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SUBUNIT COMPOSITION OF MITOCHONDRIAL F_1-ATPase ISOLATED FROM *SACCHAROMYCES CARLSBERGENSIS*

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Summary

1. The subunit stoicheiometry of mitochondrial F_1-ATPase from yeast (*Saccharomyces carlsbergensis*), grown in the presence of [^{3}H]leucine and uniformly labelled [^{14}C]glucose, has been determined.

2. The stoicheiometry on the basis of radioactivity is: $\alpha : \beta : \gamma : \delta = 3 : 3 : 1 : 1$. The amount of the smallest subunit, ϵ, could not be measured by this method.

3. The molecular weights of the subunits, determined by urea-SDS gel electrophoresis, are 53 000, 50 000, 33 000, 12 500 and 6500, respectively. The calculated molecular weight of the ATPase is 360 000, assuming the presence of one ϵ subunit per F_1.

4. The amino acid composition of the total ATPase and of the individual subunits has been determined.

5. The aurovertin-binding properties of F_1 are discussed in relation to the subunit stoicheiometry.

Introduction

Uncertainty has long existed with respect to the exact molecular weight and subunit stoicheiometry of mitochondrial F_1. Although staining of gels indicated a 3 : 3 : 1 : 1 : 1 stoicheiometry [1,2], many other data are in favour of a dimeric structure [3—6]. For bacterial F_1, especially TF_1, an $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ structure is strongly favoured [7], whereas a dimeric structure is favoured for chloroplast F_1 [8]. Recently Yoshida et al. [2] reported that F_1 from various sources all have about the same molecular weight (385 000), favouring a 3 : 3 : 1 : 1 : 1 subunit structure for all molecules.

A reliable method for the determination of the subunit stoicheiometry is to isolate F_1 from an organism grown on labelled substrates. This method has been used for *Escherichia coli* F_1 and TF_1, and in both cases a 3 : 3 : 1 : 1 : 1 stoicheiometry was the result [9—11]. However, the data on aurovertin binding, indicating a dimeric structure, were carried out on beef-heart F_1 [5,12]. To solve the question as to whether the different results are due to the method or to the organism involved, we decided to use both methods on one organism, i.e. yeast. In a previous paper [13] the aurovertin-binding data have been reported, resulting in the same conclusion as that drawn for beef-heart F_1, namely that yeast F_1 contains two sites for aurovertin, even after dissociation with LiCl, whereas the isolated β-subunit binds aurovertin in a 1 : 1 stoicheiometry. In this paper the results of the second method will be reported and the consequences of these results for the interpretation of the aurovertin-binding data will be discussed.

Methods

ATPase

For large-scale isolation of ATPase, *S. carlsbergensis* (strain NCYC 74) was grown in a semi-synthetic lactate medium (12 l) and harvested late in the exponential phase, as described by Katan et al. [14]. At harvesting, the absorbance at 545 nm equalled 16. After digestion of the cell wall with snail-gut enzyme [15], 1490 mg (protein) mitochondria were isolated from 116 g cells. ATPase (11.9 mg protein) was isolated by the chloroform extraction method [13].

For small-scale isolation of radioactively labelled ATPase, the yeast was grown for 18 h at 28°C, with continuous shaking, in 0.5 l synthetic medium [16] containing D-[U-^{14}C]glucose (230 Ci/mol stock) and L-[4,5-^{3}H]leucine (150 Ci/mol stock). After washing and helicase digestion, the cells were broken by shaking with the same volume of glass beads (0.52—0.69 mm diameter) in a Vortex mixer (maximal output). The subsequent procedure was the same as described previously [13], except that gel chromatography on ACA 34 was omitted. The ATPase was precipitated by centrifugation (30 min at 100 000 × *g*) after addition of 0.05 vol. HCl, and the pellet dissolved in 40 μl of sample buffer for gel electrophoresis. From a 0.5-l culture with an absorbance of 4, 1.5—2 g cells (wet weight) were isolated, yielding an ATPase preparation with an activity of 20—30 μmol P_i/min (corresponding to 150—200 μg protein).

The measurement of activity and protein concentration [17] and the gel electrophoresis were carried out as described previously [13].

