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OXIDATIVE PHOSPHORYLATION IN YEAST

X. PHOSPHORYLATION ABILITY OF MUTANTS DEFICIENT IN CYTOCHROMES *a* AND *b*

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SUMMARY

1. Mitochondria from three non-allelic mutants of *Saccharomyces cerevisiae*, each lacking cytochrome aa_3 as a consequence of single nuclear gene mutation, exhibited oxidative phosphorylation with ferricyanide as electron acceptor with the same efficiency as wild-type yeast mitochondria.

2. A mutant deficient in cytochrome *b* synthetized only negligible amounts of cytochrome *b*. Cytochromes *b* with absorption maxima at 561 and 565 nm seemed to be equally affected by the mutation. Respiration of mitochondria from the cytochrome *b*-deficient strain was limited by the low amount of cytochrome *b* (and was accordingly substantially enhanced by the *N,N,N',N'*-tetramethyl-*p*-phenylenediamine shunt) but the phosphorylation efficiency was the same as in wild-type yeast mitochondria.

3. Cells simultaneously deficient in cytochromes *a* and *b* were isolated as segregants from a cross between cytochrome *a*- and cytochrome *b*-deficient mutants. Unlike cytochrome *a*+*b*-deficient mutants in which the deficiency was due to cytoplasmic or nuclear single-gene mutation, mitochondria from this two-gene mutant catalyzed ATP–phosphate exchange reaction, exhibited ATPase activity sensitive to oligomycin and were able to carry out autonomous protein synthesis.

4. The results indicate that intact cytochromes *a* or *b* are required as redox carriers in the respiratory chain but the absence of the intact cytochromes does not affect the phosphorylation ability of mitochondria.

INTRODUCTION

Energy conservation in mitochondria rests upon a highly complex interplay of catalytic and structural components of the mitochondrial membrane. It is not yet clear how far the mitochondrion can be structurally simplified and still preserve its energy conservation ability[1]. Phosphorylating submitochondrial particles have been found to contain a complete respiratory chain[2,3]; their resolution into separate

Abbreviation: TMP, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

complexes containing pieces of the respiratory chain leads unavoidably to the loss of the capacity for respiration-linked ATP synthesis[4,5]. Extraction of cytochrome *c* from mitochondria has been found to reduce not only overall oxidation but also phosphorylation not directly linked to the span containing cytochrome *c*; addition of exogenous cytochrome *c* restored both oxidation and phosphorylation[6–9]. Mitochondria from mutants deficient simultaneously in cytochrome *a* and *b* have been shown not to catalyze ATP–phosphate exchange reaction and to exhibit ATPase activity insensitive to oligomycin, indicating a defective energy-transfer system[10,11].

On the other hand, the presence of energy-transfer reactions has recently been demonstrated in yeast promitochondria lacking functional cytochromes[12]. Also, submitochondrial particles largely devoid of cytochrome oxidase have been found to catalyze the ATP–phosphate exchange reaction[13]. Particles have been prepared from bovine heart containing no cytochromes except traces of cytochromes *b* and c_1 and exhibiting ATP–phosphate exchange[14], and it has been claimed that the exchange reaction can be catalyzed even by a soluble enzyme complex isolated from mitochondria[15]. In addition, mitochondria from cytoplasmic mutants lacking cytochromes *a* and *b* and exhibiting no ATP–phosphate exchange have been shown to maintain proton impermeability[16], a property considered to be essential for mitochondrial energy conservation through the chemiosmotic mechanism[17].

In this work, mitochondria from mutants deficient in cytochrome *a* or *b* and from a segregant simultaneously deficient in both cytochromes were studied with respect to their oxidative phosphorylation abilities. The results indicate that phosphorylating mitochondria can dispense with intact cytochrome *a* or *b*. They do not exclude the possibility that apoproteins of the cytochromes may have a structural role in the mitochondrial membrane.

