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Random deficiency mutations and reversions in the cytochrome *c* oxidase subunits I, II and III of *Saccharomyces cerevisiae*

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1. Introduction

Cytochrome *c* oxidase, the terminal acceptor of the mitochondrial respiratory chain, catalyses the reduction of oxygen to water. Electron transfer from cytochrome *c* to oxygen catalysed by this enzyme is coupled to translocation of protons across the membrane. The mitochondrial cytochrome oxidase is composed of up to 13 subunits [1], contains two haems and two or three copper atoms, and is a member of a large family of proton pumping oxidases [2]. Only three subunits (subunits I, II and III) are mitochondrially-encoded, and these appear to be constituents of the minimal catalytic core. Homologous subunits are present in the bacterial 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 [3–10]. Subunit II has two predicted transmembrane spans and a large hydrophilic domain which contains the ligands of the Cu_A and the docking site for the cytochrome *c* [11]. Subunit III does not contain redox active centre and appears not to be essential for the activity [12], although it may have a role in assembly, stability or activity modulation.

In order to obtain further insight into the structure and function of the catalytic core of the enzyme, random deficient mutants in subunits I, II and III have been isolated using the yeast, *Saccharomyces cerevisiae* as described in Ref. [13]. A rapid spectrophotometric technique has been developed to give an assessment of the level of expression of the cytochrome oxidase in the deficient mutants. More than 200 mutants in subunits I, II and III were tested. In many mutants, a failure in enzyme assembly rather than an impaired turnover seemed responsible for the respiratory growth deficiency. Only a few (about 10%) contained optically-detectable oxidase (Ref. [14]; S. Brown et al., unpublished data). These mutants are most important for further bioenergetic studies since the assembled complex is retained, resulting in limited structural alterations even although they are functionally impaired. Genetic mapping and sequence analyses have identified the nature and positions of the mutational changes responsible for the deficiency. Biochemical and biophysical characterisation of mutant forms of cytochrome oxidase obtained by random mutagenesis may allow unsuspected important regions of the protein to be identified. This approach may provide information which is complementary to the data obtained by the study of site-directed mutated bacterial oxidases.

From mutants in subunits I and II, chosen on the basis of the location of the deficiency mutation or the characteristics of the oxidase, revertants (respiratory competent clones) have been selected. Second-site in-

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tra- and extragenic reversions have been identified [15,16]. The characterisation of distant reversions allowed the detection of pairs of amino acids which are functionally complementary and potentially close in space. It provides a means of determining parts of the protein which may be close to each other in the three-dimensional structure and gives information on the interactions between the subunits.

The yeast *S. cerevisiae* offers several avantages for this approach. Since it depends on cytochrome oxidase for its growth on respiratory substrates, any mutation dramatically affecting this enzyme leads to an inability to grow on respiratory medium and revertants are easily selected on such medium as respiratory growth competent colonies. Subunits I, II and III are encoded by the mitochondrial genome and the genetic localisation of the deficient mutations and the second-site reversions are easily performed with a battery of tester strains.

In this paper, random mutations in subunits I, II and III, which lead to respiratory growth deficiency are described, together with second-site reversions responsible for the restoration of the respiratory growth ability.

2. Results and discussion

2.1. Deficient mutations

2.1.1. Subunit I

Seven amino acid replacements in subunit I, which are responsible for respiratory growth deficiency are presented in Fig. 1. Three mutations are located in the interhelical loops, on the outer side of the subunit: S140L, in loop 3–4; A361D and D369N, in loop 9–10. Four mutations are located in the putative membrane-spanning helices, in helix 2, near one of the ligands of heme *a* (I67N), in helix 6 (G253D), in helix 8 (T316K) and in helix 9 (G352V). The deficient mutants carrying these mutations contain optically-detectable oxidase except for the mutant carrying the mutation D369N. Other mutants with optically-detectable oxidase have been selected with deficiency mutations localised near the C-terminal part of the subunit I. The sequencing of these mutations is in progress.

As described in Ref. [14], the mutation T316K, located on the conserved face of helix 8 leads to a modification of the CO-recombination kinetics which could be explained by an alteration in the binuclear

