Biochimica et Biophysica Acta, 985 (1989) 127–132
Elsevier

BBAMEM 74566

Modifications of the relative proteolipid composition in the ATP synthase of a respiratory competent mutant of *Saccharomyces cerevisiae*

Stephen Manon and Martine Guerin

Institut de Biochimie Cellulaire et Neurochimie du CNRS, Bordeaux (France)

(Received 2 February 1989)
(Revised manuscript received 20 June 1989)

Key words: Proteolipid composition; Respiratory mutant; ATPase, subunit 8; (*S. cerevisiae* mitochondria)

A comparative study of the proteolipid composition of the F_0-sector of the ATP synthase of wild-type strain of *Saccharomyces cerevisiae* and of nuclear mutants, modified at the level of the oxidative phosphorylation due to an enhanced proton permeability of the inner membrane, was carried out. Analysis of the crude proteolipid extract by electrophoresis and high liquid performance chromatography showed some differences at the level of mitochondrial DNA encoded proteolipids. Subunit 6 and in particular subunit 8 were present in reduced amounts, whereas subunit 9 was present in equal amounts in both types of strain. However, the phosphate binding affinity of subunit 8 was the same in wild-type and mutant strains. The fact that subunit 6 and subunit 8 are cotranscripted on a single mRNA led to the problem of the regulation of the mitochondrial synthesis of these two proteins by a nuclear gene.

Introduction

Previous studies have described the isolation and the phenotypic and genotypic characterization of mutants from *Saccharomyces cerevisiae* modified in the modulation of ATP synthase activity by phosphate. These mutants supported several mutations, but all the modifications, phenotypic and biochemical, linked to oxidative metabolism, cosegregated with a cryosensitive phenotype (residual growth at 18°C on strictly aerobic substrate) due to a nuclear recessive mutation [1,2].

Experiments carried out on whole cells [2] as well on isolated mitochondria [1], allowed to evidence a low efficiency of the oxidative phosphorylation in these strains, which was related to an enhanced proton permeability of the inner mitochondrial membrane.

On the other hand, from measurements of the kinetic parameters of the rate of ATP synthesis as a function of phosphate concentration, great differences appeared between wild-type and mutant strains: In the parental strain, two kinetic systems were evidenced, as already described by Rigoulet et al. [3]: These systems are related to two different states of the flux control by the phosphate transport which is directly linked to ΔpH [4]: system I corresponds to a high ΔpH and a low control by phosphate transport, whereas system II is related to a low ΔpH but a high control by phosphate carrier. In mutant mitochondria, both kinetic systems were replaced by only one, the parameters of which are similar to those of system II [1].

Despite the fact that phosphate transport appeared to be mersalyl insensitive [1], the phosphate carrier did not seem modified, since, when isolated from either mutant or wild-type mitochondria, and reconstituted in phospholipid vesicles it presented the same characteristics in both cases (Guerin, B., et al., unpublished results). Consequently, we searched for possible alterations occurring in the F_1-F_0-ATPase.

Results presented in this paper show that, in mutant mitochondria, the F_0 sector of ATPase was latered since the relative composition in proteolipids encoded by mitochondrial DNA (Su6, Su8, Su9) was modified.

Material and Methods

Strains. The wild-type haploid strain of *Saccharomyces cerivisiae* AB1-4A/8 (mat a, his 4, T+,

Abbreviations: BSA, bovine serum albumin; DCCD, dicyclohexylcarbodiimide; CCCP, *p*-carbonylcyanide *m*-chlorophenylhydrazone. Enzyme: ATP synthase, ATP phosphohydrolase: EC 3.6.1.34. F_0 and F_1: integral membrane and peripheral portions of ATP synthase.

Correspondence: M. Guerin, Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 Rue Camille Saint-Saëns, F-33077 Bordeaux Cedex, France.

C+, P+) was from the collection of P.P. Slonimski. Mutant strain AB1-4A/8/100 (mat α, his4, Ts, Cs, Ps), and segregants GM16/28-2B (mat α, his4, T+, C+, Ps), GM16/28-2D and GM 16/28-6D (mat α, ade1, T+, Cs, Ps) were described in Ref. 2.

Cell cultures, mitochondria isolation and measurements of oxidative phosphorylations were performed by classical methods as previously described [1,2].

