Properties of Membranes from Mutant Strains of *Escherichia coli* in which the β -Subunit of the Adenosine Triphosphatase is Abnormal

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Five uncoupled mutant strains of *Escherichia coli* carrying mutations in the *uncD* gene have been studied. In each of these mutant strains the β -subunit of the F_1 portion of the membrane-bound adenosine triphosphatase is abnormal. In one of the mutant strains (carrying the *uncD412* allele) an F_1 -ATPase aggregate was formed which was purified and found to have low ATPase activity. ATPase activity was absent in the other four strains and the abnormal β -subunits were tightly bound to the membranes. However, membranes from these strains exhibited various proton permeabilities as indicated by NADH-dependent atebirin-fluorescence quenching and bound different amounts of normal F_1 -ATPase. The amounts of reconstitution of energy-linked reactions after the addition of normal F_1 -ATPase also varied depending on the mutant allele. It is apparent that considerable phenotypic variations can occur between strains carrying mutations in the same *unc* gene.

A mutant strain of *Escherichia coli*, in which oxidative phosphorylation is uncoupled from electron transport due to a mutation in the *uncD* gene (the *uncD409* allele), has recently been described (Cox *et al.*, 1978a). This strain fails to grow on succinate, has a low aerobic growth yield on limiting concentrations of glucose, and membrane preparations lack ATPase activity. The *uncD* gene is a component gene of the *unc* operon, the known genes of which are transcribed in the order *uncBEADC* (Gibson *et al.*, 1978; Downie *et al.*, 1979). It has also been shown that the *uncD* gene codes for the β -subunit of the ATPase since strains carrying the *uncD409* allele form a β -subunit of abnormal net charge (Fayle *et al.*, 1978).

The present paper describes the characterization of four new mutant *uncD* alleles. Membrane preparations from strains carrying these alleles have properties different from those previously described for membranes from strains carrying the *uncD409* allele.

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

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Materials and Methods

Chemicals

Acrylamide, *NN'*-methylenebisacrylamide and *NNN'*-tetramethylethylenediamine were from Eastman Kodak, Rochester, NY, USA. Sodium dodecyl sulphate (especially pure) and Nonidet P40 were from BDH Chemicals, Poole, Dorset, U.K. Coomassie Blue R250 was from Serva, Heidelberg, Germany. Ampholines were from LKB Produkter AB, Bromma, Sweden. All chemicals were of the highest purity available commercially and were not further purified.

Organisms

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

Media and growth of organisms

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

Cells for the preparation of membranes were grown in 14-litre New Brunswick Fermenters essentially as described previously (Cox *et al.*, 1970). The inoculum was grown in 1 litre of glucose/mineral-salts medium at 37°C with aeration until the cells were in mid-exponential phase. The medium in the

Table 1. *Strains of E. coli K12 used*

Bacterial strain	Relevant genetic loci	Other information
AN249	<i>uncA401, argH, entA</i>	Cox <i>et al.</i> (1973b)
AN285	<i>uncD405, argH, entA</i>	Cox <i>et al.</i> (1974)
AB3311	<i>metB</i>	Hfr Reeves
AN181	<i>metB, uncD406</i>	Isolated from strain AB3311 after treatment with <i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
AN248	<i>ilvC, argH, entA</i>	Butlin <i>et al.</i> (1973)
AN342	<i>uncD406, argH, entA</i>	Isolated after transduction with strain AN181 as donor and strain AN248 as recipient
M54	<i>uncD409</i>	Cox <i>et al.</i> (1978a)
AN463	<i>uncD409, argH, entA</i>	Isolated after transduction with strain M54 as donor and strain AN248 as recipient
AB259		Hfr Hayes
M75	<i>uncD412</i>	Isolated from strain AB259 after treatment with <i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
AN484	<i>uncD412, argH, entA</i>	Isolated after transduction with strain M75 as donor and strain AN248 as recipient
AN357	<i>metE, argH, entA</i>	
D1275	<i>uncD427, metE, argH, entA</i>	Isolated from strain AN357 after treatment with <i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
AN346	<i>ilvC, argH, pyrE, entA</i>	Gibson <i>et al.</i> (1977a)
AN934	<i>uncD427, argH, pyrE, entA</i>	Isolated after transduction with strain D1275 as donor and strain AN346 as recipient
AN875	<i>unc-413::Mu argH, pyrE, entA</i>	Gibson <i>et al.</i> (1978)
AN862	<i>F' (pANII), ilvC, argH, pyrE, purE, recA, nalA</i>	Cox <i>et al.</i> (1978a)

fermenter vessels was supplemented with 5% (v/v) Luria broth (Luria & Burrous, 1957).

