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Purification from a yeast mutant of mitochondrial F_1 with modified β-subunit. High affinity for nucleotides and high negative cooperativity of ATPase activity

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Mitochondrial F_1 containing genetically modified β-subunit was purified for the first time from a mutant of the yeast *Schizosaccharomyces pombe*. Precipitation by poly(ethylene glycol) allowed us to obtain a very stable and pure enzyme from either mutant or wild-type strain. In the presence of EDTA, purified F_1 retained high amounts of endogenous nucleotides: 4.6 mol/mol and 3.7 mol/mol for mutant and wild-type F_1, respectively. The additional nucleotide in mutant F_1 was ATP; it was lost in the presence of Mg^{2+}, which led to a total of 3.4 mol of nucleotides/mol whereas wild-type F_1 retained all its nucleotides. Mutant F_1 bound more exogenous ADP than wild-type F_1 and the same total nucleotide amount was reached with both enzymes. Kinetics of ATPase activity revealed a much higher negative cooperativity for mutant than for wild-type F_1. Bicarbonate abolished this negative cooperativity, but higher concentrations were required for mutant F_1. The mutant enzyme was more sensitive than the wild-type one to azide inhibition and ADP competitive inhibition; this indicated stronger interactions between nucleotide and F_1 in the mutant enzyme. The latter also showed increased sensitivity to N,N'-dicyclohexylcarbodiimide irreversible inhibition.

Introduction

The F_1 moiety of mitochondrial or bacterial ATPase-ATPsynthase contains five types of subunit, namely α, β, γ, δ and ε. F_1, when solubilized from the membrane, cannot synthesize ATP but exhibits a high hydrolytic activity. The essential role of β-subunits in catalysis and regulation [1] has been demonstrated by a series of chemical modifications (for a review, see Refs. 2, 3). More recently, β-defective bacterial mutants have been obtained and characterized [4,5]. In a number of mutant strains, the F_1 moiety was unstable and therefore difficult to solubilize and purify as an active enzyme. Similarly, F_1 isolated from two yeast mutants obtained by site-directed mutagenesis of *Saccharomyces cerevisiae* showed high instability and could only be partially purified [6]. In contrast, a number of stable mutants, unable to growth on a respiratory medium, were obtained in vivo from the 'petite-negative' yeast, *Schizosaccharomyces pombe* [7]. One of these mutants lacking detectable β-subunit was used to select a phenotypic revertant, R4.3, which possessed an altered ATPase activity [8].

The present paper describes the purification, in very stable form, of F_1 from the R4.3 mutant. One essential step of the purification procedure concerned poly(ethylene glycol) precipitation, which resulted in a pure and very stable enzyme containing high amounts of endogenous nucleotides. The mutant F_1 was characterized by increased interactions with nucleotides, increased negative cooperativity of ATP hydrolysis and increased sensitivity to azide and to N,N'-dicyclohexylcarbodiimide inhibitions, as compared to the wild-type F_1.

Materials and Methods

Chemicals. Nucleotides were from Boehringer-Mannheim; their purity was controlled by HPLC (see below).

Abbreviations: DCCD, N,N'-dicyclohexylcarbodiimide; F_1, solubilized mitochondrial ATPase; Mops, 4-morpholinepropanesulfonic acid; PAB, *p*-aminobenzamidine; PEG, poly(ethylene glycol).

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[2-^{3}H]ADP (22 Ci/mmol) was purchased from Amersham. Phenylmethylsulfonyl fluoride and ε-amino-*n*-caproic acid were from Sigma. DCCD and PAB came from Aldrich. Poly(ethylene glycol) (PEG) 6000 was purchased from Prolabo, PEG 4000 from Merck, and Ultrogel AcA34 from IBF.

Yeast strains and culture. The R4.3 mutant was obtained from the 972 h$^-$ wild strain of *S. pombe* as previously described [8]. The yeasts were grown in a glycerol-yeast extract medium as described by Goffeau et al. [9] using a 20 litre fermenter. The harvested yeasts were washed in cold distilled water, frozen in liquid nitrogen and stored at −80 °C.

