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Characterization of the Mutant-*uncD*-Gene Product in a Strain of *Escherichia coli* K12

AN ALTERED β-SUBUNIT OF THE MAGNESIUM ION-STIMULATED ADENOSINE TRIPHOSPHATASE

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Membranes from a mutant strain of *Escherichia coli* K12 carrying the *uncD409* allele were washed in low-ionic-strength buffers in the presence or absence of the proteinase inhibitor *p*-aminobenzamidine. Unlike membranes from a normal strain, those from strain AN463 (*uncD409*) did not become proton-permeable, as judged by NADH-induced atebrin-fluorescence quenching, when the membranes were washed in the absence of *p*-aminobenzamidine. Furthermore, ATP-dependent atebrin-fluorescence quenching in such washed membranes could not be reconstituted by the addition of solubilized Mg^{2+}-stimulated adenosine triphosphatase preparations. The examination by two-dimensional polyacrylamide-gel electrophoresis of the polypeptide composition of the washed membranes from strain AN463 (*uncD409*) indicated the presence of a polypeptide of similar molecular weight to the normal β-subunit of the Mg^{2+}-stimulated adenosine triphosphatase, but with an altered isoelectric point. Both the normal and abnormal β-subunits were identified in membranes prepared from a partial diploid strain carrying both the *unc*$^+$ and *uncD409* alleles. It is concluded that the *uncD* gene codes for the β-subunit of the Mg^{2+}-stimulated adenosine triphosphatase.

Mutants of *Escherichia coli* in which the Mg-ATPase complex is affected (*unc* mutants) have been isolated in many laboratories (see Simoni & Postma, 1975). Four *unc* genes (*A*, *B*, *C* and *D*) have been identified by genetic complementation tests by using strains diploid for the *unc* region of the chromosome (Gibson *et al.*, 1977*b*; Cox *et al.*, 1978*b*). Gene–polypeptide relationships have not yet been reported for any of these four genes. There have, however, been reports of altered or missing polypeptides in the membrane or in the F_1 portion of the Mg-ATPase in uncoupled mutant strains. Bragg *et al.* (1973) reported that a strain of *E. coli* isolated by Hong & Kaback (1972) and carrying the *etc-15* allele had an abnormal γ-subunit of the F_1 portion of the Mg-ATPase. Reconstitution experiments indicated, however, that the abnormal subunit was probably not responsible for the mutant phenotype. Simoni & Shandell (1975), working with an *E. coli* strain carrying an amber mutation in an unspecified *unc* gene, demonstrated the loss of a membrane polypeptide of about 54000 mol.wt.

In the present work, an altered β-subunit of the Mg-ATPase is identified in strains carrying the *uncD409* allele, and the effect of this altered β-subunit on the proton-permeability of the membrane is described.

Abbreviations used: Mg-ATPase, Mg^{2+}-stimulated adenosine triphosphatase (EC 3.6.1.3); Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and Methods

Materials

Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Eastman Kodak, Rochester, NY, U.S.A. Sodium dodecyl sulphate (especially pure) and Nonidet P4 were from BDH Chemicals, Poole, Dorset, U.K. Coomassie Blue R250 was from Serva, Heidelberg, Germany. *p*-Aminobenzamidine hydrochloride was from Sigma Chemical Co., St. Louis, MO, U.S.A., and ε-amino-n-hexanoic acid was from Fluka A. G., Buchs, Switzerland. Ampholines were from LKB Produkter AB, Bromma, Sweden. All chemicals were of the highest purity available commercially and were not further purified.

Organisms

The strains used were all derivatives of *E. coli* K12 and are described in Table 1.

Media and growth of organisms

The mineral-salts minimal medium used and additions, unless otherwise indicated, were as described previously (Gibson *et al.*, 1977*b*).

Table 1. *Strains of E. coli K12 used*
The gene designations follow those of Bachmann *et al.* (1976) and the plasmid nomenclature is that of Novick *et al.* (1976).

Bacterial strain	Relevant genetic loci	Other information
AN248	F⁻,*ilvC,argH,entA*	Butlin *et al.* (1973)
AN463	F⁻,*uncD409,argH, entA*	Isolated after transduction with strain M54 (Cox *et al.*, 1978*b*) as donor and strain AN248 as recipient
AN821	F′(pAN7),*ilvC, argH, pyrE, entA,recA,nalA*	Isolation described previously (Cox *et al.*, 1978*b*)
Plasmid		
pAN7	*uncD409,ilvC*⁺, *argH*⁺,*pyrE*⁺	Cox *et al.* (1978*b*)

Cells for the preparation of membranes were grown in 14-litre New Brunswick Fermenters essentially as described by Cox *et al.* (1970). The inoculum was grown in 1 litre of glucose-mineral salts medium at 37°C overnight with shaking. The inoculum was then added to 10 litres of medium in the fermenter and incubated at 37°C with aeration until the cells were in mid-exponential phase. The medium in the fermenter vessels was supplemented with 5% (v/v) Luria broth (Luria & Burrous, 1957).

