THE SULPHYDRYL CONTENT OF YEAST MITOCHONDRIAL F_1-ATPase AND THE STOICHIOMETRY OF SUBUNITS

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1. Introduction

Mitochondrial coupling factor F_1-ATPase catalyzes the hydrolysis of ATP. The structure of this multi-subunit enzyme is complex [1,2] and the exact stoichiometry of the 5 subunits is still not clear, the choice lying between $\alpha_3\beta_3\gamma\delta\epsilon$ and $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ (or similar) [2,3]. A stoichiometry of 3:3:1:1:1 for α:β:γ:δ:ϵ, respectively, is likely in the case of yeast F_1-ATPase [4–6], F_1-ATPase of thermophilic bacterium [7] and *Escherichia coli* [8], and this stoichiometry may be universal for F_1-ATPase [9].

Because the sulphydryl content of a protein is usually very low, titration or specific labelling of sulphydryl side-chain groups offers a means of determining their number and distribution, and hence obtaining chemically the subunit stoichiometry, in the case of a multi-subunit enzyme. Using this approach with the yeast F_1-ATPase we show that:

(1) The F_1-ATPase contains no disulphide linkages;
(2) Only the α-, γ- and δ-subunits contain sulphydryl groups;
(3) The stoichiometry of α:γ:δ is 3:1:1.

From this distribution and from the yeast mitochondrial F_1-ATPase M_r of 390 000 [10], a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ is obtained.

2. Materials and methods

Saccharomyces cerevisiae mitochondrial F_1-ATPase was prepared as in [4]. Urea and reagents for electrophoresis were obtained from Serva (Heidelberg), DTNB from Sigma Chemical Co., Sephadex G-25 (fine) from Pharmacia and other biochemicals from Merck or Baker Chemicals. *N*-Ethyl [2,3-^{14}C]maleimide and *n*-[^{14}C]hexadecane were obtained from the Radiochemical Centre, Amersham, *N*-ethyl[1-^{14}C]-maleimide and Aquasol from New England Nuclear, while Quickszint 212 came from Koch-Light Labs.

Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); M_r, relative molecular mass; NEM, *N*-ethylmaleimide; SDS. sodium dodecylsulphate

2.1. *Titration with DTNB*

The method is based on those in [11,12]. Sulphydryls were estimated with F_1-ATPase in the (i) native, (ii) unfolded and (iii) unfolded reduced states. For (i), the F_1-ATPase (~1 mg) was dissolved in 2 ml 50 mM Tris–SO_4 buffer (pH 8.0) containing 1 mM EDTA. In (ii), the buffer contained 2% SDS in addition, and the dissolved sample was warmed at 50°C for 10 min to promote dissociation. The reduced F_1-ATPase in (iii) was obtained by dissolving the enzyme in 0.5 ml 50 mM Tris–SO_4 buffer (pH 8.0) containing 1 mM EDTA, 2% SDS and 50 mM dithioerythritol and incubating for 3 h at room temperature under nitrogen. The sample was then desalted of dithioerythritol by twice centrifuging through Sephadex G-25(fine) into Tris–SO_4–EDTA–SDS buffer [13]. A control with no protein was similarly treated. Titration with DTNB followed within 12 min. In (ii) and (iii) the maximum change (within 20 min) in absorbance at 412 nm was noted after addition of 0.1 ml 10 mM DTNB. For the native enzyme, the change was followed over 4 h. In calculating the number of sulphydryl groups a molar absorptivity of 13 600 was taken for the *p*-nitrothiophenol anion [14].

2.2. *Labelling with [^{14}C]NEM*

The method was based on [15] and labelling of F_1-ATPase was done on the native or unfolded (reduced and non-reduced) states. Reduction of the

enzyme (250 μg/assay) was achieved in 40 mM Tris–SO_4 buffer (pH 8.0) containing 1 mM EDTA, 50 mM dithioerythritol and 1% SDS, incubated for 3 h at room temperature under nitrogen. Desalting into buffer (pH 7.0) containing 10 mM sodium phosphate, 1% SDS, 0.05 mM dithioerythritol was as above. Unfolded enzyme (non-reduced) was achieved by dissolving a sample in 250 μl 10 mM sodium phosphate, 1% SDS (pH 7.0). The native enzyme was dissolved in 10 mM phosphate (pH 7.0) also at 1 mg/ml.

