The **uncA** Gene Codes for the α-Subunit of the Adenosine Triphosphatase of *Escherichia coli*

**ELECTROPHORETIC ANALYSIS OF uncA MUTANT STRAINS**

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Four mutant strains of *Escherichia coli* which lack membrane-bound adenosine triphosphatase activity have been isolated (Senior et al., 1979). Genetic complementation tests have been used to establish the link between the *uncA* gene and ATPase activity. The *uncA* gene codes for the α-subunit of the ATPase and the properties of the membranes vary depending on the particular mutant α-subunit present (Senior et al., 1979).

Evidence has been obtained (Dunn, 1978; Kanazawa et al., 1978) that the *uncA* gene codes for the α-subunit of the ATPase. Thus ATPase activity could be restored to the inactive F₁-ATPase from a mutant strain carrying the *uncA4401* allele by reconstitution of the dissociated complex with an excess of the normal α-subunit. The work described in the present paper confirms the conclusions of Dunn (1978) and Kanazawa et al. (1978) in that two of four strains carrying mutations in the *uncA* gene are found to form an α-subunit of abnormal net charge.

**Materials and Methods**

**Chemicals**

Poly(ethylene glycol) 6000 was obtained from BDH Chemicals, Poole, Dorset, U.K. All chemicals used were of the highest quality available.

**Organisms**

All the bacterial strains used were derived from *E. coli* K12 and are described in Table 1 or in the text.
Table 1. Strains of E. coli K12 used

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant genetic loci</th>
<th>Other information</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN249</td>
<td>uncA401, argH, entA</td>
<td>Cox et al. (1973)</td>
</tr>
<tr>
<td>AN346</td>
<td>ilvC, argH, pyrE, entA</td>
<td>Isolated from strain AN732 after treatment with N-methyl-N'-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>AN732</td>
<td>argH, pyrE, metE, entA</td>
<td>Isolated after transduction with strain G31 as donor and strain AN346 as recipient</td>
</tr>
<tr>
<td>G31</td>
<td>uncA447, argH, pyrE, metE, entA</td>
<td>Isolated after transduction with strain AN732 after treatment with N-methyl-N'-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>AN1111</td>
<td>uncA447, argH, pyrE, entA</td>
<td>Isolated after transduction with strain G65 as donor and strain AN346 as recipient</td>
</tr>
<tr>
<td>G65</td>
<td>uncA453, argH, pyrE, metE, entA</td>
<td>Isolated after transduction with strain AN732 after treatment with N-methyl-N'-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>AN1137</td>
<td>uncA453, argH, pyrE, entA</td>
<td>Isolated after transduction with strain G88 as donor and strain AN346 as recipient</td>
</tr>
<tr>
<td>G88</td>
<td>uncA450, argH, pyrE, metE, entA</td>
<td>Cox et al. (1978a); the plasmid pAN11 carried a normal unc operon</td>
</tr>
<tr>
<td>AN1113</td>
<td>uncA450, argH, pyrE, entA</td>
<td>Prepared by the method described by Gibson et al. (1977b)</td>
</tr>
<tr>
<td>AN862</td>
<td>F' (pAN11), ilvC, argH, pyrE, purE, recA, nalA</td>
<td></td>
</tr>
<tr>
<td>AN1164</td>
<td>F' (pAN11), uncA450, argH, pyrE, entA, recA, nalA</td>
<td></td>
</tr>
</tbody>
</table>

Other methods

Media and growth of organisms, genetic techniques, preparation of cell membranes, washing of cell membranes in low-ionic-strength buffer, assay of ATPase activity, determination of protein, two-dimensional gel electrophoresis and measurement of aetrin-fluorescence quenching are described in Senior et al. (1979).

Purification of F1-ATPase

The methods of Vogel & Steinhart (1976), Bragg & Hou (1972) and Futai et al. (1974) were used in attempts to purify F1-ATPase from partial diploid strain AN862 (unc+/-unc+) and from the normal strain AN248. None of these methods gave a pure preparation which was capable of reconstituting ATP-dependent aetrin-fluorescence quenching or ATP-dependent transhydrogenase activity in F1-ATPase-depleted membranes. This confirms the findings of others (Sternweis & Smith, 1977; Larson & Smith, 1977) that a given purification procedure may give varying results when applied to different E. coli strains, or to the same strain grown in different media. However, by combining features of these methods, a rapid method was devised, which can be used with equal confidence for the preparation of either normal F1-ATPase or inactive F1-ATPase from mutant strains. All steps were carried out at 4°C.

