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THE RESPIRATORY CHAIN IN A UBIQUINONE-DEFICIENT MUTANT OF *SACCHAROMYCES CEREVISIAE*

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SUMMARY

1. Two allelic mutants of *Saccharomyces cerevisiae* with a deficiency in the biosynthesis of ubiquinone have been isolated. The properties of one particular mutant strain were investigated. Submitochondrial particles of this strain contain maximally 3 % of the amount of ubiquinone in wild-type particles; the amounts of other components of the respiratory chain are essentially normal.

2. The respiratory rates of mutant cells, mitochondria and submitochondrial particles are low with ubiquinone-dependent substrates, but are restored to normal levels by addition of Q-1; the restored respiration is antimycin sensitive. Intact cells and mitochondria show respiratory control both in the absence and presence of Q-1.

3. The NADH:Q-1 oxidoreductase of submitochondrial particles of the mutant follows pseudo first-order kinetics in [Q-1]. QH_2-1 inhibits competitively with respect to Q-1, the K_i for QH_2-1 being equal to the K_m for Q-1.

4. Succinate dehydrogenase in both wild-type and mutant submitochondrial particles can be activated by NADH.

5. The turnover number of succinate dehydrogenase in the mutant, measured with phenazine methosulphate as primary electron acceptor, is about one-half that of wild-type particles.The turnover numbers measured with Q-1 as electron acceptor are about the same in the two types of particles.

6. The kinetics of redox changes in cytochrome *b*, in the presence of antimycin and oxygen, are distinctly different in the mutant and wild-type particles. They indicate that ubiquinone plays an important role in the phenomenon of the increased reducibility of cytochrome *b* induced by antimycin plus oxygen.

INTRODUCTION

The facultative anaerobic character of the yeast *Saccharomyces cerevisiae*

Abbreviations: PMS, phenazine methosulphate; DCIP, 2,6-dichlorophenolindophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

permits the isolation of mutants with defects in mitochondrial metabolism. Mutants of nuclear origin with defects in the cytochromes of the respiratory chain have been intensively studied [1–4].The present study deals with a ubiquinone-deficient mutant.

Experiments involving extraction of ubiquinone from beef-heart submitochondrial particles have made it clear that ubiquinone is an obligatory component of the respiratory chain, its place of action lying between the flavoprotein dehydrogenases and cytochrome *b* [5, 6]. However, it has not proved possible to extract ubiquinone from mitochondria or submitochondrial particles with preservation of an intact phosphorylating system. The use of mutants specifically lacking ubiquinone should, in principle, overcome this difficulty.

Ubiquinone-deficient mutants of *Escherichia coli* have been described by Ccx and coworkers [7, 8], who proposed, on the basis of the properties of these mutants, that ubiquinone functions at more than one site of the bacterial respiratory chain. Since, however, the respiratory chain in bacteria differs considerably from that in mammalian mitochondria it would be useful also to have available mutants of yeast, the mitochondria of which resemble rather closely those of mammalian tissues. In the present paper two such mutants are described. Mitochondria and submitochondrial particles of one of these have been used to investigate some roles of ubiquinone that have been proposed on the basis of experiments with mammalian mitochondria.

A preliminary account of this work has been published [9].

METHODS AND MATERIALS

Growth media and growth conditions

Complete media used for selection of mutants and for genetic studies contained 2 % peptone, 1 % yeast extract, 2 % agar and either 2 % glucose, 3 % glycerol or 3 % ethanol. Medium Go, described by Jakob [10], supplemented with 2 % glucose and 2 % agar, served as synthetic minimal medium. The pH was adjusted to pH 6.5. Liquid sporulation medium contained 1 % potassium acetate, 0.1 % yeast extract and 0.05 % glucose.

Cells used for preparation of mitochondria or submitochondrial particles were grown in liquid medium containing per liter: 5 g glucose, 4 g yeast extract, 5 g peptone, 2 g KH_2PO_4, 0.5 g NaCl. 2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 5 mg $FeCl_3$. The cells were grown in 20-l vessels under vigorous aeration. The cells were harvested in early stationary phase 24 h after inoculation. Cultures of mutant strains were checked for reversion by plating cells on complete glycerol medium.

All cultures were grown at 30°C.

Strains, mutagenesis and selection

The parent strain DW_3 (*a, leu, his, ade*) was obtained from Dr D. Wilkie. Strain 17 (α, *ura, ade*), obtained from Dr H. Jakob and strain DP_1-1B (α, *his, try*), obtained from Dr P. P. Slonimski, served as test-strains in genetic studies. Strain 17 was also used as a normal wild-type strain in biochemical studies.