Mitochondria were labeled according to Douglas and Butow [5] and SDS-PAGE according to the method of Laemmli modified according to Esparza et al. [6].

Extraction of proteolipids, and HPLC analysis was performed as described by Michon et al. [7]. Phosphate binding activity of the organic extract and phospholipid content were measured according to Guerin and Napias [8]. The organic extract was prepared from the strains as for HPLC analysis, 0.05 to 0.4 ml of the extract were completed up to 0.4 ml with chloroform/methanol (2:1, v/v), and then added with 0.4 ml of chloroform and 0.4 ml of a mixture of 50 mM Tris-maleate (pH 7.3)/methanol (1:1) containing 10 to 1000 nmol of [^{32}P]phosphate (10000 cpm/nmol). The mixture was vigorously stirred for 3 min; after centrifugation, 0.5 ml of the organic phase was taken out and the radioactivity was counted. It was verified that the dependence of the phosphate binding on the protein concentration was linear.

Reagents.

Mersalyl, dicyclohexylcarbodiimide, oligomycin and carbonyicyanide *m*-chlorophenylhydrazone were from Sigma. [^{32}P]Phosphate was from the CEA and [^{35}S]sulfate from Amersham.

Results

Experiments carried out on whole cells have shown a low efficiency of oxidative phosphorylations, resulting in a lower energetic yield in strains supporting the nuclear mutation inducing a cryosensitive phenotype [2]. This phenomenon was related on an enhanced proton permeability of the inner membrane in these strains, evidenced on isolated mitochondria by swelling experiments in potassium salt solutions [2]. Possible alterations in the F_1-ATPase were ruled out in measuring ATPase activities which were similar in mutant and parental strains (see legend of Fig. 3).

On the other hand, it appeared that, if at low phosphate concentration mutant mitochondria differed greatly from the wild-type in the V_{max} of ATP synthase activity, at high phosphate concentration (> 5 mM), kinetic parameters were of the same order of magnitude in both strains [1].

A consequence of this proton leakage can be seen in Fig. 1 where the respiratory rate (second state 4, Fig. 1A), the respiratory control (Fig. 1B) and the ratio ATP/O (Fig. 1C), are reported as a function of the temperature of the experiment. In wild-type mitochondria, the oxidation rate of ethanol was very temper-

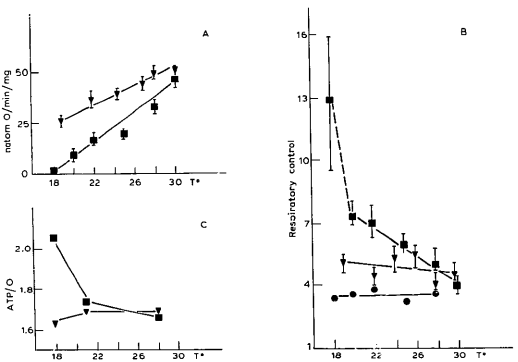

Fig. 1. Effect of the temperature on oxidative phosphorylation in mutant and wild-type mitochondria. Mitochondria were isolated in buffers containing 0.5 mM phosphate [1], from cells grown at 28° C. Respiration rates were measured with 5 mM ethanol as substrate, 5 mM phosphate and 0.17 mM ADP. Measurements were done in triplicate. (A) Respiration rate, second state 4. (B) Respiratory control was calculated on the second state 4. (C) Initial rate of [^{32}P]ATP synthesis, carried out in the presence of 5 mM [^{32}P]phosphate, and respiration rate were measured simultaneously. ■, AB1-4A.8; ▼, ▲B1-4A/8/100; ●, GM16/28-2D.

ature sensitive, corresponding to a decrease of the intrinsic permeability of the inner membrane since respiratory control evoluted in the opposite way. It should be noted that at 18° C, the actual value of the respiratory control was difficult to ascertain, due to the very low oxidation rate. On the opposite, in mutant mitochondria, the oxidation rate was less temperature sensitive and the respiratory control was relatively constant (and lower than that of the wild-type) along the range of the temperature. As a matter of fact, ATP/O ranged to the same values in mutant mitochondria whereas in wild-type mitochondria this ratio increased when the temperature decreased. An explanation of this result could be that, in wild-type mitochondria, at low temperature, the decrease of the enzyme activities (electron transfer and ATP synthesis) is compensated by the coupling between both systems; in mutant mitochondria, this compensating coupling mechanism should be absent.

