Journal of Bioenergetics and Biomembranes, Vol. 14, No. 3, 1982

The Reconstitution of Oxidative Phosphorylation in Mitochondria Isolated from a Ubiquinone-Deficient Mutant of *Saccharomyces cerevisiae*

A. De Santis,[1,3] E. Bertoli,[2] A. Di Gioia,[1] B. A. Melandri,[1] and A. Baccarini Melandri[1]

Received November 17, 1981; accepted December 10, 1981

Abstract

Mitochondria, isolated from the ubiquinone-deficient nuclear mutant of *Saccharomyces cerevisiae* E3-24, are practically unable to oxidize exogenous substrates. Respiratory activity, coupled to ATP synthesis, can, however, be reconstituted by the simple addition of ethanolic solutions of ubiquinones. A minimal length of the isoprenoid side chain (≥ 3) was required for the restoration. Saturation of the reconstitution required a large amount of exogeneous ubiquinone, in excess over the normal content present in the mitochondria of the wild type strain. A similar pattern of reconstituted activities could be also obtained using sonicated inverted particles. Mitochondria and sonicated particles are also able to carry out a dye-mediated electron flow coupled to ATP synthesis in the absence of added ubiquinone, using ascorbate or succinate as electron donor. This demonstrates that the energy conserving mechanism at the third coupling site of the respiratory chain is fully independent of the presence of the large mobile pool of ubiquinone in the membrane.

Key Words: Respiratory chain; ATP synthesis; mitochondria; ubiquinone; *Saccharomyces cerevisiae;* cytochrome oxidase.

Introduction

The involvement of quinones in respiratory and photosynthetic electron transport chains has been firmly established (for reviews see Crane, 1977; Trumpower and Katki, 1979; Wraight, 1979); however, its localization in the membrane and its role in energy transduction is still a matter of intensive

[1]Istituto ed Orto Botanico dell'Università, Via Irnerio 42, 40126 Bologna, Italy.
[2]Istituto di Chimica Biologica dell'Università, Via Irnerio 48, 40126 Bologna, Italy.
[3]Send correspondence to: Dr. Aurelio De Santis, Istituto Botanico, Università di Bologna, Via Irnerio 42, 40126 Bologna, Italy.

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investigation and debate (Mitchell, 1976; Trumpower, 1981). Especially controversial is whether quinones act as a kinetically homogeneous pool, shunting reducing equivalents from negative electron transport donors to more positive acceptors, or whether specialized molecules of quinones act also in specific redox sites of the electron transport chains.

In mitochondria several lines of evidence (Kröger and Klingenberg, 1977a,b; Schneider *et al.*, 1980) are in favor of a homogeneous pool of UQ,[4] required for the interaction of dehydrogenase and cytochrome complexes; however, the observation of ubisemiquinone radicals in the cytochrome b–c_1 region of mitochondria (Onhishi and Trumpower, 1980) and the isolation of UQ-binding proteins (Yu *et al.*, 1977, 1980a,b) can be suggestive of the involvement of specialized forms of ubiquinone.

Solvent extraction of lyophilized mitochondria, which removes endogenous UQ, and reconstitution by exogenous UQ homologs and analogs has been a widely utilized technique to study the role of this electron transport carrier (Crane, 1977). However, this extraction causes a partial inactivation of NADH dehydrogenase (Gutman *et al.*, 1971), a modification of the catalytic properties of succinate dehydrogenase (Rossi *et al.*, 1970), and alterations of ATPase activity (Degli Esposti *et al.*, 1981); in addition it has not been possible to preserve an intact electron transport chain coupled to ATP synthesis. In extracted mitochondria succinate oxidase and NADH oxidase activities differ also with respect to the stimulatory and inhibitory effects of analogs and homologs of ubiquinone, suggesting a functional heterogeneity and compartmentation of ubiquinone in the respiratory chain (Lenaz *et al.*, 1975).