Strain DW_3 was mutagenized with nitrous acid [11]. The mutagenized cells were spread on complete glucose medium and, after 4 days incubation, the colonies were replica-plated onto complete glucose medium and complete glycerol

medium. Those colonies were selected that were unable to grow on complete glycerol medium but able to stain with 2, 3, 5-triphenyltetrazolium chloride when grown on complete glucose medium [12]. Two mutants, Nos A26 and A46 out of thirty isolated in this way, were deficient in ubiquinone. In biochemical experiments the mutant strain 26H was used. This is a diploid strain, prototrophic and homozygous for the mutant allele of strain A26.

Genetic analysis

Crosses were performed by the mass-mating technique. Diploids were isolated by prototrophic selection on minimal medium. Sporulation was performed by growing diploid cells on sporulation medium for 3 days. After digestion of the ascus wall with snail digestive enzyme, asci were dissected by micromanipulation. Segregants of complete tetrads were analyzed with respect to their ability to grow on complete glycerol and ethanol media and with respect to mating type and nutritional markers.

Preparation of mitochondria and submitochondrial particles

Mitochondria were prepared according to Kováč et al. [13]. Submitochondrial particles were prepared as follows. Cells were harvested by centrifugation, washed once with distilled water and twice with 0.25 M mannitol–1 mM EDTA–10 mM potassium phosphate (pH 7.5). The washed cells (50–100 g wet wt) were mixed with 200 ml glass beads (diameter 0.5–0.7 mm). The mannitol–EDTA–phosphate buffer was added in such an amount that the volume of the yeast suspension just exceeded the void volume between the beads. The suspension was homogenized for 10 min with a Silverson mixer-emulsifyer (Silverson Machines, S.E.1, London). This treatment resulted in about 50 % cell breakage. The contents of the vessel were maintained at 0–3°C during homogenization by placing the vessel in an ice–salt bath. All subsequent steps were carried out at 0–4°C. The beads were washed several times with the buffer and the collected washings were centrifuged for 15 min at $6000 \times g$. The turbid supernatant was centrifuged for 40 min at 75 000 $\times g$ in a 8×50 angle rotor of an MSE high-speed centrifuge. The pellet was resuspended in the buffer and centrifuged again at 75 000 $\times g$ for 40 min. This step was repeated once and the pellet was suspended in the buffer at a protein concentration of 20–30 mg/ml.

Determination of ubiquinone

Ubiquinone was extracted from 10–20 mg submitochondrial particles with light petroleum (b.p. 40–60°C)–methanol (30:70, by vol.)[14]. The extract was evaporated at reduced pressure at room temparature. The residue was dissolved in a small volume of light petroleum and chromatographed on thin-layer silica gel (Merck F254) with light petroleum (b.p. 40–60°C)–chloroform (30:70, by vol.) [8]. The ubiquinone band was eluted with heptane–abs. ethanol (20:80, by vol.). The concentration of ubiquinone was determined from the difference in absorbance at 280–289 nm before and after addition of KBH_4, using $8.8\ mM^{-1} \cdot cm^{-1}$ as absorbance coefficient [14]. In some experiments ubiquinone of mutant particles was estimated by adaptation of a fluorimetric method [15]. To the evaporated extract (see above) was added 3 ml ethyl cyanoacetate–20 mM KOH in abs. ethanol (4:25, by vol.). After standing for 5 days at 5°C in glass–stoppered tubes, the fluorescence was mea-

sured with an Eppendorf fluorimeter with excitation filter 405+436 nm and emission filter 500–3000 nm. After subtraction of a blank (no extract added) the fluorescence was compared with that of samples of extracts of wild-type particles. Quenching by other components present in the extract was corrected for by using pure ubiquinone added to appropriate amounts of the extract of mutant particles as internal standard.

Determination of covalently bound flavin

Covalently bound flavin was determined by adaptation of published methods [16–18]. Samples of particles containing 10–30 mg protein were precipitated with 5 % trichloroacetic acid and washed five times with this solution. Following proteolytic digestion and hydrolysis to the mononucleotide, fluorescence was measured at both pH 3.5 and pH 7.5 before and after reduction by $Na_2S_2O_4$. The fluorescence (oxidized minus reduced) at pH 7.5 was subtracted from the fluorescence (oxidized minus reduced) at pH 3.5. This value was compared with the fluorescence (oxidized minus reduced) of standard solutions of FMN. An Eppendorf fluorimeter was used with excitation filter 405+436 nm and emission filter 500–3000 nm.

Assay of succinate dehydrogenase activity

The succinate dehydrogenase was first activated by incubating the particles for 10 min at 30°C in cuvettes containing 40 mM succinate, 1 mM EDTA, 50 mM potassium phosphate (pH 7.5) and 2 mM cyanide in a total volume of 3 ml. The reaction was started by addition of electron acceptor (either phenazine methosulphate (PMS) plus 2,6-dichlorophenolindophenol (DCIP), or Q-1). Succinate:PMS oxidoreductase activity was measured at 600 nm in the presence of 0.2–2 mM PMS and approx. 0.05 mM DCIP. After subtraction of a blank, where succinate was replaced by 40 mM malonate, the activity was calculated using 21 $mM^{-1} \cdot cm^{-1}$ (oxidized minus reduced) as absorbance coefficient for DCIP [17]. Succinate: Q-1 oxidoreductase activity was measured in the presence of 12–120 μM Q-1 by following the absorbance decrease at 275 nm, using 12.5 $mM^{-1} \cdot cm^{-1}$ (oxidized minus reduced) as absorbance coefficient for Q-1 (see below).