Preparation of cell membranes

Membranes were prepared as described previously (Cox *et al.*, 1973) except that ε-amino-n-hexanoic acid (40mM) was included in all buffers. Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator, cell debris was removed by centrifugation, and the membranes were separated by ultracentrifugation. The membranes were then resuspended in 0.1M-Tes buffer, pH7.0, containing magnesium acetate, sucrose, EGTA and ε-amino-n-hexanoic acid. The proteinase inhibitor *p*-aminobenzamidine was added to buffers as indicated at a final concentration of 6mM, including the Tes buffer system in which the cells were suspended before disintegration.

Solubilization of Mg-ATPase

Membrane-bound Mg-ATPase was solubilized, as described previously (Cox *et al.*, 1978*a*), by successive washes of the membrane preparations by low-ionic-strength buffer containing ε-amino-n-hexanoic acid. The washes were pooled and concentrated by using a Diaflo XM-50 filter. If the buffers contained *p*-aminobenzamidine, the Mg-ATPase was solubilized, as described previously (Cox *et al.*, 1978*a*), by the chloroform treatment described by Beechey *et al.* (1975).

Determination of protein

Protein concentrations were determined by using Folin's phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Assay of Mg-ATPase

Assays for Mg-ATPase activity were carried out as described previously (Gibson *et al.*, 1977*a*).

Measurement of atebrin fluorescence

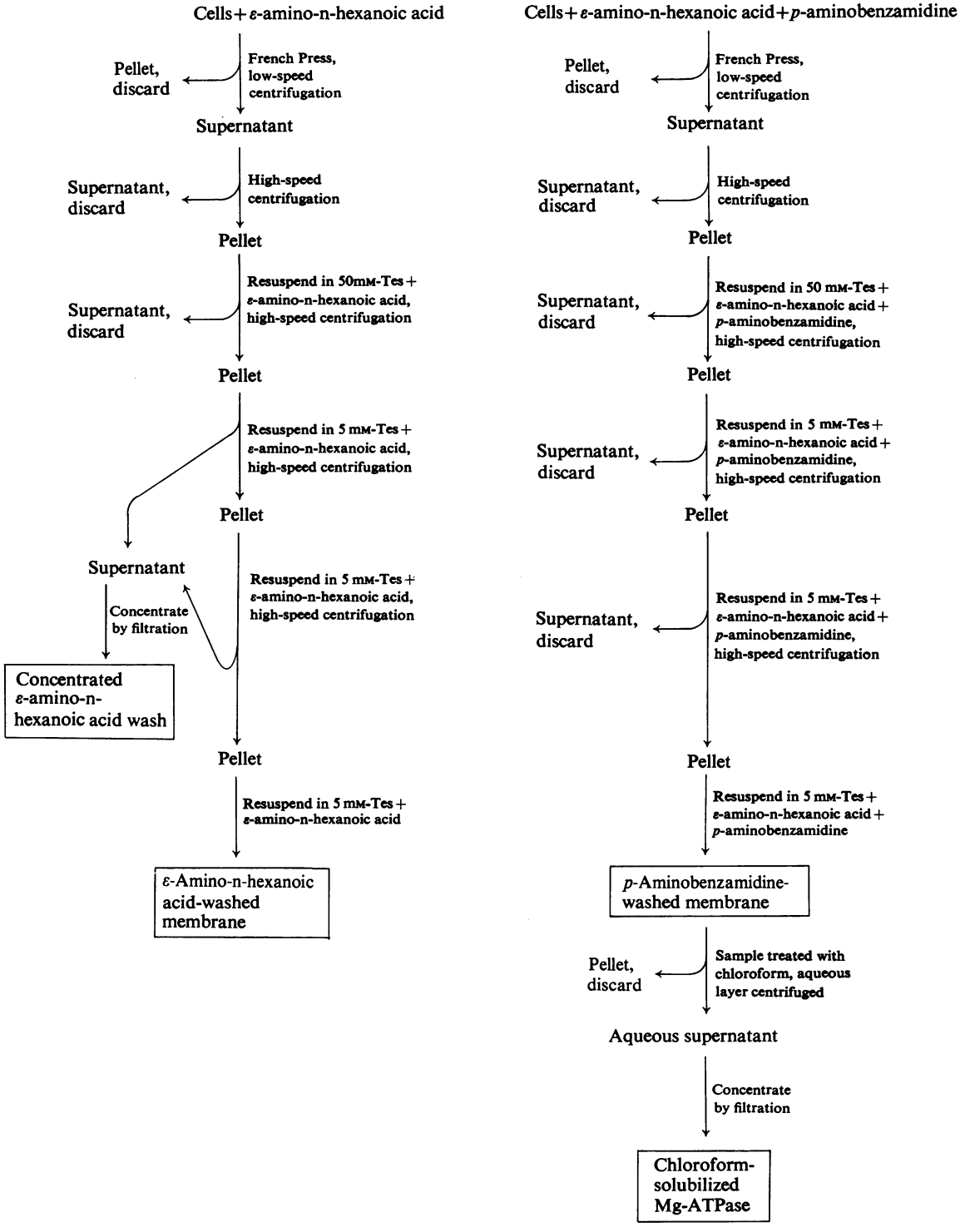
Atebrin fluorescence was measured at 30°C as described by Gibson *et al.* (1977*a*).