Preparation of F_1-ATPase. Mitochondria, mitoplasts and submitochondrial particles were obtained from the R4.3 mutant exactly as described for the wild strain [10]. However, several modifications were necessary in order to obtain pure and stable F_1 from either mutant or wild-type strain. Firstly, chloroform saturated with Tris buffer at neutral pH [11] was used to solubilize the enzyme [12]; secondly, ammonium sulfate was replaced by PEG (as detailed in Results) to precipitate and concentrate the chloroform extract before Ultrogel AcA 34 filtration in 100 mM Tris-HCl/50% glycerol/5 mM EDTA/10 mM PAB (pH 7.5) [10]. As previously, ATP was only present during chloroform extraction. All other steps were performed in the absence of any added nucleotide.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [13] was performed as previously described [14]. Protein concentration was determined by the biuret method [15] for the particulate fractions or by a modified Lowry procedure [16] for the soluble enzyme. When PEG was present, proteins were estimated by the bicinchoninic acid method [17], the samples being heated for 20 min at 50 °C prior to absorbance measurements at 562 nm.

Nucleotide titration. Endogenous nucleotides of pure F_1 were titrated by HPLC after heat denaturation of the enzyme [14,18]. A molecular mass of 380 kDa was assumed for pure F_1 [10].

The binding of [^{3}H]ADP was measured after incubation of F_1 at 0.5 mg protein/ml of 0.25 M sucrose/50 mM Tris-acetate/1.5 mM $MgCl_2$ (pH 7.5) for 30 min at 30 °C, in the presence of increasing concentrations of [^{3}H]ADP (160 dpm/pmol) up to 330 μM. Unbound nucleotide was eliminated by centrifugation-elution through Sephadex G50-fine columns [19]. The radioactivity recovered in the eluates was estimated by scintillation counting in HP/b (Beckman) medium.

Assay of ATPase and ITPase activities. The activity was routinely measured spectrophotometrically at 30 °C in the presence of an ATP, or ITP, regenerating system in 50 mM Hepes-KOH (pH 8.0) [14]. The assay medium contained 1 mM $MgCl_2$ and in addition equal concentrations of $MgCl_2$ and ATP or ITP, up to 4 mM. Where indicated, effectors were added: sodium bicarbonate (60 mM for the mutant or 20 mM for the wild strain) or sodium azide (up to 3 mM). When submitochondrial particles were assayed, 1.5 mM KCN was added to the medium. The hydrolysis traces were recorded for several minutes. For ADP inhibition studies, ATPase and ITPase activities were measured by a colorimetric phosphate assay [20]. Both assay methods gave identical rates. The activity was expressed as units/mg protein (1 unit = 1 μmol ATP or ITP hydrolyzed/min).

Irreversible inhibition by DCCD. F_1 at 0.5 mg/ml of 50 mM Mops/4 mM ATP/1 mM EDTA (pH 7.0) was incubated at 30 °C with 2 mM DCCD in solution of methanol (2% final concentration). Controls were conducted in the presence of 2% methanol but in the absence of DCCD. At given intervals, 1 μl aliquots were withdrawn and the residual activity was spectrophotometrically assayed in the presence of either 3.3 mM Mg-ATP or 3.3 mM Mg-ITP.

Results

Preparation of pure and stable F_1 from the mutant strain

The purification procedure developed-which included recent modifications used for the wild strain of *S.pombe* [10] involved the following steps: (1) preparation of mitoplasts before sonication to eliminate proteases located in the intermembranous space or linked to the external membrane of mitochondria; (2) addition of a protease inhibitor cocktail containing phenylmethylsulfonyl fluoride, ε-aminocaproic acid, PAB and EDTA; (3) F_1 extraction by neutralized chloroform; (4) PEG – instead of ammonium sulfate – precipitation to concentrate F_1 before loading on the final gel filtration in the presence of 50% glycerol. Indeed, with mutant F_1, only 3% of the activity was recovered after ammonium sulfate precipitation and gel filtration: the bulk of protein was present in the void volume as inactive F_1, probably due to aggregation. Fig. 1 shows that increasing PEG 6000 concentrations quantitatively precipitated the solubilized mutant enzyme under a fully active form. The precipitation was achieved at approx. 25% (w/v) PEG 6000 and about 95% of both ATPase activity (panel A) and protein (panel B) initially present were recovered in the pellets obtained after centrifugation. A half-maximal effect was obtained at about 15% PEG 6000. Similar precipitation patterns were obtained for the wild-type enzyme although the initial specific activity was much higher. Furthermore, PEG 4000 could also be efficiently used, but slightly higher concentrations were required, with a half-maximal precipitation at 20% (data not shown).

