Oxidative phosphorylation by mutant *Escherichia coli* membranes with impaired proton permeability

Graeme B. COX, David A. JANS, Frank GIBSON, Lyndall LANGMAN, Alan E. SENIOR* and Anthony L. FIMMEL

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia

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The effect on the function of the *Escherichia coli* F_1F_0 -ATPase of the substitution of leucine-31 by phenylalanine in the *c*-subunit of the enzyme was examined. The assembly of the mutant *c*-subunit requires an increased gene dosage [Jans, Fimmel, Langman, James, Downie, Senior, Ash, Gibson & Cox (1983) *Biochem. J.* **211**, 717–726], and this was achieved by incorporation of the *uncE408* or *uncE463* alleles on to F-plasmids or multicopy plasmids. Membranes from strains carrying either the *uncE463* or *uncE408* alleles on F-plasmids or multicopy plasmids were capable of carrying out oxidative phosphorylation. In particular, membranes from strain AN1928 (pAN162, *uncE463*) gave phosphorylation rates and P/O ratios equal to or greater than those obtained for the control strain AN1460 (pAN45, *unc*⁺). However, the mutant membranes, on removal of the F_1 -ATPase, appeared to be proton-impermeable. The ATPase activity of the mutant membranes was also resistant to the inhibitor dicyclohexylcarbodi-imide.

The genes forming the *unc* operon in *Escherichia coli* code for the components of the energy-coupling ATPase complex and map at about 82.5 min on the *E. coli* chromosome (Downie *et al.*, 1979a). Genetic studies (Gibson *et al.*, 1978; Downie *et al.*, 1981) and DNA sequencing (Gay & Walker, 1981) have established that the *unc* operon consists of nine genes in the order *uncIBEFHAGDC*, the *uncl* gene being closest to the promoter. The protein coded for by the *uncl* gene does not appear to be a structural component of the F_1F_0 -ATPase complex (Gay & Walker, 1981). The *uncBEF* genes code for the *a*-, *c*- and *b*-subunits respectively of the membrane sector of the ATPase (Downie *et al.*, 1981), whereas the *uncHAGDC* genes code for the δ -, α -, γ -, β - and ϵ -subunits respectively of the F_1 -ATPase (Downie *et al.*, 1980; Gay & Walker, 1981; Gunsalus *et al.*, 1982). Mutations affecting either assembly or function of the F_1F_0 -ATPase have been reported in seven of these genes, the exceptions being the *uncl* and *uncH* genes.

Mutations in which the aspartic acid-61 of the

c-subunit is replaced by glycine or asparagine (Hoppe *et al.*, 1980b; Wachter *et al.*, 1980) have confirmed studies with the inhibitor DCCD that this amino acid plays a key role in proton translocation and oxidative phosphorylation. The examination of mutations in the *uncA* gene coding for the α -subunit of the F_1 -ATPase have also given insight into the mechanism of oxidative phosphorylation (Wise *et al.*, 1981, 1982). These mutations have been shown to block interaction between the aurovertin-binding site on the β -subunit and an exchangeable nucleotide-binding site, which was postulated to involve both the α - and β -subunits. The authors concluded that the *uncA* mutations did not affect the catalytic site but prevented ATPase activity and ATP synthesis by preventing co-operativity between the catalytic sites on the F_1 -ATPase.

The present paper examines the effect of the substitution of leucine-31 by phenylalanine that occurs in the mutant *c*-subunits of the F_1F_0 -ATPase formed in strains carrying multicopies of the *uncE408* or *uncE463* alleles.

Materials and methods

Enzymes and chemicals

Restriction endonucleases and T4-bacteriophage DNA-ligase were obtained from New England

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

* Present address: Department of Biochemistry, University of Rochester Medical Center, School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

Table 1. *Bacterial strains and plasmids used*

Chromosomal gene nomenclature is that of Bachmann *et al.* (1976). Plasmid nomenclature is that of Novicek *et al.* (1976).

