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Oxidative phosphorylation in *Escherichia coli*

Characterization of mutant strains in which F_1-ATPase contains abnormal β-subunits

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To facilitate study of the role of the β-subunit in the membrane-bound proton-translocating ATPase of *Escherichia coli*, we identified mutant strains from which an F_1-ATPase containing abnormal β-subunits can be purified. Seventeen strains of *E. coli*, characterized by genetic complementation tests as carrying mutations in the *uncD* gene (which codes for the β-subunit), were studied. The majority of these strains (11) were judged to be not useful, as their membranes lacked ATPase activity, and were either proton-permeable as prepared or remained proton-impermeable after washing with buffer of low ionic strength. A further two strains were of a type not hitherto reported, in that their membranes had ATPase activity, were proton-impermeable as prepared, and were not rendered proton-permeable by washing in buffer of low ionic strength. Presumably in these two strains F_1-ATPase is not released in soluble form by this procedure. F_1-ATPases of normal molecular size were purified from strains AN1340 (*uncD478*), AN937 (*uncD430*), AN938 (*uncD431*) and AN1543 (*uncD484*). F_1-ATPase from strain AN1340 (*uncD478*) had 15% of normal specific Mg-dependent ATPase activity and 22% of normal ATP-synthesis activity. The F_1-ATPase preparations from strains AN937, AN938 and AN1543 had respectively 1.7%, 1.8% and 0.2% of normal specific Mg-dependent ATPase activity, and each of these preparations had very low ATP-synthesis activity. The yield of F_1-ATPase from the four strains described was almost twice that obtained from a normal haploid strain. The kinetics of Ca-dependent ATPase activity were unusual in each of the four F_1-ATPase preparations. It is likely that these four mutant *uncD* F_1-ATPase preparations will prove valuable for further experimental study of the F_1-ATPase catalytic mechanism.

In *Escherichia coli* the membrane-bound proton–ATPase complex catalyses ATP-driven proton translocation and the terminal stages of oxidative phosphorylation. The complex contains eight different subunits, coded for by genes named *uncA–uncH* (Downie *et al.*, 1979, 1980, 1981; Gunsalus *et al.*, 1982). These genes are arranged in an operon (Downie *et al.*, 1979). The first three genes of the operon (*uncB,E,F*) code for membrane sector proteins, which together catalyse proton translocation across the membrane to the F_1-sector (Fillingame, 1981). The F_1-sector is the catalytic unit where ATP synthesis or hydrolysis occurs (Senior, 1979*a*). It is made up of five different subunits, termed α–ε, and coded for by the last five genes in the operon, *uncHAGDC* (Downie *et al.*, 1979, 1980; Gunsalus *et al.*, 1982). The *uncD* gene codes for the β-subunit (Fayle *et al.*, 1978).

Work using the affinity label *p*-fluorosulphonyl benzoyladenosine (an ATP analogue) has strongly suggested that a particular tyrosine residue in the β-subunit of F_1-ATPase interacts with the β–γ-phosphate region of ATP during hydrolysis (Esch & Allison, 1978). Similarly, the inhibitor dicyclohexylcarbodi-imide reacts with two glutamic acid residues in the β-subunit. These glutamic acid residues are also therefore implicated in catalysis, perhaps by liganding with Mg^{2+} in the MgATP substrate (Yoshida *et al.*, 1981; Esch *et al.*, 1981). These and other experiments (see Senior, 1979*a*; Cross, 1981) have indicated that the β-subunits contribute structurally toward the catalytic sites in the F_1-ATPase.

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For this reason, strains of *E. coli* carrying mutations in the *uncD* gene, and which form an F_1-aggregate, are of interest because they present the possibilities that the structure of the catalytic site, or features of the catalytic mechanism may be modified. Advantages of using mutant *uncD* F_1-ATPase preparations for mechanistic and structural studies are that they are homogeneous and are specifically modified at single, functionally important, residues in the β-subunit.

Previously we have described five strains of *E. coli* carrying mutations in the *uncD* gene (Senior *et al.*, 1979*b*), and the conclusion from that work was that such mutants may display a wide range of properties. Only one out of the five strains actually formed an F_1-aggregate; this was the strain carrying the *uncD412* allele. We purified the *uncD412* F_1-ATPase and showed that it has normal molecular size and subunit structure. The β-subunit had an isoelectric point lower than normal, and the specific ATPase activity of the F_1-ATPase was only one-tenth of normal.