Table I reports the yields of the main purification steps of mutant F_1, starting from 240 g wet weight cells produced by a 15 litre yeast culture. The enzyme solu-

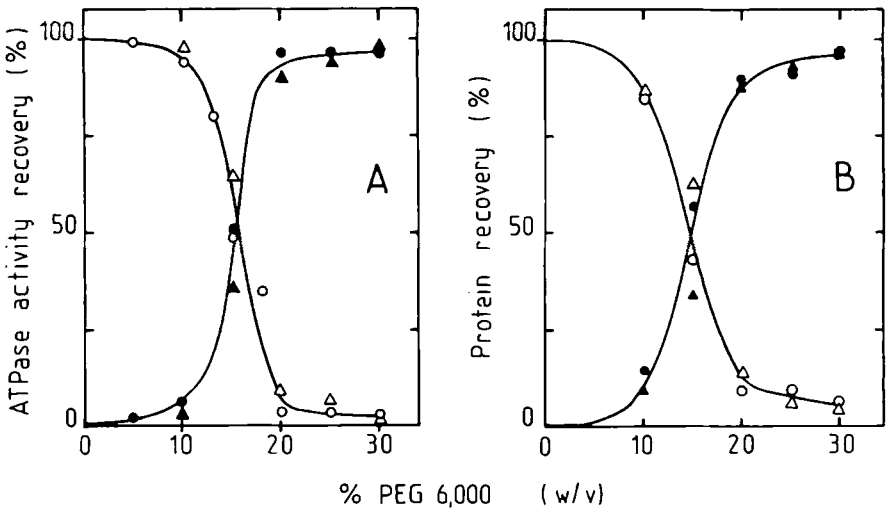

Fig. 1. PEG precipitation of mutant and wild-type soluble enzymes obtained after chloroform extraction. Aliquots of mutant or wild-type submitochondrial particles (4.8–6 mg protein/400 μl) were mixed with equal volumes of chloroform, vigorously shaken for 20 s at room temperature and centrifuged at 9000 × *g* for 5 min. The supernatants were centrifuged again to completely eliminate chloroform and then divided into 70 μl aliquots. To aliquots of mutant (△, ▲) or wild-type (○, ●) solubilized enzymes were added increasing amounts of PEG 6000 up to 30% (w/v) final concentration. After 15 min, the samples were centrifuged at 15000 × *g* for 20 min and the pellets were solubilized in 20 ml of 0.3 M sorbitol/1 mM EDTA/10 mM PAB/10 mM ε-amino-*n*-caproic acid/1 mM phenylmethylsulfonyl fluoride/2 mM ATP/20 mM Tris-HCl (pH 7.5). The ATPase activity (A) was measured both in supernatants (○, △) and in solubilized pellets (●, ▲) by the spectrophotometric assay and the proteins (B) were estimated by the bicinchoninic acid method (see Materials and Methods). Results are expressed as ATPase activity or protein recovery with respect to initial values in the absence of PEG. Initial values for mutant and wild-type enzymes were, respectively: 0.27 and 2.68 units/70 μl; 35 and 22 μg protein/70 μl.

bilization by chloroform extraction was quite efficient, since a 14-fold increase in specific ATPase activity was obtained. The precipitation by 25% PEG 6000, as mentioned above, resulted in 95% concentration of a fully active enzyme prior to loading on the Ultrogel AcA 34 column. A final amount of 11.8 mg of pure F_1 was collected in the enzyme pool with a specific ATPase

TABLE I

Purification steps of mutant F_1

Submitochondrial particle preparation, chloroform extraction and Ultrogel AcA 34 filtration were performed as described in Materials and Methods. The chloroform extract (21 mg protein/59 ml) was mixed with PEG 6000 (25% (w/v) final concentration). After 15 min incubation at room temperature and 30 min centrifugation at 100000 × *g*, the pelleted enzyme (19.7 mg protein) was solubilized in a minimal volume of 100 mM Tris-HCl/10 mM PAB/5 mM EDTA (pH 7.5) and submitted to Ultrogel AcA 34 filtration in the same buffer containing 50% (w/v) glycerol. The ATPase activity was measured spectrophotometrically (see Materials and Methods).