In each case, [^{14}C]NEM was added to 1.7 mM and after 20 min, the reaction terminated by addition of 10 vol. ice-cold 96.5% ethanol. The protein pellet was washed twice more with 10 vol. ethanol, then dried under a stream of nitrogen. The dried material was taken up in 300 μl 3% SDS containing 0.2 M sucrose. Alternatively, excess [^{14}C]NEM was removed by 2 centrifugations through Sephadex gel as above. A control was done omitting protein.

Samples were counted to determine total NEM bound and others were subjected to SDS–polyacrylamide gel electrophoresis [16] to determine the distribution of the label amongst the subunits. After staining with Coomassie brilliant blue R-250 gels were scanned and sliced laterally into 1 mm slices and extracted in 1 ml 3% SDS for 20 h at 65°C. Finally 10 ml Aquasol or Quickszint 212 were added for counting ^{14}C. Counting efficiency was 87–88% as determined with an internal standard (*n*-[^{14}C]hexadecane).

2.3. *Protein determination*

Protein was assayed by the Coomassie blue method [17] or by the Lowry procedure [18].

2.4. *Assay of ATPase activity*

ATPase activity was assayed according to the coupled enzyme system [19].

2.5. *Amino acid analysis*

Protein samples containing a norleucine internal standard were hydrolysed in 6 N HCl at 110°C for 24, 48 or 72 h. Triplicate amino acid analyses were then performed on a Biotronik amino acid analyzer. For the determination of cysteine as cysteic acid, protein samples were first oxidized according to [20].

3. Results

3.1. *Total SH-content of F_1-ATPase*

In table 1 the numbers of sulphydryl groups

Table 1
Sulphydryl content of F_1-ATPase (mol –SH/mol F_1-ATPase)

Method	Mean	SD	No.
(i) Amino acid analysis after performic acid oxidation of F_1-ATPase	7.39	(0.39)	3
(ii) Labelling of unfolded F_1-ATPase with [^{14}C]NEM	6.06	(1.46)	6
(iii) Titration with DTNB of:			
(1) unfolded F_1-ATPase;	6.23	(0.74)	13
(2) unfolded and reduced F_1-ATPase	6.83	(1.25)	11

Listed are the mean values together with standard deviations and the number of different F_1-ATPase preparations used. Values were calculated using 390 000 M_r for F_1-ATPase [10]. Corrections were made for appropriate controls

Table 2
Amino acid composition of F_1-ATPase

Amino acid	Mol/mol F_1-ATPase	mol%
Asx	317.6	8.8
Thr	211.3	5.8
Ser	235.9	6.5
Glx	479.7	13.3
Pro	200.4	5.5
Gly	320.0	8.8
Ala	360.0	9.9
Cys	7.4	0.2
Val	251.0	6.9
Met	35.0	1.0
Ile	207.3	5.7
Leu	352.4	9.7
Tyr	82.8	2.3
Phe	120.2	3.3
Lys	210.9	5.8
His	52.6	1.5
Arg	177.3	4.9
Trp	n.d.	n.d.

A pellet of ~1.1 mg F_1-ATPase, as prepared in [4], was desalted twice through Sephadex G-25 gel (fine) into 10 mM sodium phosphate (pH 7.0), then precipitated with 10% trichloroacetic acid. The precipitated protein, with added norleucine as an internal standard, was hydrolyzed in 6 N HCl and analyzed as in section 2. Values for serine and threonine were obtained by extrapolation to zero time. The value for cysteine was obtained as cysteic acid after performic acid oxidation of F_1-ATPase and subsequent HCl hydrolysis; N.D., not determined; F_1-ATPase was taken as 390 000 M_r [10]

found/per F_1-ATPase molecule are given, as determined by 3 independent methods with the amino acid composition shown in table 2 (see also [4,6]). The numbers of sulphydryl groups yield an average value that is close to 6.6 sulphydryls/F_1-ATPase. The close agreement of the two DTNB-titration figures for unfolded F_1-ATPase, with and without reduction, indicates that the F_1-ATPase does not contain disulphide linkages, which is further verified below. Also, similar results were obtained if the DTNB-titration was carried out in 6 M guanidine–HCl, 10 mM EDTA, 50 mM Tris–SO_4 (pH 8.0).