Amino acid analysis

ATPase was precipitated, dried and, after evaporation to remove O_2, hydrolysed in 6 M HCl for 24 h at 110°C. After lyophilizing the hydrolysate, the sample was dissolved in 0.2 M citrate brought to pH 2.2.

Samples containing 2—15 nmol of each amino acid were analysed on a Beckman Multichrome M 4327 amino acid analyser as described in Refs. 18 and 19.

Subunit isolation

The five subunits of the F_1-ATPase were separated by urea-SDS polyacryl-

amide gel electrophoresis. After staining with Coomassie blue, the coloured bands were cut out, dried, weighed, and dissolved in 6 M HCl. After evaporation the gel slices were heated for 24 h at 110°C. After removal of the gel material by centrifugation, the supernatant containing the extracted and hydrolysed material was lyophilized and dissolved in 0.2 M citrate brought to pH 2.2 for amino acid analysis.

Determination of radioactivity

For determination of the amount of ^{3}H and ^{14}C in the separated subunits, a quantity of ATPase corresponding to an activity of 15—20 μmol P_i/min (about 100 μg) was applied to a urea-SDS gel. The gel was sliced without staining. Gel slices of 1.0 mm were dissolved in protosol/H_2O (9 : 1, v/v) for 48 h at 37°C. After addition of 10 ml scintillation liquid (Packard Scintillator 299 TM) the number of disintegrations per minute was determined during 10 min by a Packard Tricarb Liquid Scintillation Spectrometer 3255, using the $^{3}H/^{14}C$ setting. The assumption was made that no counts of ^{3}H appear in the ^{14}C channel. Two independent quench curves were made for ^{3}H and ^{14}C, and the distribution of the ^{14}C counts over the ^{14}C and ^{3}H channels was determined. By making use of the external standard ratio, the numbers of disintegrations per minute of ^{3}H and ^{14}C were calculated.

Results

Amino acid composition of ATPase of Saccharomyces cerevisiae and S. carlsbergensis and its subunits

Table I shows the amino acid composition of ATPase from *S cerevisiae* and *S. carlsbergensis*, and Table II the amino acid composition of the subunits of the ATPase of *S. carlsbergensis* (extracted from the gels). Interference by material extracted from the gel itself was allowed for by extracting blank gel slices. The large amount of ammonia freed from acrylic acid made it impossible to determine histidine. Tyrosine was not detectable in any of the subunits, although it is present in the intact ATPase. A band, seen between the β and γ subunits on the gel, with a R_F corresponding to a polypeptide of 42 000 daltons contained only a low amount of protein, as judged from the freed amino acids. Since its amount diminishes upon further purification, it is presumably a contaminant.

Since the recovery of protein after extraction from the gel is only 31—53%, assuming a subunit structure of $\alpha_3\beta_3\gamma\delta\epsilon$, these data cannot be used for the determination of the subunit structure.

Since leucine is present in each subunit to an extent of about 10%, it was chosen as a label to determine the subunit stoicheiometry.

Subunit stoicheiometry based on radioactive labelling of the subunits

In preliminary experiments, designed to find conditions in which the effect of an internal leucine pool or pools would be minimalised, *S. carlsbergensis* was grown in the presence of 0, 0.01, 0.1, 1 and 10 mM leucine, respectively, containing 1.1 μCi ^{3}H ($2.5 \cdot 10^{6}$ dpm). After removal of the cells by centrifugation, the amount of radioactivity in the supernatant was determined at various times,

TABLE I

AMINO ACID COMPOSITION OF F_1-ATPase FROM *S. CEREVISIAE* AND *S. CARLSBERGENSIS*

250 μg ATPase of *S. cerevisiae*, with a specific activity of 130 μmol · mg^{-1} · min^{-1}, in 174 μl buffer containing 20 mM Tris-H_2SO_4 buffer, 1 mM EDTA, 1 mM ATP, 10% methanol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 33% glycerol, pH 7.5, and 250 or 1000 μg ATPase of *S. carlsbergensis*, with a specific activity of 148 μmol · mg^{-1} · min^{-1}, in 82 and 330 μl, respectively, of a buffer containing 20 mM Tris-H_2SO_4 buffer, 1 mM EDTA, 1 mM ATP, 10% methanol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM 6-aminocaproic acid and 5 mM *p*-aminobenzamidine, pH 7.5, were precipitated by centrifugation after addition of 0.05 vol. HCl, as described in Methods.