Assay of NADH:Q-1 oxidoreductase activity

A modification of the method described by Singer et al. [18] was used. The activity was measured at 30°C in 3 ml reaction medium containing 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 2 mM cyanide and varying concentrations of NADH and Q-1. The reaction was started by addition of NADH and the decrease in absorbance at 340 nm was followed. Activities were calculated using an absorbance coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ (reduced minus oxidized) for NADH [19] and 0.58 $mM^{-1} \cdot cm^{-1}$ (oxidized minus reduced) for Q-1 (see below).

Determination of absorbance coefficients of ubiquinone-1

The absorbance decreases at 275 nm and 340 nm were measured after reduction with KBH_4 of a solution of Q-1, the concentration of which was determined by titration with NADH in the presence of particles under the conditions used to measure the NADH:Q-1 oxidoreductase.

Miscellaneous procedures

Protein was determined by the biuret method after trichloroacetic acid precipitation, using egg albumin as standard [20]. Enzyme activities were measured with a Zeiss PMQ spectrophotometer. Redox changes of cytochromes were followed with an Aminco–Chance spectrophotometer. Cuvettes of 1 cm light path were used throughout. Oxygen uptake was measured with a Clark-type electrode.

Q-1 was kindly donated by Dr O. Isler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) by Dr P. G. Heytler and bongkrekic acid by Dr G. W. M. Lijmbach. QH_2-1 was prepared according to Rieske [21], ethanol being replaced by methanol. All other chemicals were commercially available products. Q-1, QH_2-1, CCCP and antimycin were added as methanolic solutions.

RESULTS

Isolation of mutants and genetic analysis

The respiration of all 30 mutants that were isolated according to a criterium set up to find mutants with a deficiency in oxidative phosphorylation [12] was decreased compared with the parent strain. Insofar as the respiration was cyanide sensitive it was always strongly inhibited by bongkrekic acid, an inhibitor of the adenine nucleotide translocator [22, 23]. This was taken as an indication that the oxidative phosphorylation machinery in these mutants was functional. Inhibition by bongkrekic acid of the respiration of yeast cells has been described by Šubik et al. [24]. Upon closer examination it appeared that a number of these mutants have deficiencies in Krebs-cycle enzymes. Two mutants, Nos A26 and A46, showed a rate of ethanol respiration of 20–30 natoms $O \cdot min^{-1} \cdot mg^{-1}$ dry weight. This rate was increased by Q-1 to 60–100 natoms $O \cdot min^{-1} \cdot mg^{-1}$ dry weight, which is in the same range as the respiratory rate of the parent strain DW_3. This indicated that the mutants A26 and A46 have a deficiency in ubiquinone. Fig. 1 shows the stimulation by Q-1 of the respiration of cells of strain 26H.

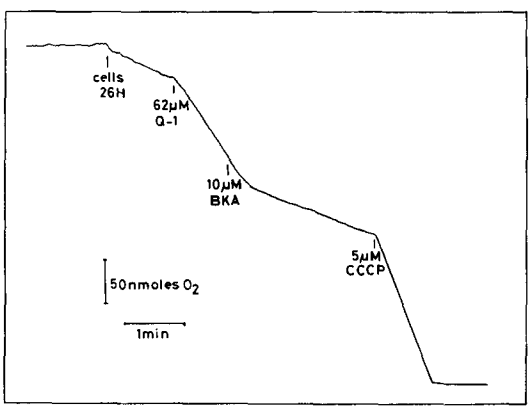

Fig. 1. The effects of consecutive additions of Q-1, bongkrekic acid (BKA) and CCCP on ethanol respiration of intact cells of mutant strain 26H. The reaction medium contained 50 mM phosphate–citrate buffer (pH 4.2) and 50 mM ethanol in a total volume of 1.6 ml. The temperature was 25 °C. The reaction was started by addition of cells (3.8 mg dry weight).

The Mutants A26 and A46 were crossed with wild-type strain 17. In all 15 tetrads derived from the hybrid A26 × 17 and in all 10 tetrads derived from the hybrid A46 × 17 the inability to grow on non-fermentable carbon sources segregated in a 2:2 fashion. This indicates that the deficiencies of both mutants are the result of single gene mutations. The mutants do not complement each other, as judged by crossing Mutant A46 with a ubiquinone-deficient segregant, derived from the hybrid A26 × 17. The resulting diploid did not grow on non-fermentable carbon sources and was deficient in ubiquinone. It may be concluded that the Mutants A26 and A46 have deficiencies in the same cistron.