The effect of long term exposure of native F_1-ATPase to DTNB was also measured (fig.1). Exposure over 4 h resulted in a gradual loss of ATPase activity relative to a control. Apart from an initial sharp increase, the rise in absorbance at 412 nm coincided roughly with the fall in activity. This pointed to differential reaction of DTNB with ~1 of the sulphydryl groups in 1 of the subunits.

3.2. *Distribution of sulphydryl groups*

The distribution of the sulphydryl groups of F_1-ATPase among the 5 subunits was determined from the radioactivity pattern obtained upon SDS–polyacrylamide gel electrophoresis of F_1-ATPase that had been labelled with [^{14}C]NEM (fig.2). Of the 5 subunits of F_1-ATPase, only the α-, γ- and δ-subunits bound the label. The β- and ϵ-subunits did not bind significant quantities of label, but NEM was bound to the protein of M_r ~42 000 [4], an extra protein seen variably in our preparations. Fig.2 shows the labelling of F_1-ATPase in the unfolded state. The same picture is obtained if the F_1-ATPase is, in addition, reduced before reaction with NEM (not shown). Reaction of native F_1-ATPase with [^{14}C]NEM yielded the same

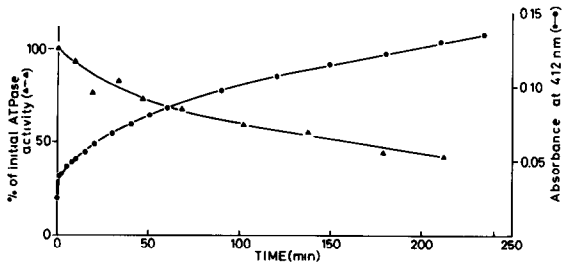

Fig.1. Titration of native F_1-ATPase with DTNB. The titration was done as in section 2. The percentage of enzymic activity and the absorbance at 412 nm (a measure of the degree of DTNB reaction) are plotted against time.

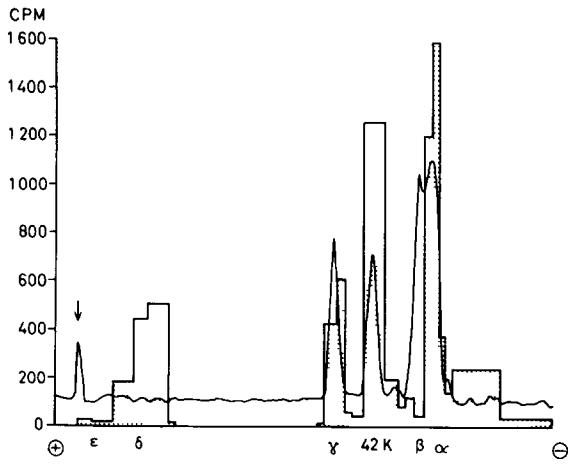

Fig.2. Labelling of F_1-ATPase subunits by [^{14}C]NEM. The solid trace shows absorbance at 540 nm of the stained gel and the shaded areas indicate radioactivity. Reaction with [^{14}C]NEM in 1% SDS, desalting and subsequent SDS electrophoresis on 10% gels were done as in section 2. The sample contained 10 μg F_1-ATPase. The δ- and ϵ-subunits are not visible at this loading of the gel and their positions were identified from other gels carrying more protein. The arrow indicates the position reached by the bromophenol blue marker dye.

degree of labelling of both the γ- and δ-subunits as for the unfolded enzyme, but the amount of label bound to the α-subunits was very low (not shown).