Kanazawa *et al.* (1980) have described a mutant strain of *E. coli* (KF11) which also forms an F_1-aggregate containing an altered β-subunit; they reported the purification and preliminary characterization of the KF11 F_1-ATPase in the same paper. In fact, comparison of our data on *uncD412* F_1-ATPase (J. G. Wise, L. R. Latchney, D. Cox & A. E. Senior, unpublished work) with the data reported by Kanazawa *et al.* (1980) showed that the two mutant F_1-ATPase preparations resembled each other closely.

We considered that progress toward understanding the mechanism of ATP synthesis and hydrolysis by F_1 would be further expedited if several more *uncD* mutant F_1 preparations were available for study. We therefore carried out a search for appropriate mutant strains, and in this paper we report the properties of 17 new *uncD* mutants of *E. coli*. Four of these were found to assemble an F_1-aggregate of normal molecular size, but with abnormal catalytic activity. The purification of each of the four mutant *uncD* F_1-ATPase preparations was accomplished, and preliminary characterization of each is reported.

Methods

Strains of E. coli used

The strains of *E. coli* used are listed in Table 1. The general procedure for isolation and charac-

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

Bacterial strain	Relevant genetic loci	Other information
AN1110	*uncD404,argH,pyrE,entA*	An isogenic set of strains carrying the *uncD* alleles shown. Strain AN346 was transduced with a P1 phage lysate of the original Suc$^-$ isolate selected after treatment of strain AN732 with nitrosoguanidine (Cox & Downie, 1979)
AN1114	*uncD411,argH,pyrE,entA*	
AN937	*uncD430,argH,pyrE,entA*	
AN938	*uncD431,argH,pyrE,entA*	
AN1109	*uncD451,argH,pyrE,entA*	
AN1118	*uncD457,argH,pyrE,entA*	
AN1160	*uncD459,argH,pyrE,entA*	
AN1168	*uncD460,argH,pyrE,entA*	
AN1215	*uncD466,argH,pyrE,entA*	
AN1420	*uncD470,argH,pyrE,entA*	
AN1509	*uncD472,argH,pyrE,entA*	
AN1511	*uncD474,argH,pyrE,entA*	
AN1352	*uncD477,argH,pyrE,entA*	
AN1340	*uncD478,argH,pyrE,entA*	
AN1543	*uncD484,argH,pyrE,entA*	
AN1544	*uncD485,argH,pyrE,entA*	
AN1573	*uncD496,argH,pyrE,entA*	
AN346	*ilvC,argH,pyrE,entA*	
AN732	*argH,entA,pyrE,metE,strR*	
AN862	*pAN11/ilvC,argH,pyrE,purE,recA,nalA*	Partial diploid strain with F′ carrying the *unc* operon (Cox *et al.*, 1978).
AN1461	*pAN45/unc416::Mu B*$^+$EFHAGDC *argH,pyrE,entA,recA,nalA*	A polar mutant in the *unc* operon isolated as described (Gibson *et al.*, 1978) carrying *unc* operon on a multicopy plasmid
Plasmids		
pAN11	*pyrE*$^+$,*unc*$^+$,*ilvC*$^+$,*argH*$^+$	F′ plasmid carrying the *unc* operon (Cox *et al.*, 1978).
pAN45	Cm^r,Tc^s,*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	A multicopy plasmid carrying the *unc* operon (Downie *et al.*, 1980).

terization of the mutants followed that of Cox & Downie (1979). After mutagenesis, with nitrosoguanidine, Suc$^-$ strains with low aerobic growth yield on limiting glucose were isolated. Mutations affecting the *unc* genes were then transduced by using phage P1 into strain AN346 (*ilv, argH,entA,pyrE*) and selecting for Ilv$^+$, Suc$^-$ transductants. Complementation tests were done by using cross-streaking procedures with strains carrying known mutant *unc* alleles or phage-Mu-induced *unc* mutations (Gibson *et al.*, 1977, 1978). The 17 new mutants studied in this work, listed in Table 1, were all shown by complementation analysis to carry mutations in the *uncD* gene.

Growth of cells

Cells were grown in either small batches of 20g wet wt. (for characterization of the membranes) or large batches of around 300g wet wt. (for purification of F_1-ATPase) as described previously (Cox *et al.*, 1970). Tests to show that reversion to normal phenotype had not occurred were routinely performed at the time the cells were harvested.