The percentage of double mutants pet $[\varrho^-]$ in cultures of ubiquinone-deficient mutants, as estimated with the tetrazolium overlay method, was always below 2 %.

The content of ubiquinone and other components of the respiratory chain in submitochondrial particles

Extracts of submitochondrial particles in methanol–light petroleum contain, besides ubiquinone, other ultraviolet-absorbing material, mainly sterol compounds, that interfere with the spectrophotometric determination of ubiquinone of wild-type particles and even more with the detection of small amounts of ubiquinone that may be present in mutant particles. For this reason, the extracts were first chromatographed on thin-layer silica gels. Ubiquinone is easily identified as a yellow spot on the chromatogram of the extract of wild-type particles. No spot was visible in the corresponding area on the chromatogram of the extract of mutant particles. The amount of ubiquinone in the latter area is at or below the limit of detection in a series of spectrophotometric determinations. This limit corresponds to approx. 0.05–0.1 nmole Q/mg protein. This could, however, be an underestimation, since small amounts of ubiquinone may not be quantitatively recovered in the chromatographic procedure. For this reason, the amount of ubiquinone in the extract of mutant particles was also estimated by adaptation of a fluorimetric procedure recently described by Rokos [15]. This method, which is based on the fluorescence of end products of the Craven reaction, could, however, result in an overestimation since compounds of related structure might also give rise to fluorescent products.

TABLE I

CONTENT OF UBIQUINONE AND CYTOCHROME *b* OF SUBMITOCHONDRIAL PARTICLES

Ubiquinone was determined as described in the section Methods and Materials. The cytochrome *b* content was determined from the difference in absorbance ($Na_2S_2O_4$ reduced minus oxidized) at 562–575 nm, using an absorbance coefficient of 25.6 $mM^{-1} \cdot cm^{-1}$ [25].

Strain	Content of component (nmoles/mg protein)	
	Ubiquinone	Cytochrome *b*
17	7.0*	0.35
26H	≦0.2**	0.32

* Determined spectrophotometrically.
** Determined fluorimetrically.

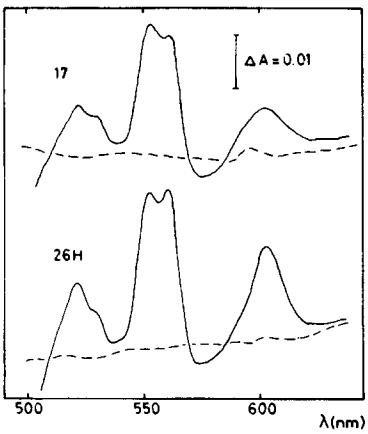

Fig. 2. Difference spectra of submitochondrial particles. Spectra were measured at 22 °C with a Perkin–Elmer spectrophotometer, Model 356 with split-beam attachment. Particles were suspended at a protein concentration of 2.0 mg/ml in 50 mM phosphate buffer (pH 7.5), containing 1 mM EDTA. - - -, baseline; —, $Na_2S_2O_4$-reduced minus oxidized.

Table I shows that particles of mutant strain 26H contain at most 3 % of the amount of ubiquinone present in wild-type particles. The latter contain approx. 20 times more Q than cytochrome *b*, whereas mutant particles contain less Q than cytochrome *b*.

Fig. 2 shows that the cytochrome spectrum of mutant particles is essentially normal.

Respiratory activities

Table II compares the respiratory activities of mutant and wild-type particles. The activities of mutant particles are low with NADH, glycerol 1-phosphate and succinate, but can be stimulated to values comparable with wild-type activities by addi-

TABLE II

RESPIRATORY ACTIVITIES OF WILD-TYPE AND MUTANT SUBMITOCHONDRIAL PARTICLES

Oxygen uptake was measured at 25 °C in a medium containing 50 mM potassium phosphate buffer (pH 7.5) and 1 mM EDTA. 10 μM bovine cytochrome *c* was present. Substrate concentrations were: 1 mM NADH, 20 mM glycerol 1-phosphate, 10 mM succinate, 10 mM ascorbate and 50 μM TMPD.