These disadvantages can be overcome using mitochondria isolated from mutant yeast cells specifically lacking the ubiquinone pool. Various mutant of *Saccharomyces cerevisiae,* which are specifically altered in the ubiquinone content, have been utilized to study some aspects of the role of UQ in electron transport (Tzagoloff *et al.*, 1975; De Kok and Slater, 1975; Brown and Beattie, 1977). Strains of *E. coli* impaired in the biosynthesis of quinones have been also used in bioenergetic studies (Cox and Gibson, 1974; Stroobant and Kaback, 1979).

In this paper we have examined in detail the properties of mitochondria and submitochondrial particles from the ubiquinone-deficient mutant of *Saccharomyces cerevisiae,* strain E3-24 (Tzagoloff *et al.*, 1975), with special attention to the restoration of coupled ATP synthesis by different UQ-homologs in specific segments of the respiratory chain, which might involve also specialized UQ populations.

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[4]Abbreviations used: UQ, ubiquinone; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine.

A preliminary report of this work has been presented (De Santis *et al.*, 1980).

Materials and Methods

Strain of Yeast

The ubiquinone-deficient *Saccharomyces cerevisiae* mutant, strain E3-24, which is incapable of growth on nonfermentable substrates, was obtained by mutagenization with ethylmethanesulfonate from the prototrophic haploid strain D273-10B (Tzagoloff *et al.*, 1975). This mutant has been shown to exhibit an intact *in vivo* mitochondrial protein synthesis activity and was considered to be a nuclear mutant on the basis of the complementation with a cytoplasmic petite tester strain and 2:2 segregation of tetrads (Tzagoloff *et al.*, 1975).

Cell Growth

Cells were grown aerobically at 30°C in a rotary shaker using a medium containing 1% yeast extract, 0.2% peptone, the basic salts of Wicherham (1946), and 2% galactose as carbon source. The absence of spontaneous revertants in the cultures of mutant cells was routinely checked using nonfermentable carbon source as 2% ethanol and 3% glycerol in the medium of parallel cultures.

Preparation of Protoplasts

The cells were harvested at the end of the logaritmic phase of growth. Cell walls were digested according to Schatz and Kováč (1974), using Helicase (L'industrie biologique Francaise, Gennevilliers) or lyophilized β-glucuronidase (Sigma, St. Louis). The formation of protoplasts was followed spectrophotometrically at 540 nm and was continued until 50% decrease in the absorbance of the original suspension was observed.

Isolation of Mitochondria

Mitochondrial fractions were isolated from partially digested yeast cells essentially as reported by Schatz and Kováč (1974). For cell breakage a French pressure cell (Aminco) operating at 3200 psi was used instead of a blendor. Following this method of breakage mitochondria from aerobically grown wild-type *Saccharomyces cerevisiae* cells (strain D273-10B) had good respiratory control and ADP/O ratios. Mitochondria were finally suspended in 0.5 M sorbitol, 1 mM EDTA, and 10 mM Tris SO_4, pH 7.5, at a protein concentration of 20–40 mg protein/ml.

Protein was determined by the Lowry method, using albumin as standard (Lowry *et al.*, 1951).

Preparation of Submitochondrial Particles

Submitochondrial particles were prepared from mitochondrial suspension by sonic irradiation for 30 sec using an MSE sonifier. Particles were collected by centrifugation of the clarified supernatant at 218,000 *g* for 30 min and were suspended in the same medium used for mitochondria.

Oxygen Uptake and ATP Synthesis

Oxygen uptake was measured polarographically at 30°C with a Clark electrode (Yellow Spring). The reaction medium contained 0.5 M sorbitol, 1 mM EDTA, 10 mM Tris SO_4, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KPi, 2 mM ADP, and 1 mg/ml bovine serum albumin. Ubiquinone homologs, FCCP, and antimycin were added as ethanolic solutions. ATP synthesis was measured in the same oxygen electrode vessel with $^{32}P_i$ utilizing a glucose–hexokinase trap, as reported previously (Melandri *et al.*, 1971).