Reconstitution of atebrin-fluorescence quenching

Electron-transport- and ATP-induced atebrin-fluorescence quenching were reconstituted by mixing ε-amino-n-hexanoic acid-solubilized Mg-ATPase [5mg of protein containing 5.5 units of Mg-ATPase activity (1 unit of activity hydrolyses 1 μmol/min)] with ε-amino-n-hexanoic acid-washed membranes (about 2mg of protein). After incubation for 10min at 30°C the volume was made up to 2.5ml by the addition of 10mM-Hepes buffer containing 300mM-KCl and 20mM-$MgCl_2$.

Gel electrophoresis

The polypeptide compositions both of washed membrane preparations and of soluble protein fractions were analysed by two-dimensional electrophoresis under dissociating conditions, by the method of O'Farrell (1975) with some modifications. Briefly, proteins were solubilized by using sodium dodecyl sulphate and mercaptoethanol (Cox *et al.*, 1978*a*), and applied to a cylindrical isoelectric-focusing gel. The proteins, separated according to isoelectric point, were then electrophoresed into a polyacrylamide-gradient slab gel containing sodium dodecyl sulphate, and separated according to molecular weight. In the first dimension, Ampholines of pH range 4–6 and pH range 3.5–10 were present at 1.6% (w/v) and 0.4% respectively. The isoelectric-focusing gels were not pre-run; samples were electrophoresed at an initial current of 1mA/tube for 3h, during which time the voltage increased to 500V. The voltage was then set at 600V and electrophoresis continued for a further 3h. To determine the pH gradient in a particular gel, 5mm sections were added to 2.5ml of water, mixed and the pH was measured. Focusing gels were shaken in 0.12M-Tris/HCl, pH6.8, containing 2% (w/v) sodium dodecyl sulphate for 15min, before they were sealed to the second-dimension slab by using 1% agarose dissolved in 'running buffer'. An acrylamide gradient



Scheme 1. *Flow diagram for membrane fractionation*
Details are given in the Materials and Methods section and in Cox *et al.* (1978*a*).

of 7.5–22.5% (w/v), formed as described previously (Cox *et al.*, 1978*a*), was used in the second dimension, and the gels were run at 14mA for 13.5h without a dye marker. Proteins were stained with Coomassie Blue R250 (Cox *et al.*, 1978*a*).

Results

Fractionation of membranes

Membranes from strains AN463 (*uncD409*), AN821 (*uncD409/unc*$^+$) and AN248 (*unc*$^+$) were fractionated as outlined in Scheme 1. The fractionation procedure is based on the observation that the presence of the proteinase inhibitor *p*-aminobenzamidine prevented the solubilization, by low-ionic-strength washing, of the membrane-bound Mg-ATPase activity from strain AN248 (*unc*$^+$) (Cox *et al.*, 1978*a*). The Mg-ATPase activity could be subsequently solubilized by the chloroform-treatment method of Beechey *et al.* (1975). Membranes from strain AN463 (*uncD409*) were fractionated by the same procedure, even though these membranes did not have Mg-ATPase activity. The specific activity and percentage recovery of the Mg-ATPase in each of the four fractions (see Scheme 1) obtained from strains AN248 (*unc*$^+$) and AN821 (*uncD409/unc*$^+$) are summarized in Table 2.

In the presence of *p*-aminobenzamidine the membranes from the diploid strain AN821 (*uncD409/unc*$^+$) lost more Mg-ATPase activity during washing than did the membranes from strain AN248 (*unc*$^+$). The recovery of Mg-ATPase activity after chloroform treatment was also lower for the diploid strain (Table 2).

