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THE YEAST MITOCHONDRIAL ATPase COMPLEX

SUBUNIT COMPOSITION AND EVIDENCE FOR A LATENT PROTEASE CONTAMINANT

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Summary

1. The subunit compositions of the F_1 (oligomycin-insensitive) and F_1—F_0 (oligomycin-sensitive) mitochondrial ATPase complexes from *Saccharomyces cerevisiae* have been examined by the highly resolving technique of sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis using a discontinuous buffer system. When isolated in the presence of protease inhibitors, F_1 and F_1—F_0 contained five and twelve bands, respectively; this contrasts with the four- and ten-band patterns seen previously using the less resolving disc gel method. When isolated in the absence of protease inhibitors both F_1 and F_1—F_0 contain spurious polypeptides produced by proteolytic modification.

2. Endogenous protein turnover in *S. cerevisiae* was impaired in the presence of protease inhibitors. F_1—F_0 isolated from cells grown in the presence and absence of inhibitors contained an identical polypeptide composition, suggesting that the subunits are not significantly modified by endogenous proteases prior to cell harvesting.

3. Yeast F_1—F_0 prepared in the presence of protease inhibitors contains a latent, sodium dodecyl sulphate-activated protease contaminant. Sodium dodecyl sulphate-induced proteolysis is largely confined to the 52 000 dalton α subunit which degrades into polypeptides of 40 000 and 10 700 daltons. The 40 000 dalton band is apparently equivalent to the polypeptide previously designated subunit 3.

4. Both F_1 and F_1—F_0 were isolated from *Torulopsis glabrata*, a yeast with considerably shorter mitochondrial DNA than that in *S. cerevisiae*. F_1—F_0 catalysed high rates of ATP—$^{32}P_i$ exchange when reconstituted into phospholipid

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Abbreviations: SDS, sodium dodecyl sulphate; F_1—F_0, mitochondrial oligomycin-sensitive ATPase complex; F_1, oligomycin-insensitive ATPase complex, also termed coupling factor 1; F_0, hydrophobic sector required to confer oligomycin sensitivity on F_1; TEMED, *N,N,N',N'*-tetramethylethylene-diamine.

vesicles, thus demonstrating the presence of a complete coupling mechanism. F_1—F_0 contained approximately twelve subunits and F_1 five, like the *S. cerevisiae* complexes. It therefore appears that the shorter mitochondrial DNA length does not produce a significantly simpler ATPase subunit structure.

Introduction

The properties of the yeast mitochondrial F_1—F_0 ATPase complex, particularly its subunit composition, are of considerable current interest. The biogenesis of F_1—F_0 is under intense study, firstly because it is a major constituent of the inner mitochondrial membrane, but more especially because several of the hydrophobic F_0 polypeptides are thought to be encoded as well as synthesized within the mitochondrion, whereas those of the F_1 sector are not [1]. Thus the complex represents an intriguing problem in the assembly of membrane proteins. From a different standpoint, F_1—F_0 is important because of its central role in the mechanism of oxidative phosphorylation. Reconstitution using phospholipids yields vesicles capable of ATP—$^{32}P_i$ exchange [2], ATP-driven proton uptake [3], uncoupler-stimulated ATPase activity [2], and net [^{32}P]ATP formation [3], thus demonstrating that the complex itself contains the complete coupling mechanism required for ATP formation.

Yeast F_1—F_0 was originally considered to contain eight subunits as seen on SDS-polyacrylamide gels, with a further proteolipid polypeptide (subunit 9) which failed to stain. The F_1 sector was suggested to contain five of these [4]. Recent work in this laboratory, however, (ref. 5 and the present study) has shown that this interpretation is oversimplified. At least two bands in the F_1—F_0 complex and one in the F_1 sector are apparently produced by proteolytic modification during isolation. Also, the 10% polyacrylamide disc gels used originally [4,5] do not resolve sufficiently, particularly in the low molecular weight region, thus causing underestimation of the true number of polypeptides. More bands are seen using a more highly resolving slab gel technique. Further, work reported here shows that the F_1—F_0 complex contains a latent, SDS-activated protease contaminant which can be activated and produce spurious polypeptides under conditions used to dissociate the protein prior to electrophoresis. A modified and more detailed picture of the structure and some properties of the yeast complex is presented in this paper.

Materials and Methods

Growth of yeast cells and preparation of the ATPase. Yeast cells were grown in 1-l batches on a rotary shaker in a medium [6] normally containing 1.25% (w/v) galactose as carbon source. Unless otherwise stated the F_1 and F_1—F_0 ATPase complexes were prepared as described, using protease inhibitors throughout [5].

Effect of protease inhibitors on in vivo protein degradation. Saccharomyces cerevisiae was grown to early stationary phase in a medium containing 2% galactose, then quickly cooled, harvested by centrifugation, and washed twice at 4°C in a solution containing only the salts of the growth medium [6].