Results

Mitochondria isolated from the ubiquinone-deficient mutant are practically unable to utilize substrates like malate or succinate, which are oxidized by dehydrogenases localized on the internal face of the inner membrane. However, these two respiratory activities are promptly restored to physiological values by addition of solutions of exogenous ubiquinones in ethanol, which *per se* does not act as a respiratory substrate in reconstituted preparations. The activities restored are antimycin A and cyanide-sensitive (not shown), indicating that added UQ does not bypass segments of the electron transport chains (Fig. 1).

As reported by Tzagoloff *et al.* (1975), these mitochondria are also able to oxidize ascorbate at very high rate in the presence of redox mediators (as TMPD or DAD), which are believed to enter the respiratory chain at the level of cytochrome *c* (Sanadi and Jacobs, 1967; Hauska *et al.*, 1977). The addition of UQ homologs or of antimycin A has no effect on this reaction, which is on the contrary almost completely inhibited by cyanide.

Table I reports the results of experiments in which oxidative phosphorylation coupled to the oxidation of malate plus pyruvate or of succinate was measured in mitochondria obtained from mutant cells. Different UQ homologs are able to reconstitute electron transport activities and coupled ATP synthesis. In the presence of UQ_1 much lower rates of stimulated activities

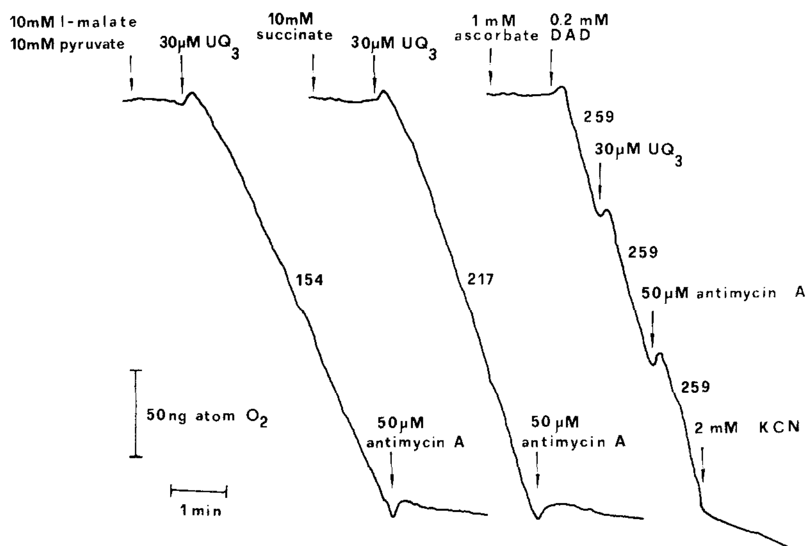

Fig. 1. The reconstitution of respiration by exogenous ubiquinone in mitochondria from E3-24 mutant. Conditions as described in Materials and Methods. Additions as indicated. Figures along the traces are ng atom · min^{-1} · mg^{-1} protein.

were found as compared with those obtained utilizing longer-chain homologs like UQ_3 or UQ_5. It should be recalled that the natural quinone present in membrane from prototrophic strain is UQ_6 (Ohnishi *et al.*, 1966).

No difference in effectiveness in reconstituting the activities dependent on malate plus pyruvate or on succinate was observed using different quinone

Table I. The Reconstitution of Oxidative Phosphorylation by Exogenous Ubiquinones in Mitochondria from E3-24 Mutant[a]

Substrate	Additions	ATP yield (nmol · min^{-1} · mg^{-1} protein)	Oxygen uptake (ng-atom · min^{-1} · mg^{-1} protein)	ATP/O
Malate + pyruvate	—	0	0	—
	UQ_1	72	81	0.89
	UQ_3	190	154	1.24
	UQ_5	191	147	1.30
	UQ_5 + FCCP	9	154	0.06
Succinate	—	0	0	—
	UQ_1	59	68	0.87
	UQ_3	198	207	0.96
	UQ_5	194	158	1.23
	UQ_5 + FCCP	0	108	0

[a]Conditions as described in Materials and Methods. Reaction medium contained also 10 mM substrate, 30 μM UQ, and 2.5 μM FCCP, where used.

homologs, in agreement with reconstitution of extracted yeast mitochondria (Lenaz *et al.*, 1971), but in contrast to that reported for beef heart mitochondria (Lenaz *et al.*, 1975). As can be seen, the P/O ratio values obtained after reconstitution are comparable to those reported for mitochondria isolated from prototrophic strains of *Saccharomyces cerevisiae* (Guérin *et al.*, 1979).