Preparations of membranes

Membranes were prepared from the cells as described by Senior *et al.* (1979*b*). After washing in buffer containing 50mM-Tes (2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid), the membranes were treated differently according to whether they were to be used for F_1-ATPase purification or for membrane characterization. The small batches (for membrane characterization) were split into equal portions. One half was washed once in 5mM-Tes buffer containing *p*-aminobenzamidine as described previously (Senior *et al.*, 1979*b*); the other half was washed twice in 5mM-Tes buffer containing no *p*-aminobenzamidine. When this procedure is applied to normal strains, half (*p*-aminobenzamidine present) would be enriched in F_1-ATPase and the other half (*p*-aminobenzamidine absent) would be depleted of F_1-ATPase. The large batches of membranes (for F_1-ATPase purification) were treated exactly as described by Senior *et al.* (1979*b*).

Purification of F_1-ATPase

This was done as described by Senior *et al.* (1979*a*). The buffer for the ion-exchange chromatography and Sepharose CL-6B gel-filtration steps contained 2mM-$MgCl_2$ in the present work instead of 2mM-EDTA as previously. This was to stabilize any F_1-ATPase preparations which might have a tendency to depolymerize during purification (see Kanazawa *et al.*, 1980; Wise *et al.*, 1981).

Two-dimensional gel electrophoresis

Membranes washed in 5mM-Tes buffer containing *p*-aminobenzamidine were examined by two-dimensional electrophoresis as described by Senior *et al.* (1979*b*). This technique is essentially that of O'Farrell (1975), involving isoelectric focusing in the first dimension and sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis in the second dimension.

Other procedures

Oxidative phosphorylation was assayed as described by Cox *et al.* (1973), ATPase activity as described by Senior (1979*b*), and protein as described by Lowry *et al.* (1951). Quenching of atebrin fluorescence was assayed as described by Senior *et al.* (1979*b*). Growth yields were measured as final turbidities after aerobic growth in media containing 5mM-glucose and required supplements as described by Cox *et al.* (1970).

Results

General characteristics of the 17 uncD mutant strains

The strains examined were selected on the basis of their slow growth or inability to grow on succinate-containing media and sub-normal aerobic growth yield on limiting glucose. The properties of their membranes are described in Table 2, where we have divided the mutant strains into four groups.

Group I. These strains have membranes which are not leaky to protons (NADH causes a full quenching of atebrin fluorescence in the 'plus *p*-aminobenzamidine' washed membranes). Moreover, washing the membranes in low-ionic-strength (5mM-Tes) buffer devoid of *p*-aminobenzamidine did not render them leaky to protons. Strains in Group I lacked membrane ATPase activity. Group I strains therefore resembled the previously characterized *uncD409* mutant strain in their properties (Fayle *et al.*, 1978; Senior *et al.*, 1979*b*). In the *uncD409* mutant an F_1-aggregate does not form. Instead an abnormal β-subunit and a normal α-subunit bind to the membrane sector (Cox *et al.*, 1981), rendering it impermeable to protons, and are not removed by washing in low-ionic-strength buffer devoid of *p*-aminobenzamidine. Strains in Group I were therefore not characterized further in the present work.

Group II. These strains had membranes which were not leaky or only slightly leaky to protons (again NADH caused complete or almost complete quenching of atebrin fluorescence in *p*-aminobenzamidine-washed membranes). Here the membranes did become leaky to protons when washed in low-ionic-strength buffer devoid of *p*-aminobenzamidine, suggesting that in these strains an F_1-aggregate is washed off under these conditions. These strains therefore resembled the *uncD412* mutant, which we had characterized previously

Table 2. *Properties of uncD mutant strains*

For atebrin-fluorescence-quenching measurements, washed membranes (about 2 mg of protein) were diluted in 2.0 ml of 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.5) containing 300 mM-KCl and 5 mM-$MgCl_2$. Atebrin was added to give a final concentration of 4 μM, NADH to 1 mM, NaCN to 1 mM, ATP to 0.8 mM, and finally carbonyl cyanide *m*-chlorophenylhydrazone to 20 μM. 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 was calculated from the amount of fluorescence restord after the addition of carbonyl cyanide *m*-chlorophenylhydrazone. ATPase activity was measured in 100–200 μg of membranes, which were washed once in 5 mM-Tes buffer containing *p*-aminobenzamidine. Abbreviation: ND, not determined.