EXPERIMENTAL

The following strains of *Saccharomyces cerevisiae* were used. Cytochrome *a*-deficient: Z 8 (haploid, α ad_1 lys_2), S 10 (haploid, α ad_1 lys_2), S 10/10 (diploid, ad_1 lys_2/ad_1 his) and S 18 (haploid, α try). Cytochrome *b*-deficient: 681-9 D (haploid, a his_1 tr_1 cy_1) and 681-9A X D (diploid, his_1 tr_1 cy_1/his_1 tr_1 cy_1). These strains were prepared from wild-type strain D 225-5A by ultraviolet light or nitrite mutagenesis by Lachowicz *et al.*[18]. Cytochrome *b*-deficient strain 4-4-C, and strain 4-9-D simultaneously deficient in cytochromes *a* and *b* were isolated as segregants from crosses between strains Z 8 and 681-9 D by Dr V. Kováčová.

For comparison, diploid wild-type strain DT XII was employed.

Methods of culture, preparation of mitochondria, determination of oxidative phosphorylation, of ATP–phosphate exchange and of protein synthesis *in vitro*, other auxiliary methods and materials were the same as used previously[10,12,19,20]. Oxidative phosphorylation with ferricyanide as electron acceptor was measured according to the method of Estabrook[21]. The quantity of cytochromes in mitochondria was determined by the procedure of Vanneste[22], except that the extinction coefficient for cytochrome aa_3* as given by Van Gelder[23] was used in the calculation. Spectra were recorded in the Hitachi–Perkin Elmer 356 split-beam spectrophotometer.

* Cytochrome aa_3 $(a+a_3)$: based on a molecule containing 2 haem *a* molecules.

RESULTS AND DISCUSSION

Mutants deficient in cytochrome a

The three mutant strains studied, Z 8, S 10 and S 18, were each deficient in cytochrome *a*. The mutated genes were non-allelic, *e.g.* an independent gene was responsible for the cytochrome *a* deficiency in each strain[18]. The mutated gene of the strain Z 8 was found to be allelic with the gene p_3 previously described by Sherman[24].

Cytochrome aa_3 was completely absent from absolute and difference (reduced by endogenous substrates plus dithionite minus oxidized by H_2O_2) spectra of the three strains. Mitochondria isolated from the three mutant strains exhibited no cytochrome oxidase activity (measured as oxidation of TMPD *plus* ascorbate). The content of cytochromes *b* and $c + c_1$ in the mutant mitochondria is shown in Table I.

As reported previously, mitochondria from the cytochrome *a*-deficient mutants displayed ATPase activity sensitive to oligomycin[10]. This implies that the energy-transfer system may be preserved in the mutant mitochondria. In fact, ATP–P_i exchange activity was revealed in the mutant mitochondria with specific activities in the range of 10.5 to 43.5 nmoles $P_i \cdot min^{-1} \cdot mg^{-1}$ protein which may be compared with that of wild-type mitochondria (141 nmoles $P_i \cdot min^{-1} \cdot mg^{-1}$ protein).

Table II presents oxidative phosphorylation data obtained with mutant mitochondria with ferricyanide as electron acceptor. $P:2e^-$ values with succinate or pyruvate+malate approach the theoretical value of 1, indicating the operation of Site 2 phosphorylation. Similar values (0.6 to 0.9) were obtained with mitochondria isolated from wild-type strain DT XII. Site 1 phosphorylation has not been found in *Saccharomyces* mitochondria isolated under our conditions. Specific activities of ferricyanide reduction were lower in the mutants than in the wild-type strain (the latter displaying activities of 276 and 388 nmoles ferricyanide reduced $\cdot min^{-1} \cdot mg^{-1}$ with succinate and citrate, respectively) which may be explained by a higher catabolic repression to which cytochrome-deficient mutants were subjected when compared with the respiration-competent strain. The activities of strains S 10 and S 18 with

TABLE I

CONCENTRATION OF CYTOCHROMES IN MITOCHONDRIA ISOLATED FROM CYTOCHROME-DEFICIENT MUTANTS

The method of analysis is described in the Experimental section. Mitochondria isolated from wild-type strain DT XII were used for comparison.

