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ENERGY CONSERVATION IN MEMBRANES OF MUTANTS OF *ESCHERICHIA COLI* DEFECTIVE IN OXIDATIVE PHOSPHORYLATION

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SUMMARY

1. Energy conservation in membranes of *Escherichia coli* was measured using 9-amino-6-chloro-2-methoxyacridine fluorescence.

2. Energy conservation in membranes, depleted of coupling factor, can be restored either by the isolated crude coupling factor or by *N,N'*-dicyclohexylcarbodiimide (DCCD) but not by Dio-9 or oligomycin.

3. Several mutants, defective in different components of the ATP-synthesizing complex, are described.

4. Mutants possessing a DCCD-insensitive ATPase have been isolated which can or cannot be recoupled by DCCD.

INTRODUCTION

Recently we reported the isolation and partial characterization of two mutants of *Escherichia coli* K12, deficient in oxidative phosphorylation[1–3]. Similar mutants have been isolated by other groups[4–8]. All of the mutants isolated thus far appear to be affected in the membrane–ATPase complex of *E. coli* such that either the catalytic function of the enzyme[1–5,7,8] or its resistance to the energy-transfer inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD)[3] have been genetically altered.

The isolation and partial purification of the ATPase of *E. coli* has recently been reported[9]. In addition, the reconstitution of *E. coli* membranes depleted of the ATPase with the purified coupling factor has also been achieved[9,10].

This report describes the properties of various mutants blocked in energy conservation with respect to their ability to couple either the oxidation of substrates or the hydrolysis of ATP to the energization of the membrane. Both the energy-linked transhydrogenase reaction and the change in fluorescence of an acridine dye, 9-amino-6-chloro-2-methoxyacridine (ACMA) have been measured. Fluorescent acridine dyes have been used as a probe for the energized state of the membrane by Kraayenhof

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

et al.[11,12] in chloroplasts, by Eilermann[13] in phosphorylating particles of *Azotobacter vinelandii*, and by Lee[14] in submitochondrial particles.

In addition, we will show that the respiratory-linked functions in membrane particles of some of the mutants can be stimulated by the addition of very small concentrations of DCCD, but not by the addition of coupling factor isolated from the parent, while these functions in depleted particles from the parent can be restored either by DCCD or by soluble ATPase.

METHODS

Strains

E. coli strain A428 (F^-, *pro*$^-$, *lac*$_1^-$, T_6^R, *gal*$_2^-$, *ara*$^-$, *his*$^-$, *xyl*$^-$, *man*$^-$, B_1^-, *str*^{R}) was used as a parent organism. The strains N_{144}, K_{11} and A_{144} were derived from A428 as described previously[1]. The isolation and further characterization of strain B_{V4} will be the subject of a separate communication.

Preparation of particles

Cells were grown in 1-l cultures in a rich medium (0.5% yeast extract, 0.8% Bacto Tryptone and 0.5% NaCl) as previously described[3]. For the preparation of membrane particles (P^+) according to Fisher *et al.*[15] and Kobayashi and Anraku[16], 60 g cells were suspended in 350 ml 50 mM Tris–HCl (pH 7.0) containing 15 mM $MgCl_2$, and sonicated for 2×1 min with a Branson sonifier (maximal output). After centrifugation at $30\,000 \times g$ for 10 min the supernatant was centrifuged at $150\,000 \times g$ for 30 min. The pellet contained particles (P^+) which were suspended to a final concentration of 25 mg/ml in 50 mM Tris–HCl (pH 7.5). To obtain particles depleted of ATPase, P^+ was washed twice with 100 ml 50 mM Tris–HCl (pH 7.5). After further washing with 10 ml 1 mM Tris–HCl (pH 7.5) and centrifugation at $200\,000 \times g$ for 180 min, the pellet contained depleted particles (P^-), while the supernatant contained the crude coupling factor. The coupling factor (about 1 mg protein/ml) was stored in 20% methanol in liquid N_2.

ATPase, transhydrogenase and oxidative capacity of membrane particles

ATPase and energy-linked transhydrogenase were measured as described earlier[3]. Oxidation velocity in membrane particles was measured polarographically in a medium containing 50 mM Tris–HCl (pH 7.5) and 2.5 mM $MgCl_2$ together with either 7 mM succinate or 1.5 mM NADH at 25 °C.