Solid media were prepared by the addition of 2% (w/v) agar to the medium described above. For agar plates containing succinate as sole source of carbon, glucose was replaced by sodium succinate (30 mM) and, in addition to the appropriate growth requirements, the medium was supplemented with 0.06% acid-hydrolysed casein (Difco, Detroit, MI, U.S.A.).

The nutrient broth used for genetic experiments was Luria broth, with glucose (10 mM) included.

Growth yields were measured as described previously (Cox *et al.*, 1970) in glucose/mineral-salts medium supplemented with Luria broth 5% (v/v). Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Genetic techniques

The techniques used for genetic experiments were as outlined previously (Gibson *et al.*, 1977a,b).

Preparation of cell membranes

Membranes were prepared as described previously (Cox *et al.*, 1973a). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator, cell debris removed by centrifugation and the membranes separated by ultracentrifugation. The membranes were then resuspended in a 0.1 M-Tes buffer system (pH 7.0) containing magnesium acetate, sucrose and EGTA. The proteinase inhibitors 6-

aminohexanoic acid and *p*-aminobenzamidine were included at final concentrations of 40 mM and 6 mM respectively (Cox *et al.*, 1978b). All procedures were carried out at 4°C.

Washing of cell membranes in low-ionic-strength buffer

All steps were carried out at 4°C. Membranes were washed first in a buffer containing Tes (50 mM), glycerol (15%, v/v), 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM), and collected by ultracentrifugation (254 000g for 2 h, Beckman 60 Ti rotor). The membranes were then washed twice in low-ionic-strength buffer [5 mM-Tes/15% (v/v) glycerol/0.5 mM-dithiothreitol/40 mM-6-aminohexanoic acid] which contained *p*-aminobenzamidine (6 mM). 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. Subsequently, washing twice with low-ionic-strength buffer which does not contain *p*-aminobenzamidine releases all the ATPase activity into the supernatant. 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).

Purification of soluble F_1 -ATPase

Pure F_1 -ATPase was prepared from the supernatant obtained by washing the membranes in low-ionic-strength buffer (above). The purification technique used was that described by Senior *et al.* (1979). Briefly, it consisted of precipitation of the F_1 -ATPase by poly(ethylene glycol), followed by ion-exchange chromatography on Whatman DE52 cellulose and gel filtration on Sepharose CL-6B. All steps were carried out at 4°C. The ATPase specific activity of F_1 -ATPase prepared from the partial diploid strain AN862 (*unc*⁺/*unc*⁺) varied from 8.5 to 12 μ mol/min per mg (six different preparations) when assayed at 30°C, pH 8.0, in the presence of 50 mM-Tris/HCl, 5 mM-MgCl₂ and 10 mM-ATP as described by Senior & Brooks (1970). All five subunits of the F_1 -ATPase were always present and the soluble enzyme fully reconstituted energy-dependent reactions in washed membranes (see below).

Determination of protein

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

Measurement of atebtrin-fluorescence quenching

Atebtrin-fluorescence quenching was measured at 30°C as described by Gibson *et al.* (1977a). The assumption is made that loss of NADH-induced atebtrin-fluorescence-quenching activity of membranes that have a normal oxidase activity is due to increased proton permeability. Although Deamer *et al.* (1972) have shown a relationship between quenching of atebtrin-fluorescence and formation of a transmembrane pH gradient, a quantitative relationship between the two parameters has been criticized (see Kell *et al.*, 1978).