Strain	Relevant genotype	Notes and references
Bacterial strains		
AN1330	pAN34 <i>uncE463/uncE463 argH pyrE entA recA</i>	Jans <i>et al.</i> (1983)
AN1332	pAN10 <i>uncE408/uncE408 argH pyrE entA recA</i>	Jans <i>et al.</i> (1983)
AN1331	pAN34 <i>uncE463/uncE408 argH pyrE entA recA</i>	Jans <i>et al.</i> (1983)
AN1333	pAN10 <i>uncE408/uncE463 argH pyrE entA recA</i>	Jans <i>et al.</i> (1983)
AN1871	pAN128 <i>uncE408/unc416::MuB⁺E⁻F⁻H⁻A⁻G⁻D⁻C⁻ argH pyrE entA recA</i>	Obtained by transformation of AN888 with pAN128
AN888	<i>unc416::MuB⁺E⁻F⁻H⁻A⁻G⁻D⁻C⁻ argH pyrE entA recA</i>	Strain isolated as described by Gibson <i>et al.</i> (1978); similar genetically to <i>unc418::Mu</i> in strain AN884
AN1570	<i>uncG428 thr leu rk mk⁺ recA</i>	<i>uncG428</i> allele transduced from original isolate into <i>E. coli</i> C600 <i>rk⁻ mk⁺</i> (laboratory stock)
AN1927	pAN162/ <i>uncG428 thr leu rk mk⁺ recA</i>	Derivation described in the present paper
AN1928	pAN162/ <i>unc416::MuB⁺E⁻F⁻H⁻A⁻G⁻D⁻C⁻ argH pyrE entA</i>	AN888 transformed by pAN162 (Downie <i>et al.</i> , 1979b)
AN795	<i>uncE408 argH pyrE entA</i>	Downie <i>et al.</i> (1979b)
AN1115	<i>uncE463 argH pyrE entA</i>	Downie <i>et al.</i> (1979b)
AN862	pAN11 <i>unc⁺/argH ilv pyrE purE recA</i>	Partial diploid strain carrying <i>unc⁺</i> operon on F-plasmid (Cox <i>et al.</i> , 1978)
AN1460	pAN45 <i>unc⁺/unc413::MuB⁺E⁻F⁻H⁻A⁻G⁻D⁻C⁻ argH pyrE entA recA</i>	Obtained by transforming AN887 (Gibson <i>et al.</i> , 1978) with pAN45 (Downie <i>et al.</i> , 1980)
AN1897	<i>argH pyrE entA</i>	Spontaneous revertant of AN795 (Jans <i>et al.</i> , 1983)
AN1871	pAN128 <i>uncE408/unc416::MuB⁺E⁻F⁻H⁻A⁻G⁻D⁻C⁻ argH pyrE entA recA</i>	AN888 transformed by pAN128
AN1961	pAN2 <i>uncA401/uncE408 argH pyrE entA recA</i>	Obtained by mating AN780 (Cox <i>et al.</i> , 1978) with AN845 (Jans <i>et al.</i> , 1983)
AN1962	pAN2 <i>uncA401/uncE463 argH pyrE entA recA</i>	Obtained by mating AN780 (Cox <i>et al.</i> , 1978) with AN1307 (Jans <i>et al.</i> , 1983)
Plasmids		
pAN128	<i>Cm^rTc^suncB⁺E408 F⁺H⁺A⁺G⁺D⁺C⁺</i>	pACYC184 derivative carrying <i>uncE408</i> allele on insert (Jans <i>et al.</i> , 1983)
pAN162	<i>Cm^rTc^suncB⁺E463 F⁺H⁺A⁺G⁺D⁺C⁺</i>	pACYC184 derivative carrying <i>uncE463</i> allele on insert; derivation described in the present paper
pAN159	<i>Cm^rTc^suncB⁺E463 F⁺H⁺A⁺G⁺D⁺C⁺</i>	Original plasmid isolated carrying <i>uncE463</i> allele (Jans <i>et al.</i> , 1983)
pAN45	<i>Cm^rTc^sunc⁺</i>	pACYC184 derivative carrying <i>unc</i> operon (Downie <i>et al.</i> , 1980)
pAN34	<i>pyrE⁺uncE463 ilvC⁺ argH⁺</i>	F-plasmid carrying <i>uncE463</i> allele prepared as described previously (Gibson <i>et al.</i> , 1977b)
pAN10	<i>pyrE⁺uncE408 ilvC⁺ argH⁺</i>	F-plasmid carrying <i>uncE408</i> allele prepared as described previously (Gibson <i>et al.</i> , 1977b)
pAN11	<i>pyrE⁺unc⁺ ilvC⁺ argH⁺</i>	F-plasmid carrying <i>unc</i> operon (Cox <i>et al.</i> , 1978)
PACYC184	<i>Cm^rTc^s</i>	Chang & Cohen (1978)

Biolabs (Beverly, MA, U.S.A.). All chemicals used were of the highest quality available.

