SPECIFIC IMMUNOPRECIPITATION OF ATPase FROM *ESCHERICHIA COLI*

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1. Introduction

Energy-transducing membrane ATPases in mitochondria [1], chloroplasts [2] and bacteria [3,4] exhibit a large degree of similarity with respect to function, subunit composition and stoichiometry, although some variability exists with different systems. It might be expected, therefore, that among bacterial mutants defective in ATPase activity, certain classes would exhibit alterations in the latter two properties. In order to examine the possibility of such modifications in mutants, it is necessary to isolate the defective ATPase using a procedure which does not depend on assay of catalytic activity. One assay frequently employed in the characterization of mutant protein involves the isolation of material which cross-reacts with specific antibody prepared against the purified parental protein [5]. This report describes a relatively simple immunological procedure designed to selectively precipitate active or defective ATPase from a crude Triton extract of *E. coli* membranes using antibody prepared against the purified wild-type enzyme. The cross-reacting material in the immuno-precipitate is subsequently analysed by SDS–gel electrophoresis.

Abbreviations: Ab, antibody; ATPase, Mg^{2+}-Ca^{2+} adenosine triphosphatase; BSA, bovine serum albumin; cpm, counts per minute; CRM, cross-reacting material; EDTA, ethylenediamine tetrasodiumacetate; IgG, gamma globulin; OVA, ovalbumin; PMSF, phenyl methyl sulfonyl fluoride; POP, 2, 5-diphenyloxazol; POPOP, 2, 2-*p*-phenylen-bis-(4-methyl-5-phenyloxazol); SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Strains*

Parental strains A428 [6], ML 308–225 [7], S7 [8] and the ATPase-defective mutant NR70 [8].

2.2. *Materials*

Rabbit antiserum was prepared as in [6]. Two kinds of antisera were prepared using:

(i) Purified ATPase from A428, anti BF_1;

(ii) A trypsin-treated preparation of purified ATPase consisting exclusively of $\alpha + \beta$ subunits; this preparation retains catalytic activity [6], anti $(\alpha + \beta)$.

^{14}C- and ^{3}H-labeled L-amino acid mixtures with spec. act. 0.1 mCi/ml in 0.1 N HCl were purchased from New England Nuclear. Soluene-350 was purchased from Packard. All other reagents were of the highest quality available.

2.3. *Growth of cells*

Cells were grown on Davis minimal medium supplemented with 0.5% glucose 50 μg/ml each of L-proline and L-histidine and 1 μg/ml vitamin B_1. For labeling experiments 0.1 mCi ^{14}C or 0.4 mCi ^{3}H of an L-amino acid mixture was added, per liter of culture. Cells, 500 ml, at late log phase were harvested in a Sorvall RC2B centrifuge and washed once with STNT buffer (0.04 M tricine, 8 mM NaCl, 0.3 M sucrose, pH 8.0).