Deficient cytochrome	*Strain*	*Cytochrome concentration (μmoles$\cdot g^{-1}$ protein)*		
		aa_3	*b*	$c + c_1$
O (wild type)	DT XII	0.045	0.156	0.666
a	Z 8	0	0.071	0.145
	S 10	0	0.090	0.394
	S 10/10	0	0.110	0.615
	S 18	0	0.130	0.464
b	681-9D	0.111	<0.010	0.282
	4-4-C	0.040	<0.010	0.194
a + *b*	4-9-D	0	0	0.581

TABLE II

OXIDATIVE PHOSPHORYLATION WITH FERRICYANIDE AS ELECTRON ACCEPTOR IN MITOCHONDRIA ISOLATED FROM CYTOCHROME *a*-DEFICIENT MUTANTS

The reaction mixture contained in 2.3 ml: 0.56 M mannitol, 1.3 mM EDTA, 8.7 mM Tris maleate, 17.4 mM KCl, 1.3 mM $MgCl_2$, 8.7 mM potassium phosphate labelled with ^{32}P (45–60 cpm per nmole P), 21.8 mM glucose, 0.87 mM KCN, 1.75 mM potassium ferricyanide, 5 mg bovine serum albumin, 5 units hexokinase (Sigma, Type III), substrate as indicated in the table and 1.2 to 2.5 mg mitochondrial protein; final pH 6.4. After 5 min preincubation at 30 °C in the absence of ferricyanide, the reaction was started by adding ferricyanide and, after 5 to 15 min of additional incubation, terminated by adding 0.2 ml 5 M $HClO_4$. The values are corrected for activities resistant to 4 $\mu g \cdot ml^{-1}$ antimycin A.

Strain	*Substrate*	*ΔFerricyanide* ($nmoles \cdot min^{-1} \cdot mg^{-1}$)	*ΔP$_i$* ($nmoles \cdot min^{-1} \cdot mg^{-1}$)	*P:2e$^-$*
Z 8	8.7 mM succinate	50	15	0.6
	8.7 mM citrate	30	12.7	0.85
	4 mM pyruvate + 4 mM malate	82	21.5	0.52
S 10	17.4 mM citrate	146	48.9	0.67
	13 mM pyruvate + 4.4 mM malate	183.2	109.7	1.19
	8.7 mM α-ketoglutarate	59.2	45.1	1.52
S 10/10	17.4 mM citrate	85.9	20.4	0.48
	13 mM pyruvate + 4.4 mM malate	105.0	27.5	0.44
	8.7 mM α-ketoglutarate	56.9	27.9	0.98
S 18	17.4 mM citrate	69.0	17.6	0.51
	13 mM pyruvate + 4.4 mM malate	56.6	9.1	0.32
	8.7 mM α-ketoglutarate	34.0	15.3	0.90

succinate as substrate were too low to be reliably measurable; however, the reaction between succinate dehydrogenase and phenazine methosulphate has been demonstrated to be present in all the strains. Specific activity of succinate–phenazine methosulphate reductase of strain S 10, 15.3 $nmoles \cdot min^{-1} \cdot mg^{-1}$ and of strain S 18, 22 $nmoles \cdot min^{-1} \cdot mg^{-1}$ may be compared with that of wild-type strain, 170.0 $nmoles \cdot min^{-1} \cdot mg^{-1}$.

Mutants deficient in cytochrome b

Contrary to the strains deficient in cytochrome *a*, the two mutants deficient in cytochrome *b*, 681-9 D and 4-9-D, were not entirely devoid of the deficient cytochrome. As shown already in a previous report[10], a tiny amount of cytochrome *b* could be detected in cytochrome spectra of intact cells of strain 681-9 D, and a little more in a homozygous diploid 681-9 A X D derived from this strain. It has been concluded that the synthesis of cytochrome *b* in this mutant has not been arrested completely but only slowed down[10].