Bacterial strains and plasmids

All of the bacterial strains used were derived from *E. coli* K-12 and are described, together with the plasmids used, in Table 1.

Genetic techniques

The techniques used for genetic experiments were as outlined previously (Gibson *et al.*, 1977a,b, 1978; Downie *et al.*, 1980).

Preparation of plasmids

Plasmid DNA was prepared as described by Selker *et al.* (1977).

Media and growth of organisms

The mineral-salts minimal medium used and additions were as described previously (Gibson *et al.*, 1977b). Cells for the preparation of membranes were grown in 14-litre fermenters essentially as described previously (Cox *et al.*, 1970). The medium in the fermenter vessels was supplemented with 5% (v/v) Luria broth (Luria & Burrous, 1957). Growth yields were measured as described previously (Cox *et al.*, 1970). Turbidities of cultures were measured with a Klett–Summerson colorimeter.

Preparation of cell membranes

Membranes were prepared as described previously (Cox *et al.*, 1973a; Senior *et al.*, 1979). Briefly, washed cells were suspended in a 0.1 M-Tes buffer system (pH 7.0) containing magnesium acetate, sucrose, EGTA, 6-aminohexanoic acid and *p*-aminobenzamidine. The 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 washed four times. The buffer system described above was used in the first wash; in the second wash the buffer system used contained Tes (50 mM), glycerol (15%, v/v), 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM); in the third and fourth washes the buffer system used contained Tes (5 mM), glycerol (15%, v/v), dithiothreitol (0.5 mM), 6-aminohexanoic acid (40 mM) and *p*-aminobenzamidine (6 mM). The washed membrane preparation was resuspended in the last of these buffer systems. The F_1F_0 -ATPase was removed from the membranes by dialysing about 10 ml of membrane preparation against 2 litres of a buffer system similar to that used for the washed membrane suspension, except that *p*-aminobenzamidine was not included. The dialysis was maintained at 4°C for 24 h with a buffer change after 8 h.

Purification and radioactive labelling of the *c*-subunit of the F_1F_0 -ATPase

The *c*-subunit of the F_1F_0 -ATPase was purified from chloroform/methanol extracts (Fillingame, 1976) of membranes by chromatography on CM-cellulose (Graf & Sebald, 1978; Hoppe *et al.*, 1980a). For trace-labelling of the *c*-subunit, membranes (200 mg of protein) were treated with [14 C]DCCD (5 μ Ci, 0.1 μ mol) for 24 h at 4°C. The bulk of the membranes (6 g of protein) was similarly treated with excess unlabelled DCCD (1 mM). The treated membranes were pooled and the *c*-subunit was extracted and purified.

Assay of ATPase activity and DCCD inhibition

ATPase activity was measured by using the method described by Gibson *et al.* (1977a). For the

determination of the DCCD-sensitivity of the ATPase activity, membranes (0.3 mg of protein) were incubated in 5 ml of the ATPase assay mixture together with the indicated amount of DCCD at 30°C. Samples were taken at 0, 3, 6, 9 and 12 min, and the rate was determined, for each DCCD concentration, from the linear portion of the curve, and the percentage inhibition was calculated. Linearity was normally achieved after about 3 min.

Measurement of atebrin-fluorescence quenching

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

Oxidative phosphorylation

Oxidative phosphorylation was measured as described by Cox *et al.* (1973b).

Protein determination

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.

Results

Preparation of strains and their growth characteristics

The *uncE408* and *uncE463* alleles both code for mutant *c*-subunits in which the leucine at position 31 is replaced by phenylalanine (Jans *et al.*, 1983). The assembly of significant amounts of this mutant *c*-subunit requires an increased gene dosage above that found in a haploid strain (Jans *et al.*, 1983). Thus the partial diploid strains AN1330 (*uncE463/uncE463*), AN1332 (*uncE408/uncE408*), AN1331 (*uncE463/uncE408*) and AN1333 (*uncE408/uncE463*) all assemble sufficient F_1F_0 -ATPase to allow these strains to utilize succinate as energy source (Jans *et al.*, 1983). In addition, strains were prepared in which high-copy-number plasmids carrying either the *uncE463* allele or the *uncE408* allele were present. The chromosomal *unc* operon in these strains has a Mutator bacteriophage inserted in the *uncE* gene, ensuring that only the plasmid-encoded mutant *c*-subunits are formed. Thus strain AN1871 was isolated after transformation of strain AN888 (*unc-416::MuB⁺EFHAGDC*) with the pre-

Table 2. Growth yields of mutant strains with plasmids carrying either the *uncE463* or the *uncE408* allele

Growth yields were measured as turbidities (Klett units) after growth in limiting concentrations of glucose. For the partial diploid strains, the *unc* alleles shown first in parentheses are those on the plasmid and those shown second are on the chromosome.