Atebrin-fluorescence quenching

As observed previously (Cox *et al.*, 1978*a*), membranes prepared from the normal strain AN248 become permeable to protons after ε-amino-n-hexanoic acid washing, as judged by the lack of NADH-induced atebrin-fluorescence quenching (Fig. 1*b*). Such membranes have also lost Mg-ATPase activity (Table 2) and ATP-induced atebrin-fluorescence quenching (Fig. 1*b*). The solubilization of the Mg-ATPase and concomitant increase in proton permeability can be prevented by preparation and washing of the membranes in the presence of the proteinase inhibitor *p*-aminobenzamidine (Fig. 1*a*, Table 2).

In contrast, neither ε-amino-n-hexanoic acid-washed nor *p*-aminobenzamidine-washed membranes prepared from strain AN463 (*uncD409*) are proton-permeable, as judged by the NADH-dependent atebrin-fluorescence quenching (Figs. 1*d* and 1*e*). As reported previously (Cox *et al.*, 1978*b*), membranes prepared from strains carrying the *uncD409* allele lack Mg-ATPase activity. Furthermore, ATP-dependent atebrin-fluorescence quenching in ε-amino-n-hexanoic acid-washed membranes prepared from strain AN463 (*uncD*$^-$) cannot be reconstituted by the addition of the concentrated ε-amino-n-hexanoic acid wash, containing Mg-ATPase activity, obtained from strain AN248 (*unc*$^+$) membranes (Fig. 1*f*). The same solubilized Mg-ATPase preparation reconstitutes both NADH- and ATP-dependent atebrin-fluorescence quenching with ε-amino-n-hexanoic acid washed membranes from the normal strain AN248 (Fig. 1*c*).

The *p*-aminobenzamidine-washed membranes prepared from the diploid strain AN821 (*uncD*$^-$*/unc*$^+$) have normal NADH-induced atebrin-fluorescence quenching, but the ATP-induced fluorescence quenching is lower than in a normal strain (Fig. 1*g*). The ε-amino-n-hexanoic acid-washed membranes prepared from strain AN821 (*uncD*$^-$*/unc*$^+$) have lost both the ATP- and NADH-induced atebrin-fluores-

Table 2. *Mg-ATPase activities and recoveries in membrane fractions from strains of Escherichia coli*

Percentage recoveries are expressed relative to values obtained for Mg-ATPase activities of membranes washed in the 50mM-Tes buffer system (see Scheme 1).

	Mg-ATPase in membrane fractions from strain AN821 (*uncD409/unc*$^+$)		Mg-ATPase in membrane fractions from strain AN248 (*unc*$^+$)	
Membrane fraction	Specific activity (μmol of P_i/min per mg of protein)	Percentage recovery	Specific activity (μmol of P_i/min per mg of protein)	Percentage recovery
ε-Amino-n-hexanoic acid-washed membranes	0.06	7	0.14	3
ε-Amino-n-hexanoic acid-washed membranes	0.37	49	1.6	70
Concentrated ε-amino-n-hexanoic acid washes	0.51	60	1.1	57
Chloroform-solubilized Mg-ATPase	0.42	12	11	40