Our data confirm also the general notion that *Saccharomyces* cells, at the late exponential phase of growth, are incapable of phosphorylation at the site I level, owing to the lack of essential components within the NADH dehydrogenase complex (Ohnishi, 1973).

The saturation kinetics related to the reconstitution of succinate oxidation by exogenous UQ_5 in intact mitochondria from E3-24 mutant is shown in Fig. 2. Half maximal rates were reached at a concentration of about 3 nmol UQ_5/mg protein, and maximum reconstitution was achieved at 30–40 nmol UQ_5/mg protein. The total amount of UQ extractable with apolar solvents in mitochondria from wild type cells is on the order of 7 nmol/mg protein (De Kok and Slater, 1975), while extracts of mutant mitochondria do not contain spectrally detectable ubiquinone (Brown and Beattie, 1977).

Table II shows that intact mitochondria isolated from ubiquinone-deficient mutant are able to oxidize succinate in the presence of DAD or TMPD; this effect was saturated by 0.2 mM DAD or TMPD. This indicates that succinate dehydrogenase is able to reduce, even in the absence of the UQ pool, DAD or TMPD, which in turn feed electrons to the cytochrome oxidase system. Under these conditions the P/O ratio is about one-half of that observed in the presence of succinate plus UQ_5.

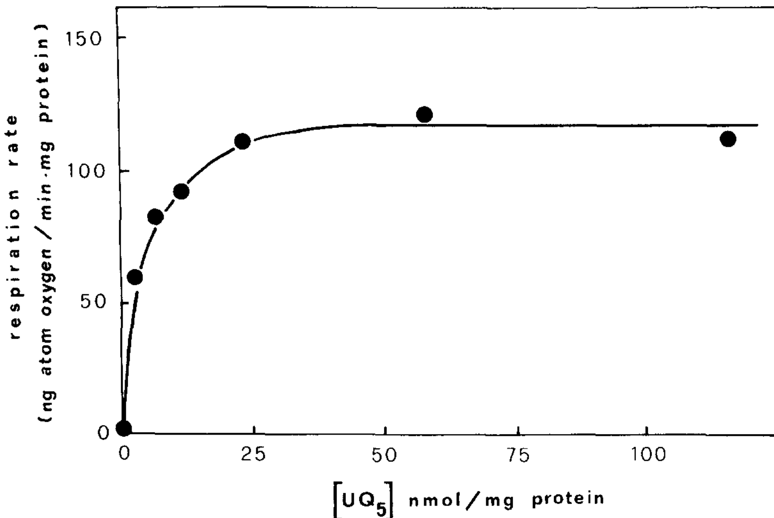

Fig. 2. The saturation kinetics of the reconstitution by exogenous UQ_5 of succinate oxidation in intact mitochondria from E3-24 mutant. Conditions as in Fig. 1.

Table II. Dye-Mediated Electron Flow in Mitochondria from E3-24 Mutant[a]

Substrate	Additions	ATP yield (nmol · min^{-1} · mg^{-1} protein)	Oxygen uptake (ng-atom · min^{-1} · mg^{-1} protein)	ATP/O
Succinate	—	0	0	—
	UQ_5	137	149	0.92
	DAD	72	163	0.44
	TMPD	83	243	0.34
Ascorbate	DAD	119	243	0.49
	TMPD	211	502	0.49
	DAD + UQ_5	120	242	0.50
	DAD + UQ_5 + FCCP	17	193	0.09

[a]Conditions as in Table I. Additions: 10 mM succinate, 1 mM ascorbate, 30 μM UQ_5, 0.2 mM DAD, 0.2 mM TMPD, 2.5 μM FCCP.

A similar pattern of response was obtained using ascorbate instead of succinate as electron donor, in the absence of added quinones (see also Fig. 1). DAD- or TMPD-mediated oxidative phosphorylation is completely insensitive to the addition of exogenous ubiquinones.