Strain	Growth yield	
	3 mM-Glucose	6 mM-Glucose
AN1115 (<i>uncE463</i>)	86	142
AN795 (<i>uncE408</i>)	90	147
AN1897 (<i>unc</i> ⁺)	147	235
AN1330 (<i>uncE463/uncE463</i>)	124	215
AN1331 (<i>uncE463/uncE408</i>)	122	220
AN1332 (<i>uncE408/uncE408</i>)	117	222
AN1333 (<i>uncE408/uncE463</i>)	125	217
AN1871 (pAN128, <i>uncE408</i>)	120	203
AN1928 (pAN162, <i>uncE463</i>)	124	208

viously described (Jans *et al.*, 1983) plasmid pAN128 (*uncE408*), and selection was made for growth on chloramphenicol-containing medium. Plasmid pAN162 (*uncE463*) was prepared from plasmid pAN159 (Jans *et al.*, 1983). The plasmid pAN159 (*uncE463*) and the vector pACYC184 were digested with the restriction endonuclease *Hind*III and mixed, and the mixture was religated with T4 DNA-ligase. Strain AN1570 (*uncG428, recA*) was transformed with the ligation mixture, and selection was made for growth on succinate/chloramphenicol-containing medium. One colony was purified, and this strain (AN1927) was shown to carry a plasmid (pAN162) corresponding in size to the plasmid pAN45 (Downie *et al.*, 1980). Strain AN1928 was isolated after transformation of strain AN888 (*unc-416::MuB⁺EFHAGDC*) with the plasmid pAN162 (*uncE463*), and selection was made for growth on chloramphenicol-containing medium. Strains AN1871 (pAN128, *uncE408*) and AN1928 (pAN162, *uncE463*) were both able to grow on media with succinate as carbon source.

The growth yields of strains AN1871 (pAN128, *uncE408*) and AN1928 (pAN162, *uncE463*) were measured as turbidities in a Klett–Summerson colorimeter, after growth had ceased in media containing 3 mM- or 6 mM-glucose. The values obtained were similar to those obtained for the partial diploid strains carrying the *uncE408* or *uncE463* alleles and were about 80–90% of the growth yield obtained for a normal strain (Table 2). These results indicated that oxidative phosphorylation was occurring in strains carrying more than one copy of the *uncE408* or *uncE463* alleles, but was occurring with diminished efficiency.

ATPase activities

The ATPase activities of membranes prepared from the partial diploid strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) indicated that, even with the increased gene dosage, the amount of F_1F_0 -ATPase assembled was only about 30% of that found in membranes from the haploid revertant strain AN1897 (Table 3). A further increase in gene dosage by using the high-copy-number plasmids pAN128 (*uncE408*) and pAN162 (*uncE463*) resulted in a further increase in the amount of F_1F_0 -ATPase assembled, particularly in strain AN1928 (pAN162, *uncE463*). The ATPase activities of membranes prepared from this strain grown either on glucose or succinate were similar to the ATPase activities of membranes prepared from the corresponding *unc*⁺ strain AN1460 (Table 3). It would appear from the lower ATPase activities of membranes from strain AN1871 (pAN128, *uncE408*) that, although the *uncE408* and *uncE463* alleles have identical DNA sequences (Jans *et al.*, 1983), strains carrying the *uncE408* allele may also carry an additional mutation in another *unc* gene (see the Discussion section).

Inhibition of ATPase activities by DCCD

The ATPase activities of membranes prepared from the mutant strains carrying either the *uncE408* or *uncE463* alleles were resistant to DCCD. The ATPase activity of membranes from strain AN1928 (pAN162, *uncE463*) was 20–30% inhibited at DCCD concentrations of about 3 µg/ml, but was about 10% inhibited at higher (10 µg/ml) concentrations (Fig. 1). The same pattern of DCCD inhibition was obtained with membranes from strain AN1871 (pAN128, *uncE408*) (results not shown). The ATPase activities of membranes from normal strains were about 80–90% inhibited by the higher DCCD concentrations (see Fig. 1).