Sensitivity to inhibitors of ATP synthase

Proton transport through F_0-ATPase can be inhibited either by DCCD, which binds to Asp-59 of subunit 9 [9], or by oligomycin which required an adequate arrangement of subunits 6 and 9 [10,11]. Both inhibitors were assayed on wild-type and mutant strains. It can be seen in Fig. 2 that the DCCD-sensitivity of the respiration (state 3) was identical in both strains. On the contrary, mutant mitochondria appeared more sensitive to oligomycin than wild-type mitochondria, since 50% inhibition were obtained for 0.7 and 1.25 μg oligomycin per mg protein for mutant and wild type mitochondria, respectively. However, the observed difference in oligomycin-sensitivity could be due to the different proton permeability of the membrane since effect of this inhibitor is dependent on the protonmotive force. In this way, titration of the ATPase activity was performed under conditions of zero protonmotive force, i.e. in the presence of an uncoupler. It can be seen in Fig. 3 that under these conditions mutant mitochondria always exhibited a higher oligomycin-sensitivity than wild-type mitochondria. This relative oligomycin sensitivity of the mutant strain could be related to the observation that growth of mutant cells were more sensitive to oligomycin than that of the parental strain (unpublished results).

Analysis of the relative proteolipid composition of the F_0-ATPase

Since the F_0 sector of ATP synthase is considered as a proton channel, analysis of its proteolipid composition was carried out in mutant and wild-type strains.

The yeast F_0-ATPase contains several proteolipids (see Refs. 12 and 13 for review), among them three proteins are encoded by mitochondrial DNA: subunits 6, 8 and 9 which are encoded by the genes *oli2*, *aap1* and *oli1*, respectively [14,15,16]. Subunits 9, 6 and 4 (a nuclear DNA encoded protein) [17,18] are assumed to form the proton channel [17] because of their structural analogies with subunits c, a and b, respectively of the

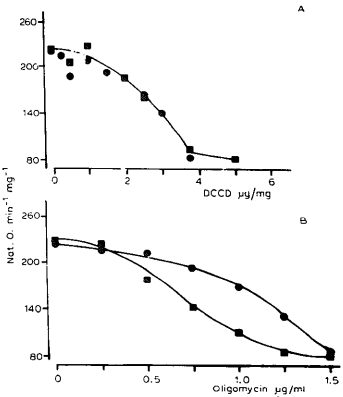

Fig. 2. Sensitivity of state 3-respiration to ATP synthase inhibitors. Mitochondria were suspended at 28° C in the respiration buffer added with 5 mM Tris-phosphate, 4 mM ethanol and 0.34 mM ADP. (A) Mitochondria were preincubated for 4 min in the presence of the indicated amounts of DCCD. (B) Mitochondria were suspended in the buffer added with the indicated amounts of oligomycin, for 3 min before ADP addition. ●, Wild-type; ■, mutant. Results are representative data of two (A) and four (B) independent experiments.

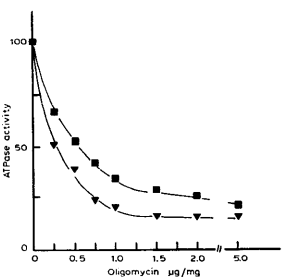

Fig. 3. Oligomycin-sensitivity of the ATPase activity in the presence of uncoupler. ATPase activity was measured at 28° C in the presence of 1 μM CCCP. Actual values: $V = 1100 \pm 100$ and 1000 ± 100 nmol P_i/mg per min for AB1-4A/8 (wild-type) and AB1-4A/8/100, respectively. ■, Wild-type; ▼, mutant.