Two-dimensional gel electrophoresis

The polypeptide compositions both of washed membranes and of purified F_1 -ATPase preparations were analysed by two-dimensional electrophoresis under dissociating conditions, by the method of O'Farrell (1975). Briefly, proteins were solubilized by using sodium dodecyl sulphate and mercaptoethanol (Cox *et al.*, 1978b), and applied to cylindrical isoelectric-focusing gels. The isoelectric focusing gels were not pre-run; samples were electrophoresed at an initial current of 1 mA/tube for 2 h, during which time the voltage increased to 500 V. The voltage was then set at 800 V and electrophoresis continued for a further 3 h. Focusing gels were shaken for 15 min in 0.12 M-Tris/HCl, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate and were then sealed to the second-dimension slab by using 1% agarose dissolved in

'running buffer'. An acrylamide gradient of 7.5–22.5% (w/v) was used in the second dimension, and the gels were run at 8 mA for 16 h. Proteins were stained with Coomassie Blue R250.

Measurement of binding of purified F_1 -ATPase to washed membranes

Various amounts of purified F_1 -ATPase were added to low-ionic-strength washed membrane samples (0.5 mg of protein) and incubated for 15 min at 30°C in 50 mM-Tris/HCl/5 mM-MgCl₂ buffer (pH 7.5), at a final membrane protein concentration of 1 mg/ml. The samples were then diluted 20-fold with the 50 mM-Tris/HCl/5 mM-MgCl₂ buffer (pH 7.5) at 4°C and centrifuged for 1 h at 260 000g. The membranes were then resuspended by gentle homogenization in the same buffer at 4°C to a final concentration of 0.5 mg of protein/ml. Samples (15–30 μ g of protein) were then assayed for ATPase activity at 30°C in 1 ml of assay solution containing 10 mM-ATP, 5 mM-MgCl₂ and 50 mM-Tris/HCl, pH 8.0, as described by Senior & Brooks (1970). Sensitivity of the ATPase activity to dicyclohexylcarbodi-imide was determined after preincubating samples of resuspended membranes (0.5 mg/ml) at 20°C for 30 min with 50 μ M-dicyclohexylcarbodi-imide and then assayed for ATPase activity as described above. Controls contained equivalent amounts of ethanol (1%, v/v).

Results

Genetic characterization of strains carrying mutations in the *uncD* gene

Three mutant strains of *E. coli* K12 (AN181, M75 and D1275) unable to grow on succinate were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (Butlin *et al.*, 1971). Strains AN181, M75 and D1275 gave uncoupled growth yields (Butlin *et al.*, 1971) and carried the mutant alleles *unc*-406, *unc*-412 and *unc*-427 respectively. These mutant alleles were co-transduced with the *ilb* genes into either strain AN248 or AN346 (see Table 1) to give strains AN342 (*unc*-406), AN484 (*unc*-412) and AN934 (*unc*-427). Membrane preparations from strains AN342 (*unc*-406) and AN934 (*unc*-427) were found to lack ATPase activity and therefore resembled strains carrying the *uncA401* or *uncD409* alleles. Strain AN484 (*unc*-412) differed from the others in that membrane preparations had a low ATPase specific activity of 0.09 μ mol/min per mg of protein, which could be compared with about 0.7 μ mol/min per mg of protein for a normal strain grown under the same conditions. The three new mutant *unc* alleles were incorporated into F-plasmids, using the method described previously (Gibson *et al.*, 1977b), in order to carry out

genetic complementation tests. The *unc-405* mutant allele, previously reported (Cox *et al.*, 1974) as causing the loss of ATPase activity, was also incorporated into a F-plasmid and included in the tests for genetic complementation. The partial diploid strains carrying the *unc-405*, *unc-406*, *unc-412*, *unc-427* or *uncD409* alleles on the F-plasmids were then used as donor strains in complementation tests on succinate medium with female strains carrying the *uncA401*, *uncB402*, *uncC424*, *uncD409* or *uncE429* alleles, as well as with female strains carrying the *unc-405*, *unc-406*, *unc-412* or *unc-427* alleles. In such tests it has been found that, if genetic complementation occurs, colonies will be formed on the succinate medium in the region where the donor and recipient strains mix in the cross-streaking test described previously (Cox *et al.*, 1978a). In addition, the reverse crosses were made in which the previously characterized mutant *unc* alleles, *uncA401*, *uncB402*, *uncC424* or *uncE429* were present on the F-plasmid and the new mutant *unc* alleles were carried in the female strains. Complementation was observed between the previously uncharacterized mutant *unc* alleles and the *uncA*, *uncB*, *uncC* or *uncE* mutant alleles. Complementation was not observed between the previously uncharacterized mutant *unc* alleles and the *uncD409* allele. Complementation was also not observed between any of the new mutant *unc* alleles. It is concluded that the *unc-405*, *unc-406*, *unc-*