Amino acid	mol%	
	S. cerevisiae	*S. carlsbergensis*
Asp	9.8	8.4
Thr	6.3	5.7
Ser	8.6	6.8
Glu	7.5	12.5
Pro	4.1	4.2
Gly	10.1	9.4
Ala	11.0	10.8
Val	5.9	6.6
Met	1.5	1.4
Ile	4.1	5.3
Leu	10.6	10.5
Tyr	3.0	2.7
Phe	4.1	3.6
Lys	6.6	6.1
His	1.5	1.3
Arg	5.1	4.9

TABLE II

AMINO ACID COMPOSITION OF THE SUBUNITS OF F_1-ATPase FROM *S. CARLSBERGENSIS*

From the same preparation from *S. carlsbergensis* as used for the experiment described in Table I, 280 166 and 82.8 μg protein of ATPase, respectively, were separated and analysed as described in Methods.

Amino acid	Mol %				
	α	β	γ	δ	ε
M_r	53 000	50 000	33 000	12 500	6500
Asp	7.9	8.1	12.6	6.1	5.7
Thr	4.8	6.8	6.6	3.8	12.3
Ser	7.1	4.7	6.9	7.8	9.6
Glu	12.6	12.5	8.9	14.0	11.1
Pro	6.3	5.4	5.1	5.5	1.7
Gly	10.6	10.3	3.5	5.4	6.3
Ala	10.4	10.8	11.5	12.4	13.9
Val	8.2	8.5	3.6	9.3	15.5
Met	0.48	0.92	1.6	0	0
Ile	5.0	6.3	8.7	3.2	4.6
Leu	11.5	9.7	8.5	10.2	8.8
Phe	3.0	4.0	2.9	5.3	2.2
Lys	6.0	5.9	11.3	6.1	5.3
Arg	5.7	5.7	8.8	11.0	3.1
Recovery of protein (%)	53	48	41	52	31

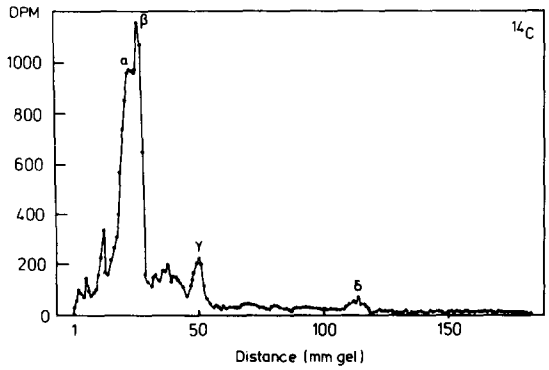

Fig. 1. Distribution of radioactivity in an urea-sodium dodecyl sulphate polyacrylamide rod gel of F_1-ATPase. *S. carlsbergensis* was grown in 500 ml synthetic medium, containing 0.3 mCi [^{14}C]glucose (0.3% w/v) and 1 mCi [^{3}H]leucine (0.8 mM), as described in Methods. The cells were harvested at A = 3.09 and the ATPase was isolated as described (small-scale isolation). An amount of ATPase corresponding to an activity of 18 μmol · min^{-1} was applied on an urea-sodium dodecyl sulphate polyacrylamide (8%) gel and electrophoresed for 16 h. The gel was cut into 1.0-mm slices and counted for ^{3}H and ^{14}C. Only the ^{14}C is shown. The sample put on th gel contained 1.6 · 10^{4} dpm ^{14}C and 1.42 · 10^{5} dpm ^{3}H. For both labels the recovery was about 95%.

up to the beginning of the stationary phase. With up to 0.1 mM leucine the radioactivity was completely incorporated within 3 h. With 10 mM leucine less than 10% was incorporated. With 1 mM leucine uniform incorporation of the labelled leucine during the whole growth period of 12—15 h was obtained. A concentration of 0.8 mM was chosen for further experiments.