The reactivity of the mutant *c*-subunit with DCCD was determined by treating a small amount of membrane with a trace amount of [¹⁴C]DCCD and including the radioactively labelled membranes with the bulk of unlabelled-DCCD-treated membranes before extraction with chloroform/methanol. The DCCD-treated *c*-subunit was then purified by CM-cellulose chromatography. A total of 0.91×10^5 c.p.m. was recovered with the purified DCCD-treated *c*-subunit from strain AN1332 (*uncE408/uncE408*), and 0.94×10^5 c.p.m. was recovered from strain AN1330 (*uncE463/uncE463*). The same experiment was performed with membranes from strains AN1460 (pAN45, *unc*⁺) and AN1897 (*unc*⁺), with 1.5×10^5 c.p.m. and 1.2×10^5 c.p.m. respectively being recovered from the purified DCCD-treated *c*-subunits. The yield of protein from strain AN1460 (pAN45, *unc*⁺) was 1.2 times that

Table 3. *ATPase activities and oxidative phosphorylation by membranes from normal and mutant strains*

Membranes were washed twice in low-ionic-strength buffer in the presence of *p*-aminobenzamidine, and the values are the averages of determinations on at least three membrane preparations. Oxygen uptake by the membrane preparations was measured by using an oxygen electrode. ATP formed from ADP in the presence of $^{32}\text{P}_i$ was trapped as glucose 6-phosphate. The reaction was stopped by the addition of trichloroacetic acid, and P_i was extracted after the addition of molybdate.

Membranes from strain	Growth substrate	ATPase activity ($\mu\text{mol/min per mg of protein}$)	NADH or D-lactate oxidase activity (ng-atoms of O/min per mg of protein)		ATP formation (nmol/min per mg of protein)	P/O
AN1897 (<i>unc</i> ⁺)	Glucose	1.4	NADH	106	18.5	0.18
			D-Lactate	7.8	1.1	0.14
AN1115 (<i>uncE463</i>)	Glucose	0	NADH	192	<0.1	0
AN795 (<i>uncE408</i>)	Glucose	0	NADH	183	<0.1	0
AN862 (<i>unc</i> ⁺ / <i>unc</i> ⁺)	Glucose	2.2	NADH	117	21	0.18
			D-Lactate	6.2	1.1	0.19
AN1460 (pAN 45, <i>unc</i> ⁺)	Glucose	1.9	NADH	49	9.1	0.19
			D-Lactate	3.3	0.6	0.18
AN1460 (pAN45, <i>unc</i> ⁺)	Succinate	3.8	NADH	61	23	0.39
			D-Lactate	12	4.8	0.42
AN1330 (<i>uncE463/uncE463</i>)	Glucose	0.42	NADH	74	6.3	0.09
			D-Lactate	14	0.7	0.05
AN1332 (<i>uncE408/uncE408</i>)	Glucose	0.34	NADH	82	7.2	0.09
			D-Lactate	5.9	0.3	0.06
AN1871 (pAN128, <i>uncE408</i>)	Glucose	0.64	NADH	109	11	0.10
			D-Lactate	22	2.1	0.10
AN1871 (pAN128, <i>uncE408</i>)	Succinate	1.1	NADH	71	12	0.17
			D-Lactate	13	1.7	0.13
AN1928 (pAN162, <i>uncE463</i>)	Glucose	1.9	NADH	56	24	0.42
			D-Lactate	7.2	2.2	0.31
AN1928 (pAN162, <i>uncE463</i>)	Succinate	4.0	NADH	89	30	0.34
			D-Lactate	19	5.5	0.29

obtained from an equivalent amount of membranes from strain AN1897 (*unc*⁺) and 3.5 times that obtained from strains AN1332 (*uncE408/uncE408*) and AN1330 (*uncE463/uncE463*). It is concluded therefore that the mutant *c*-subunits from strains AN1332 (*uncE408/uncE408*) and AN1330 (*uncE463/uncE463*) have about a 2-fold increase in reactivity with DCCD compared with a normal *c*-subunit.