412 and *unc-427* mutant alleles affect the *uncD* gene and accordingly will be referred to as the *uncD405*, *uncD406*, *uncD412* and *uncD427* alleles. In the positive complementation tests between the *uncA401* allele and the *uncD405*, *uncD406*, *uncD412* or *uncD409* alleles only small colonies were obtained. Such poor complementation between mutant *uncA* and mutant *uncD* alleles has been described previously (Cox *et al.*, 1978a).

Identification of abnormal β -subunits

Mutant strains carrying the *uncD409* allele have been shown to form an abnormal β -subunit of the ATPase. The mutant β -subunit is membrane-bound and has an apparent isoelectric point about 0.1 of a pH unit higher than the normal β -subunit (Fayle *et al.*, 1978). Membrane preparations from strains AN285 (*uncD405*), AN342 (*uncD406*), AN463 (*uncD409*), AN484 (*uncD412*) and AN934 (*uncD427*) were washed in low-ionic-strength buffer in the presence of 6-aminohexanoic acid and *p*-amino-benzamidine. The washed membranes were solubilized in buffer containing sodium dodecyl sulphate and mercaptoethanol and then analysed by two-dimensional gel electrophoresis. Abnormal β -subunits could be detected in each of the *uncD* mutant strains. These abnormal β -subunits have the same apparent molecular weights, but either higher isoelectric

Fig. 1. Two-dimensional gel electrophoresis of membranes from strains AN862 (*unc⁺/unc⁺*), AN285 (*uncD405*) and AN934 (*uncD427*)

Low-ionic-strength washed membranes (about 350 μ g of protein of each) from strains AN862 (a), AN285 (b) and AN934 (c) were solubilized and electrophoresed as described in the Materials and Methods section. Only a section of each gel (corresponding to the pH range of about 5.7–4.8) is shown. The polypeptides a, b, c, d and e could always be seen on the electrophoretograms and are used for reference. The normal β - and the two abnormal ' β '-subunits of the F_1 -ATPase are indicated by vertical arrows. The apparent isoelectric points of the unknown marker polypeptides (a, b, c, d, and e) of the normal β -subunit and of the abnormal ' β '-subunits from each of the strains carrying the mutant *uncD* alleles, are indicated on the pH scale (d).

points (*uncD409*, *uncD427*, *uncD406*) or lower isoelectric points (*uncD412*, *uncD405*) than that of the normal β -subunit (Fig. 1). The abnormal β -subunits were also shown to be present on membranes from strains AN285 (*uncD405*), AN342 (*uncD406*), AN463 (*uncD409*) and AN934 (*uncD427*) that had been washed in low-ionic-strength buffer in the absence of *p*-aminobenzamidine (results not shown). Membranes from strain AN484 (*uncD412*) were different in that low-ionic-strength washing in the absence of *p*-aminobenzamidine caused the loss of the abnormal β -subunit from the membrane (see below).

Binding of F_1 -ATPase to *uncD* mutant membranes

Membrane preparations from each of the *uncD* mutant strains were washed in low-ionic-strength buffer in the presence of 6-aminohexanoic acid and *p*-aminobenzamidine and then in the absence of *p*-aminobenzamidine. Membrane preparations from the normal strain AN248 and from the mutant strain AN875 (*unc-413::Mu*) (which carries a mutator phage inserted in the *unc* operon such that the *uncB*, *uncE*, *uncD* and *uncC* genes are not expressed, see Gibson *et al.*, 1978) were washed in the same way as the *uncD* mutant strains and included as controls. The washed membrane preparations from the seven strains were then tested for their ability to bind purified normal F_1 -ATPase prepared from the partial diploid strain AN862 (*unc⁺/unc⁺*). A range of binding activities was obtained depending on the particular mutant allele present in the cells from which the membranes were prepared. The binding of F_1 -ATPase to membranes prepared from strains AN484 (*uncD412*) and AN285 (*uncD405*) was similar to the binding of F_1 -ATPase to membranes from the normal strain AN248, whereas membranes from

strain AN463 (*uncD409*) were similar to those from AN875 (*unc-413::Mu*) in that the binding of F_1 -ATPase was greatly decreased (Fig. 2). In all cases the