Cells (100 g wet weight) were then suspended in 1-l of a medium containing the salts and 2% galactose. The suspension was incubated for 15 min at 30°C under an oxygen atmosphere, then 200 μC_i of L-[^{3}H]leucine (Amersham) was added and incubation was continued for 60 min. L-Leucine was then added to 20 mM and after 15 min the cells were cooled, harvested and washed twice in a medium containing only the salts. Cells (50 g wet weight) were then resuspended in a medium containing the salts, 2% galactose, 10 mM L-leucine and 100 μg/ml chloramphenicol (to prevent bacterial growth) and divided into two 900-ml batches. Protease inhibitors in either aqueous or ethanolic stock solution were added to one batch (5 mM ϵ-amino-*n*-caprioc acid, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM diphenylcarbamyl chloride and 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone) with a second addition after 3 h. The final concentration of ethanol did not exceed 0.4%. The solutions were shaken at 30°C in an air atmosphere and at preselected times 300-ml aliquots were removed and mitochondrial membranes were prepared as described [6].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis was performed in slab gels using a method modified from Laemmli [7] and Laemmli and Favre [8]. All acrylamide-methylene-bisacrylamide stock solutions were made up fresh. The separating gel was 15 X 15 X 0.15 cm in dimensions and contained 15% acrylamide (Bio-Rad electrophoresis grade), 0.2% methylene-bisacrylamide (Eastman), 0.1% SDS (Sigma, recrystallized from ethanol) and 0.75 M Tris (Sigma Trizma base), pH 8.9. For polymerization 25 μl of *N,N,N',N'*-tetramethylethylene-diamine (TEMED) and 0.5 ml of 5% $(NH_4)_2S_2O_8$ were added per 50 ml. The mixture was overlaid with 0.1% SDS and allowed to polymerize for 20—30 min at 23°C. The overlay was then removed and a solution of the stacking gel components, containing 6% acrylamide, 0.08% methylene-bisacrylamide, 0.1% SDS and 0.062 M Tris, pH 6.7, was introduced. Polymerization was initiated with 20 μl of TEMED and 0.2 ml of 5% $(NH_4)_2S_2O_8$ per 20 ml. Before gel formation began, a perspex comb was introduced which formed ten sample wells 2 cm deep X 0.6 cm wide. The bottom of the wells were 2.5 cm from the separating gel. The running buffer contained 0.025 M Tris, 0.1% SDS and 0.2 M glycine, pH 8.6. All gels were pre-electrophoresed for 1—2 h at 25 mA/gel before addition of the sample.

Samples for electrophoresis were suspended at 1—2 mg protein/ml in the running buffer containing 1% SDS, 1% dithiothreitol, 10 mM *p*-aminobenzamidine, 10 mM ϵ-amino-*n*-caproic acid, and 2 mM phenylmethylsulfonyl-fluoride, then heated at 100°C for 3 min. One-tenth volume of 50% sucrose was then added to allow introduction of the sample under the running buffer. No more than 50 μl of sample should be applied, otherwise blurring of the faster moving bands is observed. Where necessary, the ion front was monitored by addition of 2 μg of bromophenol blue to the sample.

Electrophoresis was normally carried out at 23°C ambient temperature for 15—17 h using 12 mA/gel (runs performed at 25 mA/gel for 8 h produced identical subunit patterns). Electrophoresis was stopped when the marker dye had migrated 10 cm into the separating gel. The supporting glass plates were carefully removed and the gel was 'fixed' by incubation at 60°C for 30 min in an aqueous solution containing 5% trichloroacetic acid, 5% sulfosalicylic acid

and 5% methanol. Residual SDS, which impairs staining, was completely removed by further overnight incubation at 4°C in an excess volume of methanol/acetic acid/water (5 : 1 : 5, v/v). Gels were stained for 2—3 h at 23°C in the same medium containing 0.3% Coomassie Brilliant Blue, then diffusion destained.

Other methods. Reconstitution of ATPase proteoliposomes [3] and measurement of protein [6] and ATPase [6] and ATP—$^{32}P_i$ exchange activities [2] were as described previously.

Results

The yeast F_1—F_0 ATPase complex

As result of this work the technique of SDS-polyacrylamide disc gel electrophoresis, as used both in this [5] and other [4] laboratories to elucidate the subunit structure of the yeast ATPase complex, is now known to be inadequate for complete resolution of the ATPase polypeptides. As shown in Fig. 1, the F_1 and F_1—F_0 complexes, which show four and eight bands, respectively, on disc gels (gels 1 and 2) reveal five and twelve bands using the newer slab gel technique (gels 3 and 4). Comparison of the two techniques (Fig. 1), and of the apparent molecular weights of the subunits obtained by calibrating the gels

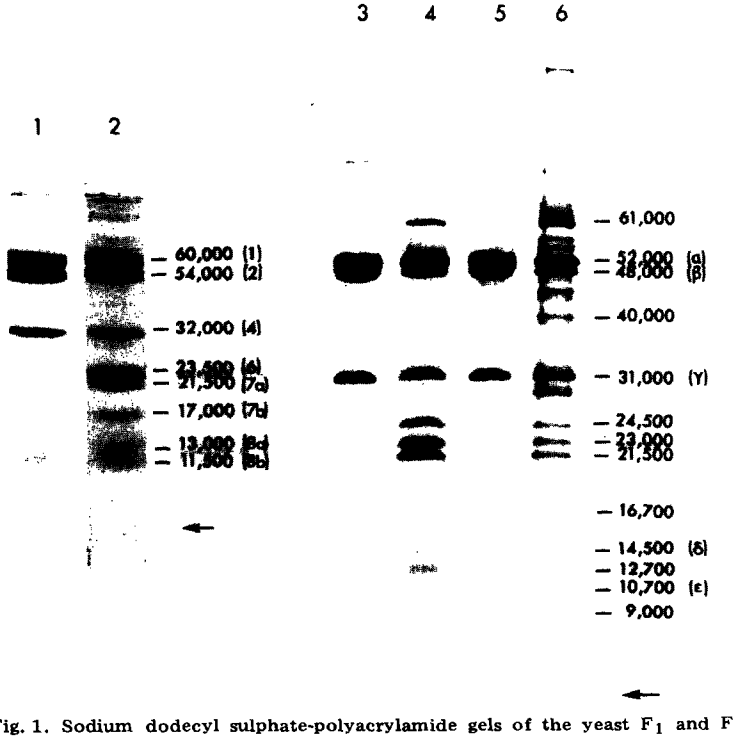

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gels of the yeast F_1 and F_1—F_0 ATPase complexes. Electrophoresis was performed either in cyclindrical disc gels containing 10% acrylamide [5] (gels 1 and 2) or in a slab gel containing 15% acrylamide (gels 3—6). Gels 1 and 3, 30 μg F_1; gels 2 and 4, 40 μg F_1—F_0; gels 5 and 6, 30 and 40 μg of F_1 and F_1—F_0, respectively, prepared in the absence of protease inhibitors. The arrows indicate the positions of the marker dye.