	Atebrin-fluorescence quenching in membranes washed in 5 mM-Tes buffer (%)				Membrane ATPase activity (μmol of ATP hydrolysed/min per mg of protein)
	Plus *p*-aminobenzamidine		Minus *p*-aminobenzamidine		
Strain	NADH	ATP	NADH	ATP	
AN346 (*unc*$^+$)	82	86	10	0	0.7
Group I					
AN1544 (*uncD485*)	82	0	86	0	0
AN1160 (*uncD459*)	82	0	84	0	0
AN1109 (*uncD451*)	80	0	90	0	0
AN1573 (*uncD496*)	82	0	90	0	0
AN1118 (*uncD457*)	82	0	89	0	0
AN1509 (*uncD472*)	86	0	92	0	0
AN1511 (*uncD474*)	84	0	88	0	0
AN1352 (*uncD477*)	78	0	72	0	0
AN1420 (*uncD470*)	84	0	65	0	0
AN463 (*uncD409*)*	72	0	82	0	0
Group II					
AN1340 (*uncD478*)	92	68	16	ND	0.15
AN937 (*uncD430*)	84	0	20	ND	0.06
AN938 (*uncD431*)	78	0	26	ND	0.06
AN1543 (*uncD484*)	78	0	27	ND	0
AN484 (*uncD412*)*	44	13	27	0	0.09
Group III					
AN1215 (*uncD466*)	25	0	ND	ND	0
AN1114 (*uncD411*)	24	0	ND	ND	0
AN285 (*uncD405*)*	33	0	35	0	0
AN342 (*uncD406*)*	21	0	20	0	0
AN934 (*uncD427*)*	32	0	25	0	0
Group IV					
AN1168 (*uncD460*)	83	0	60	ND	0.06
AN1110 (*uncD404*)	88	14	71	ND	0.10

* Taken from Senior *et al.* (1979*b*) and included here for comparative purposes.

(Senior *et al.*, 1979*b*). Moreover, several of the strains in Group II had membrane-ATPase activity, and one had ATP-dependent quenching of atebrin fluorescence. These strains were therefore of interest for further study, and we will return to them later in this paper.

Group III. These strains had membranes which were leaky to protons and had zero ATPase activity, even when *p*-aminobenzamidine was included in the washing medium. Therefore it was concluded that no F_1-aggregate was present on membranes in these strains. These strains resembled the *uncD405*, *uncD406* and *uncD427* mutants described previously (Senior *et al.*, 1979*b*). It was found that full atebrin-fluorescence-quenching responses (approx. 80% on addition of NADH or ATP) could be reconstituted by preincubating membranes of strains AN1215 or AN1114 (Group III), which had been washed in 'plus *p*-aminobenzamidine' buffer, with normal (*unc*$^+$) F_1-ATPase. In view of the previously defined pathway of assembly of the *E. coli* ATPase *in vivo* (Cox *et al.*, 1981), it seems likely that strains

in this group do actually assemble an F_1-aggregate on the membrane initially, but that the F_1-aggregate is then unstable, and dissociates partly or totally from the membrane. The reason for concluding this is that a functional proton pore is present in the membranes, and therefore the initial binding of α- and β-subunits must have occurred normally to incorporate the *uncF* protein (mol.wt. 18000) and form a functional proton pore (Cox *et al.*, 1981).

Group IV. These strains had membranes which were not leaky to protons, and which did not become leaky to protons on washing in buffer devoid of *p*-aminobenzamidine. They had significant membrane ATPase activity, and in one case had ATP-dependent quenching of atebrin fluorescence. They seem to represent an hitherto unrecognized type of *unc* mutant in which an abnormal F_1-aggregate is formed which is bound so tightly to the membrane that it is not released by washing conditions that normally do release F_1-ATPase in soluble form. These strains were not further characterized in the present work, but are clearly of interest for future study.