2.4. *Isolation of cross-reacting material*

About 0.8 g (wet wt) of washed cells were sus-

pended in 8 ml STNT buffer. The suspended cells were disrupted either by sonification with Labsonic 1510 Brown sonifier or by French Pressure cell single passage 20 000 p.s.i. Cell-free extract was obtained following centrifugation at 10 000 rev./min for 10 min and spun for 3 h at 130 000 × g in Beckman L3-50 ultracentrifuge. The high speed supernatant thus obtained is termed 'Sup I'. Sup I contained ~2 mg protein/ml determined by the method in [9]. The pellet was suspended in 3.5 ml STN buffer (0.4 M sucrose, 10 mM tricine, 10^{-2} M NaCl, pH 8.0) supplemented with 1% Triton X-100 and 0.5 mM EDTA, and incubated on ice for 30 min. The suspension was centrifuged for 2 h at 130 000 × g in Beckman L3-50 ultracentrifuge to yield a supernatant fraction termed 'Triton extract'. The Triton extract fraction contained ~0.5 mg protein/ml. The Triton-extracted membrane pellet was suspended in 0.5 ml STN buffer and residual ATPase activity determined as in [10]. Inorganic phosphate was measured according to [11]. Either 0.2 ml anti BF_1 or 0.2 ml anti ($\alpha + \beta$) were added to Sup I and the Triton extract and incubated for 30 min at 37°C. The Ab–CRM complex was obtained in the pellet following centrifugation at 15 000 rev./min for 10 min. The complex was washed once with 10 mM Tris, 100 mM NaCl, 1% Triton X-100, pH 7.8. Following a second centrifugation the washing procedure was repeated twice with the same buffer in the absence of Triton. The complex was solubilized overnight in 0.2 ml mixture of STN buffer containing 3% SDS and 0.1 M mercaptoethanol. The radioactivity of the complex was determined in scintillation fluid (0.04 g POP/liter toluene and 0.4 g POPOP/liter toluene) in a Packard 3320 Tri-carb scintillation spectrometer.

2.5. *SDS–gel electrophoresis*

SDS–gel electrophoresis was carried out on 9% acrylamide gels according to the procedure in [12]. Gels were loaded with 10–50 μl of the solubilized Ab–CRM complex. The gels were stained with Coomassie brilliant blue and destained according to the procedure in [13]. Stain intensity at A_{620} was monitored in a Gilford 240 spectrophotometer equipped with special adaptor (Model 2410) for gel scanning. For radioactive determination, the gels were cut into slices 1 mm thick and each slice was incubated in 0.5 ml soluene-350 for 2 h at 80°C. The radioactivity of each slice was determined in a Packard 3320 Tri-carb scintillation spectrometer, after the addition of scintillation fluid.

3. Results and discussion

Measurements of the residual ATPase activity on the membranes following extraction indicated that 85 ± 5% of the ATPase was solubilized. Since considerable activity was detected in the Sup I fraction, cross-reacting material (CRM) was precipitated with anti BF_1 from both Sup I and the Triton extract. As can be seen in fig.1, SDS–gel electrophoresis of the CRM antibody complex obtained from either fraction, showed 4 distinct ATPase subunits, α, β, γ and ϵ. In additon, the light and heavy chain of the antibody (mol. wt 23 000 and 53 000, respectively) were also observed. The same profile was found when the CRM–antibody complex isolated from Sup I or the Triton extract was analyzed from 3 different parental strains, A428 [6], ML308-225 [7] and S7 [8]. Moreover, identical profiles were observed when antibody prepared against trypsin-treated purified ATPase, which retains catalytic activity, anti ($\alpha + \beta$) was used in place of anti BF_1. This suggests that the CRM is precipitated as a complex, since the anti ($\alpha + \beta$) does not precipitate the isolated γ subunit (B. I. Kanner, unpublished observations). The same subunit distribution was also observed when a purified preparation of ATPase, containing four subunits, from a parental strain A428 was analyzed in the same fashion (data not shown). Finally, extraction of the CRM in the presence of the protease inhibitor PMSF (10^{-3} M) did not alter the gel profile.

In order to estimate the relative amount of the subunits in the CRM antibody complex, cells were grown in the presence of a mixture of radioactive amino acids, as in section 2, and the radioactive CRM in both Sup I and the Triton extract was precipitated with anti BF_1. The complexes were subjected to SDS gel electrophoresis. Table 1 demonstrates the relative amount of radioactivity appearing in the CRM precipitated from Sup I and the Triton extract. Although the absolute amount of radioactivity in the different fractions varied with different experiments, the relative amount which precipitated with the anti BF_1 was found to be constant. The radioactive CRM–