Fluorescence assay

The fluorescence of ACMA was measured with an Eppendorf fluorimeter (filters: excitation 405+436 nm, emission 500–3000 nm) in a medium containing 50 mM Tris–HCl, 2.5 mM $MgCl_2$, 1 μM ACMA and particles (0.4 mg protein/ml), final pH 7.8. The temperature of the reaction was 22 °C. 5 mM succinate or 0.5 mM ATP (final concentrations) were added if required. Inhibitors such as DCCD, Dio-9 or oligomycin were added as an ethanolic solution.

Ammonia particles

Cells were suspended in a medium containing 50 mM Tris–HCl, 1 mM EDTA

and brought at pH 9.2 with ammonia. After sonication for 2×1 min the solution was treated as described earlier for P^+ particles.

Chemicals

DCCD was obtained from Koch-Light Laboratories.

The following compounds were gifts: Dio-9, Gist-Brocades N.V.; oligomycin, Upjohn Chem. Co.; carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), Dr P. Heytler; ACMA, Dr R. Kraayenhof.

RESULTS

Energization in membranes of A428 (parent)

Fig. 1 shows that upon the addition of an oxidizable substrate to membrane particles of the parent A428, the fluorescence of the fluorescent acridine derivative ACMA is quenched. Addition of KCN, an inhibitor of the respiratory chain, or anaerobiosis restores the fluorescence to the original level. Subsequent addition of ATP induces again quenching which is relieved by DCCD, an inhibitor of the membrane-bound bacterial ATPase. Both oxidation-driven and ATP-driven quenching of ACMA fluorescence are sensitive to the uncoupler FCCP (2.5 μM). Fig. 1 also shows that in membrane particles depleted of the coupling factor, no quenching of ACMA fluorescence is observed neither upon addition of succinate nor ATP. Quenching in coupled particles (P^+) can be observed with either succinate, NADH or D-lactate as substrates, although the rate and the extent of quenching may be different as a result of the different oxidation rates. NADH is oxidized much faster than the other substrates, and the rate and extent of quenching are larger.

Upon addition of the crude coupling factor to depleted membranes, the succinate- and ATP-driven quenching is restored (Fig. 2) as observed previously in particles from *A. vinelandii*[13]. Separate experiments show that the soluble ATPase which is resistant to DCCD can be bound to the particles, resulting in its sensitivity to

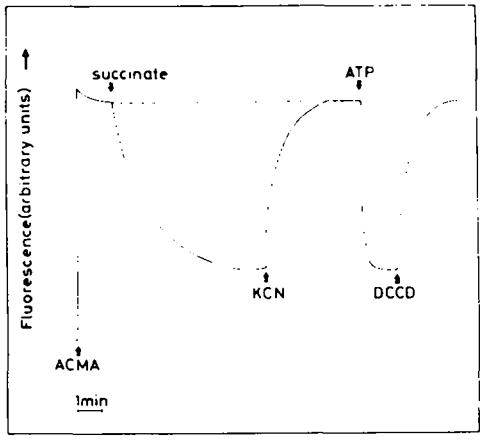

Fig. 1. Quenching of ACMA fluorescence by P^- and P^- particles of A428 upon energization. Fluorescence quenching was measured as described in Methods. Where indicated, succinate (5 mM), KCN (3 mM), ATP (0.5 mM) and DCCD (60 μM) were added. — · — ·, P^+; - - - -, P^-.

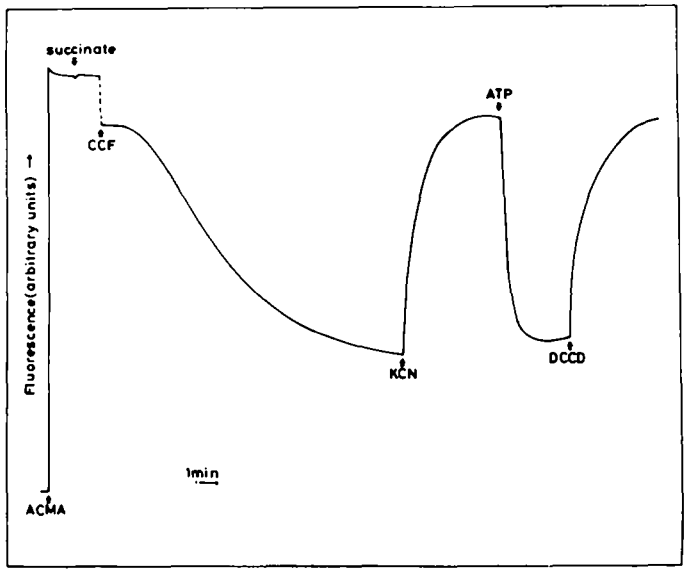

Fig. 2. Reconstitution of P⁻ (A428) with parental crude coupling factor. Fluorescence quenching was measured as described in Methods. The reaction was started by the addition of 5 mM succinate and 0.1 mg parental crude coupling factor (CCF, spec. act. 5 μmoles P_i released/min per mg protein at 37 °C). KCN (3 mM), ATP (0.5 mM) and DCCD (60 μM) were added at the arrows, respectively.