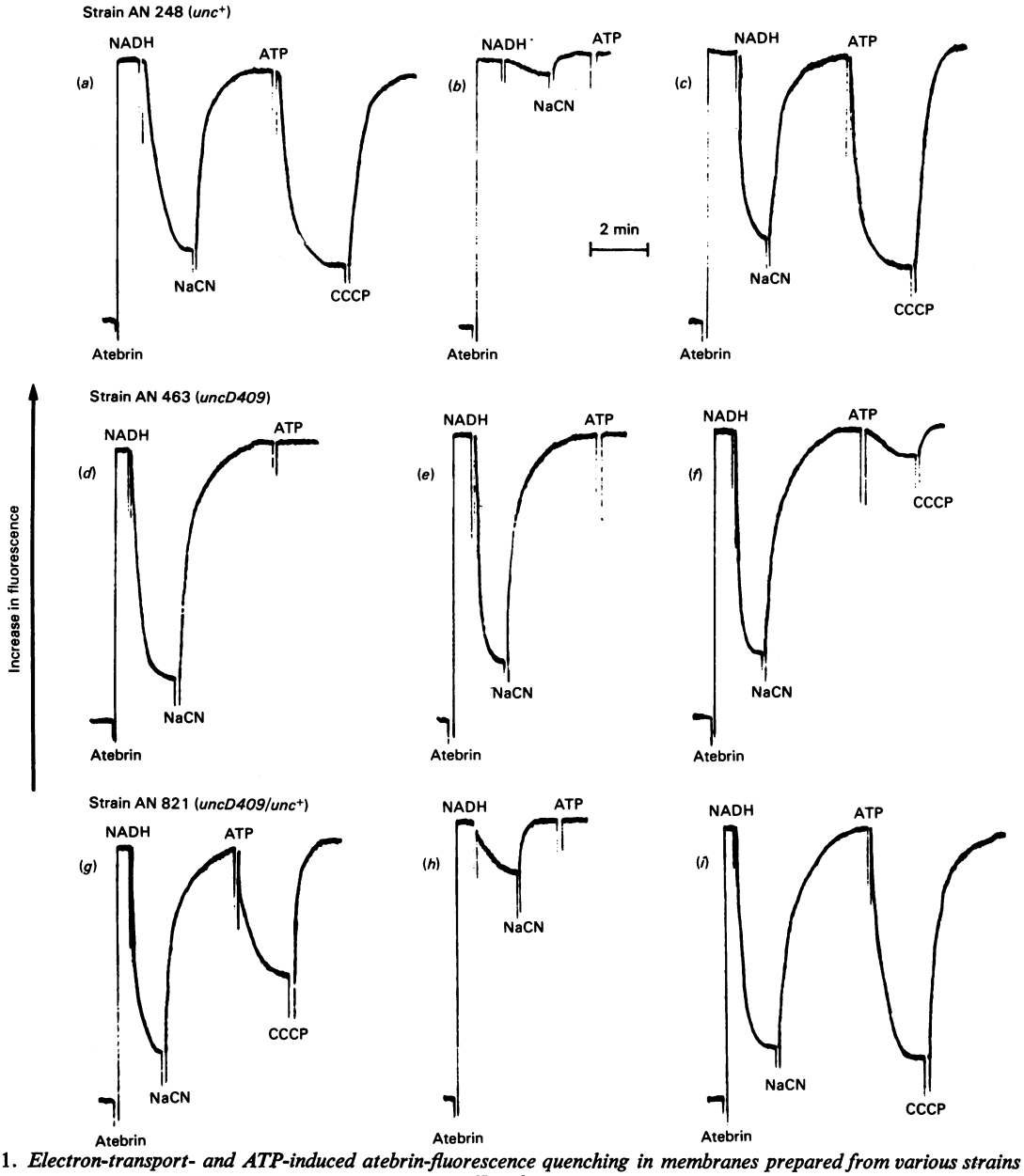


Fig. 1. *Electron-transport- and ATP-induced atebrin-fluorescence quenching in membranes prepared from various strains of E. coli*

Atebrin-fluorescence quenching was measured as described previously (Gibson *et al.*, 1977*a*). Conditions of reconstitutions are as described in the Materials and Methods section. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to 20 μM. (*a*) *p*-Aminobenzamidine-washed membranes from strain AN248 (*unc*+). (*b*) ε-Amino-n-hexanoic acid-washed membranes from strain AN248 (*unc*+). (*c*) ε-Amino-n-hexanoic acid-washed membranes from strain AN248 (*unc*+) reconstituted with ε-amino-n-hexanoic acid-solubilized Mg-ATPase prepared from strain AN248 (*unc*+). (*d*) *p*-Aminobenzamidine-washed membranes from strain AN463 (*uncD409*). (*e*) ε-Amino-n-hexanoic acid-washed membranes from strain AN463 (*uncD409*). (*f*) ε-Amino-n-hexanoic acid-washed membranes from strain AN463 (*uncD409*) reconstituted with ε-amino-n-hexanoic acid-solubilized Mg-ATPase prepared from strain AN248 (*unc*+). (*g*) *p*-Aminobenzamidine-washed membranes from strain AN821 (*uncD409/unc*+). (*h*) ε-Amino-n-hexanoic acid-washed membranes from strain AN821 (*uncD409/unc*+). (*i*) ε-Amino-n-hexanoic acid washed membranes from strain AN821 (*uncD409/unc*+) reconstituted with ε-amino-n-hexanoic acid-solubilized Mg-ATPase prepared from strain AN248 (*unc*+).

cence quenching (Fig. 1*h*). Furthermore, normal reconstitution of both NADH- and ATP-induced atebrin-fluorescence quenching occurs after addition of the concentrated ε-amino-n-hexanoic acid wash from strain AN248 (*unc*$^+$) to the ε-amino-n-hexanoic acid-washed membranes from strain AN821 (*uncD*$^-$/*unc*$^+$)