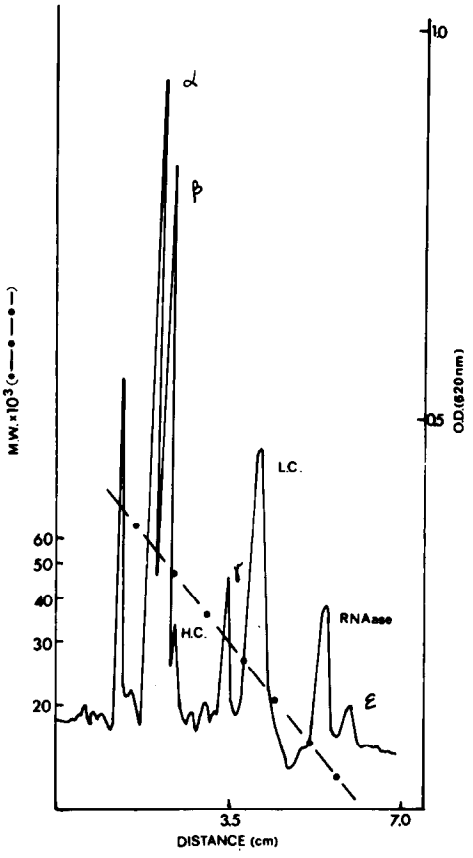

Fig.1. Gel profile of Ab–CRM complex from parental strain A428. The CRM was isolated from Triton extract, precipitated with anti BF_1 and washed as in section 2. Solubilized Ab–CRM complex, 20 μl, was subjected to SDS–gel electrophoresis and stained with Coomassie brilliant blue. RNase, 10 μg, was included as internal standard and a gel containing 10 μg each of BSA (mol. wt 68 000), OVA (mol. wt 43 000), IgG (light chain, mol. wt 23 000 and heavy chain mol. wt 53 000) and RNase (mol. wt 13 700), was run in parallel, for estimation of molecular weights.

Table 1
The distribution of radioactivity in cross-reacting material (CRM) precipitated from Sup I and Triton extract

Preparation	% Radioactivity in CRM
Sup I	1.0 ± 0.2
Triton extract	1.3 ± 0.3
Total	1.0 ± 0.2

Radioactive Sup I and Triton extract were prepared as in section 2. Following precipitation with anti BF_1 and subsequent washing of the CRM, the amount of radioactivity was determined. The % radioactivity in the CRM refers to the fraction of radioactivity from either Sup I or Triton extract which was precipitated by the antibody (× 100). The total % radioactivity in CRM is the sum of cpm in CRM from each preparation divided by the sum of radioactivity in each extract prior to precipitation (× 100). The data are the average of 10 independent experiments