Step 1: solubilization of F1-ATPase. The F1-ATPase was solubilized by washing the membranes in low-ionic-strength buffer as described by Senior et al. (1979). Briefly, membranes were washed twice in low-ionic-strength buffer containing 5 mM-Tes, 15% (v/v) glycerol, 0.5 mM-dithiothreitol, 40 mM-6-aminoheptanolic acid and 6 mM-p-aminobenzamidine. As shown previously (Cox et al., 1978b) about 70% of the protein is washed from the membranes by this procedure, but the ATPase activity remains membrane-bound due to the presence of p-aminobenzamidine. It had been concluded (Cox et al., 1978b) that the effect of the p-aminobenzamidine was due to inhibition of a proteinase, but subsequent work has indicated that the retention of the F1-ATPase on the membrane is due to binding of the p-aminobenzamidine to the membrane-bound ATPase complex (J. A. Downie, unpublished observation). Subsequent washing twice with the same buffer system, but lacking p-aminobenzamidine, releases all the ATPase activity into the supernatant. The 6-aminoheptanolic acid, another known proteinase inhibitor, is included in the buffer system in order to stabilize the F1-ATPase (see Cox et al., 1978b).

Step 2: treatment with MgCl2 followed by precipitation with poly(ethylene glycol). Tes buffer (pH 7.0) was added to the crude soluble F1-ATPase preparation from a 1 M stock solution to give a final concentration of 50 mM, dithiothreitol was added from a 0.5 M stock solution to 1 mM and MgCl2 was added from a 1 M stock solution (pH 7.0) to 50 mM. The solution was centrifuged for 1 h at 8000g and the pellet discarded. Poly(ethylene glycol) (PEG 6000; 50%, w/v, in water) was added to give 10% (w/v) final concentration and the turbid solution was centrifuged for 1 h at 8000g. The white pellet was retained and dissolved in 'column buffer', which contained Tris/HCl (50 mM, pH 7.4), glycerol (10%, v/v), EDTA (2 mM), ATP (1 mM), dithiothreitol (1 mM) and 1979
6-aminohexanoic acid (40mM); 50 ml of column buffer was used to dissolve the protein obtained from 200g of cells. Just before use, phenylmethylsulphonyl fluoride was added from an ethanolic stock solution (40mM) to the column buffer to give 1mM final concentration. The solution was centrifuged for 15 min at 50000g and the brown pellet discarded.

**Step 3: Ion-exchange chromatography on Whatman DES2 cellulose.** The cellulose column (3.1 cm × 4.5 cm) was equilibrated with the column buffer described above. The enzyme solution was applied and 50 ml of column buffer was used to wash through unadsorbed proteins; 100 ml of column buffer fortified with 40 mM-Na₂SO₄ (pH 7.4) was applied and the effluent discarded. Then 200 ml of column buffer fortified with 80 mM-Na₂SO₄ (pH 7.4) was passed through the column and the eluate concentrated to about 5 ml using an Amicon Diaflo pressure filtration system and UM 10 membranes. The concentrate was centrifuged for 20 min at 40000g and the pellet discarded.

**Step 4: Gel filtration.** A Sepharose CL-6B column (2.5 cm × 95 cm) was equilibrated with column buffer fortified with 40 mM-Na₂SO₄ (pH 7.4), which was passed upwards through the column at a flow rate of 12-15 ml/h. Only one protein peak was obtained, eluting at about 270 ml. The peak fractions were concentrated by ultrafiltration as above. The enzyme was stored frozen at −20°C and remained stable for at least 3 months.