The yeast was grown in 0.5 l medium, containing 0.3 mCi [^{14}C]glucose (0.3%, w/v) and 1 mCi [^{3}H]leucine (0.8 mM). Of the radioactivity present in the isolated yeast cells 0.041% of the ^{3}H and 0.009% of the ^{14}C was recovered in the isolated ATPase. The isolated ATPase (about 200 μg protein, containing 3.2 · 10^{4} dpm ^{14}C and 2.9 · 10^{5} dpm ^{3}H) was separated into its subunits by gel electrophoresis, and the amount of radioactivity in 1-mm slices determined. Since it is difficult to purify this low amount of protein by molecular sieving

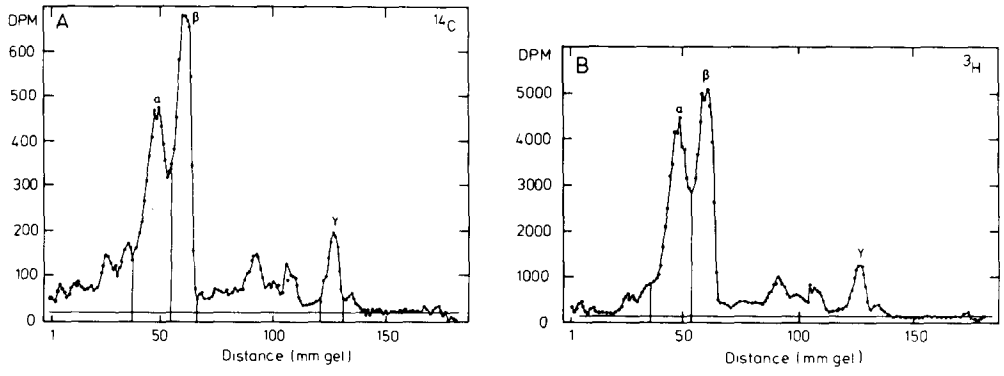

Fig. 2. Distribution of radioactivity in an urea-sodium dodecyl sulphate polyacrylamide rod gel of F_1-ATPase. The experiment was identical with that shown in Fig. 1, apart from the electrophoresis time. This was increased to 24 h to obtain a separation between the α and β-subunits. As a consequence the δ-subunit migrated off the gel. A : ^{14}C. B : ^{3}H.

TABLE III

DISTRIBUTION OF RADIOACTIVITY OVER THE SUBUNITS OF ATPase FROM *S. CARLSBERGENSIS*

The data are obtained from the experiment, partly shown in Fig. 1 (gel 1) and the experiment shown in Fig. 2 (gel 2).

Subunit	dpm		M_r	Leucine (mol%)	Stoicheiometry	
	Gel 1	Gel 2			Gel 1	Gel 2
α			53 000	11.5		
^{14}C	—	5 469			—	3.03
^{3}H	—	47 626			—	2.86
β			50 000	9.7		
^{14}C	—	5 062			—	2.97
^{3}H	—	37 686			—	2.84
$\alpha + \beta$			103 000	10.6		
^{14}C	10 493	—			2.8	—
^{3}H	89 263	—			2.87	—
γ			33 000	8.5		
^{14}C	1 200	1 124			1	1
^{3}H	7 996	7 703			1	1
δ			12 500	10.2		
^{14}C	329	—			0.72	—
^{3}H	5 306	—			0.97	—

(ACA 34), the preparation was not completely pure. The subunits were identified by comparing their migration rates with those of a pure preparation. No accurately measurable radioactivity could be detected in the smallest subunit (ϵ). The recovery of radioactivity was about 95% for both ^{14}C and ^{3}H. The proposed subunit stoicheiometry of ATPase of *S. carlsbergensis* is $\alpha : \beta : \gamma : \delta = 3 : 3 : 1 : 1$, based on the results given in Table III. These results are derived from the experiments shown in Figs. 1 and 2. The calculated molecular weight is 360 000. In a second experiment these results were confirmed. Staining intensity, measured at 496 nm, results in a subunit stoicheiometry of $(\alpha + \beta) : \gamma : \delta : \epsilon = 4.6 : 1 : 0.65 : 1$.