with standard proteins, shows that the slab gels are much more effective in resolving the faster moving bands. The previously designated subunit 6 of F_1—F_0 (gel 2) is resolved into two bands of 24 500 and 23 000 daltons on slab gels, and subunits 8a and 8b are resolved into four bands of apparent molecular weights 14 500, 12 700, 10 700 and 9 000 (gel 4). Also notable is that the two most prominent high molecular weight subunits (the α and β subunits of F_1) display lower apparent molecular weights on slab gels (52 000 and 48 000) than on disc gels (60 000 and 54 000) and that the slab gels clearly reveal a previously unidentified band of 61 000 daltons above the α subunit (gel 4).

A pleasing feature of the slab gels is the demonstration of five bands in the F_1 sector (Fig. 1, gel 3). Disc gels reveal only four (gel 1) owing to a lack of resolution of band 8b into its two components. By convention, the F_1 subunits are designated α, β, γ, δ, and ϵ in order of decreasing mobility.

For comparison Fig. 1 also shows the polypeptide compositions of F_1 and F_1—F_0 prepared in the absence of protease inhibitors. Several prominent bands and some minor ones are present in F_1—F_0 (gel 6) which are almost absent where protease inhibitors are included during isolation (gel 4). One of these, the 40 000 dalton band, previously shown [5] to correspond to subunit 3 of the Tzagoloff and Meagher preparation [4], is also visible to a varying degree in F_1 preparations isolated without inhibitors. We now believe this band is a proteolytic breakdown product of the α subunit (see below). Also obvious is the 'blurring' of the fastest moving bands in gel 6; this may also result from proteolytic modification during purification.

The effectiveness of protease inhibitors in minimising proteolytic modification during isolation of the ATPase raised the difficult problem of whether significant in vivo modification might occur before cell harvesting. Our current isolation method involves inclusion of protease inhibitors from the time of cell breakage. However, proteolysis during growth could conceivably produce a heterogeneous population of ATPase molecules, some with 'nicked' polypeptides, which would likely copurify together with the unmodified molecules and thus produce an artificially complex subunit pattern on gels. Since a mutant lacking all of the cellular proteases is unavailable, an attempt was made to impair in vivo proteolysis by growing cells in the presence of protease inhibitors. Growth occurred normally in the presence of 0.3 mM phenylmethylsulfonyl fluoride, as shown by Luzikov et al. [9], and also in the presence of 5 mM ϵ-amino-*n*-caproic acid, 5 mM *p*-aminobenzamidine, 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone or 2 mM diphenylcarbamyl chloride (results not shown). Prompted by this, growth was measured in the presence of combinations of protease inhibitors. Adequate growth still occurred even with five inhibitors present together (Fig. 2). To determine whether protease inhibitors impair protein breakdown in vivo, the cellular protein was labelled with [^{3}H]leucine and the loss of label from the mitochondrial fraction was monitored under non-growing conditions as an index of protein decay. As shown in Table I, the specific radioactivity decayed markedly over 20 h but was maintained when the cell suspension contained inhibitors. Even after 20 h in the presence of protease inhibitors the respiratory capacity was 140 ng atoms oxygen consumed/min per mg dry weight (measured polarographically at 30°C in a medium containing 20 mM galactose and 50 mM sodium phosphate, pH