TABLE I

BINDING OF PARENTAL COUPLING FACTOR TO P⁻ MEMBRANES OF PARENT AND MUTANTS OF *E. COLI* K12

Crude coupling factor of parent (A428), P^+ and P⁻ membranes were isolated as described in Methods. To measure binding, P⁻ particles (about 3 mg protein) and 0.2 mg parental crude coupling factor (spec. act. 6 μmoles P_i released/min per mg protein at 37 °C) were incubated in a medium containing 50 mM Tris-HCl (pH 7.8) and 2.5 mM $MgCl_2$, final volume 2 ml, for 5 min at 37 °C. After centrifugation at 200000 × *g* for 20 min, the pellet was resuspended in about 0.5 ml 50 mM Tris-HCl (pH 7.8) and 2.5 mM $MgCl_2$. The ATPase activity of P^+, P⁻, the crude coupling factor and reconstituted membranes was measured as described in Methods. DCCD, when used, was added at a concentration of 60 μM.

Strain	*ATPase (nmoles P_i released/min per mg protein)*							
	P+		*P−*		*P− + crude coupling factor*		*Crude coupling factor*	
	−DCCD	*+DCCD*	*−DCCD*	*+DCCD*	*−DCCD*	*+DCCD*	*−DCCD*	*+DCCD*
A428	230	40	30	18	230	65	6000	6000
N_{I44}	30	27	0	0	63	22	—	—
Bv_4*	12	13	0	0	29	17	- -	- -
K_{I1}	130	135	5	4	49	35	- -	—
A_{I44}	100	100	2.5	2.5	22	17	- -	- -

* In some experiments the ATPase activity of Bv_4 particles was much lower than reported earlier[3]. It appeared that the ATPase activity was released upon strong sonication in the supernatant.

DCCD (Table I). In similar experiments, restoration of the energy-linked transhydrogenase has been observed by Bragg and Hou[9] in *E. coli*. Fig. 2 shows also the time dependency of reconstitution of depleted particles with the crude coupling factor. The coupling factor from chloroplasts (CF_1)[25] or from mitochondria (F_1 or F_1 *plus* oligomycin-sensitivity conferring protein)[26,27] was unable to replace the bacterial ATPase in the reconstitution.

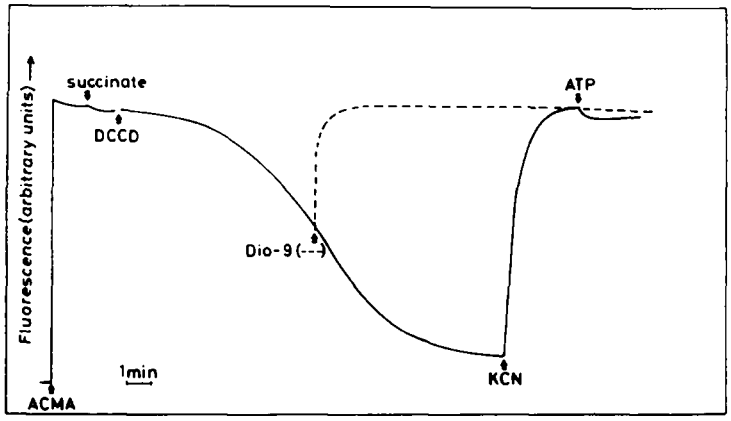

Fig. 3. Stimulation by DCCD of quenching of ACMA fluorescence by P^- particles from A428. Fluorescence quenching was measured as described in Methods. The reaction was started by adding 5 mM succinate and 10 μM DCCD to P^- particles. Subsequently, 3 mM KCN and 0.5 mM ATP were added. In a separate experiment Dio-9 (15 μg/ml) was added instead of KCN (- - - - -).