Step	Proteins		ATPase activity	
	mg	%	units/ fraction	units/mg protein
Submitochondrial particles	720	(100)	432	0.6
Chloroform extraction	21	2.9	179	8.5
PEG precipitation	19.7	2.7	169	8.6
Ultrogel AcA 34 filtration	11.8	1.6	110	9.3

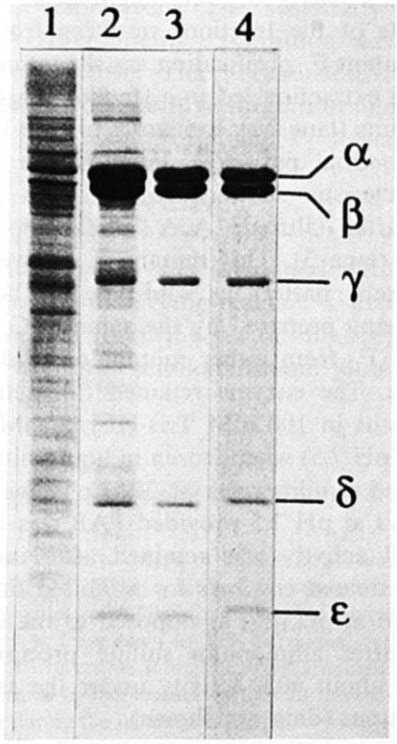

Fig. 2. Protein analysis of fractions obtained during the purification procedure of mutant F_1; comparison of mutant purified F_1 with the wild-type enzyme. The fractions obtained in Table I were analyzed by SDS-polyacrylamide gel electrophoresis: lane 1: submitochondrial particles (100 μg); lane 2: chloroform extract (20 μg); lane 3: purified mutant F_1 (10 μg); lane 4: wild-type F_1 (10 μg) purified by the same procedure.

TABLE II

Endogenous nucleotide content of purified F_1 as obtained in the presence of EDTA. Effects of Mg^{2+} addition

Purified F_1 was prepared from the wild strain or the R4.3 mutant in 100 mM Tris-HCl/50% glycerol/5 mM EDTA/10 mM PAB (pH 7.5). It was either directly assayed (EDTA columns) or equilibrated for 30 min under hydrolysis conditions in the presence of 1.5 mM excess $MgCl_2$, in 0.25 M sucrose/50 mM Tris-acetate (pH 7.5), and then submitted to centrifugation-elution in the same Mg^{2+} containing buffer (Mg^{2+} columns). The nucleotide amounts of samples were assayed by HPLC after enzyme thermal denaturation (see Materials and Methods). Results are the mean of at least three different experiments; the experimental precision was in the 3–5% range.

Strain	Endogenous nucleotides (mol/mol F_1)					
	ATP		ADP		ATP+ADP	
	EDTA	$+Mg^{2+}$	EDTA	$+Mg^{2+}$	EDTA	$+Mg^{2+}$
Wild-type	1.79	0.96	1.91	2.67	3.70	3.63
R4.3 mutant	2.99	1.15	1.63	2.23	4.62	3.38

activity of 9.3 units/mg protein as measured in 50 mM Hepes (pH 8.0). The enzyme pool was then routinely concentrated by ultrafiltration through an Amicon PM 10 membrane without any loss of specific activity and with approx. 70% protein recovery. The final enzyme solution at 4–8 mg protein/ml of 100 mM Tris-HCl/5 mM EDTA/10 mM PAB/50% glycerol (pH 7.5) was stored frozen in liquid nitrogen. A similar yield in enzyme protein recovery was obtained when wild-type F_1 was prepared by the same procedure: 21.6 mg of pure enzyme was obtained after Ultrogel AcA 34 filtration starting from 440 g of wet weight cells. The protein components of the fractions resulting from the main steps of mutant F_1 purification are shown in Fig. 2. The chloroform extraction led to a fraction largely enriched in F_1 subunits (lane 2 as compared to lane 1 concerning submitochondrial particles). Pure mutant F_1 with the five characteristic subunits, α, β, γ, δ and ε, was obtained after Ultrogel AcA 34 filtration and concentration (lane 3). This mutant F_1 showed the same electrophoretic pattern as wild-type F_1 (lane 4), both enzymes being prepared by the same PEG procedure.

