Volume 255, number 1, 5–9 FEBS 07561 September 1989

Electron-transfer restoration by vitamin K_3 in a complex III-deficient mutant of *S. cerevisiae* and sequence of the corresponding cytochrome *b* mutation

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Received 13 July 1989

The yeast box-mutant W7 exhibits deficiencies in cytochrome *b* and in nuclear coded complex III subunits, a phenotype observed previously in a patient with mitochondrial myopathy. DNA sequence analysis of mutant W7 revealed a single base transition in the cytochrome *b* gene; the mutated residue Gly 131 is perfectly conserved in all known cytochromes *b* and belongs to the Q_o domain. Mutant W7 provides a model system for evaluating the action of therapeutic agents, such as vitamin K_3 which restored NADH-oxidase activity in the mutant as well as in the antimycin-inhibited wild type. However, with the mutant, a greater quantity of menadione was necessary due to a decrease in other complex activities, and a much lower electron-flow fraction passed through cytochrome oxidase.

Complex III deficiency; Bioenergetics; Cytochrome *b* mutant; Vitamin K_3 action; DNA sequence, mutation; Mitochondria

1. INTRODUCTION

Numerous apocytochrome *b* mutants defective in the ubiquinol-cytochrome *c* reductase segment (complex III) of the mitochondrial respiratory chain have been isolated in the yeast *S. cerevisiae* [1]. The mutant W7, some of the biochemical properties of which have been described previously [2], is particularly interesting in that it is characterized by a low cytochrome *b* level, a loss of activity in the *b*-c_1 segment of the respiratory chain and a decrease in other complex III subunits coded for by the nuclear genome: core protein I, non-heme Fe-S and subunit VI. A similar phenotype has been described for the first time in a patient with mitochondrial myopathy [3]; the question as to whether the primary site of the mutation in this patient might be mitochondrial or nuclear has been discussed previously [4].

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This analogy suggests the possibility of using the yeast mutant to test the effects of products liable to bypass this genetic defect. The complex III-deficient patient has been favorably treated with vitamins K_3 and C and the rationale for this drug therapy was based on known in vitro results with antimycin-inhibited yeast [5].

We report here the DNA sequence analysis of mutant W7 and data on the respiratory activity restoration induced by vitamin K_3, in antimycin-inhibited wild type strain and mutant W7.

2. MATERIALS AND METHODS

2.1. *Strains*

The diploid wild-type strain PS409 was derived from the haploid wild strain of *S. cerevisiae* 777-3A crossed with the rho° IL126-1C/52; in a similar manner, the isogenic strain PS429 was derived from the original cob-box mutant of 777.3A, named W7, crossed with the same rho° [6]. Isonuclear genotype: −/a, op/+, ade./+, +/ura.

Cultures, preparation of mitochondria, spectral analysis, protein, succinate dehydrogenase activity, cytochrome-*c* oxidase and CoQ_2H_2-cytochrome *c* reductase activities have been

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00145793/89/$3.50

described previously [2,7]. Complex I activities were determined spectrophotometrically as in [8].

2.2. *Sequence analysis*

Mitochondrial RNA isolation and RNA sequencing were performed as in [9]. Three primers synthesized by Dr Javer Perea (Centre de Génétique Moléculaire, Gif-sur-Yvette) were used for the sequencing of the first exon of the cytochrome *b* gene. The position of the primers is given on the basis of their first and last bp numbers on the cytochrome *b* split gene: P1, 109–128; P2, 270–298; P3, 2636–2655.

3. RESULTS AND DISCUSSION

The respiratory deficient W7 mutant has been previously localized at the end (interval 7) of the first exon (B1) of the split mitochondrial COB-BOX gene coding for apocytochrome *b* [10]. The nucleotidic sequence of the mutated exon was analysed here by reverse transcription of the cytochrome *b* mRNA from crude mitochondrial RNA preparations as described in section 2. The only base change affecting the wild-type sequence was a G to A transition near the end of exon B1, leading to the replacement of Gly 131 by serine (GGT→AGT). In view of the perfect agreement between the genetic mapping and sequencing data, it can be concluded that this alteration is in fact responsible for the respiratory deficient phenotype of mutant W7.