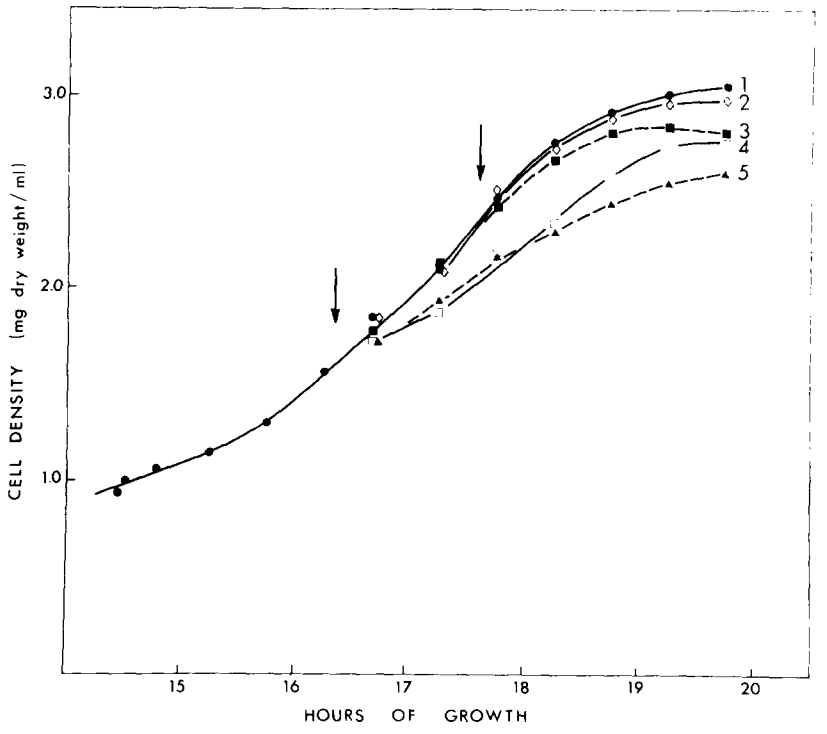

Fig. 2. Effect of protease inhibitors on the growth of *S. cerevisiae*. Cells were grown in 100-ml aliquots on a rotary shaker using 1.25% galactose as carbon source. Protease inhibitors were added at the times indicated by the arrows. 1, no inhibitors; 2, phenylmethylsulfonyl fluoride and *p*-aminobenzamidine; 3, phenylmethylsulfonyl fluoride, *p*-aminobenzamidine and ϵ-amino-*n*-caproic acid; 4, L-1-tosylamide-2-phenylethylchloromethyl ketone, and diphenylcarbamyl chloride; 5, phenylmethylsulfonyl fluoride, *p*-aminobenzamidine, ϵ-amino-*n*-caproic acid, L-1-tosylamide-2-phenylethylchloromethyl ketone and diphenylcarbamyl chloride. At each addition, the concentrations of the inhibitors were 0.2 mM phenylmethylsulfonyl fluoride, L-1-tosylamide-2-phenylethylchloromethyl ketone and diphenylcarbamyl chloride; 5 mM *p*-aminobenzamidine and ϵ-amino-*n*-caproic acid.

TABLE I

THE EFFECT OF PROTEASE INHIBITORS ON THE IN VIVO BREAKDOWN OF YEAST MITODRIAL PROTEINS

Yeast cells were labelled with [^{3}H]leucine, harvested, then resuspended in two equal volumes of a medium containing salts and 2% galactose. To one aliquot was added 5 mM ϵ-amino-*n*-caprioc acid, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM diphenylcarbamyl chloride and 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone, with second additions after 3 h. The cells were then shaken in an air atmosphere at 30°C and at preselected times aliquots were removed and a labelled mitochondrial fraction was isolated. Details are given in the text.

Time of incubation (h)	Specific radioactivity (cpm/mg mitochondrial protein)	
	Control	+ Protease inhibitors
0	3183	—
2	2378	2979
6	2197	2899
20	1785	3724

6.0) compared with 200 in controls lacking inhibitors. The respective rates measured in the presence of the uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (50 μM) were 330 and 295 ng atoms oxygen consumed/min per mg dry weight. Thus, like growth, respiratory development is little affected by the presence of protease inhibitors.

In the light of these findings, cells were grown in the presence or absence of phenylmethylsulfonyl fluoride, ϵ-amino-*n*-caproic acid, L-1-tosylamide-2-phenylethylchloromethyl ketone and diphenylcarbamyl chloride (*p*-aminobenzamidine was omitted due to its prohibitive cost). F_1—F_0 was then isolated in the presence of inhibitors and examined by SDS-polyacrylamide gel electrophoresis. Fig. 3 shows the SDS gels of both F_1—F_0 (gels 4—6) and the parent mitochondrial membranes (gels 7 and 8) and of a series of standard proteins