Purified F_1 from either mutant or wild strain was very stable. The enzyme retained full activity during many months in 100 mM Tris-HCl/5 mM EDTA/10 mM PAB (pH 7.5) when frozen in liquid nitrogen. After thawing and equilibration at 30 °C, F_1 was stable for several days at pH 7.5 provided PAB was still present. Nearly full activity was retained after incubation of both PEG-treated enzymes for a limited time at acidic pH (20 min at pH 4.5) as opposed to the wild-type F_1 prepared after ammonium sulfate precipitation [10], which lost about 60% activity under the same incubation conditions (data not shown).

Interactions with nucleotides

Table II (last two columns) shows that both enzymes purified after PEG precipitation retained high amounts of endogenous nucleotides. Mutant F_1 (4.62 mol/mol) retained one extra nucleotide as compared to wild-type F_1 (3.70 mol/mol) in EDTA containing medium (no Mg^{2+}). This additional nucleotide was present as ATP (2.99 as compared to 1.79 mol/mol), whereas the amount of ADP was not markedly changed (1.63 mol/mol as compared to 1.91 mol/mol). The presence of 1.5 mM Mg^{2+} produced a significant loss of total nucleotides (1.24 mol/mol) from mutant F_1: as much as 1.84 mol ATP/mol was lost, whereas only 0.60 mol of extra ADP/mol was recovered. On the contrary, the ATP lost from wild-type F_1 (0.83 mol/mol) was almost completely recovered as ADP (0.76 mol/mol). Therefore, the Mg^{2+} treatment strongly decreased the nucleotide amount of mutant F_1 to reach a value slightly lower than that of wild-type F_1 (3.38 mol/mol as compared to 3.63 mol/mol). For both enzymes, it is clear that one endogenous ATP is hydrolyzed at catalytic site, maybe two in the case of mutant F_1, in the presence of Mg^{2+} but in the absence of exogenous ATP.

When tritiated ADP (0–20 μM) was added during the Mg^{2+} treatment and free nucleotides were eliminated

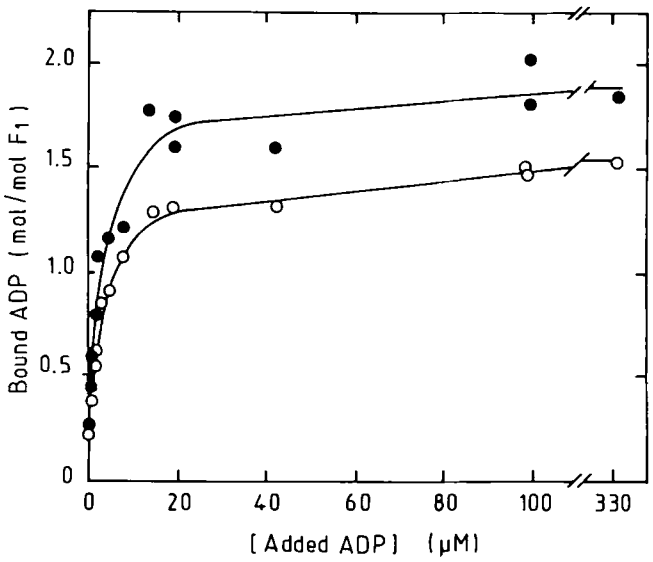

Fig. 3. Concentration-dependent ADP binding to purified F_1. The wild-type (○) or mutant (●) purified enzyme was incubated with increasing concentrations of [^{3}H]ADP up to 330 μM. The ADP binding was estimated after elimination of free nucleotides by centrifugation-elution (see Materials and Methods).

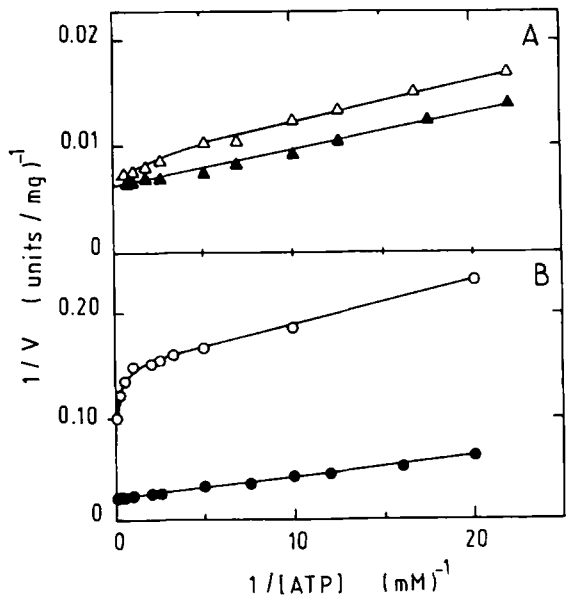

Fig. 4. Kinetics of F_1-ATPase activity and activation by bicarbonate. Purified F_1 from wild-type (A) or mutant (B) strain was assayed in the absence (empty symbols) or the presence (closed symbols) of 60 mM $NaHCO_3$, at increasing $MgCl_2$ and ATP concentrations up to 4 mM.