Purification of F_1 aggregates from the strains in Group II

Large batches of cells of each of the strains in Group II (Table 2) were grown and harvested, membranes were prepared and F_1-ATPase was purified as described in the Methods section. An F_1-aggregate with ATPase activity which was eluted from the Sepharose CL-6B column in the same volume as normal (*unc*$^+$) F_1-ATPase during the last step of purification was obtained from each of the strains in Group II. Therefore it can be concluded that an F_1-ATPase of normal molecular size is formed in each of these strains. In each case only a single peak was eluted from the Sepharose CL-6B, indicating that depolymerization of the F_1-ATPase into subunits was not occurring (Wise *et al.*, 1981). The specific ATPase activities and yield of F_1-ATPase obtained from each strain are shown in Table 3, with values from normal strains shown for comparison. The specific ATPase activity varied very widely, with one strain (AN1543, *uncD484*) hydrolysing ATP at only one-five-hundredth of the normal rate. Each of the four new F_1-ATPase preparations was abnormal in specific ATPase activity. In each strain an increase in yield of F_1-ATPase was seen (Table 3). However, in no case did the yield of F_1-ATPase per g wet wt. of cells exceed that found in the partial diploid strain AN862 (*unc*$^+$/*unc*$^+$). In strains carrying multiple copies of the *unc*$^+$ operon on plasmids [e.g. AN1461 (pAN45)] yields of F_1-ATPase of 0.2 mg/g wet wt. of cells have been obtained (Table 3). Therefore, it appears that *E. coli* has some ability to increase the amounts of the proton-ATPase synthesized to compensate for impaired catalytic capability, but the true extent of this increase cannot be determined from our data, because the assembly of the ATPase complex may be impaired in the *uncD* mutants (Cox *et al.*, 1981).

Table 4 shows the growth yields obtained for the strains of Group II (Table 2) when the strains were grown aerobically on 5 mM-glucose. Three of the strains (AN937, AN938 and AN1543) had growth yields characteristic of fully uncoupled strains. Strain AN1340 had an intermediate growth yield.

Comparison of pH-dependence of uncD F_1-ATPase activities

The velocity of the ATPase reaction catalysed by each of the *uncD* F_1-ATPase preparations at pH 7.3–9.3 was compared with that catalysed by normal (*unc*$^+$) F_1-ATPase (see Fig. 1). The curves

Table 3. *Yield and specific activities of purified F_1 enzymes from uncD mutants*

The ATPase assay was performed at pH 8.0, 30°C in 50 mM-Tris/H_2SO_4/10 mM-ATP/5 mM-$MgCl_2$, by varying the time of incubation from 1 to 30 min and the amount of F_1-ATPase from 26 to 49 μg/ml as required. The values in parentheses are percentages of normal activity.

Strain	Wet wt. of cells (g)	Yield of F_1-ATPase (mg)	Yield of F_1-ATPase (mg/g of cells)	ATPase activity (μmol of ATP hydrolysed/min per mg of protein)
AN1340 (*uncD478*)	330	27.2	0.082	3.4 (15)
AN937 (*uncD430*)	320	24.0	0.075	0.37 (1.7)
AN938 (*uncD431*)	350	26.8	0.077	0.40 (1.8)
AN1543 (*uncD484*)	380	28.0	0.074	0.04 (0.18)
AN346 (*unc*$^+$)			0.043*	
AN862 (*unc*$^+$/*unc*$^+$)			0.09*	
AN1461 (*unc*$^{+n}$)			0.20	22.0† (100)

* Typical values noted previously in our laboratory.
† Normal value, under assay conditions as described.

Table 4. *Growth yields of uncD mutant strains*
Turbidities were measured in a Klett–Summerson colorimeter after aerobic growth in 10ml of minimal medium with 5mM-glucose at 37°C. Control experiments with a strain known to give uncoupled growth yields gave values of 117.

Strain	Growth yield (Klett units)
AN1340 (*uncD478*)	137
AN937 (*uncD430*)	117
AN938 (*uncD431*)	118
AN1543 (*uncD484*)	117
AN1461 (*unc*$^+$)	201

