THE SULPHYDRYL CONTENT OF YEAST MITOCHONDRIAL F$_1$-ATPase AND THE STOICHIOMETRY OF SUBUNITS

Roland GREGORY and Benno HESS
Max-Planck-Institut für Ernährungsphysiologie, 4600 Dortmund, Rheinlanddamm 201, FRG

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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 $\alpha_2\beta_2\gamma\delta\varepsilon$ and $\alpha_2\beta_2\gamma\delta\varepsilon$ (or similar) [2,3]. A stoichiometry of 3:3:1:1:1 for $\alpha:\beta:\gamma:\delta:\varepsilon$, 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 $\alpha$, $\gamma$ and $\delta$-subunits contain sulphydryl groups;
(3) The stoichiometry of $\alpha:\gamma:\delta$ is 3:1:1.
From this distribution and from the yeast mitochondrial F$_1$-ATPase $M_r$ of 390 000 [10], a stoichiometry of $\alpha_2\beta_2\gamma\delta\varepsilon$ 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.

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), 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 $^{14}$CNEM

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-
SO₄ 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 phos-
phate, 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, [¹⁴C]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 [¹⁴C]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–polyacryl-
amide gel electrophoresis [16] to determine the dis-
tribution 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 ¹⁴C. Counting efficiency was 87–88% as
determined with an internal standard (n-[¹⁴C]hex-
adecane).

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 F₁-ATPase

In table 1 the numbers of sulphydryl groups

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>SD</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Amino acid analysis after performic acid oxidation of F₁-ATPase</td>
<td>7.39</td>
<td>(0.39)</td>
<td>3</td>
</tr>
<tr>
<td>(ii) Labelling of unfolded F₁-ATPase with [¹⁴C]NEM</td>
<td>6.06</td>
<td>(1.46)</td>
<td>6</td>
</tr>
<tr>
<td>(iii) Titration with DTNB of: (1) unfolded F₁-ATPase; (2) unfolded and reduced F₁-ATPase</td>
<td>6.23</td>
<td>(0.74)</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mol/mol F₁-ATPase</th>
<th>mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>317.6</td>
<td>8.8</td>
</tr>
<tr>
<td>Thr</td>
<td>211.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Ser</td>
<td>235.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Glx</td>
<td>479.7</td>
<td>13.3</td>
</tr>
<tr>
<td>Pro</td>
<td>200.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Gly</td>
<td>320.0</td>
<td>8.8</td>
</tr>
<tr>
<td>Ala</td>
<td>360.0</td>
<td>9.9</td>
</tr>
<tr>
<td>Cys</td>
<td>7.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Val</td>
<td>251.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Met</td>
<td>35.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ile</td>
<td>207.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Leu</td>
<td>352.4</td>
<td>9.7</td>
</tr>
<tr>
<td>Tyr</td>
<td>82.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Phe</td>
<td>120.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Lys</td>
<td>210.9</td>
<td>5.8</td>
</tr>
<tr>
<td>His</td>
<td>52.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Arg</td>
<td>177.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Trp</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

A pellet of ~1.1 mg F₁-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 F₁-ATPase and subsequent HCl
hydrolysis; N.D., not determined; F₁-ATPase was taken as 390 000 Mr [10].
Found/ per F

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 per ATPase. The close agreement of the two DTNB titration figures for unfolded F

ATPase, with and without reduction, indicates that the F

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

(pH 8.0).

The effect of long term exposure of native F

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 different 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

ATPase among the 5 subunits was determined from the radioactivity pattern obtained upon SDS—polyacrylamide gel electrophoresis of F

ATPase that had been labelled with [14C]NEM (fig. 2). Of the 5 subunits of F

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

κ 42 000 [4], an extra protein seen variably in our preparations. Fig. 2 shows the labelling of F

ATPase in the unfolded state. The same picture is obtained if the F

ATPase is, in addition, reduced before reaction with NEM (not shown). Reaction of native F

ATPase with [14C]NEM yielded the same degree of labelling of both the γ- and δ-subunits as for the unfolded enzyme, but the amount of label bound to the α-subunit was very low (not shown).

In fig. 2, the protein band with M

κ 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

ATPase, again indicating the absence of disulphide bonds, and a stoichiometry of α3γδ. 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 [14C] 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

<table>
<thead>
<tr>
<th>Form of $F_1$-ATPase</th>
<th>No.</th>
<th>$\alpha : \gamma : \delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native $F_1$-ATPase</td>
<td>(6)</td>
<td>0.79 : 1.00 : 0.95</td>
</tr>
<tr>
<td>Unfolded $F_1$-ATPase</td>
<td>(9)</td>
<td>3.39 : 1.00 : 0.85</td>
</tr>
<tr>
<td>Unfolded and reduced $F_1$-ATPase</td>
<td>(6)</td>
<td>3.40 : 1.00 : 0.92</td>
</tr>
</tbody>
</table>

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 sulphhydryl 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 sulphhydryl-containing protein of $M_f \sim 42 000$, and which is not considered a subunit of $F_1$-ATPase. Indeed, a protein of similar $M_f$ 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 sulphhydryl groups found for the subunits of $F_1$-ATPase.

The α-, γ- and δ-subunits bind [14C]NEM when $F_1$-ATPase is unfolded (fig.2), indicating that these but not the β- and ε-subunits possess sulphhydryl groups. The absence of any significant increase in the total sulphhydryls 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 sulphhydryl 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 sulphhydryls and 2 disulphide bonds/$F_1$-ATPase was proposed. In the case of the chloroplast reduced $F_1$-ATPase, 11.6-14 sulphhydryl 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 sulphhydryl 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 sulphhydryls/$F_1$-ATPase molecule in the absence of any 42 000 $M_f$ 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 $M_f$ 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 sulphhydryl residue. Up to 1 more sulphhydryl residue/$F_1$-ATPase is provided by a
contaminating protein of $M_c$, 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 $M_c$-values in [4], a stoichiometry of $αβγδε$ agrees best with the $M_c$-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 ε-subunits. Interestingly, native F$_1$-ATPase from chloroplasts [28] and beef-heart mitochondria [11] also contains 2 accessible sulphydryl groups.

### Acknowledgements

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### References