Strain 4-4-C, also cytochrome *b* deficient, carries the same mutated gene as strain 681-9 D. However, probably due to the presence of different genes in the two strains which are not directly related to the cytochrome deficiency, the amount of cytochrome *b* in strain 4-4-C was consistently found to be more diminished than in strain 681-9 D, being at the limit of spectral detection. When cytochrome content was measured in mitochondria isolated from the two mutant strains, cytochrome *b* could not be discerned in deoxycholate-clarified mitochondrial suspensions. The content of other cytochromes is shown in Table I.

Recently, the existence of at least two cytochrome *b* species in mitochondria from various sources has been surmised[25–29]. The two species were reported to differ in their absorption maxima and their response to antimycin A. It has been shown that, in intact yeast cells, antimycin A induces oxidation of the cytochrome *b* with absorption maximum at 562 nm under strictly anaerobic conditions while, in the presence of air, it causes an increased reduction of the species absorbing at 565 nm (ref. 30). This provides a procedure to find out which of the two cytochromes *b* has been affected in our cytochrome *b*-deficient strains.

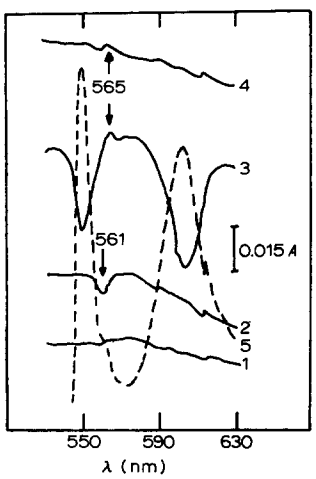

Fig. 1. Evidence for the presence of 561- and 565-nm absorption bands in the cytochrome *b*-deficient mutant. Spectrophotometric cuvettes contained cells of cytochrome *b*-deficient diploid strain 681-9 AXD grown for 24 h aerobically on a semi-synthetic medium with 0.5% glucose as carbon source (about 50 mg dry weight cells·ml^{-1} of 0.1 M citrate phosphate buffer, pH 4.3). The suspension was prebubbled with argon and covered with a layer of liquid paraffin. 1, base line; 2, antimycin A (50 μg·ml^{-1}) was added to the sample cuvette and the difference spectrum recorded after 20 min; the trough at 561 nm indicates anaerobic oxidation of cytochrome b_{561}; 3, a short pulse of air was introduced into the sample cuvette; 4, the same spectrum after 2 min; 5, difference redox spectrum (cells in the sample cuvette reduced by dithionite and in the reference cuvette oxidized by 60 mM H_2O_2).

As shown in Fig. 1, differential behaviour of cytochrome *b* in the presence of antimycin A under anaerobic and aerobic conditions could be observed with the cytochrome *b*-deficient strain just as with the wild-type strain except for the obvious quantitative differences between the mutant and wild-type strains. Thus, the two cytochromes *b* seem to be preserved in minute quantities in the cytochrome *b*-deficient mutant, the mutation probably affecting the synthesis of the two cytochromes to the same extent. This indicates that the synthesis of the two presumed species may be closely linked. Before a clear-cut physical separation of the two cytochromes *b* is accomplished, the possibility cannot be excluded that only one species of cytochrome *b* exists and its kinetic and spectral heterogenity is due to its inhomogenous distribution within the mitochondrial membrane[29].

Mitochondria from the two cytochrome *b*-deficient strains catalyzed the ATP–P_i exchange reaction with the specific activities of about 11 nmoles $P_i \cdot min^{-1} \cdot mg^{-1}$ protein. This is in line with the previous finding that the ATPase activity of mito-

chondria from a cytochrome *b*-deficient mutant was oligomycin-sensitive[10]. As shown in Table III, mitochondria of cytochrome *b*-deficient strains could oxidize various substrates with oxygen as electron acceptor but at a much lower rate than mitochondria from wild-type yeast. The low rate was obviously due to the limited availability of cytochrome *b* as it was considerably enhanced when the cytochrome *b* region was circumvented by the N,N,N′,N′-tetramethyl-*p*-phenylenediame (TMPD) shunt[31]. In addition, the rates of L-lactate and of TMPD + ascorbate oxidation which furnished reducing equivalents to the respiratory chain above the level of cytochrome *b* were commensurate with the rates found in wild-type mitochondria.