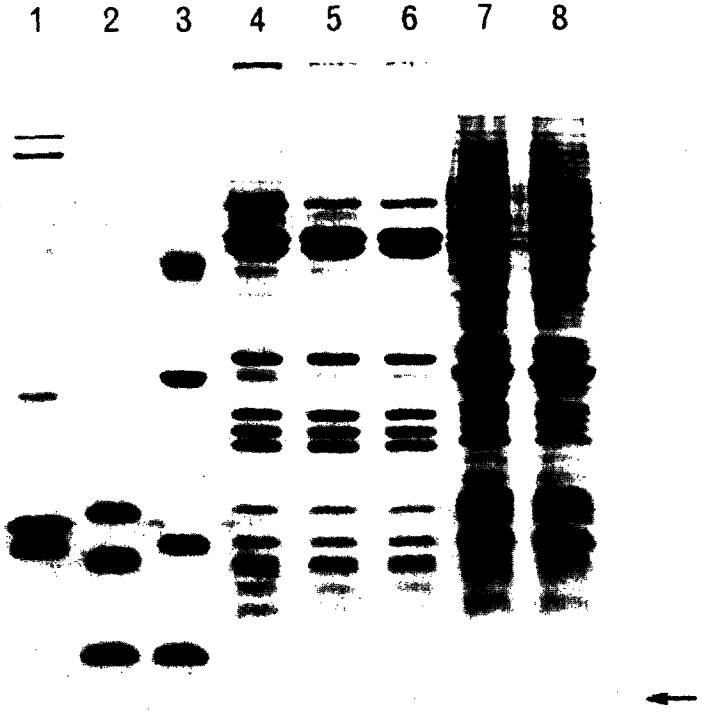

Fig. 3. Effect of growing yeast cells in the presence of protease inhibitors on the polypeptide compositions of the mitochondrial membranes and the F_1—F_0 complex. Cells were grown in 1-l batches and protease inhibitors (5 mM ϵ-amino-*n*-caproic acid, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone and 0.1 mM diphenylcarbamyl chloride) were added to selected flasks 2 h before the cessation of growth. Gels 1—3, 5 μg of a series of standard proteins; gel 1 (from top to bottom) β-galactosidase (130 000), phosphorylase *A* (94 000), myoglobin (17 600), cytochrome *c* (11 700); gel 2, hemoglobin (15 500), ribonuclease A (13 700), insulin (5700); gel 3, ovalbumin (46 000), carbonic anhydrase (29 000), lysozyme (14 400), insulin (5700); gel 4, 40 μg F_1—F_0 isolated in the presence of *p*-aminobenzamidine, ϵ-amino-*n*-caproic acid and phenylmethylsulfonyl fluoride from cells grown without inhibitors; gels 5 and 7, 40 μg F_1—F_0 and 100 μg of mitochondrial membranes, respectively, isolated in the presence of 5 mM *p*-aminobenzamidine, 5 mM ϵ-amino-*n*-caproic acid, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone and 0.1 mM diphenylcarbamyl chloride from cells grown without inhibitors; gels 6 and 8, 40 μg F_1—F_0 and 100 μg of mitochondrial membranes, respectively, isolated in the presence of five protease inhibitors from cells grown in the presence of inhibitors.

used as molecular weight markers (gels 1—3). The staining patterns of F_1—F_0 and the membranes were in no way altered by inclusion of inhibitors during cell growth (gel 6 versus 4 and 5, gel 8 versus 7) thus suggesting that endogenous proteolysis does not complicate the polypeptide compositions. Further, comparison of gels 4 and 5 shows that the inclusion of L-1-tosylamide-2-phenylethylchloromethyl ketone and diphenylcarbamyl chloride together with phenylmethylsulfonyl fluoride, ϵ-amino-*n*-caproic acid, and *p*-aminobenzamidine during purification is unnecessary; the subunit pattern is identical where only phenylmethylsulfonyl fluoride, ϵ-amino-*n*-caproic acid, and *p*-aminobenzamidine were used.

It is noteworthy that microorganisms often manifest increased levels of proteases and enhanced protein turnover during prolonged incubation in stationary phase [10]. In all of our studies, cells were cooled and harvested within 30 min of cessation of growth.

Latent, sodium dodecyl sulphate-activated protease contamination in purified preparations of the yeast F_1—F_0 complex

In early work it was observed that dissociation of F_1—F_0 at 70°C for 30 min in the presence of 1% SDS occasionally resulted in formation of a 40 000 dalton band on SDS-polyacrylamide gels *. This did not occur where dissociation was carried out by the normal procedure at 100°C for 3 min. This band corresponds to the previously designated subunit 3 [4,5] which is present in F_1 and F_1—F_0 preparations prepared in the absence, but not the presence, of protease inhibitors (ref. 5 and Fig. 1). This indicates that subunit 3 is a breakdown product.

As shown in Fig. 4, gels 1—6, incubation of F_1—F_0 at 50°C in 0.1% SDS induced an increasing production of the 40 000 dalton band over 2 h; also apparent, particularly in gels 5 and 6, is the simultaneous loss of the 52 000 dalton α band and the appearance of a faster moving band of 10 700 daltons which comigrates with the ϵ subunit. A further band of 35 000 daltons is also noticeable. Degradation of the α subunit is more clearly shown in gels 7—10 which were run for longer times to achieve better separation (faster migrating bands have electrophoresed from the gel). Besides the 40 000 dalton band, prolonged incubation also produced other more minor degradation products in this high molecular weight region (47 500, 39 500, 35 000, 30 000, 29 000 and 26 500) and an eventual decline in the 31 000 and 23 000 dalton subunits.

Fig. 5, gels 1—4, shows that the appearance of the 40 000 dalton band at 50°C was optimal at about 0.03% SDS with a lesser amount produced at 0.3%. At 0.1% SDS, breakdown was almost completely prevented by *p*-aminobenzamidine (gel 7) or by the combination of *p*-aminobenzamidine, phenylmethylsulfonyl fluoride and ϵ-amino-*n*-caproic acid (gel 9). Neither phenylmethylsulfonyl fluoride nor ϵ-amino-*n*-caproic acid alone were effective (gels 6 and 8) although phenylmethylsulfonyl fluoride did prevent formation of the

* The initial observation of this was made by Mr. D.R.H. Fayle (John Curtin School of Medical Research, this University) using the F_1—F_0 complex prepared in this laboratory.