Reconstitution of respiratory activities coupled to phosphorylation was also obtained with different substrates in sonicated particles prepared from E3-24 mitochondria. Table III shows that the restoration of respiratory activities and ATP synthesis in submitochondrial particles by addition of exogenous UQ_5 is similar to that observed in the intact mitochondria, suggesting that the polarity of the coupling membrane has hardly any relevance in the reconstitution of the mechanism of energy transduction.

It is noteworthy that also in submitochondrial particles, in the absence of exogenous ubiquinone, dye-mediated electron transport coupled to phospho-

Table III. Oxidative Phosphorylation in Sonicated Submitochondrial Particles from E3-24 Mutant[a]

Substrate	Additions	ATP yield (nmol · min^{-1} · mg^{-1} protein)	Oxygen uptake (ng-atom · min^{-1} · mg^{-1} protein)	ATP/O
Succinate	—	0	0	—
	UQ_5	64	77	0.83
	UQ_5 + antimycin A	nd	8	nd
	DAD	36	112	0.32
	TMPD	58	177	0.33
	DAD + UQ_5 + antimycin A	68	248	0.27
Ascorbate	DAD	77	248	0.31
	TMPD	103	310	0.33
	DAD + UQ_5 + antimycin A	81	269	0.30

[a]Additions: 10 mM succinate, 1 mM ascorbate, 40 μM UQ_5, 0.4 mM DAD, 0.8 mM TMPD, 5 μM antimycin A.

rylation can be observed. Moreover, a bypass of coupling site II of the respiratory chain can also be demonstrated in the reconstituted particles oxidizing succinate plus DAD in the presence of antimycin A. Unlike for mitochondria, maximal rates with succinate as electron donor were obtained in sonicated particles with 0.4 mM DAD or 0.8 mM TMPD.

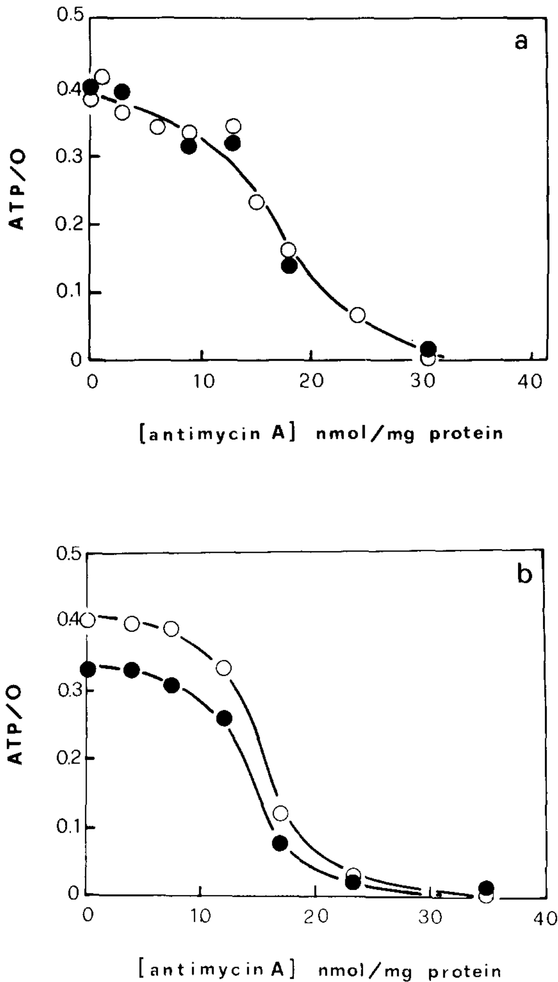

Fig. 3. The inhibitory effect of increasing concentrations of antimycin A on the ATP/O ratio with ascorbate plus TMPD (●) or ascorbate plus DAD (○) in whole mitochondria (a) or inverted submitochondrial particles (b) from E3-24 mutant. Respiration of the ascorbate–dye couple was not inhibitited by antimycin A up to 150 nmol/mg protein. Conditions as in Materials and Methods. Additions: 1 mM ascorbate, 0.1 mM TMPD, 0.2 mM DAD.