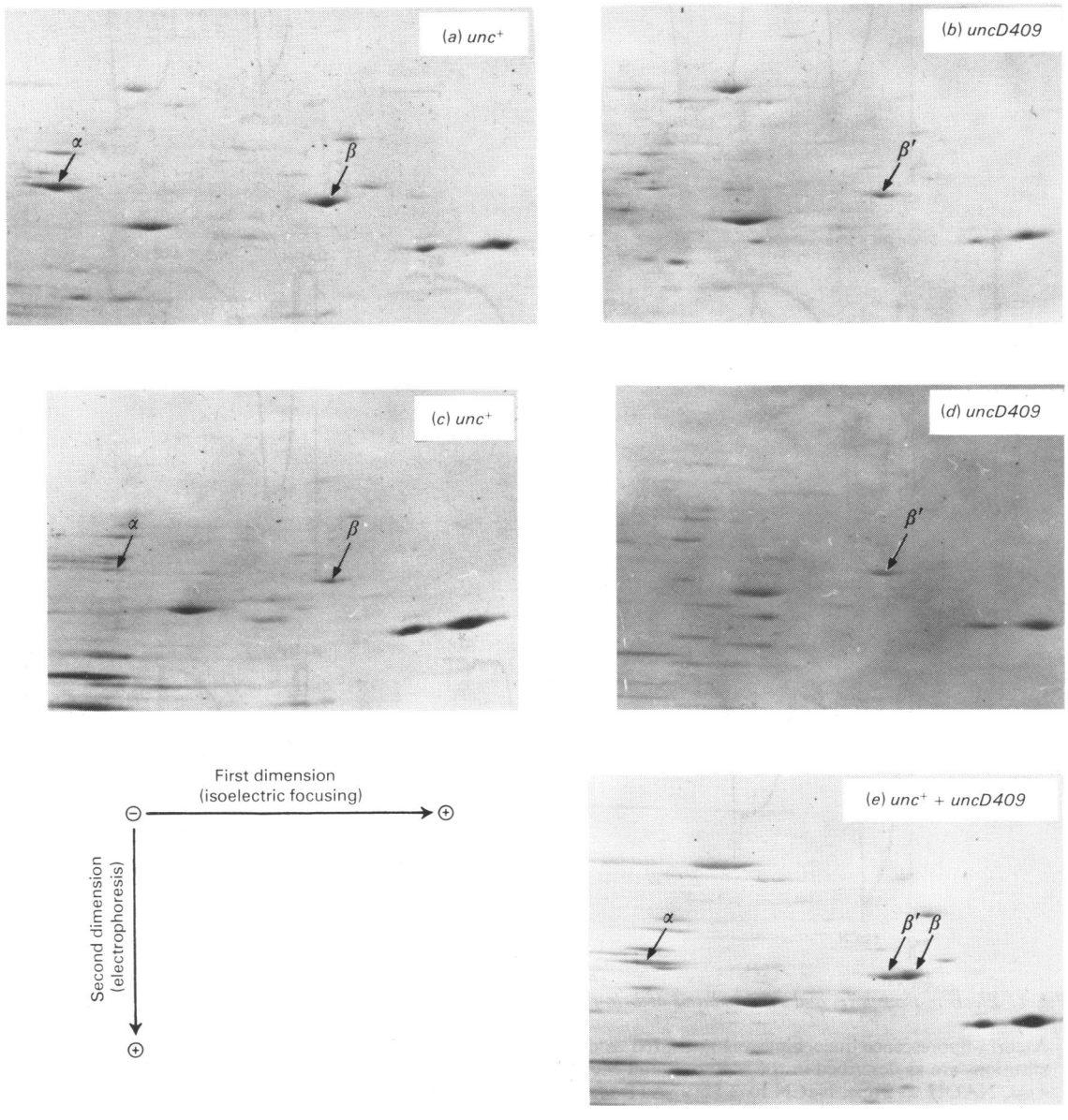


Fig. 2. *Two-dimensional gel electrophoresis of washed membranes from strains AN*248 (*unc*$^+$) *and AN*463 (*uncD*409) (*a*) *p*-Aminobenzamidine-washed membranes of strain AN248 (92 μg of protein); (*b*) *p*-aminobenzamidine-washed membranes of strain AN463 (108 μg of protein); (*c*) ε-amino-n-hexanoic acid-washed membranes of strain AN248 (134 μg of protein); (*d*) ε-amino-n-hexanoic acid-washed membranes of strain AN463 (135 μg of protein); (*e*) mixture of *p*-aminobenzamidine-washed membranes from strain AN248 (92 μg of protein) and strain AN463 (123 μg of protein). The section of gel shown encompasses a pH range from left to right of about 6.5 to 4.8 and a molecular-weight range from top to bottom of about 170000 to 23000. The normal α- and β-subunits of the Mg-ATPase and the abnormal β-subunit are indicated by the arrows marked α, β and β′ respectively.

(Fig. 1*i*). A similar reconstitution to that shown in Fig. 1(*i*) could also be achieved by using the concentrated ε-amino-n-hexanoic acid wash from strain AN821 (*uncD*$^-$/*unc*$^+$). Strain AN821 (*uncD*$^-$/*unc*$^+$) is also similar to strain AN248 (*unc*$^+$) in that the *p*-aminobenzamidine-washed membranes from strain AN821 are not proton-permeable and have retained Mg-ATPase activity.