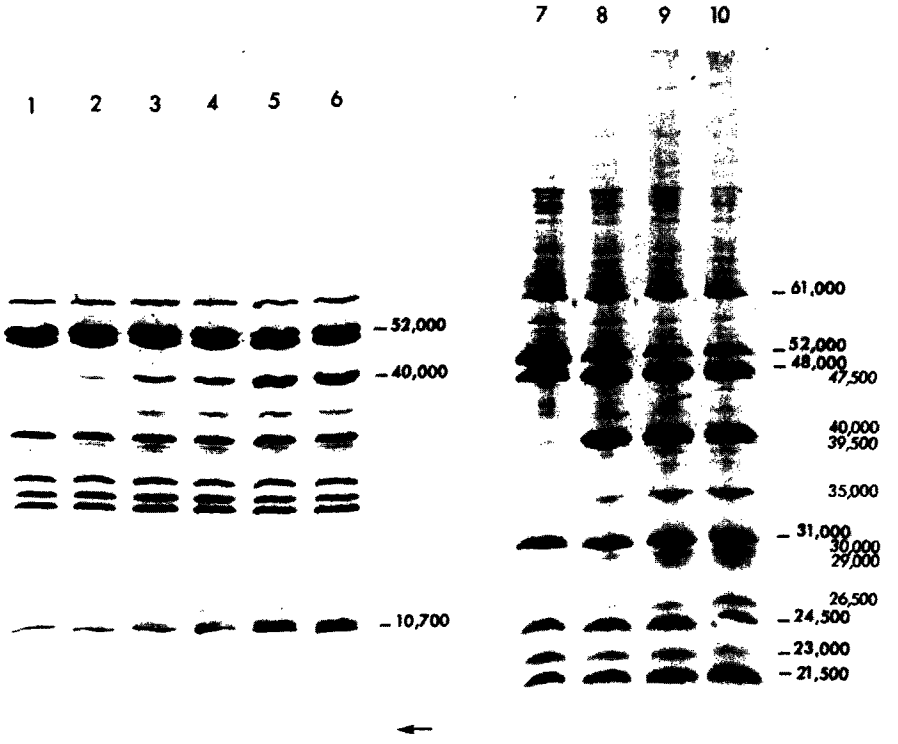

Fig. 4. The latent, sodium dodecyl sulphate-activated protease contaminant in yeast F_1—F_0 which degrades the α subunit. F_1—F_0 was prepared in the presence of protease inhibitors, reconcentrated twice in a medium containing 25 mM mannitol, 0.5 mM EDTA, 5% methanol and 10 mM Tris/acetic acid, pH 7.5, then resuspended at 500 μg of F_1—F_0 protein/ml in the same medium. Sodium dodecyl sulphate was added and after incubation for preselected times at 50°C the samples were fully dissociated by boiling with 1% SDS and electrophoresed in slab gels containing 0.1% SDS. Gels 1—6, 40 μg of F_1—F_0 incubated with 0.1% SDS for 0, 5, 15, 30, 80 and 120 min, respectively (note the compacting of the faster moving band; this occurs where gels are not pre-electrophoresed before sample additions). Gels 7—10, 40 μg of F_1—F_0 incubated with 0.03% SDS for 0, 10, 20 and 40 min, respectively. This gel was electrophoresed at twice the normal current; lower molecular weight bands have thus migrated from the gel. Molecular weights of the bands produced by protease activity are offset to the right.

35 000 dalton band (gel 6). Though degradation was detectable after 5 min in 0.1% SDS at 50°C (Fig. 4, gel 2), none was observed even after 60 min at 0 or 23°C. Neither repeated freeze-thawing, 2 M urea, 0.1% cetyltrimethylammonium bromide, 0.1% sodium cholate, nor 0.1% Triton X-100 substituted for 0.1% SDS, although 0.1% sodium deoxycholate did so but with much less effectiveness.

The mitochondrial ATPase complex of Torulopsis glabrata

The yeast *T. glabrata* contains circular mitochondrial DNA of much shorter length (6 μm, $12.8 \cdot 10^6$ daltons) than does *S. cerevisiae* (25 μm, $50 \cdot 10^6$—$54 \cdot 10^6$ daltons) [11]. Several of the F_0 membrane sector polypeptides in *S. cerevisiae* are encoded in this DNA and synthesized within the mitochondrion [1]. It thus seemed worthwhile to ask whether the shorter mitochondrial genome in *T. glabrata* might result in an ATPase complex with fewer subunits than that in *S. cerevisiae*.

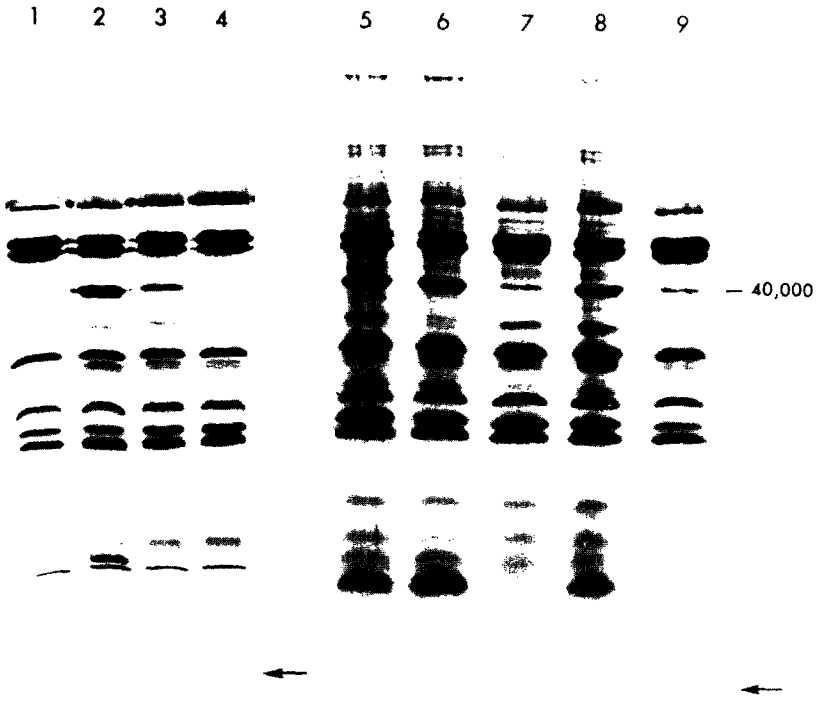

Fig. 5. The effect of sodium dodecyl sulphate concentration and protease inhibitors on production of the 40 000 dalton band in the yeast F_1—F_0 complex. Incubation of F_1—F_0 together with SDS was basically as described in the legend to Fig. 4. Gels 1—4, 40 μg of F_1—F_0 incubated for 20 min at 50°C in a medium containing 0, 0.03, 0.1 and 0.3% SDS, respectively; gels 5—9, 40 μg F_1—F_0 incubated for 15 min at 50°C in a medium containing 0.05% SDS and either no inhibitors (5), 2 mM phenylmethylsulfonyl fluoride (6), 20 mM *p*-aminobenzamidine (7), 20 mM *ε*-amino-*n*-caproic acid (8), or all three inhibitors (9).

