L	–	1	1
Revertant	AAA	T316K	TTA > TTT	L184F	–	–	4
Revertant	AAA	T316K	TCA > ACA	S256T	–	–	1
Revertant	AAA	T316K	CTG > CAG	T326Q	–	1	–
Revertant	AAA	T316K	TCT > ACT	S322T	–	9	–
Wild type	GGT	G352					
L53	GTT	G352V					
Revertant	GCT	G352A			1		–
Revertant	GTT	G352V	GTA > TTA	V267L	1		2
Revertant	GTT	G352V	TTT > TTA	F321L	–		10
Wild type	GAT	D369					
L45	AAT	D369N					
Revertant	AAT	D369N	ATT > GTT	I294V	6		
Revertant	AAT	D369N	TCA > GCA	S289A	3		

A: independent spontaneous revertants
B: independent mutagenesis
C: revertants obtained from a mutagenized culture
The types of selection were described in section 2.

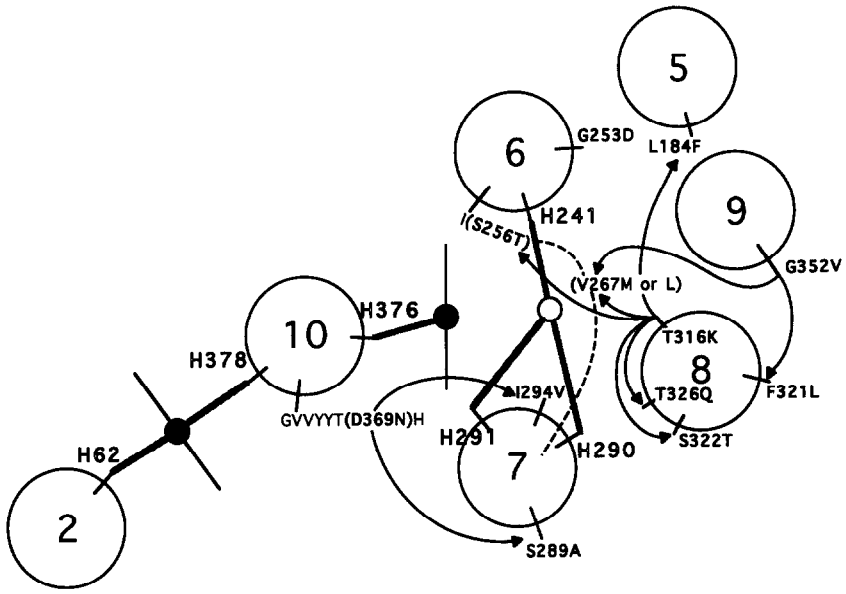

Fig. 2. Localisation of amino acid substitutions in the model of the cytochrome oxidase subunit I. The figure was based on the models proposed in [7] and [13]. Six of the 12 helices are presented. Each helix is indicated as a helical wheel. H62 and H378, H376, H291 and H290 are the histidine ligands of, respectivaly, haem *a*, haem a_3 and Cu_B. The loop between helices 6 and 7, where are located the mutations S256T and V267M or L, is indicated by a dash line. The distant reversions of the deficiency mutations G253D (L13), D369N (L45), T316K (L29) and G352V (L53) are indicated by arrows.

pared to the T316K (L29) mutation and its reversions, S322T and T326Q (Fig. 2). This may be correlated to the observation that mutant L53 (G352V) has normal CO recombination kinetics suggesting that unlike L29 (T316K), the binuclear centre or its accessibility is not modified in L53. The effect of the lesion seems thus to be different in L53 and L29. The locations of these deficiency mutations and their distant reversions argue in favour of a functional and maybe a structural relation between helices 8, 9 and the connecting loop between helices 6 and 7. It is interesting that the same change at the same position, V267L, is able to compensate the deficiency of mutant L53 (G352V), carrying a mutation in helix 9, as well as that of mutant L29 (T316K), carrying a mutation within helix 8.

Two long distant reversions, the replacement of an isoleucine by a valine in position 294 (I294V) and the replacement of a serine by an alanine in position 289 (S289A), are compensatory to the L45 deficiency mutation (D369N) which is located in the loop connecting helices 9 and 10. This mutation is proximal to the haem a_3 ligand, histidine-376. Interestingly, the reversions are in close vicinity of the two Cu_B ligands in helix 7. This represents an independent argument in favour of a topological proximity between helices 7 and 10 for the binuclear centre formation involving haem a_3 and Cu_B.

Acknowledgements: This work was partly supported by a European Community Stimulation Contract (CT90-0476) for European collaboration and by the Belgian F.R.F.C. A.-M.C. is Research Associate to the National Fund for Scientific Research (Belgium). B.M. has a grant of the National Fund for Scientific Research (Belgium). P.L acknowledges the Ministère français de la Recherche et des Technologies for financial support. Dr. Peter Rich (Glynn Research Institute, UK) is greatly acknowledged for critical reading of the manuscript.

REFERENCES

[1] Taanman, J.-W. and Capaldi, R. (1992) J. Biol. Chem. 267, 22481–22485.
[2] Saraste, M., Holm, L., Lemieux, L., Lübben, M. and van der Oost, J. (1991) Biochem. Soc. Trans. 19, 608–612.
[3] Lemieux, J.L., Calhoun, M.W., Thomas, J.W., Inglew, W.J. and Gennis, R.B. (1992) Biol.Chem. 267, 2105–2113.
[4] Shapleigh, J.P., Hosler, J.P., Tecklenburg, M.M.J., Kim, Y., Babcock, G.T., Gennis, R.B. and Ferguson-Miller, S. (1992) Proc. Natl. Acad. Sci. USA 89, 4786–4790.
[5] Minagawa, J., Mogi, T., Gennis, R.B. and Anraka, Y. (1992) J. Biol. Chem. 267, 2096–2104.
[6] Brown, S., Moody, A.J., Jeal, A.E., Bourne, R.M., Mitchell, J.R. and Rich, P.R. (1992) Short Reports of the 7th EBEC, Elsevier, Vol. 7, II-49, 39.
[7] Brown, S., Moody, A.J., Mitchell, R. and Rich, P.R. (1993) FEBS Lett. 316, 216–223.
[8] Holm, L., Saraste, M. and Wikström, M. (1987) EMBO J. 6, 2819–2823.
[9] Haltia, T., Saraste, M. and Wikström, M. (1991) EMBO J. 10, 2015–2021.
[10] Meunier, B., Lemarre, P. and Colson, A.-M. (1993) Eur. J. Biochem. (in press).
[11] Brown, S., Colson, A.-M., Meunier, B. and Rich, P.R. (1993) Eur. J. Biochem. (in press).
[12] Dujardin, G., Pajot, P., Groudinsky, O. and Slonimski, P.P. (1980) Mol. Gen. Genet. 179, 469–482.
[13] Gennis, R.B. (1992) Bioch. Biophys. Acta 1101, 184–187.