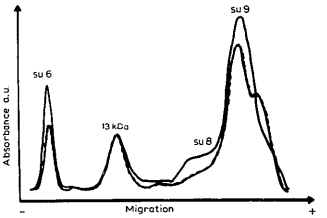

Fig. 4. SDS-PAGE profile of the crude organic extract from ^{35}S-labeled mitochondria. Cells were grown until the early-exponential phase in 100 ml of a complete glycerol medium and then washed and resuspended in 10 ml of a minimal medium (according to 5) without cycloheximide. After the addition of [^{35}S]sulfate (0.5 mCi, specific activity; 1372 Ci/mmol), cells were grown aerobically for 24 h. Then, mitochondria were isolated using the glass-beads method and proteolipids were extracted as described under Materials and Methods. The electrophoresis was performed according to the classical methods [6] and a X-ray film (Ilford) was placed on the dried gel for 15 days. The absorbance of the labeled proteins was recorded with a Vernon densitometer coupled to a Delsi integrator. Full line, wild-type; dashed line, mutant.

bacterial H^+-ATPase [19]. At this time the actual role of subunit 8 remains unclear, although it was suggested that it could be implicated in the high-affinity phosphate transport [3,20].

Preliminary experiments indicating some modifications at the level of mtDNA encoded protein synthesis (not shown), analysis of the relative composition of the purified ATPase isolated from wild-type and mutant strains was carried out. However, results were difficult to interpret due to the bad separation between proteolipids. Consequently, proteolipids were extracted from mitochondria and the relative composition of the crude extracts was analyzed.

TABLE I

Quantification of the labeling of the proteolipids present in the crude organic extract

Quantification was done from results similar to that reported in Fig. 4 for independent experiments carried out on the different strains. The ratios between the analogous proteins wild-type/mutant were calculated, assuming a ratio of 1 for the 13 kDa protein.

Protein	Ratio (wild-type/mutant)		
	Expt. 1	Expt. 2	Expt. 3
13 kDa protein	1	1	1
Subunit 9	0.86	1.08	1.16
Subunit 6	1.55	1.38	1.34
Subunit 8	1.53	2.30	2.09

Total proteins were labeled by culture of the cells for 24 h in a minimal medium added with [^{35}S]sulfate, in the absence of cycloheximide and then, proteolipids were extracted from isolated mitochondria. Fig. 4 shows an autoradiogram scanning of a SDS-PAGE of the organic extract. It contained essentially the three mitochondrial subunits 6, 8 and 9 and also a nuclear DNA-encoded subunit of 13 kDa (this protein was not labeled when the experiment was performed in the presence of cycloheximide). This protein, assumed to have an equivalent labeling in both strains, was used as internal

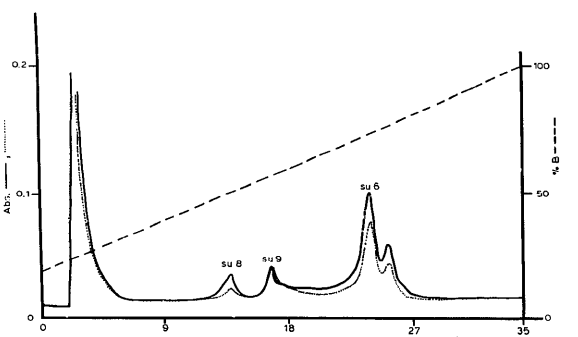

Fig. 5. Chromatographic analysis of the crude organic extract. The same amount of unlabeled mitochondria for both strains (300 mg of proteins) was treated and analyzed by HPLC as described under Materials and Methods. Full line, wild-type; dashed line, mutant.

TABLE II

Quantification of the absorbance of the proteolipids present in the retentate

Quantification was performed from the results similar to that reported in Fig. 5, for the indicated independent experiments. n.d., not detectable.

Strains	Genotype	Expt. No.	Absorbance		
			Subunit 6 / Subunit 9	Subunit 8 / Subunit 9	Subunit 8 / Subunit 6
AB1-4A/8	T^+C+P^+	3	3.4 ± 0.3	1 ± 0.2	0.29
GM16/28-2B [a]	T^+C+P^s	1			0.35
Yeast foam	T^+C+P^+	1	3.2	1.1	0.34
AB1-4A/8/100	$T^sC^sP^s$	2	2.8 ± 0.2	0.4 ± 0.1	0.14
GM16/28-2D	$T^+C^sP^s$	1	1.4	n.d.	n.d.
GM16/28-6D	$T^+C^sP^s$	2	2.4 ± 0.1	0.3 ± 0.05	0.125

[a] In this experiment, subunit 9 was difficult to measure correctly.

standard. As compared to this protein, subunits 6 and 8 but not subunit 9 appeared to be present in reduced amounts in mutant mitochondria. Results from three independent experiments are reported in Table I. Ratios between wild-type and mutant strains varied always in the same way: if the equivalent amount of subunit 9 could be depicted in both strains, subunit 6 and namely subunit 8 were in reduced amounts in mutant mitochondria.