Gly 131 is perfectly conserved in all the cytochromes *b*, including mammalian and yeast, which have been sequenced so far [11]. This residue might be located in the eight transmembrane-helix cytochrome *b* folding model, proposed recently on the basis of different calculations [12–14], near the presumed electropositive side of the membrane at the NH_2 terminal of the membrane spanning segment number 3 which connects the two heme-binding helices, numbers 2 and 4 (fig.1). The W7 mutation affects one of the two cytochrome *b* regions (extending from residues 129 to 147 and 256 to 275) which were found to confer upon amino acid replacement an increased resistance to inhibitors acting at the Q_o site of the bc_1 complex (mucidin, myxothiazol and stigmatellin) [15].

The non-respiratory growing W7 mutant has been shown previously to be strongly affected in the assembly of the bc_1 complex, as indicated by the loss of ability to bind the high-affinity inhibitor antimycin [16] and by the drastic reduction in several of the enzyme polypeptide subunits it contains; the spectral cytochrome *b* content of mutant W7 amounted to only 15% of that of the wild type while those of cytochromes *c* and aa_3 were 65 and 37%, respectively [2]. Activity measurements at the mitochondrial level revealed the nearly complete absence of QH_2-cytochrome *c* reductase activity; succinate-Q, NADH-Q and cytochrome *c* oxidase activities were substantially reduced by about 83, 38 and 64%, respectively, vs the wild type (see table 1). As a result of the loss of activity in the bc_1 segment of the electron-transfer chain, the respiratory measurements showed no succinate oxidase activity, and a low NADH oxidase activity which was of the same order as the antimycin-inhibited wild-type activity (6%, table 2).

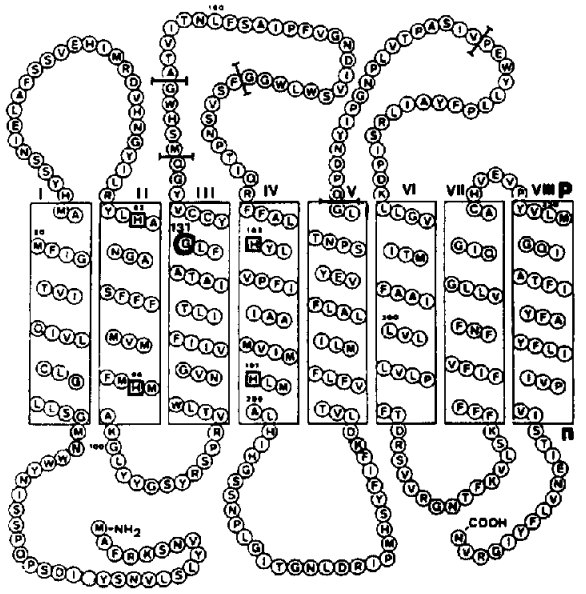

Fig. 1. Cytochrome *b* folding model in the mitochondrial inner membrane. This model represents the height transmembrane helices model recently proposed by Brasseur [9,14]. Subscript numbers, amino acid positions: mutation W7 is noted in position 131. (——) Position of the corresponding exons limits of the split cytochrome *b* gene.

The phenotype of mutant W7 shows great similarities with that of a patient with a deficit in complex III of skeletal muscle mitochondria as reported by Darley-Usmar et al. [3]. This patient's mitochondria exhibited, like mutant W7 mitochondria, a low reducible level of cytochrome *b*, a deficiency in several polypeptides of complex III and a severe decrease in ubiquinol-cytochrome *c* reduc-

Table 1

Comparative activities in the four different segments of the respiratory chain of the wild-type strain and box-mutant W7 mitochondria

Mutation (strain)	Succinate dehydrogenase activity	NADH-CoQ_2 activity	CoQ_2H_2-cytochrome *c* activity	Cytochrome *c* oxidase activity
box$^+$ (PS409)	100	100	100	100
W7 (PS429)	17	62	2	36

Activities are expressed as a percentage of box$^+$

tase activity. The possibility of improving oxidative phosphorylation has been investigated in this patient using therapeutic agents which bypass the cytochrome-deficient site. Therapy with menadione and ascorbate was found to improve phosphorylation in the patient, and concomitantly to somewhat improve the functional activity [5].

Owing to its similarities with this case of mitochondrial myopathy due to complex III deficiencies, the yeast W7 mutant constitutes a model system for evaluating the mode of action and the efficiency of therapeutic agents.

The action of vitamin K_3 was studied in the deficient mutant and antimycin inhibited wild-type strain. In both strains, with NADH as substrate, the respiratory activity accelerated considerably after the addition of vitamin K_3 (fig.2) and this recovery of NADH oxidase activity induced by vitamin K_3 was correlated in the low-temperature spectra with the reduction of cytochromes *c* and c_1 (not shown). These results suggest that menadione may establish a bridge between NADH and cytochromes *c* and c_1 so as to bypass cytochrome *b*, which is non-functional either because of the presence of antimycin or because of the modification induced by the mutation.