Binding of aurovertin to ATPase of S. carlsbergensis

Since in previous experiments [13] only two aurovertin-binding β-subunits were detected in LiCl-dissociated F_1 isolated from *S. cerevisiae*, the aurovertin-binding experiments were repeated with *S. carlsbergensis* F_1. Using the same procedure as described previously, intact F_1 (specific activity 149 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was found to bind 1.6—1.7 mol aurovertin/mol F_1 and, in the presence of LiCl, 1.9—2.0 mol aurovertin/mol F_1. Thus, in this respect, F_1 from the two strains of yeast is similar.

Discussion

The amino acid composition of the ATPase isolated from *S. carlsbergensis* (Table I) is similar to that of the enzyme from *S. cerevisiae* (our results and Ref.

20). The amino acid composition of the subunits of the ATPase from *S. carlsbergensis* is in good agreement with the findings of Takeshige et al. [20] for the ATPase from *S. cerevisiae* as far as the larger subunits are concerned. In the composition of the small subunits larger differences can be seen. Although the assumption is made that the amino acids are extracted uniformly from the gel during hydrolysis, the low recovery (31—53%) does not exclude the possibility that some amino acids are specifically lost because of binding to the gel material. This probably holds for tyrosine which is present in F_1, but not recovered in the subunits. Histidine, cysteine and tryptopham were not determined. The amounts of most of the other amino acids (but not those of methionine and arginine) found in the isolated subunits agree with the amount of these amino acids in total F_1, assuming an $\alpha_3\beta_3\gamma\delta\epsilon$ structure. However, even if a dimeric structure is assumed, the deviations are too small to be conclusive for the subunit stoicheiometry. For leucine the agreement between the amounts found in total F_1 and in the individual subunits is excellent on the basis of an $\alpha_3\beta_3\gamma\delta\epsilon$ structure, and deviates 5% on the basis of a dimeric structure, so that it is justified to use the values found for the leucine content for the calculation of the stoicheiometry.

In the calculation of the subunit structure from the [^{3}H]leucine incorporation, it is not only assumed that the amount of leucine in each subunit is correctly determined, but also that the effect of the possible presence of different pools of leucine in the cells is minimized by the uniform uptake of leucine during the complete growth time so that the specific activity of these different pools is the same and that the metabolism of leucine to other products, including amino acids, is negligible. The latter assumption is supported by the experience of Sebald et al. [21] with *Neurospora crassa*. The use of uniformly labelled [^{14}C]glucose as the only source of carbon apart from leucine does not require these assumptions.

Both labels give, within experimental error, a stoicheiometry of $\alpha : \beta : \gamma : \delta = 3 : 3 : 1 : 1$. Based on Coomassie blue staining, it is probable that there is one ϵ-subunit, but the labelling experiment was insufficiently sensitive to confirm this. To calculate the stoicheiometry, we assumed for the molecular weights of the individual subunits the values obtained by urea-SDS polyacrylamide gel electrophoresis. Those values are identical with those reported for *S. cerevisiae* [13].

Due to the small amount of protein available, it was not possible to purify the F_1 over an ACA 34-column [13]. Dilution of the labelled F_1 with unlabelled F_1 would have resulted in a very low specific activity. It is unlikely, however, that the impurities present in the labelled F_1 influence our results, since we have found identical staining intensities of the subunit bands in F_1 before and after purification on an ACA column. Apparently the impurities do not contaminate to any extent the bands derived from the F_1 subunits.

It is clear that the results of the labelling experiments ask for an explanation of the aurovertin-binding data. The finding that intact F_1 contains only two aurovertin-binding sites, while the isolated β-subunit binds aurovertin stoicheiometrically can be explained by the presence of one different β-subunit. The difference between this β-subunit and the other two could be structural or could be induced by a ligand such as an adenine nucleotide [22], which cannot

be removed by LiCl-treatment. Possible explanations for the paradox that the isolated β-subunit binds aurovertin stoicheiometrically are (1) the third β-subunit sticks to the column during the isolation procedure or (2) the conformation of this subunit changes during the isolation procedure to that of the others, e.g. by dissociation of a bound adenine nucleotide or one of the other subunits of F_1.

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