T. glabrata was grown in a medium [6] containing either 4% (v/v) ethanol or 1.25% (w/v) glucose as carbon source. In the latter medium, the cells undergo a marked respiratory adaptation where glucose is depleted towards the end of growth phase [12]. Cells were harvested in early stationary phase and F_1—F_0 was prepared as described previously [5] except the linear glycerol gradient contained 5—15% (w/v) glycerol. The specific ATPase activity was 18.1 μmol P_i released/min per mg protein and this was increased to 48.7 by heating at 50°C in the presence of 20 mM ATP [6]. Comparable activities in the presence of 5 μg/ml oligomycin were 13.3 and 48.7, respectively. The complex contained the complete coupling mechanism, since proteoliposomes reconstituted with it exhibited a high rate of ATP—$^{32}P_i$ exchange (2325 nmol [^{32}P]ATP formed/10 min per mg protein) which was inhibited 94, 94 and 97% by 5 μg/ml oligomycin, 1 μg/ml nigericin and 25 μm carbonylcyanide *m*-chlorophenylhydrazone, respectively. F_1 was prepared by the chloroform method [5] except that the final Sepharose-purified protein was further purified by glycerol gradient centrifugation using the same gradient and conditions as described for F_1—F_0. The final ATPase specific activity was 209 μmol P_i released/min per mg protein.

The SDS-polyacrylamide slab gel electrophoresis patterns of *S. cerevisiae*

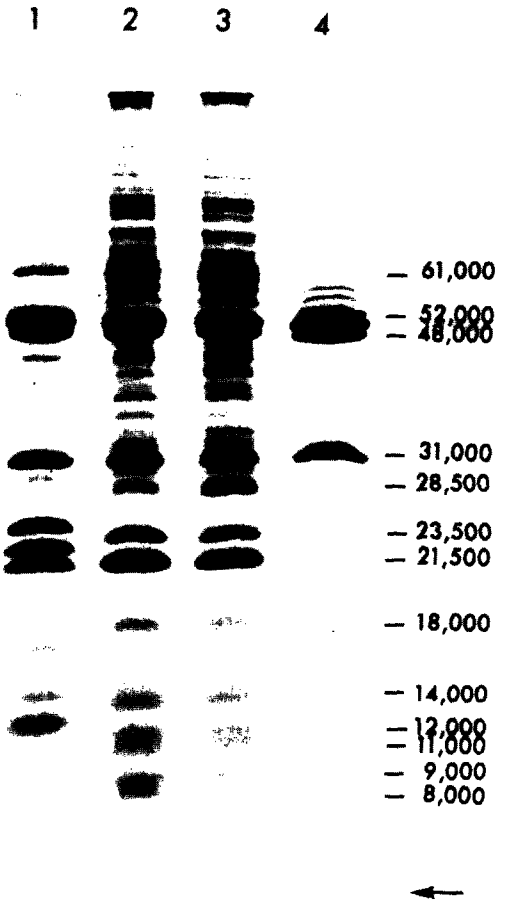

Fig. 6. Comparison of the subunit compositions of the F_1 and F_1—F_0 ATPase complexes from *S. cerevisiae* and *T. glabrata*. Details of the preparative methods are given in the text. Gel 1, 40 μg of *S. cerevisiae* F_1—F_0; gels 2 and 3, 40 μg of F_1—F_0 prepared from *T. glabrata* grown on 4% ethanol and 1.25% glucose, respectively; gel 4, 30 μg of *T. glabrata* F_1. The apparent molecular weights of the most prominent *T. glabrata* F_1—F_0 subunits are indicated. The two fastest moving bands in gel 4 are slightly retarded due to the large sample volume applied; in separate runs these bands were identified as the 14 000 and 11 000 dalton polypeptides.

F_1—F_0 and *T. glabrata* F_1 and F_1—F_0 are compared in Fig. 6. Though F_1—F_0 from *T. glabrata* appears slightly more contaminated judging by the minor high molecular weight bands (gels 2 and 3), it nonetheless seems clear that the number of major bands is similar to that in the *S. cerevisiae* complex (gel 1). F_1 from *T. glabrata* contained five subunits (gel 4) with almost identical molecular weights (52 000 (α), 48 000 (β), 31 000 (γ), 14 000 (δ), 11 000 (ϵ)) to those of *S. cerevisiae* F_1 (Fig. 1).

Discussion

An important conclusion is that proteolytic modification of the yeast F_1 and F_1—F_0 complexes can occur during purification. Other proteins from yeasts

[13] and other sources [14,15] are also known to be partially degraded during isolation and to thus have artificially complex subunit patterns on SDS-polyacrylamide gels. In some cases, limited proteolysis may not impair [13–16], and may even enhance [6,17], enzymic activity, and recognition of proteolytic artifacts in such cases can be difficult. As yeasts are particularly rich in proteases, considerable care is required in order to minimize protein degradation during purification. Since protease production is elevated during stationary phase [10] cells should ideally be harvested soon after the cessation of growth, and protease inhibitors should be included throughout the purification. Of the five protease inhibitors we have tested, *p*-aminobenzamidine is by far the most effective in protecting the F_1—F_0 complex during isolation.