by centrifugation-elution, almost maximal binding was reached but a higher amount of labeled ADP was bound to mutant F_1 as compared to wild-type F_1 (Fig. 3). When the concentration of added ADP increased, no plateau was reached. The ADP binding to both enzymes slightly increased with parallel patterns to reach 1.85 mol/mol and 1.55 mol/mol for mutant and wild-type F_1, respectively, at 330 μM ADP. No significant exchange between endogenous and exogenous nucleotides was observed.

Kinetics of ATPase activity

Fig. 4 demonstrates that mutant F_1 exhibited a much stronger negative cooperativity of ATP hydrolysis in the absence of activating anion ($h = 0.3$) than wild-type F_1 ($h = 0.7$). Estimation of apparent K_m values showed that K_{m2} was markedly increased (730 μM as compared to 92 μM) whereas K_{m1} was hardly modified (33 μM as compared to 40 μM). The ATP hydrolysis rate measured with 4 mM Mg-ATP at pH 8.0 was 94% lower for mutant than for wild-type F_1 (9.3 units/mg as compared to 159 units/mg). For both enzymes, addition of bicarbonate abolished the negative cooperativity ($h =$ 1.0) and increased the activity. However, mutant F_1 required higher bicarbonate concentrations than wild-type F_1 to show a maximal activation: half-maximal effect was obtained with 13 mM or 6 mM $NaHCO_3$ for mutant or wild-type F_1, respectively (not shown here). Kinetic parameters were extrapolated from the linear double-reciprocal plots (Fig. 4). The maximal rate V_{max} (Mg-ATP) of mutant F_1 increased about 5-fold in the presence of bicarbonate, although it was still much lower than that of the wild-type enzyme (43 units/mg as compared to 184 units/mg). K_m (Mg-ATP) values were only slightly different: 78 μM (mutant) and 58 μM (wild-type).

As indicated in Table III, the mutant F_1 was 2-fold more sensitive to azide, a potent inhibitory anion: the half-maximal inhibition at 3.3 mM Mg-ATP was obtained with 18 μM or 36 μM NaN_3 for mutant or wild-type F_1 respectively. The sensitivity of ATPase activity to ADP competitive inhibition was also 2-fold higher for mutant F_1. The ITPase activity was very sensitive to ADP competitive inhibition and even more sensitive in the case of mutant F_1 as compared to wild-type F_1.

Inactivation by DCCD

When wild-type F_1 was incubated with DCCD, a β-subunit-specific modifying reagent, a strong inactivation was observed following pseudo-first-order kinetics, as revealed by linear semilogarithmic plots of residual

TABLE III

Sensitivity of purified F_1 to azide and ADP inhibition

Strain	$I_{50\%}$ (N_3^-) (μM) [a] ATPase	K_i (ADP) (μM) [b]	
		ATPase	ITPase
Wild-type	36	450	23
R4.3 mutant	18	220	7

[a] The ATPase activity of aliquots containing 0.5–2 μg of wild-type or mutant F_1 was spectrophotometrically assayed in 50 mM Hepes-KOH (pH 8.0) in the presence of 3.3 mM Mg-ATP and increasing NaN_3 concentrations up to 3 mM.

[b] Aliquots of 1–5 μg of wild-type or mutant F_1 were assayed in 190 μl of 50 mM Hepes-KOH (pH 8.0) in the presence of either 0.2–2 mM Mg-ATP or 0.5–2 mM Mg-ITP and of 0–0.52 mM added ADP. The incubation was performed for 0.3–2 min, which gave linear kinetics of hydrolysis. The release of phosphate was colorimetrically estimated (see Materials and Methods). The K_i (ADP) values were obtained from Dixon plots.