Fig. 2. Binding of purified F_1 -ATPase to low-ionic-strength washed membranes from various *uncD* mutant strains. Low-ionic-strength washed membranes from various strains were tested for their ability to bind normal F_1 -ATPase prepared from strain AN862 (*unc⁺/unc⁺*) as described in the Materials and Methods section. ●, AN248 (*unc⁺*); ○, AN484 (*uncD412*); ▼, AN285 (*uncD405*); △, AN934 (*uncD427*); ■, AN342 (*uncD406*); □, AN463 (*uncD409*); ▲, AN875 (*unc-413::Mu*).

Table 2. Atebrin-fluorescence quenching in membranes from various *uncD* mutant strains

Washed membranes (about 2 mg of protein) were diluted in 2.5 ml of 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer containing 300 mM-KCl and 5 mM-MgCl₂. Where indicated, the washed membranes were preincubated with either dicyclohexylcarbodi-imide or purified F_1 -ATPase (about 0.4 mg of protein) for 5 min at room temperature. Atebrin was added to give a final concentration of 4 μ M, NADH to 1 mM, ATP to 0.8 mM, carbonyl cyanide *m*-chlorophenylhydrazone to 20 μ M, NaCN to 1 mM and dicyclohexylcarbodi-imide to 80 μ M. The percentage-quenching of atebrin fluorescence was determined after the addition of NADH. The fluorescence returned to normal after the addition of NaCN and then ATP was added. ATP-induced fluorescence-quenching was calculated from the amount of fluorescence restored after the addition of carbonyl cyanide *m*-chlorophenylhydrazone.

Atebrin-fluorescence quenching in membranes washed in low-ionic-strength buffer (%)

Strain	Plus <i>p</i> -aminobenzamidine		Minus <i>p</i> -aminobenzamidine			
	NADH	NADH+dicyclohexylcarbodi-imide	NADH	NADH+F ₁	ATP	ATP+F ₁
AN484 (<i>uncD412</i>)	44	82	7	84	0	75
AN285 (<i>uncD405</i>)	33	85	35	80	0	40
AN934 (<i>uncD427</i>)	32	62	25	55	0	12
AN342 (<i>uncD406</i>)	21	46	20	45	0	15
AN463 (<i>uncD409</i>)	72	82	75	81	0	13
AN248 (<i>unc⁺</i>)	78	81	7	82	0	80

membrane-bound ATPase activity was inhibited 60–80% by preincubation with 50 μ M-dicyclohexylcarbodi-imide.

Atebrin-fluorescence quenching in membranes from uncD mutants

As observed previously (Fayle *et al.*, 1978), membrane preparations from strain AN463 (*uncD409*) are not permeable to protons, as judged by the NADH-induced atebrin-fluorescence quenching (Table 2). In contrast with membrane preparations from the normal strain AN248, membranes from strain AN463 (*uncD409*) do not become permeable to protons after washing with low-ionic-strength buffer in the absence of *p*-aminobenzamidine. However, membranes from each of the other *uncD* mutant strains even when washed in the presence of *p*-aminobenzamidine are permeable to protons (Table 2). The addition of dicyclohexylcarbodi-imide decreased the proton permeability (Table 2). Further washing of the membranes in the absence of *p*-aminobenzamidine resulted in a significant increase in proton permeability in only the membranes from the normal strain AN248 and from strain AN484 (*uncD412*) (Table 2 and see below).