Both dye-mediated respiration and coupled phosphorylation are resistant to antimycin A at a concentration not exceeding 10 nmol/mg protein; at higher concentration the well-known uncoupling effect of antimycin A prevails, inhibiting ATP formation without appreciable inhibition of electron flow. The effect of antimycin A is completely independent of the polarity of the coupling membrane (compare Fig. 3a and Fig. 3b) or of the protonability of the dye feeding electrons to cytochrome *c* (Hauska *et al.*, 1977). It is also insensitive to the mechanism of reduction of the electron donor dye, either by exogenously added ascorbate or by succinate via the endogenous succinate dehydrogenase activity. It should be enphasized again that all the coupled electron flows mediated by redox dye in the E3-24 mutant are not affected at all by exogenous ubiquinones, the addition of which is, on the contrary, absolutely required for the other respiratory activities.

Discussion

The data reported herein indicate that electron transport, as well as ATP synthesis, driven by substrate oxidation at the inner face of the internal mitochondrial membrane, can be promptly restored in a UQ-deficient mutant of *Saccharomyces cerevisiae* by addition of exogenous UQ homologs. The efficiency in reconstitution, both of electron transport and of ATP synthesis, is maximal with UQ homologs having three or more isoprenoid residues in the side chain. Similar observations were also reported for the reconstitution of photophosphorylation in UQ-extracted chromatophores from photosynthetic bacteria (Baccarini Melandri *et al.*, 1980), in which a minimal length of the isoprenoid side chain (≥ 3) was required for restoration of photophosphorylation.

The reconstitution of respiratory activities in mitochondria from the E3-24 mutant requires the addition of relatively high concentrations of ubiquinone, in excess of the normal content present in the parent wild type strain. The amount of UQ_5 actually incorporated into the membrane has not, however, been determined. The requirement of a large amount of quinones for the reconstitution of steady-state energy-conserving electron flow represents another similarity with bacterial chromatophores (Baccarini Melandri *et al.*, 1980). This should indicate that the whole complement of UQ is required for maximal interaction between the dehydrogenase and the cytochrome complexes, under steady-state conditions of electron transport. Whether all the UQ added act as a kinetically homogeneous pool cannot yet be inferred from these studies.

Another interesting feature which emerges from these studies is the ability of submitochondrial particles prepared from the UQ-deficient mutant to carry on a dye (in particular TMPD-ascorbate) mediated phosphorylation.

The proton pumping activity of mitochondrial cytochrome oxidase has been a subject of considerable controversy (Wikström, 1977, Wikström and Saari, 1977; Moyle and Mitchell, 1978; Wikström and Krab, 1978).

Some of the evidence in favor of the proton translocating function of cytochrome oxidase is based on the detection of ATP synthesis, of H^+ uptake or valinomycin-dependent K^+ uptake following ascorbate + TMPD oxidation in the presence of antimycin, and of $\Delta\mu_{H^+}$ generation by the same reaction in submitochondrial particles (Siegel and Carafoli, 1978).

This observation has been confirmed by all our experiments in the E3-24 mutant either in whole mitochondria or in the sonicated inverted submitochondrial particles, and both when TMPD (an electron carrier) or DAD (a proton-electron carrier) were reduced externally by exogenous ascorbate or at the redox site of succinate dehydrogenase.

The data therefore agree with the notion of an endogenous energy-conserving mechanism within the cytochrome oxidase complex of yeast, fully independent of the presence of the large mobile pool of ubiquinone in the membrane. On the other hand, the presence of minimal amounts of UQ, not detectable by conventional procedures and present in a specialized form in the b–c_1 complex, cannot be ruled out completely and will be the subject of further investigations.

Acknowledgments

We are indebted to Prof. D. E. Griffiths (University of Warwick, Coventry, England) for the strains of yeast. Ubiquinones were kindly offered by Hoffman–La Roche, Basel, Switzerland.

This work was supported by Consiglio Nazionale delle Ricerche, Italy (Contract No. 80.00510.04).

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