Strain	Additions	Respiratory rate (natoms O · min^{-1} · mg^{-1} protein)			
		NADH	Glycerol 1-phosphate	Succinate	Ascorbate and TMPD
17	—	1630	310	346	2060
26H	—	120	12	73	2010
26H	Antimycin (1 μg/mg)	60	0	2	—
26H	Q-1 (230 μM)	1730	175	425	—

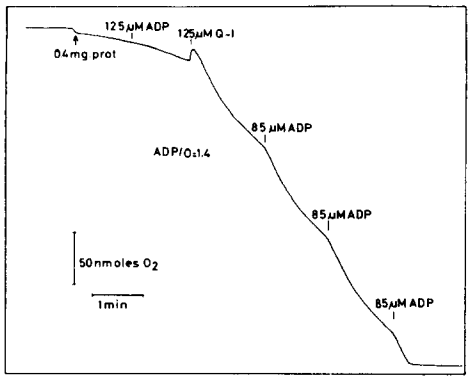

Fig. 3. NADH oxidation by intact mitochondria of strain 26H. Oxygen uptake was measured in a medium containing 0.6 M sorbitol, 10 mM potassium phosphate, 10 mM Tris–maleate, 10 mM KCl, 1 mM EDTA, 2 5 mg/ml bovine serum albumin and 1 mM NADH in a total volume of 1.6 ml. The final pH was 6.5. The temperature was 25 °C.

tion of Q-1*. The activities that are measured in the presence of Q-1 are inhibited by antimycin by more than 93 % (not shown). The NADH oxidase activity of mutant particles in the absence of Q-1 is only 50 % antimycin sensitive. The respiratory rates of particles from the mutant are comparable with those of pentane-extracted particles of wild-type yeast [26, 27].

The respiratory activities of intact mitochondria of Mutant 26H with succinate, glycerol 1-phosphate, NADH and NAD-dependent substrates are also stimulated by addition of Q-1, but to a smaller extent than with respiratory particles. The rate of NADH oxidation in State 3, for example, is stimulated only 4–6 times by addition of optimal concentrations of Q-1, and the stimulation is even less when the oxidation of other substrates is followed. Both in the absence (not shown) and presence (Fig. 3) of Q-1, respiration is coupled to phosphorylation.

TABLE III

KINETIC PARAMETERS OF NADH DEHYDROGENASE OF SUBMITOCHONDRIAL PARTICLES

Conditions were as described in Methods and Materials. Maximal velocity and K_m for NADH were measured at a fixed Q-1 concentration of 150 μM. K_m for Q-1 and K_i for QH_2-1 were measured in the presence of 133 μM NADH.

Parameter	Strain	
	17	26H
$V_{NADH \to \infty}$ (μmoles · min^{-1} · mg^{-1})	29	18
K_m for NADH (μM)	60	80
K_m for Q-1 (μM)	39	33
K_i for QH_2-1 (μM)	42	33

* With a different batch of wild-type particles, Q-1 stimulated NADH and succinate oxidation of wild-type particles by 35 % and 10 %, respectively.

Intact-cell respiration is stimulated by Q-1 by 3-fold only (see Fig. 1). However, this is in part caused by a limitation of respiration by phosphorylation. In the presence of uncoupler Q-1 stimulates 5-6 times (not shown).

Regulation of the NADH dehydrogenase of submitochondrial particles

Table III compares the NADH dehydrogenase activities of wild-type and mutant particles. These activities are extremely high compared with the activities of the other dehydrogenases, e.g. succinate dehydrogenase (see below) and compared with the activity of the terminal part of the respiratory chain. It seems improbable, therefore, that the respiration of NADH could occur in a manner coordinated with the respiration of other substrates, unless the activity of NADH dehydrogenase were restrained. Evidence was indeed found that the NADH dehydrogenase of particles is subjected to product inhibition. In the course of the reaction catalyzed by this enzyme, with Q-1 as electron acceptor, the rate slows down much faster than expected on the ground of the decrease in substrate concentrations. Upon analysis of the kinetics of this reaction it was found that the reaction is first order in [Q-1] for Q-1 concentrations up to at least 110 μM (Fig. 4). The apparent first-order rate constant decreases as the initial concentration of Q-1 increases. Moreover, at constant NADH concentration, a double-reciprocal plot of rate against Q-1 concentration showed pure competitive inhibition by QH_2-1 with respect to Q-1. It seems very likely that these rather unusual kinetics are due to the fact that the K_i for QH_2-1 is equal to the K_m for Q-1 (Table III).

The NADH dehydrogenase of particles presumably corresponds with the enzyme localized at the inner surface of the inner mitochondrial membrane. Von

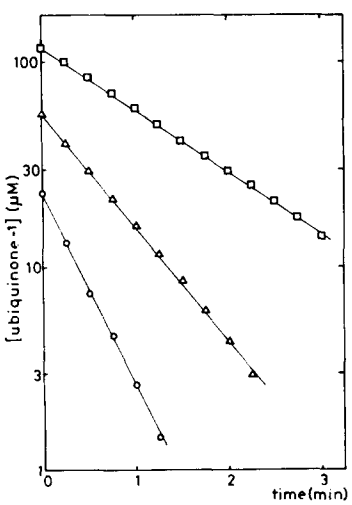

Fig. 4. Course of the reduction of Q-1 by NADH catalysed by particles of strain 26H. Measurements began approx. 15 s after starting the reaction with 0.3 mM NADH. 5.6 μg protein/ml was present. The Q-1 concentration was calculated from the absorbance decrease at 340 nm. Further experimental details in Methods and Materials. □—□, 140 μM Q-1; △—△, 70 μM Q-1; ○—○, 35 μM Q-1 (initial concentrations).