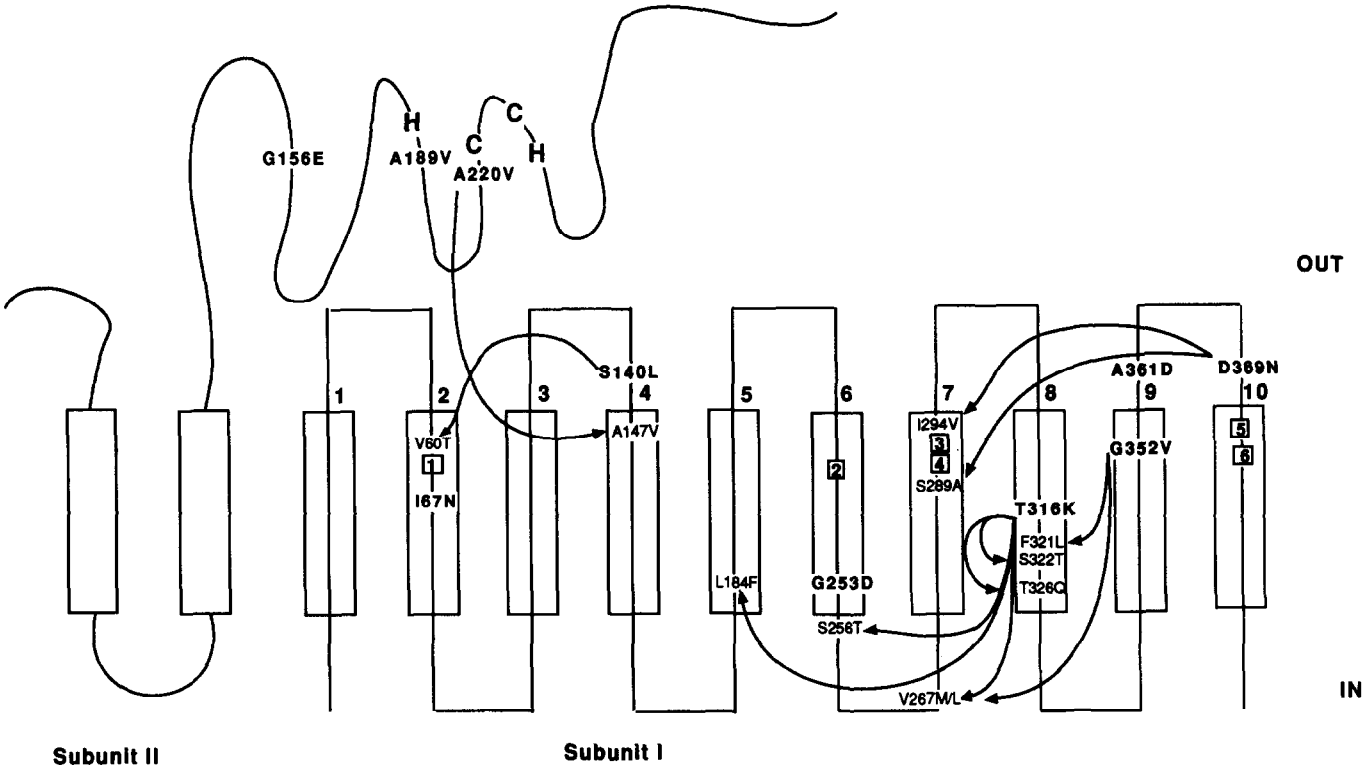

Fig. 1. Localisation of amino acid replacements in the folding model of cytochrome oxidase subunits I and II. Only helices 1 to 10 of subunit I are presented. The histidine ligands of the haems and copper B are represented by squares. Histidines 1 and 6 are proposed to be the ligands of haems *a*, histidines 2, 3 and 4, the ligands of copper B and histidine 5, a ligand of haem a_3. In the subunit II model, the histidine and cysteine ligands of the copper atom(s) are noted in bold letters. The deficiency mutations are in bold letters. The arrows indicate the second-site reversions. The deficiency mutations D369N, G352V, T316K, G253D and their reversions are from Ref. [15]. The deficiency mutations S140L and A189 + A220V and their reversions are from Ref. [16]. The other deficiency mutations, A361D and I67N in subunit I and G156E in subunit II, are from this work.

centre or in the predicted proton channel [7,14]. In the cytochrome *bo* oxidase of *E. coli*, site-directed mutations in the conserved residues in this helix have shown that helix 8 may be part of the proton channel to the binuclear centre [17]. Another mutation in helix 6 (G253D) leads to the same alteration of the CO-recombination kinetics [14]. It may be that this residue is also in the vicinity of the proton channel.

2.1.2. Subunit II

Mutants of subunit II containing optically-detectable oxidase have been sequenced. Two mutants have been characterised, carrying mutations in the hydrophilic domain of the subunit II. In one mutant, a double amino acid replacement (A189V + A220V) in the vicinity of the ligands of the copper A, has been observed. In the other mutant, an amino acid replacement, G156E, in a non-conserved region of this domain has been found (Fig. 1).

2.1.3. Subunit III

One mutation has been characterised (S203L), located in a loop on the outer side of the subunit, between helices 3–4. The mutated residue corresponds to S137 in the *E. coli* subunit III [18] and is conserved in most of species.

This deficiency mutation which does not affect the assembly of an optically detectable cytochrome oxidase may contribute to the uncovering of the possible role for subunit III, even with no metal centre.