Oxidative phosphorylation

Membranes prepared from the partial diploid strains AN1332 (*uncE408/uncE408*) and AN1330 (*uncE463/uncE463*) were capable of carrying out oxidative phosphorylation, giving P/O ratios of about 50% of those obtained for the revertant strain AN1897 and the partial diploid strain AN862 (*unc*⁺/*unc*⁺) (Table 3). However, membranes prepared from strain AN1928 (pAN162, *uncE463*) gave higher rates of phosphorylation and P/O ratios equal to or greater than those obtained with membranes from the corresponding control strain AN1460 (pAN45, *unc*⁺) (Table 3). The membranes from strain AN1871 (pAN128, *uncE408*) gave somewhat lower P/O ratios, and this could be

attributed to the lower ATPase activity of these membranes (see Table 3).

Quenching of atebrin fluorescence

The ATP-dependent atebrin-fluorescence-quenching activities of membranes from strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) were very low compared with that obtained for the revertant strain AN1897 (Fig. 2). The most surprising property of the membranes from strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*) was, however, the retention of NADH-dependent atebrin-fluorescence-quenching activity after removal of the F_1 -ATPase (Fig. 2). The loss of NADH-dependent activity accompanies the loss of ATP-dependent activity, when the F_1 -ATPase is removed from normal membranes (Fig. 2), due to the increased proton-permeability of the membranes.

The results obtained with strains AN1871 (pAN128, *uncE408*) and AN1928 (pAN162, *uncE463*) were qualitatively similar to those obtained for strains AN1330 (*uncE463/uncE463*) and AN1332 (*uncE408/uncE408*), the increased ATP-dependent atebrin-fluorescence-quenching activities

Fig. 1. *Inhibition of ATPase activity by DCCD*
Membranes (0.3 mg of protein) were incubated in 5 ml of the ATPase assay mixture together with the indicated amount of DCCD at 30°C. The mixture was sampled at intervals, and the rate was determined for each DCCD concentration and the percentage inhibition calculated. ▲, Membranes from strain AN1928 (pAN162, *uncE463*) prepared from cells grown on succinate as energy source; ■, membranes from cells of strain AN1928 (pAN162, *uncE463*) grown on glucose as energy source; ●, membranes from cells of strain AN1460 (pAN45, *unc*⁺) grown on glucose as energy source.

(Fig. 2) being due to the increased assembly of the F_1F_0 -ATPase complex (see Table 3). The membranes from strain AN1928 (pAN162, *uncE463*), though having a higher ATP-dependent atebrin-fluorescence-quenching activity than those from strain AN1460 (pAN45, *unc*⁺), obtained maximal quenching at a much lower rate (Fig. 2). The effect of removing the F_1 -ATPase from membranes of strains AN1928 (pAN162, *uncE463*) and AN1460 (pAN45, *unc*⁺) provided a striking contrast, in that the membranes from strain AN1928 (pAN162, *uncE463*) retained NADH-dependent atebrin-fluorescence-quenching activity whereas membranes from strains AN1460 (pAN45, *unc*⁺) did not. The ATP-dependent atebrin-fluorescence-quenching activity could be completely reconstituted in stripped membranes from strain AN1928 (pAN162, *uncE463*) by the addition of purified F_1 -ATPase from either strain AN1460 (pAN45, *unc*⁺) or strain AN1928 (pAN162, *uncE463*). The P/O ratios obtained with unstripped membranes from these two strains were the same (see Table 3).

Discussion

The *uncE408* and *uncE463* alleles cause the substitution of phenylalanine for leucine at position 31 in the *c*-subunit of the F_1F_0 -ATPase (Jans *et al.*, 1983). The difficulty in assembling the mutant *c*-subunit is overcome by increasing the gene dosage (Jans *et al.*, 1983). The strains carrying the *uncE408* allele appear to have a second mutation resulting in loss of the F_1 -ATPase from the membranes. This second mutation appears to lie in the *uncA* gene, since only small colonies were obtained on succinate-containing medium when testing for complementation between the *uncE408* allele and the *uncA401* allele (L. Langman, unpublished work). Normal colonies were obtained in the complementation test between the *uncE463* and *uncA401* alleles. The mutation in the *uncA* gene does not appear to have any effect in the absence of the *uncE408* allele, since membranes from the revertant strain AN1897 are normal. The *c*-subunit from strain AN1897 has been isolated and shown to be indistinguishable from a normal *c*-subunit (Jans *et al.*, 1983). In addition, the membranes from strain AN1871 (pAN128, *uncE408*) could be reconstituted with normal F_1 -ATPase, and the reconstituted membranes gave very similar activities to those obtained with membranes from strain AN1928 (pAN162, *uncE463*) (G. Cox, unpublished work).