TABLE III

RESPIRATORY ACTIVITY OF MITOCHONDRIA ISOLATED FROM CYTOCHROME *b*-DEFICIENT MUTANT

The polarographic vessel contained in 2.0 ml: 0.6 M mannitol, 20 mM KCl, 1.5 mM EDTA, 10 mM Tris maleate, 10 mM potassium phosphate and mitochondria from strain 681-9D (0.5 to 2.5 mg protein); final pH 6.4, 30 °C. The concentration of substrate is indicated in the table.

Substrate	*Respiratory activity* (*ngatoms O·min^{-1}·mg^{-1} protein*)	
	Without TMPD	*In the presence of 0.4 mM TMPD*
None	12.3	12.8
1 mM NADH	38.6	850.0
10 mM citrate	37.0	88.8
10 mM ethanol	17.4	30.9
10 mM L(+)-lactate	42.5	—
10 mM D(−)-lactate	65.7	—
7.5 mM ascorbate	—	702.5

The observation that the electron flow through the respiratory chain is limited by the diminished amount of cytochrome *b* in the mutant supports the placement of cytochrome *b* in a simple linear sequence of the respiratory carriers in mitochondria.

Table IV demonstrates that, despite a low oxidation rate, the phosphorylation efficiency of mitochondria isolated from the cytochrome *b*-deficient mutant was the same as found previously with wild-type mitochondria[19].

Mutants simultaneously deficient in cytochromes a and b

Two types of such mutants exist. In the first type, simultaneous deficiency in cytochrome *a* and *b* results from mutation of single nuclear genes[18,24,34]. Mitochondria from these mutants exhibited ATPase activity insensitive to oligomycin[10], did not catalyze the ATP–P_i exchange reaction[11], and were not able to carry on autonomous mitochondrial protein synthesis[10]. The causal relationship in these deficiencies has not been clarified. It is possible that a simultaneous deficiency in cytochromes *a* and *b* represents such an extensive modification of the mitochondrial membrane that the capabilities for protein synthesis and energy transfer are lost.

Such a possibility can be tested using the second type of cytochrome *a*- and *b*-deficient mutants as described below. In this type of mutant the simultaneous deficiency in the two cytochromes is due to the presence in a cell of two mutated genes, one responsible for cytochrome *a* and the other for cytochrome *b* synthesis.

TABLE IV

OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA ISOLATED FROM CYTOCHROME *b*-DEFICIENT STRAINS

With strain 681-9D, respiration was measured polarographically and phosphorylation determined by the radioactive phosphate extraction procedure[32]. Conditions were similar to those given in Table II except that KCN and ferricyanide were omitted and oxygen served as electron acceptor. Reaction time: 5 to 15 min; concentration of mitochondrial protein: 2.44 mg·ml^{-1}. With strain 4-4-C, respiration was measured manometrically and phosphate uptake determined by Sumner's[33] method. The reaction mixture contained: 0.42 M mannitol, 0.52 mM EDTA, 10.5 mM potassium phosphate, 10.5 mM Tris maleate, 6.8 mM $MgCl_2$, 0.18% bovine serum albumin, 0.5 mM ADP, 25 mM glucose, hexokinase (2 units·ml^{-1}), substrate as indicated in the table and mitochondria (0.6 mg protein·ml^{-1}); final pH 6.4, 30 °C. Measurement was commenced on addition of glucose and hexokinase from the side arm after 7 min of thermal equilibration and was terminated after 45 min of additional incubation by adding 2 ml 10% trichloroacetic acid.