Fig. 3. Gel filtration, on a Sepharose 6B-Cl column, of F₁-ATPase prepared from membranes of strain AN862 (*unc⁺/unc⁺*) or strain AN484 (*uncD412*)

Details of the procedure for purifying F₁-ATPase are given in Senior *et al.* (1979) and gel filtration, the elution profile of which is shown, is the final step. (a) Strain AN862 (*unc⁺/unc⁺*); (b) strain AN484 (*uncD412*). The column was 2.5 cm $\times$ 95 cm and the elution volume of the protein peak was about 270 ml. ●, Protein expressed as A₆₅₀ by the method of Lowry *et al.* (1951); ○, ATPase activity expressed as A₆₆₀ by the method of Senior & Brooks (1970).

Fig. 4. Two-dimensional gel electrophoresis of purified F₁-ATPase from strain AN484 (*uncD412*)

Purified F₁-ATPase (60 μ g of protein) from strain AN484 (*uncD412*) was electrophoresed as described in the Materials and Methods section. The α -, β' -, γ -, δ - and ϵ -subunits are marked on the electrophoretogram. The inset shows an enlarged section of a gel in which 2.5 μ g of protein of F₁-ATPase from each of strains AN484 (*uncD412*) and AN862 (*unc⁺/unc⁺*) were co-electrophoresed; only the region containing the β and β' -subunits is shown.

Reconstitution of ATP-dependent atebrin-fluorescence quenching

Membranes from a normal strain become proton permeable and lose both NADH-dependent and ATP-dependent atebrin-fluorescence-quenching activity after washing in low-ionic-strength buffer in the absence of *p*-aminobenzamidine. The washed membranes can be reconstituted, however, by the addition of purified F₁-ATPase. The washed membranes from the various *uncD* mutants were tested for reconstitution of ATP-dependent atebrin-fluorescence quenching by using purified F₁-ATPase from the partial diploid strain AN862 (*unc⁺/unc⁺*) and a range of activities was obtained (Table 2). The low-ionic-strength washed membranes from strain AN484 (*uncD412*) were reconstituted to normal activities; those from strain AN285 (*uncD405*) were reconstituted to an intermediate activity; those from the remaining *uncD* mutants gave poor reconstitution (Table 2). The amounts of reconstitution of ATP-dependent atebrin-fluorescence quenching do not correlate with the amounts of F₁-ATPase bound by the same membrane preparations from the various *uncD* mutant strains (Fig. 2 and Table 2). However, the reconstituted membranes from strains AN342 (*uncD406*) and AN934 (*uncD427*) remained permeable to protons as judged by the amounts of NADH-induced atebrin-fluorescence quenching (Table 2).

Purification of F1-ATPase from strain AN484 (uncD412)

As noted above, there was a similarity between

Fig. 5. Ability of F_1 -ATPase from strain AN484 (*uncD412*) to reconstitute ATP- and NADH-dependent atebtrin-fluorescence quenching

The conditions were as described in the legend to Table 2, but with 1.2 mg of F_1 -ATPase-depleted membranes from *uncA401* mutants. (a) No additions; (b) plus 200 μ g of F_1 -ATPase purified from strain AN484 (*uncD412*); (c) plus 200 μ g of F_1 -ATPase purified from strain AN862 (*unc⁺/unc⁺*). Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

strain AN484 (*uncD412*) and the normal strain AN248, in that the proton permeability of the membranes greatly increased when they were washed in low-ionic-strength buffer in the absence of *p*-aminobenzamidine. This suggested that an F_1 -ATPase aggregate might be released from membranes of strain AN484 (*uncD412*).

The procedure for the purification of F_1 -ATPase from strain AN862 (*unc⁺/unc⁺*) was therefore followed for strain AN484 (*uncD412*). In the terminal step of the purification procedure (Sephacose CL-6B column) a protein peak was obtained at the same elution volume as for the F_1 -ATPase from strain AN862 (*unc⁺/unc⁺*) (Fig. 3). The purified F_1 -ATPase from strain AN484 (*uncD412*) had a specific activity of 0.9 μ mol/min per mg of protein, which is about 10% of normal. Analysis of the subunit structure by two-dimensional gel electrophoresis showed that an abnormal β -subunit was present in the F_1 -ATPase from strain AN484 (*uncD412*) and that the α -, γ -, δ - and ϵ -subunits were normal (Fig. 4).