**Results**

**Characterization of uncA mutants by genetic complementation analysis**

Mutant strains carrying the uncA401 allele are unable to grow on succinate, have low aerobic growth yields, and lack membrane-bound ATPase activity (Butlin et al., 1971). Three new mutant strains unable to grow on succinate (G31, G65 and G88) were isolated after mutagenesis of strain AN732 with N-methyl-N'-nitro-N-nitrosoguanidine and found to have a similar phenotype to strains carrying the uncA401 allele. The three mutant alleles unc-447, unc-453 and unc-450 present in strains G31, G65 and G88 respectively, were transferred by cotransduction with the ilo genes into strain AN346 to give strains AN1111 (unc-447), AN1137 (unc-453) and AN1113 (unc-450). The unc-447, unc-453 and unc-450 alleles were then incorporated into F-plasmids using the method described previously (Gibson et al., 1977b). The resulting partial diploid strains were then used as donor strains in complementation tests with female strains carrying the uncA401, uncB402,

![Fig. 1. Atebrin-fluorescence quenching in membranes from various uncA mutant strains](image)

Atebrin-fluorescence quenching was measured as described by Gibson et al. (1977a). Conditions for reconstitution were as described by Senior et al. (1979), with about 1 mg of membrane protein and about 200µg of F₁-ATPase purified from strain AN862 (unc⁻/unc⁻). (a) Trace obtained with low-ionic-strength washed membranes from strain AN1111 (uncA447) prepared in the presence of p-aminobenzamidine. Similar results were obtained with membranes from strains AN249 (uncA401), AN1137 (uncA453) or AN1113 (uncA450). A similar trace was also obtained with membranes from strain AN1113 (uncA450) which had been subsequently washed in the absence of p-aminobenzamidine. (b) Trace obtained with membranes from strain AN1111 (uncA447) washed with low-ionic-strength buffer, but in the absence of p-aminobenzamidine. Similar results were obtained with membranes from strains AN249 (uncA401) or AN1137 (uncA453). (c) Trace obtained, after the reconstitution by normal F₁-ATPase, with membranes from strain AN1111 (uncA447) washed with low-ionic-strength buffer in the absence of p-aminobenzamidine. Similar reconstitution was obtained with membranes from strains AN249 (uncA401) or AN1137 (uncA453). No reconstitution of ATP-dependent atebrin-fluorescence quenching was obtained after addition of normal F₁-ATPase to low-ionic-strength washed membranes from strain AN1113 (uncA450), and the trace was essentially the same as that shown in (a).
Table 2. Purification of F1-ATPase from strain AN862 (unc+/unc+)

Details of the method of purification are given in the Materials and Methods section; 200 g of cells was used as starting material.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Protein (mg)</th>
<th>ATPase (μmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pooled low-ionic-strength washes</td>
<td>1265</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>Poly(ethylene glycol) precipitate</td>
<td>245</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>Concentrated 80 mM Na2SO4 eluate from the Whatman DE52 column</td>
<td>39</td>
<td>7.8</td>
</tr>
<tr>
<td>4</td>
<td>Sepharose CL-6B column; pooled active fractions after concentration</td>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. Gel filtration on a Sepharose CL-6B column of F1-ATPase prepared from membranes of strain AN862 (unc+/unc+) or strain AN1111 (uncA447)

Details of the procedure for purifying F1-ATPase are given in the Materials and Methods section, and the gel filtration, the elution profile of which is shown, is the final step. (a) Strain AN862 (unc+/unc+); (b) strain AN1111 (uncA447). The column was 2.5 × 95 cm and the elution volume of the protein peak was about 270 ml. ●, Protein expressed as A660 by the method of Lowry et al. (1951); ○, ATPase activity expressed as A660 by the method of Senior & Brooks (1970).

uncC424, uncD409 or uncE429 alleles as well as with female strains carrying the unc-447, unc-453 or unc-450 alleles. If genetic complementation occurs, colonies are formed on the succinate medium in the region where the donor and recipient strains mix in the cross-streaking test used. Complementation was observed between the unc-447, unc-453 or unc-450 alleles and the uncB402, uncC424, uncD409 or uncE429 alleles. Complementation was not observed between the new unc mutant alleles and the uncA401 allele, nor was complementation observed between the unc-447, unc-453 and unc-450 alleles themselves. It was concluded that the unc-447, unc-453 and unc-450 alleles affect the uncA gene and were accordingly designated uncA447, uncA453 and uncA450.