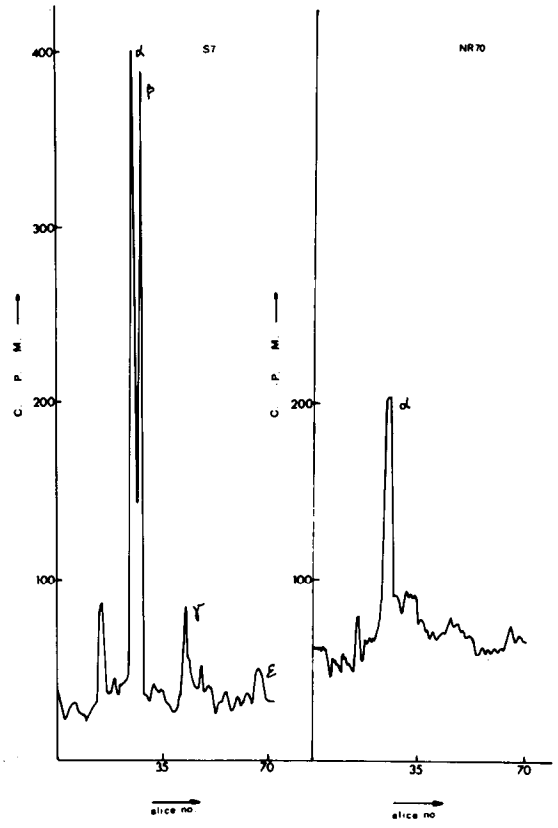

Fig.2. Distribution of radioactivity in CRM isolated from Triton extract of parental strain S7 and mutant NR70. Cells were grown in the presence of ^{14}C-labeled L-amino acid mixture and the CRM isolated from the Triton extract as in section 2. Solubilized Ab–CRM complex, 30 μl, from S7 (left-hand panel) containing 1600 cpm and 100 μl similar preparation from NR70 containing 600 cpm (right-hand panel) were loaded onto the gels and subjected to SDS–gel electrophoresis as in [12]. The gels were sliced and counted as in section 2.

antibody complexes were subjected to SDS–gel electrophoresis and the gels were sliced and counted. The results in the left-hand panel of fig.2 show the distribution of radioactivity in the CRM–antibody complex, isolated from a parental strain, S7. Four radioactive subunits, α, β, γ and ϵ were observed, and the apparent stoichiometry of the subunits was calculated from the distribution of radioactivity. When γ subunit was taken to be 1, the apparent stoichiometry was α: β: γ: ϵ, $2.4 \pm 0.4 : 2.4 \pm 0.4 : 1 : 1 \pm 0.5$, respectively. The right-hand panel of fig.2 shows the profile obtained when radioactive CRM from an ATPase-defective mutant, NR70 [8], was analyzed. This mutant which was derived from S7, yielded a total of only $15 \pm 5\%$ of the CRM found in the parent and therefore a much larger volume of material was placed on the gel. It can be seen, that in striking contrast to the parent, the mutant CRM contained only the α subunit. As illustrated in both fig.1 and 2, the δ subunit, mol. wt 18 500 [14], is missing from the parental preparation. However, when the cells were disrupted in the presence of 10 mM $MgSO_4$ prior to membrane preparation and subsequent extraction, the CRM which precipitated with anti BF_1 was found to contain the δ subunit (fig.3). Nevertheless, extraction in the presence of $MgSO_4$ yielded only 60–70% of the total CRM found in the absence of $MgSO_4$. These results may be in accordance with [15] concerning the importance of Mg^{2+} ions in the attachment of the δ subunit to the ATPase complex from *Streptococcus faecalis*. The procedure described here allows for the selective isolation of ATPase complexes without the necessity for measurement of catalytic activity, and can therefore be extended to include the analysis of mutants defective in ATPase activity. Selective antibody precipitation has been used [16] to estimate the bound nucleotide in the CRM of ATPase from mutants unc A (ATPase negative) [17] and unc B (ATPase positive) [17]. No significant difference was observed between the gel profiles of CRM from the two mutants or from the parent. Preliminary results confirm these findings. Furthermore, it is of interest that other ATPase mutants yield CRM preparations which vary both with respect to total amount of CRM as well as subunit stoichiometry (in preparation).

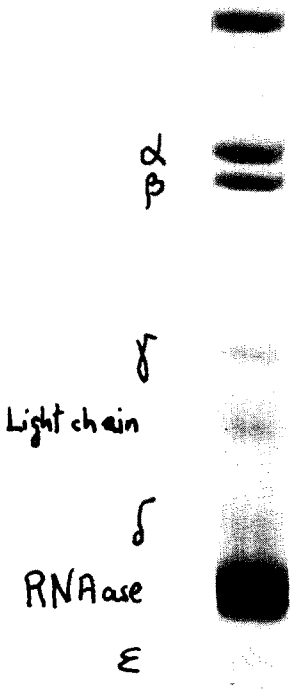

Fig.3. SDS gel of Ab–CRM complex from parental strain S7. CRM was isolated from Triton extract and subjected to SDS–gel electrophoresis as in fig.1 legend, except that the cells of S7 were disrupted in the presence of 10 mM $MgSO_4$. The gels were loaded with 20 μl solubilized Ab–CRM complex and stained with Coomassie brilliant blue.

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