In a second set of experiments, organic extracts from both strains were prepared from the same amount of unlabeled mitochondria and the three subunits of the F_0-ATPase were separated by HPLC. In Fig. 5 we compare the absorbance profiles at 280 nm obtained for mutant and parental strains, assuming that the amino-acid composition of the three proteolipids was the same in both strains: in mutant mitochondria less subunits 6 and 8 than in wild-type mitochondria were depicted while both strains exhibited the same amount of subunit 9. In Table II, the ratios subunit 6/subunit 9, subunit 8/subunit 9 and subunit 8/subunit 6 in different strains are reported. It can be seen that, relative to subunit 9, the amounts of subunit 6 and subunit 8 in mutant mitochondria were about 70% and 33%, respectively, of that of wild-type mitochondria. The ratio subunit 8/subunit 6 was about 0.30 in cryoresistant cells and 0.13 in cryosensitive cells.

Phosphate binding activity of the organic extracts

Since the subunit 8 is able to bind phosphate in organic phase [8], the phosphate binding activity of the crude organic extract from parental and mutant strains were compared. In Table III we reported different experiments carried out with crude extracts exhibiting different phospholipid/protein ratios. Relative to the protein concentration, and at an equivalent ratio of phospholipid/protein, the crude extracts from mutant mitochondria bound about 3-fold less phosphate than those from wild-type mitochondria.

However, measurements of the binding activity as a function of the external phosphate concentration demonstrated that extracts from both strains exhibited the same constant of half-saturation for phosphate: $K_{1/2} = 80 \pm 10$ μM and 70 ± 10 μM for mutant and parental strain, respectively (Fig. 6).

TABLE III

Maximal phosphate-binding activity by the crude organic extract

The phosphate-binding activity was monitored as described under materials and methods in the presence of 500 nmol [^{32}P]phosphate. It was verified that the phosphate-binding activity was maximal with this phosphate amount. The phospholipid content was monitored for each of the experiment.

Strains	Expt. No.	mg phospholipids/ mg proteins	phosphate binding (nmol/mg proteins)
AB1-4A/8	1	0.2	19
	2	2.2	51
AB1-4A/8/100	3	0.9	7
	4	2.7	17

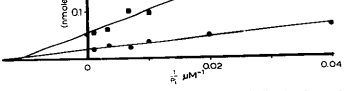

Fig. 6. Phosphate-dependence of the phosphate-binding by the crude organic extract. The crude organic extract was isolated as described under Materials and Methods and the phosphate binding activity was monitored as in Table III with various phosphate concentrations. ■, wild-type; ●, mutant.

Discussion

The mutant strain studied in this work is a respiratory competent yeast, characterized by a cryosensitive phenotype [2], which is modified at the level of oxidative phosphorylations. It presents a bad coupling, which is better evidenced at low temperature as well on the cell (growth rate and growth yield) as on isolated mitochondria (Refs. 1, 2 and this paper). The main modification concerns the phosphate dependence of the ATP synthesis. Only one kinetic system was depicted in the mutant, the parameters of which being close to the parameters of the system II depicted in wild-type mitochondria, i.e., related to a low ΔpH and a high control by phosphate carrier [1].

Experiments carried out on isolated mitochondria suggested modifications occurring at the level of the F_0 sector of the ATP synthase, the proton channel of the enzyme. Indeed, if DCCD-sensitivity was identical in both strains, the sensitive sites being saturated by the same amount of inhibitor, mutant strain exhibited an higher oligomycin-sensitivity.

Analysis of the relative stoichiometry of the mtDNA encoded proteolipids of the F_0-ATPase shows that mutant strains contain less subunit 6 and about three times less subunit 8 than the parental strain. Opposite to other mutations occurring at the level of the proteolipids [9–11,21,22] which dramatically perturbated the functioning of the ATPase-ATPsynthase, the mutation described herein only modified the ATP synthase at low external phosphate concentration and/or at low temperature. It should be noted that only the quantity, but not the enzymatic properties of the subunit 8 were altered since in both strains subunit 8 exhibited the same affinity for phosphate.