Fig. 3 shows that particles, depleted of the coupling factor (P^-), can catalyse a succinate-driven quenching of ACMA fluorescence upon addition of low concentrations of DCCD. Concentrations of DCCD below those required to inhibit maximally the membrane ATPase ($<10\,\mu$M), are able to restore the succinate-driven fluorescence quenching. The extra quenching induced by DCCD is sensitive to both FCCP and KCN. The development of the extra quenching is rather slow and depends on the DCCD concentration. The restoration of energy conservation by energy-transfer inhibitors has been observed earlier in submitochondrial particles using oligomycin[17] or DCCD[18] and in chloroplasts using DCCD[19]. Dio-9, however, an inhibitor of *E. coli* ATPase according to Roisin and Kepes[20] is unable to restore energy conservation. In fact, Fig. 3 shows that Dio-9 behaves rather like an uncoupler. In our hands, however, three different batches of Dio-9 (100 μg/ml) inhibited neither the membrane-bound ATPase nor the isolated ATPase. Oligomycin (3 μg/mg protein), which does not inhibit the bacterial ATPase, also has no effect on the energy-dependent quenching of fluorescence in depleted particles.

$ATPase^-$ mutant (N_{144})

As shown previously, the mutant N_{144} lacks ATPase activity, although the energy-linked transhydrogenase can still be driven by oxidation[2]. Fig. 4A shows that P^+ of this mutant can still support an oxidation-driven quenching of ACMA fluorescence although less than in the parent, but ATP is unable to do so. After washing

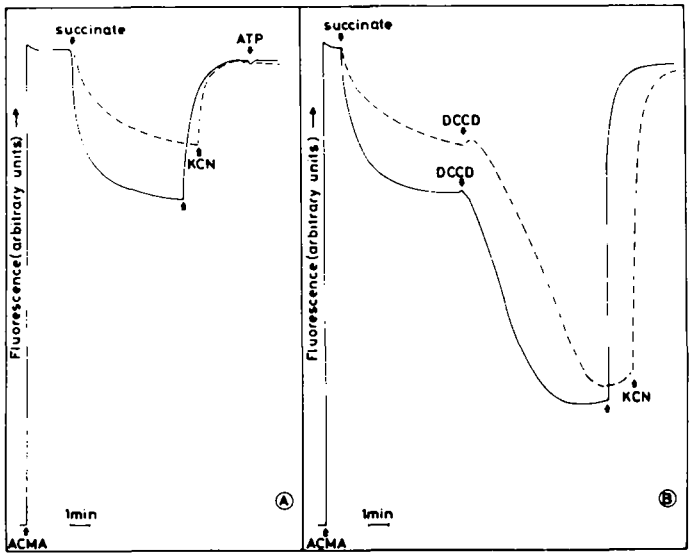

Fig. 4. Quenching of ACMA fluorescence by P^+ and P^- particles of mutant N_{144}. Fluorescence quenching was measured as described in Methods. (A) The reaction was started by the addition of 5 mM succinate. 3 mM KCN and 0.5 mM ATP were added at the arrows. ——, P^+; - - - - -, P^-. (B) The reaction was started by the addition of 5 mM succinate. When the quenching was maximal, DCCD (10 μM) was added and subsequently 3 mM KCN. ——, P^+; - - - - -, P^-.

in low salt medium, the P^- particles from N_{144} partially retain their ability to couple succinate oxidation to quenching of ACMA fluorescence, in contrast to P^- from parental cells (Fig. 1). In both P^+ and P^- particles from N_{144}, coupling between oxidation and energy conservation can be stimulated by DCCD (Fig. 4B). No stimulation was observed by the crude coupling factor isolated from the parent A428. In addition, little binding of the coupling factor from A428 to depleted particles of N_{144} was observed (Table I).

ATPase$^+$ mutants (B_{V4}, K_{11}, A_{144})

In a previous report[3] we described some properties of a mutant, B_{V4}, defective in oxidative phosphorylation. The mutant possessed an active membrane ATPase, which was insensitive to DCCD. We have isolated other mutants, K_{11} and A_{144}, with the same properties with respect to ATPase and DCCD sensitivity (Table II).

The results in Figs 5 and 6 demonstrate that the mutants blocked in oxidative phosphorylation which possess the DCCD-resistant ATPase can be further divided into 2 groups. The particles from B_{V4} (Fig. 5) still retain some of the respiratory-linked quenching of ACMA fluorescence even after washing, but no enhancement is observed by the addition of DCCD either with P^+ or P^- particles. In sharp contrast, however, is the finding that the respiratory-linked quenching of ACMA fluorescence in particles from K_{11} or A_{144} (not shown) can be stimulated by low concentrations of DCCD (Fig. 6). The addition of ATP to particles from each of the three mutants had no effect on the energization of the membranes. As observed with particles from N_{144}, the addition of crude coupling factor to preparations of depleted membranes

of either B_{V4}, K_{I1} or A_{I44} was without effect. Similarly, almost no binding of the parental coupling factor to P^- of these mutants was observed (Table I).