Membranes from each of the strains carrying either the *uncE463* or *uncE408* alleles, whether on F-plasmids or on high-copy-number plasmids, were capable of carrying out oxidative phosphorylation, even though, on removal of the F_1 -ATPase, the membranes appeared to be proton-impermeable. The growth of these strains on media containing succinate as carbon and energy source, and their growth yields on limiting concentrations of glucose, support the values for oxidative phosphorylation obtained with membrane preparations. The membranes from strain AN1928 (pAN162, *uncE463*) provide the clearest findings. The membrane ATPase activities were equivalent to those obtained for strain AN1460 (pAN45, *unc*⁺), but the ATPase activity from strain AN1928 (pAN162, *uncE463*) was only slightly sensitive to the inhibitor DCCD. The phosphorylation rates and P/O ratios obtained for the membranes from strain AN1928 (pAN162, *uncE463*) were equal to or greater than those obtained for membranes from strain AN1460 (pAN45, *unc*⁺). Remarkably, membranes from strain AN1928 (pAN162, *uncE463*) retained the NADH-dependent atebrin-fluorescence-quenching activity after removal of the F_1 -ATPase. It is inferred from this observation that the F_0 -sector of the ATPase in membranes from strain AN1928 (pAN162, *uncE463*) is impermeable to protons, or at least proton-permeability is greatly impaired. The

Fig. 2. *NADH*- 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.*, 1977a). Atebrin was added to give a final concentration of 4 μ M, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to 20 μ M. (a) Membranes from strain AN1332 (*uncE408/uncE408*). (b) Stripped membranes from strain AN1332 (*uncE408/uncE408*). (c) Membranes from strain AN1871 (pAN128, *uncE408*). (d) Stripped membranes from strain AN1871 (pAN128, *uncE408*). (e) Membranes from strain AN1330 (*uncE463/uncE463*). (f) Stripped membranes from strain AN1330 (*uncE463/uncE463*). (g) Membranes from strain AN1928 (pAN162, *uncE463*). (h) Stripped membranes from strain AN1928 (pAN162, *uncE463*). Preincubation of these membranes with F_1F_0 ATPase gave fluorescence quenching indistinguishable from that shown in (g). (i) Membranes from strain AN1897 (*unc⁺*). (j) Stripped membranes from strain AN1897 (*unc⁺*). (k) Membranes from strain AN1460 (pAN45, *unc⁺*). (l) Stripped membranes from strain AN1460 (pAN45, *unc⁺*).

oxidative phosphorylation obtained with membranes from this strain may therefore be energized by protons, generated by electron transport, that remain within the membrane.

The *E. coli* mutant F_0 -sector of the ATPase seems to be similar to the F_0 -sector found in the photosynthetic bacterium *Rhodospseudomonas capsulata*, where removal of the F_1 -ATPase does not appreciably enhance proton-permeability (Melandri *et al.*, 1970). Membranes from this organism have been used extensively to study bacterial photophosphorylation, with the general conclusion that localized coupling occurs between the redox reactions and phosphorylation (Melandri *et al.*, 1980; Baccarini-Melandri *et al.*, 1977). Nelson & Eyton (1979) have shown that the F_1 -ATPase is removed from chloroplast membranes by NaBr treatment and that, although such membranes are initially proton-permeable, this characteristic is lost after 5–8 days' incubation at 4°C. However, these membranes appear somewhat different from *E. coli* membranes in that *E. coli* membranes, from which the F_1 -ATPase has been removed, remain proton-permeable. In addition, it would seem unlikely that the substitution of leucine-31 by phenylalanine in the mutant c -subunit would cause any structural instability in the assembled F_0 -sector of the ATPase.

The secondary and tertiary structure of the c -subunit has been proposed (Sebald & Hoppe, 1981; Jans *et al.*, 1983). In the proposed 'hairpin' structure leucine-31 is juxtaposed to aspartic acid-61. The replacement of leucine-31 by the bulkier phenylalanine may cause some displacement of the aspartic acid side chain, resulting in the loss of proton-permeability through the F_0 -sector of the ATPase. The bulkier phenylalanine side chain may also prevent the conformational changes associated with the inhibition of the ATPase activity by the inhibitor DCCD.