In fig.2, the protein band with M_r 42 000 is shown to bind a total amount of label about equal to that bound by the γ-subunit (1260 cpm and 1039 cpm, respectively), and the absorbance peak heights were also about equal. The content of the 42 000 protein varied from preparation to preparation, sometimes being almost absent, at other times showing relative protein staining intensity on the gel slightly greater than that of the γ-subunit. Consequently this will cause variability in the figure found for cysteine content of the enzyme.

By plotting cpm *vs* μl of sample electrophoresed, for each subunit, it is possible to calculate the ratio of label among the subunits from the slopes of the lines generated (fig.3). The results of several experiments are shown in table 3. There is no significant difference between the ratios for unfolded (reduced or non-reduced) F_1-ATPase, again indicating the absence of disulphide bonds, and a stoichiometry of $\alpha_3\gamma\delta$. The results also show that at least 2 of the α-subunit sulphydryls react only after unfolding.

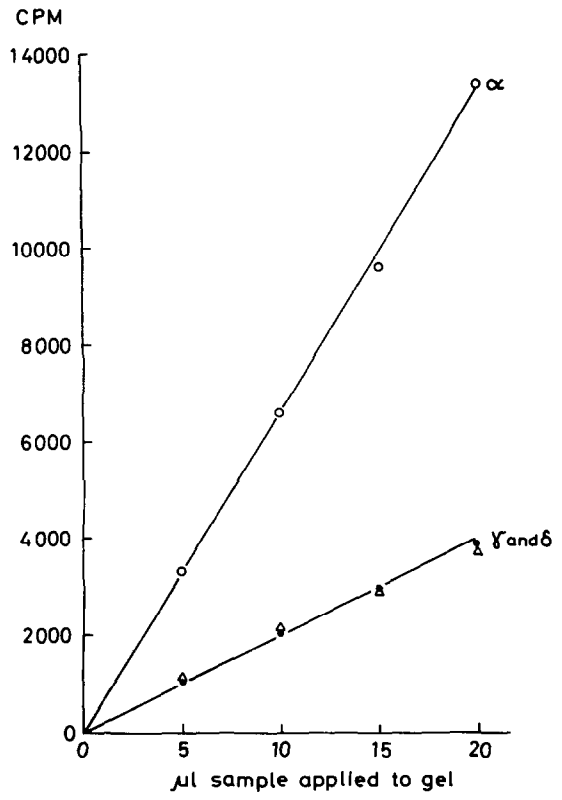

Fig.3. Distribution of sulphydryl groups in unfolded F_1-ATPase. Various aliquots (5–20 μl) of [^{14}C]NEM-labelled F_1-ATPase were subjected to SDS electrophoresis and the cpm in the indicated subunits were plotted against μl of sample. In those larger samples where it was not possible to separate the α- and β-subunit bands on the gels, the cpm in the combined (α + β) subunits were taken. Measurement of the slopes of the lines yields the ratio of labelling of sulphydryl groups by NEM; protein was ~2 mg/ml; α-subunit (○); γ-subunit (●); δ-subunit (△).

Table 3
Distribution and ratio of sulphydryl groups among the subunits of F_1-ATPase

Form of F_1-ATPase	No.	α : γ : δ
Native F_1-ATPase	(6)	0.79 : 1.00 : 0.95
Unfolded F_1-ATPase	(9)	3.39 : 1.00 : 0.85
Unfolded and reduced F_1-ATPase	(6)	3.40 : 1.00 : 0.92

These values were obtained from the slopes of plots typical of those shown in fig.3. The figures in parentheses show the number of F_1-ATPase preparations used. The values for the γ-subunit have been arbitrarily set to 1.0

4. Discussion

Using 3 different methods of determination, the number of sulphydryl groups in F_1-ATPase from yeast has been found to be ~6.6 (table 1). Some variation can be expected because of the variable content in our F_1-ATPase preparations, of a sulphydryl-containing protein of M_r ~ 42 000, and which is not considered a subunit of F_1-ATPase. Indeed, a protein of similar M_r has been observed in other preparations of yeast F_1-ATPase [6,21] and has been suggested to be a proteolytic degradation product of the α-subunit [21]. Our results show, however, that its presence did not affect the distribution of sulphydryl groups found for the subunits of F_1-ATPase.