The action of vitamin K_3 on the respiratory defective mutant and its wild type strain was studied at three different levels as shown in table 2:

(i) NADH induced a reduction in vitamin K_3 through NADH dehydrogenase (which is not inhibited by antimycin); stoichiometric quantitites of NADH and vitamin K_3 were necessary to ensure maximum activity.

(ii) The NADH cytochrome *c* activity was very low in the mutant; in the wild-type strain inhibited by antimycin, only a small quantity of vitamin K_3 was needed to restore the basal activity. A significant activity level was also recovered by mutant W7 under these conditions ($K_3 << NADH$).

(iii) The same was observed with NADH oxidase activity. This respiratory activity was studied in terms of mitochondrial concentration at two different values of vitamin K_3 (fig.3) and a hyperbolic relationship was observed; consequently, the recovery of NADH oxidase activity by vitamin K_3 in the mutant and the antimycin-inhibited wild-type strain was found to depend on the concentration of vitamin K_3 per mass of mitochondrial protein (fig.4). However, the maximum specific NADH-K_3 activity (described in [1]) was found to be independent of the mitochondrial concentra-

Table 2

Action of vitamin K_3, observed at three different levels of the respiratory chain, in the wild-type and the missense exonic cytochrome *b* mutant W7

Mutation (strain)	NADH[a]-K_3[b] activity	NADH[a]-cytochrome *c* activity			NADH[d]-oxidase activity		
			+ Antimycin	+ K_3[c]		+ Antimycin	+ K_3
box$^+$ (PS409)	224	286	50	230	95	6	232
W7 (PS429)	123	27		170	6		126

[a] NADH = 125 μM; [b] K_3 = 120 μM; [c] K_3 = 10 μM; [d] NADH = 2.5 mM

Activity assays of NADH-K_3 reductase and NADH-cytochrome *c* reductase were performed in the presence of 2 mM KCN. Activities in nmoles·min^{-1}·mg^{-1}. Antimycin, 1 μM; mitochondria, 0.03 mg of box$^+$ and 0.036 mg of W7 in the two first activities; 0.35 mg of box$^+$ and 0.5 mg of W7 in NADH-oxidase activity.

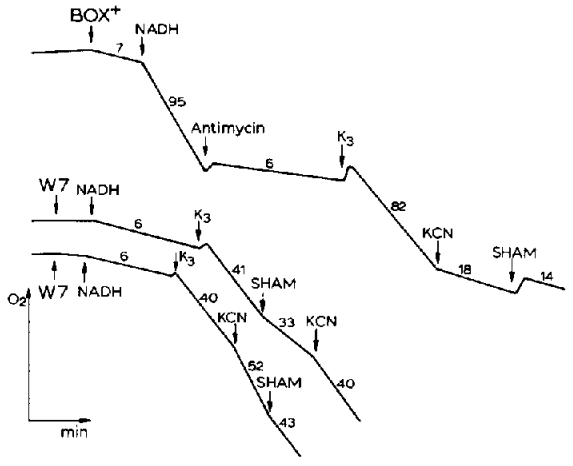

Fig. 2. Action of vitamin K_3 on NADH oxidase activity of mitochondria isolated from the wild-type strain and from the missense box mutant W7. Subscripts, respiration rates (in $nmol \cdot min^{-1} \cdot mg^{-1}$ protein). NADH, 2.5 mM; antimycin, 0.5 μM; KCN, 2 mM; SHAM, mM; mitochondria, 0.35 mg/ml. Box^+, 0.52 mg/ml W7; vitamin K_3, 20 μM with Box^+, 50 μM with W7.

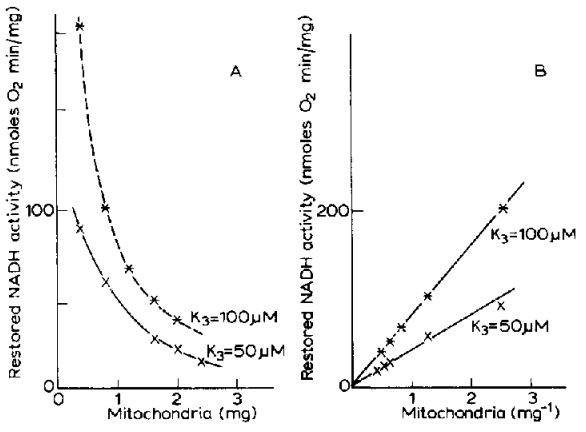

Fig. 3. Relationship between restored NADH activity and mitochondrial content in box mutant W7, for two different vitamin K3 concentrations. (A) NADH restored activity in terms of mitochondrial-protein concentration. (B) NADH restored activity in terms of inverse of mitochondrial-protein concentration. NADH, 2.5 mM.