TABLE IV

Rate constants of DCCD-irreversible inhibition of F_1-ATPase and ITPase activities

Purified F_1 from either wild-type or mutant strain was incubated up to 60 min with DCCD as described in Materials and Methods. Semi-logarithmic plots of ATPase or ITPase activity as a function of time were linear at least down to 30% of residual activity. The rate constants, k, of irreversible inhibition were graphically determined. Controls performed in the absence of DCCD showed no significant loss of activity during the incubation period.

Strain	k (min^{-1})	
	ATPase	ITPase [a]
Wild-type	0.018	0.007
R4.3 mutant	0.035	0.035

[a] The maximal rate of ITPase activity, in the absence of DCCD, was 163 or 66 units/mg protein for wild-type or mutant F_1, respectively.

activity as a function of time. The rate constant of ATPase irreversible inhibition by DCCD was 0.018 min^{-1} for wild-type F_1 or 0.035 min^{-1} for the mutant enzyme under the same conditions (Table IV). The ITPase activity of mutant F_1 was also more sensitive to DCCD than the wild-type one: rate constants of 0.035 min^{-1} and 0.007 min^{-1} were obtained respectively.

Discussion

This paper constitutes, to our knowledge, the first report of purification of a mitochondrial F_1 with genetically modified β-subunits in very stable form.

A purification procedure was recently optimized to obtain large amounts of F_1 from the wild-strain of *S. pombe* [10]. Unfortunately, it could not be applied to the R4.3 mutant strain. Therefore, a modified procedure using PEG instead of ammonium sulfate as a precipitating agent was developed to prepare F_1 from this mutant strain and it appeared to be even more successful for the wild-type F_1. The usually high ammonium sulfate concentration required for quantitative precipitation of yeast F_1 [9,21,22] leads to almost complete inactivation of our mutant enzyme, probably due to subunit dissociation and/or aggregation. This probably reveals a greater sensitivity of the mutant F_1 to high ionic strength as compared to the wild-strain enzyme. The instability of F_1 from several mutants of either bacteria [23,24] or the yeast *Saccharomyces cerevisiae* [6] might be related to similar effects. PEG is clearly shown here to substitute advantageously for ammonium sulfate and to allow precipitation of a fully active enzyme from the yeast *S. pombe* with a very high protein recovery from both mutant and wild-type strains. Interestingly, our F_1 preparations obtained after PEG precipitation and gel filtration in the presence of 50% glycerol contain high amounts of endogenous nucleotides, while the gel filtration is known to efficiently deplete from their endogenous nucleotides those enzymes previously precipitated by ammonium sulfate [25,26]. The presence of such unusually high amounts of endogenous nucleotides in these PEG-processed F_1 is likely to be responsible for the great enzyme stability, even at acidic pH values. In contrast, incubation in acidic media has been reported to favor release of endogenous nucleotides and concomitant loss of activity with bovine heart F_1 preparations [27]. It is also known that nucleotides are required for reconstitution of an active complex from the α, β and γ subunits of the *Escherichia coli* F_1 [28]. Surprisingly, F_1 of the thermophilic bacterium PS3 has particular behavior, since this enzyme can be obtained free of nucleotides without any loss of activity. In addition, reconstitution of an active complex from α, β and γ of PS3 does not require the presence of nucleotides or magnesium [29], even when modified α or β subunits are used, leading in some cases to an inactive but stable complex [30].

The β-subunit mutation of the R4.3 strain is clearly shown here to increase enzyme-nucleotide interactions:

(i) the extra endogenous nucleotide, present as ATP, is retained after PEG purification of mutant F_1 in the absence of Mg^{2+} (EDTA-containing medium). After Mg^{2+} addition, which promotes hydrolysis of at least one endogenous ATP, the extra ATP appears to be lost. However, we cannot decide whether this extra ATP is released or also hydrolyzed at the catalytic site, yielding ADP with is further released.

(ii) The slight decrease in endogenous nucleotides after the Mg^{2+} treatment of mutant F_1 is readily reversed by ADP binding upon incubation with 20 μM exogenous ADP. Under these conditions, a total amount of 5 mol nucleotides (endogenous + newly bound) is bound per mol of wild-type or mutant F_1 after centrifugation-elution. For both strains, the nucleotide affinity at the sixth site appears too low to be measured accurately by this method, since only partial saturation is observed at the highest ADP concentration tested.