Polypeptide composition of washed membranes

Since ε-amino-n-hexanoic acid-washed membranes from strain AN463 (*uncD409*) were impermeable to protons, and solubilized Mg-ATPase from the normal strain AN248 could not reconstitute ATP-dependent membrane energization, the polypeptide compositions of the washed membranes from strain AN463 (*uncD409*) and strain AN248 (*unc*$^+$) were investigated.

Membranes from the normal strain AN248, washed in the presence of *p*-aminobenzamidine, retain Mg-ATPase activity (Table 2). As expected, the polypeptides of these membranes, dissociated and analysed by two-dimensional electrophoresis, include the α- and β-subunits of the Mg-ATPase complex (Fig. 2*a*). The identification of the α- and β-polypeptides in the gels has been determined by the electrophoresis of a mixture of the membranes and a purified Mg-ATPase preparation (results not shown). The ε-amino-n-hexanoic acid-washed membranes have lost most of their Mg-ATPase activity (Table 2) and, correspondingly, only small amounts of the α- and β-subunits of the Mg-ATPase are observed in the gels after two-dimensional electrophoresis (Fig. 2*c*).

The polypeptides in *p*-aminobenzamidine-washed membranes of strain AN463 (*uncD409*) do not include the α- or β-subunits of normal Mg-ATPase (Fig. 2*b*). There is, however, a polypeptide with the same apparent molecular weight to the β-subunit, but with an apparent isoelectric point of pH 5.45, rather than pH 5.35, which is the apparent isoelectric point of a normal β-subunit (Fig. 2*b*). The difference

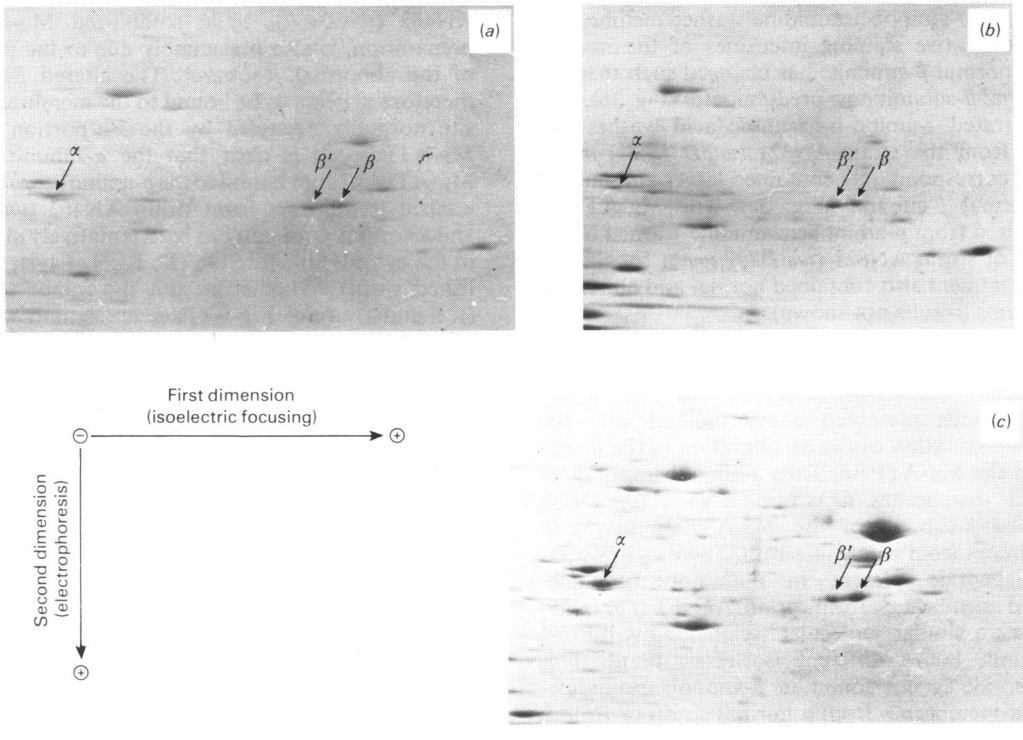


Fig. 3. *Two-dimensional gel electrophoresis of washed membranes and of the ε-amino-n-hexanoic acid wash from the partial diploid strain AN821 (uncD409/unc*$^+$)

(*a*) *p*-Aminobenzamidine-washed membranes (106 μg of protein); (*b*) ε-amino-n-hexanoic acid-washed membranes (144 μg of protein); (*c*) concentrated ε-amino-n-hexanoic acid wash (101 μg of protein). The section of gel shown encompasses a pH range from left to right of about 6.5 to 4.8 and a molecular-weight range from top to bottom of about 170000 to 23000. The normal α- and β-subunits of the Mg-ATPase and the abnormal β-subunit are indicated by the arrows marked α, β and β′ respectively.

between the isoelectric points was confirmed by electrophoresis of a mixture of washed membranes from strains AN463 (*uncD409*) and AN248 (*unc*$^+$) (Fig. 2*e*). Furthermore, as would be expected from the atebrin-fluorescence-quenching data, the abnormal β-subunit was also present in the ε-amino-n-hexanoic acid-washed membranes prepared from strain AN463 (*uncD409*) (Fig. 2*d*).