2.2. Second-site reversions

The search for second site revertants has been undertaken in order to reveal functionnally compatible amino acid pairs distantly located and affecting protein structure. Intragenic second site reversions in subunit I and extragenic reversions which put to light functional interactions between subunits I and II were found. Predicted secondary structures of subunits I and II based on molecular modelling constitute a two-dimensional map on which the position of the mutated sites can be drawn. Functional relations between the mutated sites revealed in second site revertants are interpreted as an indication for topological vicinity in the three-dimensional structure.

2.3. Choice of deficient mutants for the selection of revertants

The selection of revertants is easily performed. Respiratory growth competent colonies are selected on glycerol medium as described in [15]. But in order to obtain interesting information from the characterisation of the revertants, the choice of the deficient mutants is important. Since a mutation showing a complete failure in enzyme assembly is likely to carry a mutation in an amino acid essential for enzyme assembly or stability, it is more likely that it will be compensated by a reversion at the same site. By contrast, for deficient mutants containing optically detectable oxidase, second-site reversions at some distance from the primary mutation may be expected.

Five deficient mutants containing optically-detectable oxidase were chosen. Second-site reversions were obtained from four of them: from three mutants in subunit I, S140L (loop 3–4), T316K (helix 8), G352V (helix 9) and from one mutant in subunit II, A189V + A220V (Fig. 1). From one mutant in subunit I, G253D (helix 6), only reversions at the initial mutated locus were obtained [15].

The mutant D369N, in the interhelical loop 9–10 of subunit I was also chosen. This mutant has no optically-detectable oxidase but the mutation is located in a well conserved region proposed to be in the vicinity of the binuclear centre and the haem *a*. Site-directed mutations in the conserved residues of this loop affect the spectroscopic characteristics of both haems [19]. In addition, the residue D369 was proposed to be involved in the binding of Mn [19] and in intersubunit electron transfer between copper A in subunit II and haem *a* in subunit I [7].

The characterisation of revertants selected from other mutants I67N, A361D, in subunit I, G156E, in subunit II and S203L, in subunit III is in progress.

2.4. Characterisation of the reversions

Starting from the chosen deficient mutants in subunit I and II, spontaneous revertants have been selected and characterised. The mitochondrial heredity of the reversions was determined before sequencing, as described in Ref. [15]. The sequencing data were confirmed by genetic localisation of the reversion [15,16]. The second-site reversions are presented in Fig. 1.

2.5. Intragenic second-site reversions in subunit I

The characterisation of second-site reversions leads to a network of functional interactions between residues in the helices and the loops of subunit I, which may give clues to the three-dimensional structure of the protein.

From the S140L mutation, located in the interhelical loop 3–4, reversions in helix 2 were obtained, near the ligand of haem *a*, H62. This functional interaction may suggest a proximity of this loop and helix 2.

From the D369N mutation in the loop between helices 9 and 10, second-site reversions were obtained on the outer side of the protein, in helix 7, in the vicinity of the ligands of copper B. This suggests possi-

ble interaction between the interhelical loop 9–10 and the binuclear centre as proposed in Refs. [19,20].

From the T316K mutation located in helix 8, which may be part of the proton channel, second-site reversions were observed on the N-phase side of the subunit, at the bottom of helix 5, in loop 6–7 and in helix 8, below the deficiency mutation, on the same conserved side of the helix. It may be that loop 6–7 provides a cap on the inner site of the proton channel, as loop 9–10 may do on the outer side [21]. The putative alteration of the proton channel due to the T316K mutation can be compensated by a second amino acid replacement either on the same helix 8 (as S322T and T326Q) or in loop 6–7. It is also possible that instead of being a cap, loop 6–7 is buried between the membrane-spanning helices, as may be loop 2–3 [22]. This could explain the interaction between loop 6–7 and helices 8 and 9.

2.6. Extragenic reversions, interaction between subunits I and II

Revertants were selected from the deficient mutant carrying two amino acid replacements in subunit II, in the vicinity of the ligands of the copper A, A189V and A220V [16]. A reversion, located in subunit I, on the outer side of helix 4 was obtained (A147V), which suggests an interaction between the copper binding pocket of subunit II and helix 4 of subunit I. Helix 4 may be in the vicinity of H62, a ligand of haem *a*, because loop 3–4 and the region around H62 seem to be close in the three-dimensional structure since the intragenic reversion V60T compensates the deficiency mutation S140L, located in loop 3–4 (Fig. 1). Therefore, the copper binding pocket of subunit II is likely to be in the vicinity of haem *a*. This result is in agreement with the proposal that the electrons are transferred from the copper of subunit II to the binuclear centre via haem *a*. Characterisation of other extragenic reversions is in progress and could provide more information on the predicted interaction between subunits I and II. Further data on three-dimensional structure when available will certainly refine the interpretations of these functional relations between deficiency mutations and second site reversions.

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