The α-, γ- and δ-subunits bind [^{14}C]NEM when F_1-ATPase is unfolded (fig.2), indicating that these but not the β- and ε-subunits possess sulphydryl groups. The absence of any significant increase in the total sulphydryls or of any change in the distribution ratio among the subunits, after reduction of unfolded F_1-ATPase with dithioerythritol, show that yeast F_1-ATPase does not contain disulphide linkages.

The sulphydryl content of different F_1-ATPases varies with the source. Thus, for the beef heart enzyme [14] only the 2nd and 4th subunits did not bind NEM, i.e., β and δ in our nomenclature, and a total of 8 sulphydryls and 2 disulphide bonds/F_1-ATPase was proposed. In the case of the chloroplast reduced F_1-ATPase, 11.6–14 sulphydryl groups/F_1-ATPase have been reported, 6 of which are normally present as disulphide linkages [22–24]. The thermophilic bacterium PS3 is a simple case where only the α-subunit binds sulphydryl reagents, each of the 3 α-subunits/F_1-ATPase having 1 cysteine [25].

From the data of table 1 and table 3, it is suggested that the most likely stoichiometry in F_1-ATPase of the α-, γ- and δ-subunits, is 3:1:1. If there were 2 copies of the γ-subunit (and δ-subunit) a total of 10 sulphydryls/F_1-ATPase molecule in the absence of any 42 000 M_r protein would be found. Further evidence for a ratio of 3:1:1 is obtained by calculating the molar ratio of NEM bound to γ-subunit in fig.2. Using M_r 390 000 for F_1-ATPase and assuming 1 γ-subunit/F_1-ATPase molecule, it is shown that the γ-subunit binds ~1.1 mol NEM/mol. Thus, it is proposed that yeast F_1-ATPase contains 3 copies of the α-subunit and 1 copy each of the γ- and δ-subunits, each containing a single sulphydryl residue. Up to 1 more sulphydryl residue/F_1-ATPase is provided by a

contaminating protein of M_r 42 000. In addition, the staining intensities of the β- and ε-subunit bands relative to the staining of the α-, γ- and δ-subunit bands on polyacrylamide gels, support a stoichiometry for the 5 subunits of yeast F_1-ATPase of 3:3:1:1:1. Results of experiments with yeast grown in isotopically labelled media agree with this stoichiometry [5,6]. Further, using the subunit M_r-values in [4], a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ agrees best with the M_r-value for F_1-ATPase and its primary dissociation product determined by laser light scattering [10].

Titration of native F_1-ATPase with DTNB (fig.1) showed that in <20 min, 1.6–2.1 sulphydryl groups had been titrated, whilst the first group was titrated in <6 min, probably reflecting greater accessibility of this cysteinyl side-chain. The location of this group (either in the γ- or the δ-subunit) is not yet identified. The gradual loss of ATPase activity with time probably represents a slow denaturation of protein with subsequent exposure of sulphydryl groups to DTNB, and does not necessarily imply that such groups are involved in ATPase activity. Indeed, sulphydryls do not play a significant role in ATP hydrolysis by F_1-ATPase [26,27].

Native F_1-ATPase binds [^{14}C]NEM to the α-, γ- and δ-subunits in about equal proportion (table 3), although labelling of the α-subunit was variable (0:1:1–2.03:1:1), reflecting a variability in the accessibility to the sulphydryl group in this subunit. It is suggested on the basis of the DTNB- and NEM-data, that the γ- and δ-subunits possess accessible sulphydryls and that upon unfolding of F_1-ATPase with SDS, more sulphydryls are exposed only in the α-subunits. Interestingly, native F_1-ATPase from chloroplasts [28] and beef-heart mitochondria [11] also contains 2 accessible sulphydryl groups.

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