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References

- Baccarini-Melandri, A., Casadio, R. & Melandri, B. A. (1977) *Eur. J. Biochem.* **78**, 389–402
- Bachman, B. J., Low, K. B. & Taylor, A. L. (1976) *Bacteriol. Rev.* **40**, 116–167
- Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **135**, 1141–1156
- Cox, G. B., Newton, N. A., Gibson, F., Snoswell, A. M. & Hamilton, J. A. (1970) *Biochem. J.* **117**, 551–562
- Cox, G. B., Gibson, F., McCann, L. M., Butlin, J. D. & Crane, F. L. (1973a) *Biochem. J.* **132**, 689–695
- Cox, G. B., Gibson, F. & McCann, L. (1973b) *Biochem. J.* **134**, 1015–1021
- Cox, G. B., Downie, J. A., Gibson, F. & Radik, J. (1978) *Biochem. J.* **170**, 593–598
- Deamer, D. W., Prince, R. C. & Crofts, A. R. (1972) *Biochim. Biophys. Acta* **274**, 323–335
- Downie, J. A., Gibson, F. & Cox, G. B. (1979a) *Annu. Rev. Biochem.* **48**, 103–131
- Downie, J. A., Senior, A. E., Gibson, F. & Cox, G. B. (1979b) *J. Bacteriol.* **137**, 711–718
- Downie, J. A., Langman, L., Cox, G. B., Yanofsky, C. & Gibson, F. (1980) *J. Bacteriol.* **143**, 8–17
- Downie, J. A., Cox, G. B., Langman, L., Ash, G. R., Becker, M. & Gibson, F. (1981) *J. Bacteriol.* **145**, 200–210
- Fillingame, R. H. (1976) *J. Biol. Chem.* **251**, 6630–6637
- Gay, N. J. & Walker, J. E. (1981) *Nucleic Acids Res.* **9**, 3919–3926
- Gibson, F., Cox, G. B., Downie, J. A. & Radik, J. (1977a) *Biochem. J.* **162**, 665–670
- Gibson, F., Cox, G. B., Downie, J. A. & Radik, J. (1977b) *Biochem. J.* **164**, 193–198
- Gibson, F., Downie, J. A., Cox, G. B. & Radik, J. (1978) *J. Bacteriol.* **134**, 728–736
- Graf, T. & Sebald, W. (1978) *FEBS Lett.* **94**, 218–222
- Gunsalus, R. P., Brusilow, W. S. A. & Simoni, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 320–324
- Hoppe, J., Schraier, H. U. & Sebald, W. (1980a) *Eur. J. Biochem.* **112**, 17–24
- Hoppe, J., Schraier, H. U. & Sebald, W. (1980b) *FEBS Lett.* **109**, 107–111
- Jans, D. A., Fimmel, A. L., Langman, L., James, L. B., Downie, J. A., Senior, A. E., Ash, G. R., Gibson, F. & Cox, G. B. (1983) *Biochem. J.* **211**, 717–726
- Kell, D. B., Ferguson, S. J. & John, P. (1978) *Biochim. Biophys. Acta* **502**, 111–126
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luria, S. E. & Burrous, J. W. (1957) *J. Bacteriol.* **74**, 461–476
- Melandri, B. A., Baccarini-Melandri, A., San Pietro, A. & Gest, H. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 477–484
- Melandri, B. A., Venturoli, G., DeSantis, A. & Baccarini-Melandri, A. (1980) *Biochim. Biophys. Acta* **592**, 38–52
- Nelson, N. & Eyton, E. (1979) in *Cation Flux Across Biomembranes* (Mukohata, Y. & Packer, L., eds.), pp. 409–415, Academic Press, New York
- Novick, R. P., Clowes, R. C., Cohen, S. N., Curtiss, R., Datter, N. & Falkow, S. (1976) *Bacteriol. Rev.* **40**, 168–189
- Sebald, W. & Hoppe, J. (1981) *Curr. Top. Bioenerg.* **12**, 1–64
- Selker, E., Brown, K. & Yanofsky, C. (1977) *J. Bacteriol.* **129**, 388–394
- Senior, A. E., Fayle, D. R. H., Downie, J. A., Gibson, F. & Cox, G. B. (1979) *Biochem. J.* **180**, 111–118
- Wachter, E., Schmid, R., Deckers, G. & Altendorf, K. (1980) *FEBS Lett.* **113**, 265–270
- Wise, J. G., Latchney, L. R. & Senior, A. E. (1981) *J. Biol. Chem.* **256**, 10383–10389
- Wise, J. G., Latchney, L. R. & Senior, A. E. (1982) *Biophys. J.* **37**, 114a