Gel electrophoresis of *p*-aminobenzamidine-washed membranes from another mutant strain lacking Mg-ATPase activity (AN249; *uncA401*) showed no detectable polypeptide in the position of the abnormal β-subunit from strain AN463 (*uncD409*). They did, however, contain a β-subunit indistinguishable from that of a normal Mg-ATPase (gels not shown).

The *p*-aminobenzamidine-washed membranes of the diploid strain AN821 (*uncD*$^-$/*unc*$^+$) contain both the normal and abnormal β-subunits of the Mg-ATPase (Fig. 3*a*). The normal β-subunit appeared to be present at a higher concentration than the abnormal β-subunit, as judged by the intensity of staining in the gels (Fig. 3*a*). As expected from the fluorescence-quenching data, the amount of the β-subunit is less in the ε-amino-n-hexanoic acid-washed membranes than in the *p*-aminobenzamidine-washed membranes, but the relative staining intensities of the normal and abnormal β-subunits has changed such that the abnormal β-subunit now predominates (Fig. 3*b*). The concentrated ε-amino-n-hexanoic acid washes obtained from the strain-AN821 (*uncD*$^-$/*unc*$^+$) membranes correspondingly contained larger amounts of the normal β-subunit (Fig. 3*c*). The Mg-ATPase solubilized from *p*-aminobenzamidine-washed membranes of strain AN821 (*uncD409*/*unc*$^+$) by chloroform treatment also contained normal and abnormal β-subunits (results not shown).

Discussion

The results presented above indicate that the *uncD409* mutation causes an alteration in the β-subunit of the Mg-ATPase. Thus *p*-aminobenzamidine-washed membranes of strain AN463 (*uncD409*) lack the β-subunit of the Mg-ATPase found in membranes from a normal strain. There is, however, a polypeptide present in *p*-aminobenzamidine-washed membranes from strain AN463 (*uncD409*) that has a similar molecular weight to the normal β-subunit, but a different isoelectric point. This polypeptide is not found in *p*-aminobenzamidine-washed membranes from a normal strain or from a mutant strain (AN249) which lacks Mg-ATPase activity owing to a mutation in the *uncA* gene. Furthermore, *p*-aminobenzamidine-washed membranes from the partial diploid strain AN821 (*uncD409*/*unc*$^+$) contain both the normal and abnormal β-subunits.

The abnormal β-subunit did not appear to be removed from membranes of strain AN463 (*uncD409*) by washing in low-ionic-strength buffer in the absence of *p*-aminobenzamidine, nor could it be solubilized by chloroform treatment. Both the normal and abnormal subunits were present in the ε-amino-n-hexanoic acid wash from the diploid strain AN821 (*uncD409*/*unc*$^+$) and were also solubilized by chloroform treatment of the *p*-aminobenzamidine-washed membranes of this diploid strain. It therefore appears likely that in the diploid strain membrane-bound Mg-ATPase aggregates are formed which contain a mixture of abnormal and normal β-subunits. These Mg-ATPase aggregates behave normally (Cox *et al.*, 1978*a*) with respect to solubilization by low-ionic-strength washing in the presence or absence of *p*-aminobenzamidine.

The lack of proton permeability in ε-amino-n-hexanoic acid-washed membranes from strain AN463 (*uncD409*), as compared with similar membranes from the normal strain AN248, is presumably due to the retention on the membrane of the abnormal β-subunit. In addition, the lack of reconstitution of ATP-dependent fluorescence quenching in ε-amino-n-hexanoic acid-washed membranes from strain AN463 (*uncD409*), by a solubilized Mg-ATPase preparation, is also presumably due to the presence of the abnormal β-subunit. The altered β-subunit therefore appears to be bound to the membranes at a site normally occupied by the F_1 portion of the Mg-ATPase. It is clear that the α-subunit of the Mg-ATPase is not bound to the *p*-aminobenzamidine-washed membranes from strain AN463 (*uncD409*) and a normal α-subunit has been tentatively identified in the cytoplasmic fraction (D. R. H. Fayle, unpublished work). Whether or not the minor subunits (γ, δ and ε) of the Mg-ATPase are bound with the altered β-subunit to the membrane has not yet been determined.

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