Jagow and Klingenberg [28] found that the NADH dehydrogenase localized at the outer surface of yeast mitochondria is rather loosely bound and easily detached.

Regulation of succinate dehydrogenase

A number of different treatments effect the transition of succinate dehydrogenase from the deactivated to the activated conformation (for review see ref. 29). Gutman and coworkers [30] showed that the membrane-bound succinate dehydrogenase of beef-heart submitochondrial particles can be fully activated by incubation of the particles with NADH. Since this effect of NADH was lost after extraction of Q with pentane these authors concluded that QH_2 acts as an activator of the enzyme. Klaasse and Slater [31], however, found that the isolated mammalian enzyme can be activated merely by reduction with $Na_2S_2O_4$. Since the isolated enzyme contains no ubiquinone this finding suggests that the activation of the membrane-bound enzyme by NADH might be caused by reduction of the enzyme, via ubiquinone, rather than by QH_2 itself.

The experiments of Fig. 5 show that the particle-bound succinate dehydrogenase of yeast can also be activated by NADH, not only in wild-type particles but also in particles from Mutant 26H. Since the latter particles contain at most 3 % of the normal amount of ubiquinone it seems unlikely that QH_2 brings about activation through preferential binding to the activated form of the enzyme. This small amount of Q could, however, very well be able to bring about reduction of succinate dehydrogenase by transferring reducing equivalents from NADH dehydrogenase.

The final level of activation reached in the presence of NADH is a little lower than that reached with succinate. Possibly, this is due to denaturation of the enzyme,

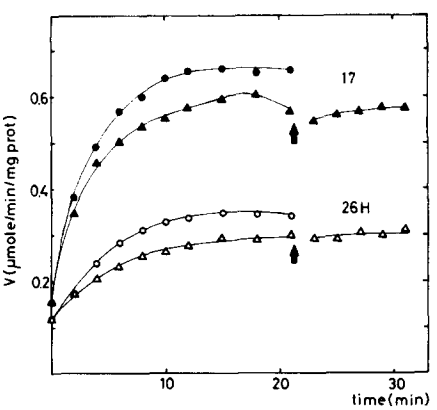

Fig. 5. Activation of particle-bound succinate dehydrogenase by succinate and NADH. Particles were first washed in 0.18 M mannitol – 50 mM Tris-acetate buffer (pH 7.5) – 1 mM EDTA. Particles of strain 17 and strain 26H were then resuspended at a protein concentration of 5.9 and 7.6 mg/ml, respectively, in the same medium supplemented with 7.5 μg/ml antimycin. Activation was started by addition of 40 mM succinate or 1 mM NADH. Activity was periodically assayed after rapid transfer of 50-μl samples to cuvettes containing the assay mixture (see Methods and Materials), including 1.1 mM PMS. The concentration of DCIP was chosen such that the absorbance at 600 nm was 0.8–1.0 at the start of the assay. Activation and assay were carried out at 25 °C. At the arrows 40 mM succinate was added to the activation mixtures containing NADH. ● — ●, strain 17, succinate; ▲ — ▲, strain 17, NADH; ○ — ○, strain 26H, succinate; △ — △, strain 26H, NADH.

TABLE IV

COVALENTLY BOUND FAD AND TURNOVER NUMBERS OF SUCCINATE DEHYDROGENASE

Strain	Covalently bound FAD (nmoles/mg)	Turnover number (s^{-1})	
		PMS plus DCIP	Q-1
17	0.128	210	120
26H	0.103	100	140

since addition of succinate, after the final level of activation has been reached in the presence of NADH, does not lead to further activation.

Rossi et al. [32] found that ubiquinone influences the kinetics of the membrane-bound succinate dehydrogenase of beef-heart submitochondrial particles. Extraction of ubiquinone caused, among other things, a decrease of about 50 % of the succinate dehydrogenase activity, measured with PMS as primary electron acceptor. A similar effect is found with the yeast enzyme. Table IV shows that the turnover number of succinate dehydrogenase of mutant particles is about half that of wild-type particles, when the activity is measured with PMS as electron acceptor. When, however, Q-1 serves as electron acceptor the turnover numbers are about equal. In contrast to