Reconstitution of ATP-dependent atebrin-fluorescence quenching

Membrane preparations from the normal strain AN248 retained ATPase activity and both NADH- and ATP-dependent atebrin-fluorescence quenching after washing in low-ionic-strength buffer in the presence of p-aminobenzamidine (Cox et al., 1978b). If these membranes were subsequently washed in low-ionic-strength buffer in the absence of p-aminobenzamidine then the membranes lost all three activities due to the removal of the F1-ATPase (Cox et al., 1978b).

Membrane preparations from strains AN249 (uncA401), AN1111 (uncA447), AN1137 (uncA453) and AN1113 (uncA450), were washed in low-ionic-strength buffer in the presence of p-aminobenzamidine. The washed membranes did not have ATP-induced atebrin-fluorescence-quenching activity, but retained the NADH-induced atebrin-fluorescence-quenching activity (Fig. 1). The addition of purified F1-ATPase from strain AN862 (unc+/unc+) did not reconstitute the ATP-dependent activity. If the membranes from strains AN249 (uncA401), AN1111 (uncA447) or AN1137 (uncA453) were subsequently washed in low-ionic-strength buffer in the absence of p-aminobenzamidine, then both the NADH- and ATP-dependent atebrin-fluorescence-quenching activities were absent; the addition of purified F1-ATPase from strain AN862 (unc+/unc+) reconstituted both activities (Fig. 1). However, if the membranes from strain AN1113 (uncA450) were also subsequently washed in the absence of p-aminobenzamidine they retained NADH-induced atebrin-fluorescence-quenching activity. Furthermore, the ATP-dependent activity could not be reconstituted by the addition of purified F1-ATPase from strain AN862 (unc+/unc+) (Fig. 1) (see below).

Purification of normal F1-ATPase and F1-ATPase from unc mutant strains

A summary of the yields and activities obtained when F1-ATPase was prepared from the partial diploid strain AN862 (unc+/unc+) is shown in Table 2. The specific activity of the ATPase is low compared with other published values (see Bragg & Hou, 1972; Vogel & Steinhardt, 1976; Futai et al., 1974). However, with respect to the reconstitution of energy-linked reactions the purified enzyme is as active, in terms of mg of protein required per mg of washed

1979
membranes, as any of the preparations referred to above (J. A. Downie, unpublished results). About 50 µg of purified F₁-ATPase gave 50% saturation of binding sites on 1 mg of F₁-ATPase-depleted normal membranes of the haploid strain AN248 (Downie et al., 1979). Two-dimensional gel electrophoresis showed five polypeptides corresponding to the α-, β-, γ-, δ- and ε-subunits of F₁-ATPase (see Fig. 3).

It was apparent from the atebrin-fluorescence-quenching data (see above) that strains AN1111 (uncA447) and AN1137 (uncA453) were similar to AN249 (uncA401) and that an inactive F₁-ATPase might be removed from the membranes of these strains by low-ionic-strength washing in the absence of p-aminobenzamidine. The method used for the solubilization and purification of normal F₁-ATPase was therefore used with membrane preparations from each of these three uncA mutant strains. A typical elution profile for the final step of the purification procedure (Sephadex CL-6B column) is shown in Fig. 2. The F₁-ATPase from each of the mutant strains had no detectable ATPase activity.

Analysis of purified F₁-ATPase from the uncA mutant strains by two-dimensional gel electrophoresis

Purified F₁-ATPase isolated from each of strains AN249 (uncA401), AN1111 (uncA447) and AN1137 (uncA453) was dissociated by heating at 100°C in the presence of sodium dodecyl sulphate and mercaptoethanol (Cox et al., 1978b) with protein concentrations of about 2.5 mg/ml. These samples were then analysed by two-dimensional gel electrophoresis. The β-, γ-, δ- and ε-subunits of the inactive ATPases from each of these uncA mutant strains had the same molecular weights and isoelectric points as the corresponding subunits from a normal F₁-ATPase (see Fig. 3). However, the α-subunit of the F₁-ATPase from strain AN1111 (uncA447) had the same molecular weight but a different isoelectric point to the α-subunit from a normal F₁-ATPase or from the other uncA mutants AN249 (uncA401) or AN1137 (uncA453) (Fig. 3). The α-subunits in strains AN249 (uncA401) and AN1137 (uncA453) are presumably abnormal, but the alteration does not result in a molecular weight, or charge, difference that can be detected by the methods used.