(iii) The affinity for ADP which produces competitive inhibition [31] of ATP or ITP hydrolysis is at least 2-fold increased in mutant F_1 as compared to the wild-type enzyme. Concerning the ATPase activity of wild-type F_1 which shows bicarbonate-sensitive negative cooperativity ($h = 0.7$), the rate-limiting step of the reaction may be assumed to be the dissociation rate of product ADP, as proposed for beef heart F_1 [32]. Our results show a strong increase in negative cooperativity of mutant F_1 ATPase activity ($h = 0.3$). Two-fold higher bicarbonate concentrations both abolish the negative cooperativity and produce a much higher activation than for the wild-type enzyme. In contrast to bicarbonate, the inhibitory anion azide is assumed to increase enzyme-ADP interactions in beef heart F_1 [33]. Our yeast mutant F_1 shows a 2-fold increase in affinity for azide which is consistent with the 2-fold increase in K_i(ADP). It should be recalled that a previous study [8] revealed that ATP hydrolysis by submitochondrial particles was also much more sensitive to azide in the R4.3 mutant than in the wild strain.

As a general conclusion, it appears that the β-mutation in the R4.3 strain increases enzyme–ADP interactions in such a way that the rate-limiting ADP dissociation in the ATPase reaction becomes even lower. It remains to be determined whether the mutation occurs inside the catalytic site itself or inside another site which regulates the hydrolytic activity. It is of interest to mention that this is, to our knowledge, the first report of a mutant with increased enzyme–nucleotide interactions.

Even in the presence of saturating bicarbonate concentrations, the maximal rate of ATP hydrolysis is markedly lower in mutant F_1 than in the wild-type

enzyme. This indicates that the mutation also alters the β–γ pyrophosphate bond splitting which is assumed by Harris et al. [32] to be the rate-limiting step of the ATPase reaction in the presence of activating anion. A similar conclusion was reached with the ATPase-ATP-synthase complex in mutant submitochondrial particles [8]. In that case, the alteration appeared less pronounced, probably due to membrane protection of the general enzyme conformation.

Another interesting effect produced by the mutation is the increase in the rate of inactivation by DCCD, which reflects a higher reactivity and/or accessibility of the glutamate carboxyl group towards the reagent. Assuming that nucleotide binding at catalytic site increases DCCD reactivity as proposed for thermophilic bacterium F_1 [34], our results would indicate that the additional ATP retained in the yeast mutant F_1 might be located at a catalytic site. The effects observed with DCCD and azide on our yeast mutant are opposite to those reported for a β-modified *Escherichia coli* mutant showing decreased sensitivity to both inhibitors [35]. It is not known, however, how the two types of inhibition are related, or whether the change in sensitivity to inhibitors is due to mutation inside the inhibitor binding site or to distant mutation producing an enzyme conformational change.

Since the nature of the mutation of the R4.3 strain has not yet been identified, we can only speculate on the structural modification of the β subunit. The R4.3 strain analyzed in this paper is derived from an original mutant (B59.1) devoid of immunodetectable β subunit and completely defective in ATPase activity [7]. The mutation was located in the β-subunit gene since the mutant could be complemented by the wild-type gene for the β subunit [36]. The reversion frequency (10^{-8}) of the initial mutation supports the hypothesis of a single-point mutation. Since some revertants like R4.3 acquired a modified activity and thus a modified structure, a mutation in the transcription promoter can be ruled out. A shift in the intron border locations is not possible, either, since, contrary to the mammalian [37] or plant [38] mitochondrial β-subunit gene, the *S. pombe* β gene does not contain any intron (P.F. and M.B., in preparation). The observation that the β subunit of R4.3 strain has a normal electrophoretic mobility [8] excludes any major structural modification. A plausible hypothesis would be to consider that a possible original nonsense-mutation is converted back to the wild-type or to a missense mutation in some revertants. Alternatively, a frameshift mutation in the original mutant could be corrected by a second frameshift at the same position or located nearby. Sequencing of both the original mutant and revertant will clarify this alternative.

The higher sensitivity of ITPase activity to ADP and its lower sensitivity to DCCD – as compared to ATPase activity – could indicate different mechanisms for both hydrolyses. This might also reflect the existence in yeast F_1 of an ADP-specific regulatory site similar to that responsible for 'hysteretic inhibition' of ATP hydrolysis by mitochondrial F_1 from pig heart [39–41] and more recently from beef heart [42].

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