Purified F_1—F_0, like some preparations of yeast hexokinase [16], contains a latent, SDS-activated protease contaminant. This latency is not unusual, since the yeast proteases which are adequetely characterized (proteases A, B and C) are known to occur naturally as masked (zymogen) forms due to association with respective protease inhibitor polypeptides [13]; activation can occur by proteolysis, a circumstance which may be optimized during vigorous cell breakage where cellular compartmentation is destroyed. Like the protease contaminant in the F_1—F_0 complex, activation of the inhibitor-protease complex in vitro often requires denaturing conditions at elevated temperature [18] which presumably causes release of the inhibitor.

It is unlikely that the protease contaminant in yeast F_1—F_0 would be visible as a band on SDS-polyacrylamide gels. Only extremely small amounts of protease are required to cause drastic modification of the substrate protein for example a subtilisin/serum albumin ratio of only $1 : 10^6$ yields extensive degradation of the albumin in 1% SDS [13].

An intriguing feature of the protease contaminant(s) is the considerable (but not absolute) specificity for the α subunit of F_1—F_0 (Fig. 4). In separate experiments, we have included either bovine serum albumin, pyruvate kinase, ovalbumin, alcohol dehydrogenase or carbonic anhydrase together with F_1—F_0 containing the activated protease contaminant. No breakdown of these proteins occurred even under conditions where much of the α subunit was degraded. Susceptibility of this subunit (and resistance of the β subunit) to breakdown by endogenous proteases has also been observed by Douglas et al. [19]. Though it is well known that the larger subunits in oligomeric proteins are more rapidly degraded than smaller ones [20], the closeness in size of the α and β subunits (52 000 versus 48 000) suggests this is not an important factor in determining their relative turnover rate. Probably the α subunit simply has more bonds which are susceptible to proteolytic cleavage. Proteolytic modification of *Micrococcus* sp. ATCC 398 F_1 during purification has also been reported [21]; again the α subunit (65 000 daltons) is specifically degraded, first to a 61 000 dalton species then to two polypeptides of 35 000 and 23 000 daltons.

Some of the cellular protease activity (up to 50% of *Escherichia coli* for example [22]) is membrane bound [23–25] and even extensive washing may not remove such activity completely. 'Intrinsic' membranes may be sterically protected by the surrounding lipid-protein milieu, but rapid proteolysis may begin when the protein (and presumably the proteases) are solubilized from the membrane during purification. An apparent example of this is the cytochrome

c_1 polypeptide from yeast mitochondria, which is degraded to lower molecular weight forms at the detergent solubilization step [26,27] unless protease inhibitors are included.

The introduction of the highly resolving SDS-polyacrylamide slab gel electrophoresis technique has revealed a more complex subunit pattern in the yeast F_1—F_0 complex than was previously recognized. We now believe that the complex, when purified without protease inhibitors, contains at least 15 separate bands (Fig. 1, gel 6) rather than the 8—10 bands seen previously. More careful isolation in the presence of protease inhibitors results in a 12 subunit pattern in slab gels (Fig. 1, gel 4; Fig. 3, gels 4—6) with sharp banding and very little background contamination. The same protein shows only eight bands on disc gels (Fig. 1, gel 2). F_1—F_0 has also been electrophoresed using a two-dimensional technique (see O'Farrell [28] as modified by Fayle et al. [29]) by electrofocusing in polyacrylamide over the pH range 3.9—10.4, then electrophoresing in an SDS gel containing a linear gradient of 7.5—22.5% polyacrylamide. Although one of the low molecular weight bands was not clearly identified due to low staining intensity, the remaining eleven appeared as clearly distinct spots thus confirming that they contain only a single polypeptide species.

Yeast F_1—F_0 contains a hydrophobic proteolipid polypeptide (or an oligomer of it) [1] which is difficult to stain and may not be seen as one of the twelve bands. A loosely attached ATPase inhibitor polypeptide of approx. 7000 daltons is also associated with the complex in situ [30,31]. This component is not required for coupling activity and is presumably lost during isolation of F_1—F_0.

Highly purified F_1, isolated by the chloroform method in the presence of protease inhibitors [5], is shown here to consist of five subunits (Fig. 1, gel 3) rather than the four seen on disc gels (Fig. 1, gel 1). This subunit pattern more closely corresponds with that of other preparations from photosynthetic [32] and respiratory [33] membranes, and from the yeast *Schizosaccharomyces pombe* [34], which, if reconstitutionally active, virtually always contains five subunits. It must be pointed out that the 'five subunit' pattern previously seen on SDS-polyacrylamide disc gels of F_1 prepared without protease inhibitors [4,5] is now considered incorrect for two reasons: firstly, the fastest moving band was not resolved into its two component subunits; six bands are visible in more highly resolving gels (ref. 35 and Fig. 1, gel 5); secondly, the present results suggest that the minor band of 40 000 daltons, originally considered an F_1 polypeptide, is a proteolytic breakdown product of the α subunit. Takeshige et al. [35] do not consider the 40 000 dalton polypeptide to be a proper subunit either, although their F_1 preparation contains it as an extra (sixth) band.

Douglas et al. [19] have recently isolated yeast F_1 containing only the α, β and γ subunits. Their method, a modification of that of Takeshige et al. [35], involves purification of the chloroform-solubilized protein on DEAE-cellulose. Interestingly, however, the F_1 prepared by Takeshige et al. [35] contains the δ and ϵ subunits, like the one reported here. It thus appears that either F_1 from the strain used by Douglas et al. [19] is inherently more labile or that differences in the purification procedure result in detachment of the δ and ϵ subunits. Clarification of this may provide a useful method for partial separation of the F_1 subunits.

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