tion, as expected; moreover, the vitamin K_3 concentration necessary for this pseudo mono-enzyme activity to be reached, was about 3000 nmol/mg protein, vs about 300 nmol/mg in the case of the other two activities (table 2, W7). This suggests that vitamin K_3 acts in NADH-K_3, as a saturating substrate, and in the other two activities as an intermediate redox component.

Recovery of normal activity occurred in wild-type mitochondria with about 90 nmol vitamin K_3/mg mitochondrial protein, which is identical to the level reported by Nosoh et al. [17]. With the box mutant, a 2-fold quantity was necessary to reach the same basal activity, which was probably related to the reduced activities observed in this mutant with the NADH-dehydrogenase and cytochrome *c*-oxidase segment (table 1).

The respiratory experiments were performed at pH 6.3 in order to minimize the autooxidation of vitamin K_3 occurring at pH values above 7. It was also noted that quinones may stimulate cyanide-resistant respiration [18] and we have observed that this phenomenon increases with the quantity of vitamin K_3 added (unpublished).

The addition of vitamin K_3 with NADH as substrate restored the electron flow through the NADH dehydrogenase segment in both antimycin-inhibited wild type and respiratory-deficient box

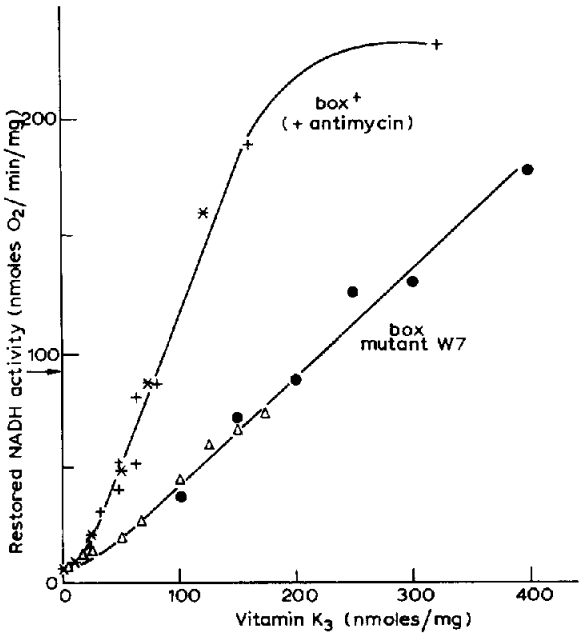

Fig. 4. NADH oxidase activity restored by vitamin K_3 in wild-type mitochondria (box^+) inhibited by antimycin and in the missense mutant of cytochrome *b* gene, W7. The arrow indicates the usual wild-type NADH oxidase activity. (+, *) Two different sets of experiments for the wild-type strain PS409 (two different mitochondrial preparations); (△, ▲, ●) different sets of experiments for mutant W7; NADH, 2.5 mM; antimycin, 0.5 μM.

mutant mitochondria; a small part of this electron flow went directly through the SHAM sensitive oxidase (fig.2) and the remainder joined the respiratory chain at the cytochrome *c* and c_1 levels. A necessary condition for phosphorylation to be restored at site III level is the passing of electron flow through cytochrome oxidase; this clearly occurred with the wild type mitochondria, as the restored activity was largely inhibited by cyanide (fig.2). With mutant W7, no such inhibition by cyanide was observed; on the contrary, cyanide appeared to stimulate the activity restored by vitamin K_3 (secondary way) (fig.2); in this case, it is difficult to estimate what proportion of electron flow may pass through cytochrome-oxidase in the absence of cyanide: it is therefore doubtful whether site III of oxidative phosphorylation is restored with this mutant. However, at the cellular level, with ethanol as substrate, the inhibition by KCN of vitamin K_3-restored respiration is nearly complete with the wild type strain (96–98%) and low (30–40%) with the mutant. This will be further investigated.

Acknowledgements: We thank Professor P. Slonimski for providing us with mutant and wild-type strains. These studies were partly supported by grants (AIP 06931) from the CNRS.

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