The purified F_1 -ATPase from strain AN484 (*uncD412*) reconstituted NADH-dependent atebtrin-fluorescence quenching in washed membranes from strain AN249 (*uncA401*) and, as expected from the low ATPase activity, only partially reconstituted the ATP-dependent activity (Fig. 5).

Discussion

The different mutations in the *uncD* gene, described in the present paper, all result in the formation of β -subunits with abnormal net charge, yet lead to considerable variation in the properties of the cell membranes. The extremes in the variation among the

strains described above are exemplified by strains AN463 (*uncD409*) and AN484 (*uncD412*). In membranes from strain AN463 (*uncD409*), the β -subunit has an isoelectric point higher than the normal β -subunit and binds to the membrane sector of the ATPase in such a way that the membrane remains impermeable to protons and purified F_1 -ATPase is unable to bind. Most of the abnormal β -subunit remains attached to the membrane after low-ionic-strength washing in the absence of *p*-aminobenzamidine. However, treatment of the membranes sequentially with 4 mM-urea and then 4 mM-sodium thiocyanate increases the F_1 -ATPase-binding capacity of the membranes to about 65% of normal (A. E. Senior, unpublished observations). Preliminary experiments suggest that the abnormal β -subunit is the only subunit of the F_1 -ATPase that remains bound to the washed membranes from strain AN463 (*uncD409*).

Strain AN484 (*uncD412*) represents the other extreme in the variation among these *uncD* mutants. The abnormal β -subunit in this strain has a lower isoelectric point than the normal β -subunit, but still becomes incorporated into an F_1 -ATPase aggregate of similar overall subunit composition to normal. The purified F_1 -ATPase from strain AN484 (*uncD412*) had a low ATPase activity and a low ATP-dependent atebtrin-fluorescence-quenching activity when used in reconstitution experiments with washed membranes.

Strains AN934 (*uncD427*) and AN342 (*uncD406*) both have abnormal β -subunits with a higher isoelectric point than the normal β -subunit and resemble strain AN463 (*uncD409*) more closely than AN484 (*uncD412*). It would appear from the F_1 -ATPase-

binding studies, that only about half the binding sites are occupied by the abnormal β -subunit in such a way as to prevent binding of purified F_1 -ATPase. However, even after binding of the F_1 -ATPase, the membranes, as judged by NADH-induced atebriin-fluorescence quenching, are still somewhat proton 'leaky'. This would account for the low ATP-dependent quenching of atebriin fluorescence obtained in the reconstituted membranes. The abnormal β -subunits, although preventing F_1 -ATPase-binding, apparently do not prevent some proton permeability through the F_0 portion of the ATPase. The presence or absence of the other F_1 subunits in washed membranes from strains AN934 (*uncD427*) and AN342 (*uncD406*) has not yet been investigated.

The membranes from strain AN285 (*uncD405*) are similar to those from strain AN484 (*uncD412*) in that the abnormal β -subunit has a lower isoelectric point than normal and purified F_1 -ATPase binds to the washed membranes to almost the same extent as to normal washed membranes. As would be expected from the binding studies the membranes from strain AN285 (*uncD405*), when reconstituted with F_1 -ATPase, did not appear to be permeable to protons, but the ATP-dependent atebriin-fluorescence quenching was only about half the control value. This could indicate that the abnormal β -subunit on the membranes from strain AN285 (*uncD405*) did not prevent binding of the purified normal F_1 -ATPase, but did affect the ATP-dependent energization of the reconstituted membranes.

The biochemical characterization of five *uncD* mutants had shown that mutations affecting the same subunit of the ATPase complex can have quite diverse effects. Variation in the extent of F_1 -ATPase-binding, energization of the membranes, ability to form an F_1 -aggregate and proton permeability of the membranes have been described. This diversity emphasizes the necessity of classifying *unc* mutant strains by genetic complementation tests. The fact that the abnormal β -subunits in some *uncD* mutant strains are tightly bound to the membrane suggests some interesting speculations regarding the interaction of normal β -subunits with the membrane sector of the ATPase complex.

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