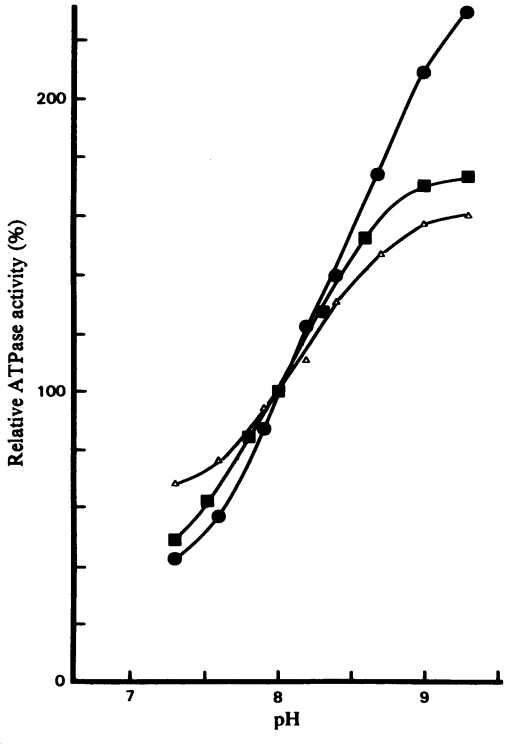

Fig. 1. *pH-dependence of ATPase activity of F_1-ATPase from uncD mutant strains*
The assay medium contained 50mM-Tris (adjusted to pH with H_2SO_4), 5mM-$MgCl_2$ and 10mM-ATP, at 30°C. Other conditions were as in Table 3. The results were expressed relative to the rate at pH8.0 (set at 100%). The results obtained with only two of the *uncD* F_1-ATPase preparations are plotted, together with those for normal (*unc*$^+$) F_1-ATPase for comparison. ■, *unc*$^+$ (AN1461); △, *uncD478* (AN1340); ●, *uncD430* (AN937). Of the two *uncD* F_1-ATPase preparations not shown on the graph, that from *uncD431* (AN938) resembled that from *uncD430* (AN937) very closely; that from *uncD484* (AN1543) followed the curve for *unc*$^+$ at pH < 8 and the curve for *uncD478* at pH > 8.

for the mutant F_1-ATPase preparations were similar to that obtained with normal (*unc*$^+$) F_1-ATPase, although not identical.

Comparison of apparent K_m values of uncD F_1-ATPase preparations

Apparent K_m values for MgATP and CaATP, deduced from Lineweaver–Burk double-reciprocal plots, are presented in Table 5. Linear double-reciprocal plots were obtained in all MgATP-hydrolysis experiments, and the K_m values found for MgATP were all similar to normal (Table 5). The $V_{max.}$ values for MgATP hydrolysis calculated from the double-reciprocal plots (results not shown) showed the same large variation from normal already evident from the data on specific ATPase activity in Table 3.

Linear double-reciprocal plots were not obtained in all the CaATP-hydrolysis experiments. Normal (*unc*$^+$) and *uncD478* F_1-ATPase preparations did give linear plots, and from these the apparent K_m values for CaATP shown in Table 5 were calculated. The value of 3.3mM for *uncD478* F_1-ATPase is significantly higher than normal. The $V_{max.}$ values for CaATP hydrolysis calculated from the linear double-reciprocal plots were: normal (*unc*$^+$) F_1-ATPase, 1.1 μmol hydrolysed/min per mg; *uncD478* F_1-ATPase, 0.5 (*uncD412* F_1-ATPase has a $V_{max.}$ for CaATP hydrolysis approximately twice that of normal F_1-ATPase; J. G. Wise, L. R. Latchney, D. Cox & A. E. Senior, unpublished work).

Curved double-reciprocal plots for CaATP hydrolysis were obtained for the three mutant F_1-ATPase preparations that had the very low rates of MgATP hydrolysis, i.e. those from *uncD430*, *uncD431* and *uncD484*. Fig. 2 shows such curved plots together with the linear plots observed with *uncD478* and *unc*$^+$ F_1-ATPase for comparison. The reason for the

Table 5. *Apparent K_m values for MgATP and CaATP of uncD mutant F_1-ATPase preparations*
The assays were performed at pH7.5 in 50mM-Tris/H_2SO_4, at 30°C. The ATP/metal-ion molar ratio was 2:1 throughout. ATP concentrations of 0.1–2.5mM were used.

Strain	K_m (mM) MgATP	K_m (mM) CaATP
AN1340 (*uncD478*)	0.35	3.3
AN937 (*uncD430*)	0.47	*
AN938 (*uncD431*)	0.48	*
AN1543 (*uncD484*)	1.25	*
AN1461 (*unc*$^+$)	0.59	0.58

* Curved double-reciprocal plots obtained; see Fig. 2 and the text.