As regards subunit 6, the problem was to know whether the relative weak modification observed in the amount of this protein, as compared to that of subunit 8, is directly linked to the mutation or is a consequence of it. Indeed, genes encoding for subunits 6 and subunit 8 (*oli2* and *aap1*, respectively) are cotranscripted on a single mRNA [23], but, it is also known that the sequence of assembly of the three proteolipids in F_0-ATPase follows the order 9, 8, 6, a defect in the assembly of subunit 8 inducing a defect of assembly of subunit 6 with subunit 9 [24]. Thus, a mutation inducing a reduced amount of subunit 8 could promote a reduction of assembly of subunit 6. Thus, it is difficult to conclude about a functional perturbation induced by a reduction and/or incorporation of subunit 8 in F_0-ATPase, or a structural modification due to the diminution of the amount of both subunits 6 and 8.

Acknowledgements

The authors wish to thank Dr. J. Velours for help in the proteolipid extraction and for stimulating discussions. This work was supported by Grants from the Centre National de la Recherche Scientifique, the University of Bordeaux II and the Conseil Régional d'Aquitaine.

References

1 Manon, S. and Guerin, M. (1988) Eur. J. Biochem. 172, 205–211.
2 Manon, S., Rakotomanama, F. and Guerin, M. (1988) Eur. J. Biochem. 174, 399–404.
3 Rigoulet, M., Guerin, M. and Guerin, B. (1977) Biochim. Biophys. Acta 471, 280–295.
4 Beauvoit, B., Rigoulet, M. and Guerin, B. (1989) FEBS Lett. 244, 255–258.
5 Douglas, M.G. and Butow, R.A. (1976) Proc. Natl. Acad. Sci. USA 73, 1083–1086.
6 Esparza, M., Velours, J. and Guerin, B. (1981) FEBS Lett. 134, 63–66.
7 Michon, T., Galante, M. and Velours, J. (1988) Eur. J. Biochem. 172, 621–625.
8 Guerin, M. and Napias, C. (1978) Biochemistry 17, 2510–2516.
9 Sebald, W. and Hoppe, J. (1981) Microbiol. Rev. 51, 477–497.
10 Jean-François, M.J.B., Luckins, H.B. and Marzuki, S. (1986) Biochim. Biophys. Acta 868, 178–182.
11 Somlo, M., Clavilier, L., Dujon, B. and Kermorgant, M. (1985) Eur. J. Biochem. 150, 89–94.
12 Nagley, P. (1988) Trends Genet. 4, 46–52.
13 Senior, A.E. (1988) Physiol. Rev. 68, 177–221.
14 Macreadie, I.G., Novitski, C.E., Maxwell, R.J., John, U., Ooi, B.G., McMullen, G.L., Lukins, H.B., Linnanae, A.W. and Nagley, P. (1983) Nucleic Acids Res. 11, 4435–4451.
15 Macino, G. and Tzagoloff, A. (1979) J. Biol. Chem. 254, 4617–4623.
16 Macino, G. and Tzagoloff, A. (1980) Cell 20, 507–517.
17 Velours, J., Arselin de Chateaubodeau, G., Galante, M. and Guerin, B. (1987) Eur. J. Biochem. 164, 579–584.
18 Velours, J., Durrens, P., Aigle, M. and Guerin, B. (1988) Eur. J. Biochem. 170, 637–642.
19 Schneider, E. and Altendorf, K. (1987) Microbiol. Rev. 51, 477–497.
20 Arselin de Chateaubodeau, G., Guerin, M. and Guerin, B. (1976) Biochimie (Paris) 64, 883–890.
21 Choo, W.M., Hadikusumo, R.G. and Marzuki, S. (1985) Biochim. Biophys. Acta 806, 290–304.
22 John, U.P. and Nagley, P. (1986) FEBS Lett. 207, 79–83.
23 Hibbs, A.R., Maheshwari, K.K. and Marzuki, S. (1987) Biochim. Biophys. Acta 908, 179–187.
24 Hadikusumo, R.G., Meltzer, S., Choo, W.M., Jean-François, M.J.B., Linnane, A.W. and Marzuki, S. (1988) Biochim. Biophys. Acta 933, 212–222.