Strain	*Substrate*	*ΔO (ngatoms O·min^{-1}·mg^{-1})*	*ΔP_i (nmoles·min^{-1}·mg^{-1})*	*P/O*
681-9D	None	6.3	10.4	1.65
	2 mM NADH	23.4	19.7	0.84
	10 mM citrate	38.7	48.5	1.25
	20 mM succinate	16.0	18.2	1.14
	7.5 mM ascorbate + 0.2 mM TMPD	48.4	38.5	0.79
4-4-C	None	4.7	8.7	1.85
	10 mM citrate	59.1	92.3	1.56
	10 mM α-ketoglutarate	58.0	106.2	1.83

TABLE V

PROPERTIES OF MITOCHONDRIA ISOLATED FROM A TWO-GENE MUTANT DEFICIENT IN CYTOCHROMES *a* AND *b*

For the ATPase assay the reaction mixture contained in 1.0 ml: 80 mM mannitol, 20 mM Tris chloride, 80 mM KCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 4 mM ATP and 0.17 mg mitochondrial protein; final pH 9.5, 30 °C, incubation time 10 min. In the ATP–P_i exchange assay, the reaction mixture contained in 1.0 ml: 80 mM mannitol, 25 mM Tris chloride, 40 mM KCl, 5 mM EDTA, 6 mM ATP, 1 mM KCN, 25 mM potassium phosphate (50–100 cpm per nmole) and 1.7 to 2.0 mg mitochondrial protein; final pH 7.0, 30 °C, incubation time 3, 6, 9, and 12 min. Protein synthesis was measured in a reaction mixture containing: 100 mM mannitol, 60 mM Tris chloride, 66 mM KCl, 1 mM EDTA, 20 mM $MgCl_2$, 2.5 mM ATP, 10 mM phosphoenolpyruvate, 50 μg pyruvate kinase, 0.1 mM cycloheximide, 50 μg of amino acid mixture without leucine[35] and 2 mg of mitochondrial protein in 1.0 ml; final pH 7.6. After 3 min of preincubation at 37 °C, 0.8 μCi [^{14}C]leucine (125 mCi·mmole^{-1}) was added and samples were taken after 0, 5, 10, and 20 min for measurement of radioactivity[20].

Property	*Value*
ATPase	
Specific activity (μmoles P_i·min^{-1}·mg^{-1})	0.92
Inhibition (%) by 4 μg·ml^{-1} oligomycin	61.5
ATP-P_i exchange	
Specific activity (μmoles P_i·min^{-1}·mg^{-1})	0.012
Inhibition (%) by 0.5 mM 2,4-dinitrophenol	70.0
Inhibition (%) by 4 μg·ml^{-1} oligomycin	44.4
Protein synthesis	
Specific activity (pmoles leucine·min^{-1}·mg^{-1})	14.7

A mutant of this type was isolated as a segregant from a cross between mutants deficient in cytochrome *a* and *b*, respectively. Cytochromes *a* and *b* were not discernible in the spectrum of cells or mitochondria isolated from the mutant; cytochrome *c* was present in amounts similar to the parent strains (Table I).

As shown in Table V, this type mutant exhibited mitochondrial oligomycin-sensitive ATPase and ATP–phosphate exchange activity as does the wild-type strain. In addition, the ability of isolated mitochondria to carry out protein synthesis was also preserved. Thus, the lesion in this two-gene mutant was but a simple combination of deficiencies of the parent strains and no additional modification of the mitochondria took place.

These observations lend support to the previous suggestion that the primary deficiency in those mutants which are cytochrome *a* and *b* deficient as a consequence of the mutation of single nuclear genes is their inability to synthetize a functional protein inside mitochondria controlling the assembling process of mitochondrial components and the pleiotropic effect observed is the secondary consequence of this deficiency[10]. The present knowledge on properties of yeast promitochondria[12,36] are consistent with this explanation and with the assumption that intact cytochromes *a* and *b* have no special role in the mitochondrial membrane apart from their carrier function in the respiratory chain. Of course, the possibility remains that apoproteins of the cytochromes may be present in promitochondria, or in mitochondria of the cytochrome deficient mutants, and be required for the assembly of other constituents in the mitochondrial membrane.

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