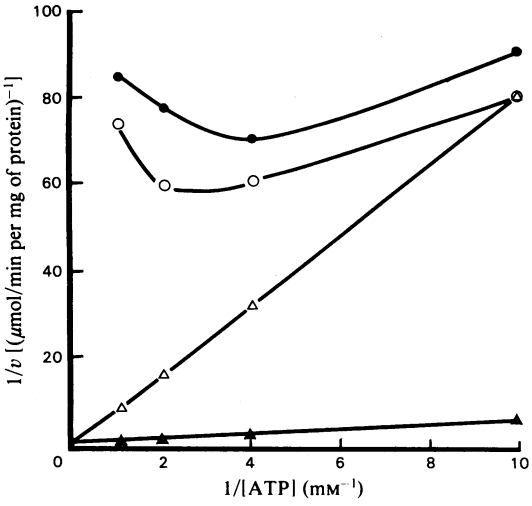

Fig. 2. *Kinetics of CaATP hydrolysis*
The assay medium contained 50mM-Tris/H_2SO_4, pH 7.5, and ATP and $CaCl_2$ in 2:1 molar ratio. F_1-ATPases: ●, *uncD430* (AN937); ○, *uncD431* (AN938); △, *uncD478* (AN1340); ▲, *unc*$^+$ (AN1461). *uncD484* (AN1543) F_1-ATPase gave a curved plot similar to that for *uncD430* F_1-ATPase (but with one-quarter of the velocity at each CaATP concentration) (results not shown).

Table 6. *Oxidative phosphorylation and ATP-dependent atebrin-fluorescence quenching catalysed by uncD mutant F_1-ATPase preparations in a reconstituted system*
Washed F_1-ATPase-depleted membranes (1mg) of strain AN1461 (pAN45,*unc*$^+$) were incubated with saturating amounts of F_1-ATPase in 0.1ml of 10mM-Hepes/5mM-$MgCl_2$/300mM-KCl, pH 7.5, for 3min. For oxidative-phosphorylation assay the reconstituted mixture was then transferred by syringe to the assay vessel. The ATP-synthesis and oxygen-uptake assays were exactly as described previously (Cox *et al.*, 1973), with NADH as substrate. ATP-driven atebrin-fluorescence quenching was measured on the reconstituted membranes as in Table 2. Where no F_1-ATPase was added, there was no ATP synthesis or ATP-driven atebrin-fluorescence quenching. Values in parentheses are percentages of normal activity.

Strain from which F_1-ATPase was purified	Rate of ATP synthesis (nmol/min per mg)	P/O ratio	ATP-driven atebrin-fluorescence quenching (%)
AN1340 (*uncD478*)	1.7 (22)	0.034	52
AN937 (*uncD430*)	0.37 (5)	0.011	6
AN938 (*uncD431*)	0.07 (1)	0.003	8
AN1543 (*uncD484*)	0.23 (3)	0.004	8
AN1461 (*unc*$^+$)	7.7 (100)	0.153	80

curvature in some of the CaATPase double-reciprocal plots is not known, but it might be due to unusual inhibition by free Ca^{2+} ions or CaATP at higher concentration.

Oxidative phosphorylation catalysed by the uncD mutant F_1-ATPase preparations

Having shown that the four new *uncD* F_1-ATPase preparations were altered in ATP-hydrolytic properties to various degrees, we now decided to study how well the forward reaction, i.e. ATP synthesis, was catalysed by each enzyme. To measure this we required an assay system that would give good rates of oxidative phosphorylation, at fairly constant rates of electron transport. In preliminary experiments we found that membranes prepared directly from the haploid strains described in Group II of Table 2 had very low rates of ATP synthesis with widely varying NADH oxidase rates.

We therefore used a technique in which the required *uncD* F_1-ATPase preparation was first re-bound to washed membranes of strain AN1461, and then the reconstituted membranes were assayed for oxidative phosphorylation. Strain AN1461 carries a multicopy plasmid containing the *unc*$^+$ operon, and membranes from this strain are calculated to contain at least five times as many membrane-sector sites for binding F_1-ATPase as in membranes from a haploid (*unc*$^+$) strain. It was necessary to wash the membranes from strain AN1461 five times in 5mM-Tes buffer lacking *p*-aminobenzamidine to release all of the F_1-ATPase.

In the reconstituted system the rate of oxidative phosphorylation should be determined by the catalytic properties of the F_1-ATPase preparation that was re-bound. To ensure that saturation of all available membrane-sector sites on the washed membranes from strain AN1461 was achieved, preliminary experiments utilizing the atebrin-fluorescence-quenching assay (as in Table 2) were performed to ascertain, for each F_1-ATPase preparation, the amount of F_1-ATPase required to saturate a given amount of membranes. This amount was found by gradually increasing the amount of F_1-ATPase added to 1mg of washed membranes and noting when full quenching response on addition of NADH was attained. The ATP-dependent atebrin-fluorescence quenching was then determined on these same samples and is presented in Table 6.