The apparent isoelectric point of the mutant uncA453 ‘α’-subunit is about 6.4, the same as the normal α-subunit, whereas the mutant uncA447 ‘α’-subunit has an apparent isoelectric point of about 6.3. The unlabelled arrow indicates a polypeptide which is found in purified F₁-ATPase preparations and which is used for reference.
Purified F₁-ATPase (15 μg of protein) from strain AN1164 (unc⁺/uncA450) was electrophoresed as described by Senior et al. (1979) over a pH range of about 7.5–4.5. The normal α-, β-, γ-, δ- and ε-subunits and the abnormal ‘α’-subunit are marked on the electrophoretogram. The abnormal ‘α’-subunit has an apparent isoelectric point of about 6.5. The unlabelled arrow indicates a polypeptide which is found in purified F₁-ATPase preparations and which is used for reference.

**Identification of an abnormal α-subunit in the purified F₁-ATPase from the partial diploid strain AN1164 (unc⁺/uncA450)**

The addition of purified F₁-ATPase from a normal strain to washed membranes from strain AN1113 (uncA450) does not result in the reconstitution of ATP-dependent atebrin-fluorescence quenching (see above). In this respect the washed membranes are similar to those from mutant strains carrying the uncD409 allele in which an abnormal β-subunit was tightly bound to the membrane (Fayle et al., 1978). However, this subunit could be solubilized from the membranes by a partial diploid strain carrying both the uncD409 allele and a normal uncD allele (Fayle et al., 1978). Membranes from the partial diploid strain AN1164 (unc⁺/uncA450) were therefore prepared and an F₁-ATPase aggregate was solubilized and purified by the method used for the normal F₁-ATPase. The purified F₁-ATPase from strain AN1164 (unc⁺/uncA450) had a specific activity of 8.5. The F₁-ATPase was dissociated as described above and the subunit structure analysed by two-dimensional gel electrophoresis. The β-, γ-, δ- and ε-subunits of the F₁-ATPase had the same molecular weights and isoelectric points as the corresponding subunits from a normal F₁-ATPase (Fig. 4). However, there were two polypeptides with the same molecular weight as the α-subunit, but with different isoelectric points. The mutant α-subunit had a higher isoelectric point than the normal α-subunit (Fig. 4).

**Discussion**

The presence of α-subunits with abnormal isoelectric points in strains AN1111 (uncA447) and AN1113 (uncA450) confirm that the uncA gene codes for the α-subunit of the ATPase (Dunn, 1978; Kanazawa et al., 1978). It would appear from the results with strains AN249 (uncA401) and AN1137 (uncA453) that mutations affecting the α-subunit leading to loss of ATPase activity do not necessarily result in an alteration of charge. This is in contrast with the effect of mutations in the uncD gene where it is notable that all five mutant strains so far examined form β-subunits with altered isoelectric points (Senior et al., 1979).

The properties of the membranes from strain AN1113 (uncA450) are similar to the properties of some uncD mutants in which abnormal β-subunits of the ATPase are attached to the membrane and cannot be removed by low-ionic-strength washing (Fayle et al., 1978; Senior et al., 1979). Such results emphasize the difficulty of determining, on the basis of biochemical studies, whether a particular mutation affects the F₀ or the F₁ portions of the ATPase (see Gibson et al., 1977b; Rosen et al., 1978). Thus, for example, the lack of reconstitution of ATP-dependent atebrin-fluorescence quenching by the addition of purified normal F₁-ATPase to washed membranes from strain AN1113 (uncA450) might lead to the incorrect conclusion that this particular mutation affected a component of the F₀ portion of the ATPase.

Analysis of membranes from strain AN1113 (uncA450), by two-dimensional gel electrophoresis, indicated that both the abnormal α-subunit and the normal β-subunit were retained on the membrane and could not be removed by low-ionic-strength washing (G. B. Cox, unpublished observations). It would appear therefore that the presence of this abnormal α-subunit prevents the loss of normal β-subunits from the membrane during low-ionic-strength washing, but abnormal β-subunits, as found in most uncD mutants, do not prevent the loss from the membrane of normal α-subunits (Senior et al., 1979).

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**References**

THE uncA GENE CODES FOR THE α-SUBUNIT OF F1-ATP-ASE


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