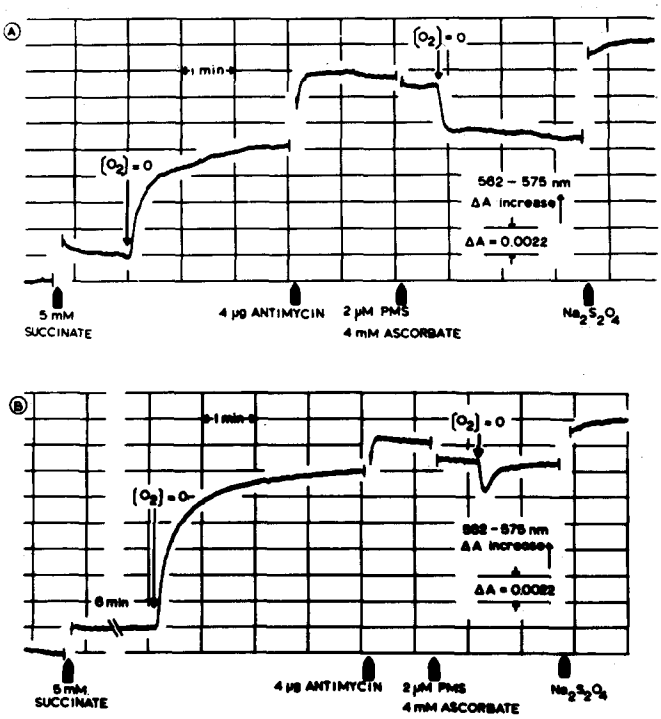

Fig. 6. Effect of antimycin plus oxygen on the redox level of cytochrome *b* in particles. Antimycin plus oxygen were added after anaerobiosis was reached by oxidation of succinate. Particles were suspended in 50 mM potassium phosphate (pH 7.5). Trace A, particles of strain 17 (2.2 mg/ml). Trace B, particles of strain 26H (2.4 mg/ml).

wild-type particles, the activity of mutant particles is higher with Q-1 as acceptor than with PMS.

Redox changes of cytochrome b

The mutant affords an opportunity to investigate the role of ubiquinone in the intriguing phenomenon that antimycin, in the presence of an oxidant such as oxygen, increases the reducibility of cytochrome *b* [33–35].

Figs 6A and 6B show the redox changes of cytochrome *b* when particles are sequentially treated with succinate, antimycin plus oxygen and PMS plus ascorbate. The introduction of antimycin (plus oxygen that is stirred in simultaneously) induces both in the wild-type (Fig. 6A) and mutant (Fig. 6B) particles an extra reduction of cytochrome *b* above the anaerobic reduction level. The extra reduction is abolished when anaerobiosis is reached in the presence of PMS [36]. The fact that cytochrome *b* of mutant particles (Fig. 6B) is initially further oxidized than in the final equilibrium may be explained by assuming that cytochrome *b* re-equilibrates with components that have lost reducing equivalents to oxygen during the aerobic phase.

The effect of antimycin plus oxygen was further investigated in a simpler system viz. in the presence of the ascorbate–dehydroascorbate couple with either PMS or TMPD as redox mediator. Fig. 7 shows that, after oxygen is exhausted by oxidation of ascorbate, the fraction of cytochrome *b* that is reducible under these conditions is much more slowly reduced in wild-type particles (Traces A and B) than in mutant particles (Traces C and D). This is presumably mainly due to simultaneous reduction of the much larger ubiquinone pool present in wild-type particles. When oxygen is added to wild-type particles, in the presence of antimycin, rapid reduction of cytochrome *b* occurs. Exhaustion of oxygen results in an extensive reoxidation. This is found both in the presence of PMS (Trace A) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (Trace B). In particles from the mutant the behaviour of cyto-

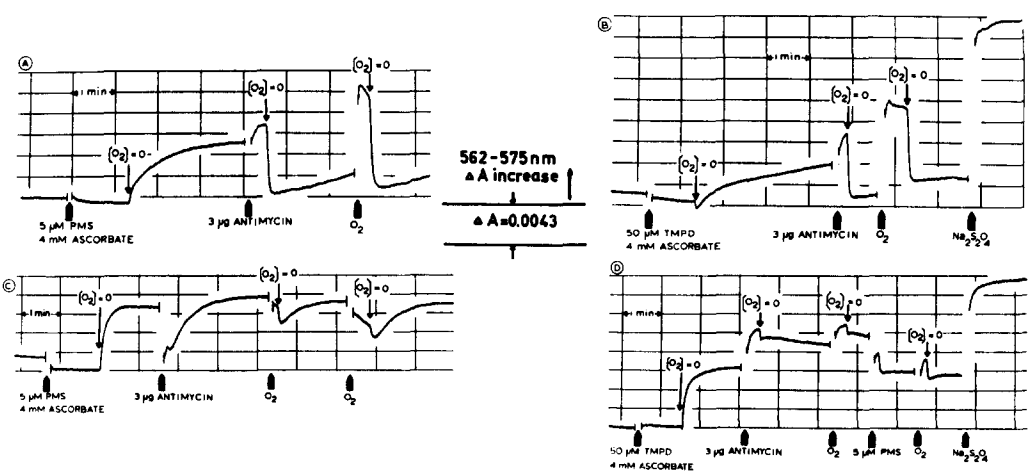

Fig. 7. Redox changes of cytochrome *b* in the presence of the ascorbate–dehydroascorbate couple with PMS or TMPD as redox mediators. Medium as in Fig. 6. Traces A and B, 2.2 mg/ml particles of strain 17; Traces C and D, 2.2 mg/ml particles of strain 26H.