The rates of ATP synthesis and the P/O ratios determined in these reconstituted membranes are also tabulated in Table 6. It is notable that the rate of ATP synthesis catalysed by F_1-ATPase from strain AN1340 was impaired to almost the same degree as ATP hydrolysis (see Tables 6 and 3). This suggests

that both 'backward' and 'forward' reactions are equally affected in this *uncD* F_1-ATPase preparation. Unfortunately the actual rates of ATP synthesis noted for *uncD430*, *uncD431* and *uncD484* F_1-ATPase preparations (Table 6) were too low to allow confidence in making comparisons, so that it is not possible to decide whether backward and forward reactions of F_1-ATPase are equally affected in these strains. We can say with confidence that ATP synthesis and hydrolysis rates are both very low, however, in these F_1-ATPase preparations.

Two-dimensional gel electrophoresis of membranes from uncD mutant strains of Group II

Our previous work (Senior *et al.*, 1979*b*) had shown that two-dimensional gel electrophoresis of membranes from *uncD* mutant strains readily allowed us to observe the altered β-subunits. We therefore examined each of the strains in Group II (Table 2) by using this procedure. The results (not shown) indicated that the β-subunits of the F_1-ATPase preparations from strains AN937 and AN938 had lower isoelectric points than the normal β-subunit, and β-subunits from strains AN1340 and AN1543 had the same isoelectric points as normal.

Discussion

Out of 17 new *uncD* mutant strains of *E. coli* studied in this work, four were found to synthesize an F_1-aggregate of normal molecular size, and the F_1-ATPase was purified in good yield from each of these four strains. On the basis of the results presented, there seem to be three distinct 'types' of F_1-ATPase among the four preparations. That from strain AN1543 (*uncD484*) was distinguished by its extremely low ATPase activity (0.2% of normal). The F_1-ATPase preparations from strains AN937 (*uncD430*) and AN938 (*uncD431*) had 1.7–1.8% of normal ATPase activity and were very similar in all properties studied. Therefore we class them together as one 'type'. F_1-ATPase from strain AN1340 (*uncD478*) had 15% of normal ATPase activity and 22% of normal ATP-synthesis activity. This was sufficient to allow this strain to grow on limiting concentrations of glucose with a growth yield intermediate between that of a normal strain and an uncoupled strain.

Comparison of the properties of F_1-ATPase preparations from strain KF11 (Kanazawa *et al.*, 1980) and from strains carrying the *uncD412* allele (Senior *et al.*, 1979*b*; J. G. Wise, L. R. Latchney, D. Cox & A. E. Senior, unpublished work) with the properties of the F_1-ATPase preparations described in the present paper suggests that the new F_1-ATPase preparations reported here are each different from either of the previously described mutant F_1-ATPase preparations. The *uncD412* and KF11 F_1-ATPase both have about 10% of normal specific MgATPase activity. The only one of the four new F_1-ATPase preparations reported here with similar MgATPase activity is the *uncD478* F_1-ATPase. However, *uncD478* F_1-ATPase differs from both *uncD412* and KF11 F_1-ATPase in that it has abnormally high K_m for CaATP (as compared with K_m values for CaATP close to normal for *uncD412* and KF11 F_1-ATPase). The *uncD478* F_1-ATPase also has a CaATPase/MgATPase ratio of less than 1 (as does normal *unc*$^+$ F_1-ATPase), whereas *uncD412* and KF11 F_1-ATPases have CaATPase/MgATPase ratios of approx. 2 and 3.5 respectively.

From the experiments described, it is not possible to draw firm conclusions regarding the likely reasons for the functional defects in any of the new *uncD* F_1-ATPases. Such conclusions must await more detailed analyses. Work on the mechanism of ATP hydrolysis by F_1-ATPase has suggested that intersubunit conformational interaction and co-operativity are of major importance in catalysis (Grubmeyer & Penefsky, 1981*a*,*b*; Wise *et al.*, 1981). F_1-ATPase preparations with low turnover rates may be of substantial assistance in experimental study of intersubunit conformational interactions, and the four new mutant *uncD* F_1-ATPase preparations described here may therefore prove valuable in studying the catalytic mechanism.

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