chrome *b* towards addition of oxygen in the presence of antimycin is quite different. Moreover, the effects are different with PMS compared with TMPD as mediator. In the presence of PMS and antimycin (Trace C), addition of oxygen results in an oxidation of cytochrome *b*, whereas in the presence of TMPD and antimycin (Trace D) cytochrome *b* is further reduced. In the former case extra reduction of cytochrome *b* is possibly obscured by a PMS-mediated transfer of reducing equivalents from the ubiquinone–cytochrome *b* pool to cytochrome *c* over the antimycin block. TMPD may not establish a shunt either because it is per se an ineffective mediator in the ubiquinone–cytochrome *b* region or because TMPD is much further reduced in the aerobic steady state due to its higher midpoint potential [36]. In the presence of PMS and antimycin a further oxidation is observed upon anaerobiosis, followed by rereduction. Interestingly, in the presence of TMPD and antimycin, reoxidation of cytochrome *b* after exhaustion of oxygen is extremely slow, at least a factor 100 slower than in wild-type particles under the same conditions (cf. Traces B and D). Rapid reoxidation to the anaerobic level occurs only after addition of PMS.

Both PMS and TMPD have the capacity rapidly to equilibrate cytochrome *c* with the ascorbate–dehydroascorbate couple. The latter observation suggests therefore that reoxidation of cytochrome *b* at anaerobiosis is not induced by reduction of cytochrome *c* or of components that are in rapid equilibrium with cytochrome *c*. The fact that reoxidation in the presence of TMPD is much slower in mutant particles than in wild-type particles indicates that reoxidation of cytochrome *b* depends either on reduction of ubiquinone itself or on reduction of another component that is reducible only via ubiquinone in the presence of TMPD.

DISCUSSION

We have not attempted to investigate which step in the biosynthesis of ubiquinone is affected in the mutants described. Both mutants isolated have a residual respiration that may be due to an incomplete block of the biosynthesis leading to the formation of a trace amount of ubiquinone. Mutants in which ubiquinone formation is completely blocked may not have been detected by the screening procedure applied since this was devised to find mutants with an ability to respire. In this connection it may not be accidental that the two independently derived mutants have defects in the same cistron.

The respiratory rates of mutant particles are in comparison with wild-type particles decreased to a lesser extent than the ubiquinone content. A similar finding has been made with a leaky ubiquinone-deficient mutant of *E. coli* [8]. This finding agrees with the proposal of Kröger and Klingenberg [37] that ubiquinone acts according to "saturation kinetics".

The metabolic versatility of yeast poses special demands on the coordination of respiration. One expression of coordination is the activation of the particle-bound succinate dehydrogenase by NADH, as also occurs in beef-heart submitochondrial particles. Another expression of coordination could be the fact that the NADH: Q-1 oxidoreductase of particles is subject to product inhibition. The fact that the K_i for the product, QH_2-1, is equal to the K_m for Q-1 is presumably responsible for the pseudo first-order kinetics of this enzyme. Pseudo first-order kinetics have also been observed with the enzymes cytochrome *c* oxidase [38] and cytochrome *c* peroxi-

dase [39]. Mechanisms for cytochrome *c* oxidase, based on the assumption that K_m is equal to K_i for product inhibition, have been proposed by Minnaert [40] and Yonetani and Ray [41]. If we may extrapolate the results obtained with Q-1 to the natural ubiquinone, Q-6 [42], it seems likely that inhibition by QH_2 contributes to the coordination of NADH oxidation with the oxidation of other substrates that are oxidized via ubiquinone-dependent dehydrogenases.

Several different mechanisms have been proposed to explain the increased reducibility of cytochrome *b* induced by antimycin plus oxidant. Wilson et al. [43] and Erecińska et al. [44] proposed that the extra reduction is caused by electron transport through the second phosphorylation site. According to this model abolition of extra reduction at anaerobiosis is caused by reduction of components on the oxygen side of the second phosphorylation site. The experiments reported here indicate, however, that extra reduction is not abolished by reduction of components that are in equilibrium with cytochrome *c* but by reduction of components that are in equilibrium with ubiquinone or by reduction of ubiquinone itself. The experiments could, therefore, be explained by the model proposed by Wikström and Berden [36], according to which abolition of extra reduction is thought to be the result of reduction of ubiquinone to the semiquinone form (see however ref. 45). Rieske [46] and Lee and Slater [47] have proposed different models, that have in common that the redox state of cytochrome *b* is thought to be controlled by the redox state of other components of the bc_1 complex. If the assumption is made that these components are, in the presence of antimycin, in equilibrium with ubiquinone but not with cytochrome *c*, the experiments could equally well be explained according to these models.

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