TABLE II

ATPase, ENERGY-LINKED TRANSHYDROGENASE AND OXIDASE ACTIVITIES IN MEMBRANE PARTICLES OF STRAINS A428, N_{I44}, B_{V4}, K_{I1} AND A_{I44} OF *E. COLI* K12

Particles (P^+) of parent and mutants were isolated as described in Methods. ATPase, energy-linked transhydrogenase and oxidase activity were measured as described in Methods. If used 60 μM DCCD was added.

Strain	*ATPase (nmoles P_i released/min per mg protein)*		*Respiration-linked transhydrogenase (nmoles NADPH/min per mg protein)*	*Oxidation rate (natoms O/min per mg protein)*	
	−DCCD	*+DCCD*		*NADH*	*Succinate*
A428	254	48	19.4	690	175
N_{I44}	30	27	17.8	250	140
B_{V4}	269	254	15.4	700	135
K_{I1}	130	135	5.0	395	205
A_{I44}	100	100	5.1	318	140

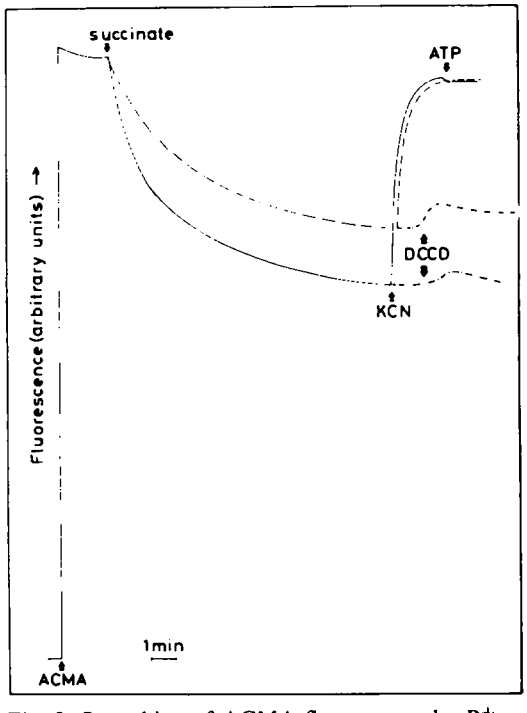

Fig. 5. Quenching of ACMA fluorescence by P^+ and P^- particles of mutant B_{V4}. Fluorescence quenching was measured as described in Methods. The reaction was started by the addition of 5 mM succinate. KCN, ATP and DCCD were added at concentrations of 3 mM, 0.5 mM and 10 μM, respectively. ·····, P^+; - - - - -, P^-; -·-·-·, P^+ or P^-, instead of KCN, DCCD was added.

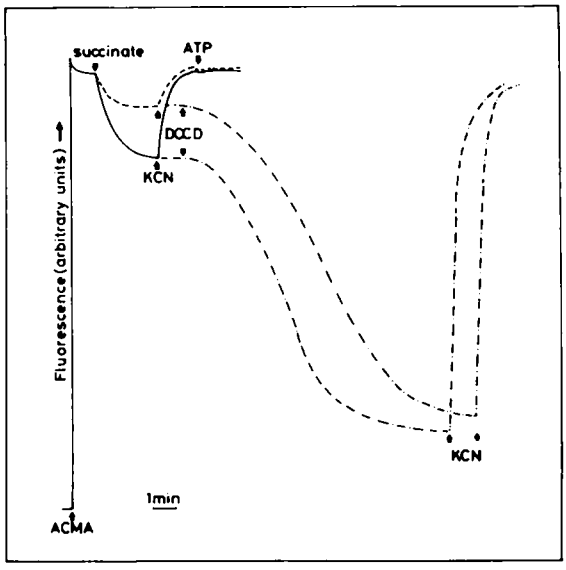

Fig. 6. Quenching of ACMA fluorescence by P^+ and P^- particles of mutant K_{11}. Fluorescence quenching was measured as described in Methods. The reaction was started by the addition of 5 mM succinate. KCN, ATP and DCCD were added at concentrations of 3 mM, 0.5 mM and 10 μM, respectively. ———, P^+; – – – –, P^-; -·-·-·, P^+ or P^-, instead of KCN, DCCD was added. When the quenching of ACMA fluorescence was maximal, 3 mM KCN was added.

Ammonia-treated membrane particles

Since no reconstitution between washed depleted mutant particles and parental coupling factor could be observed, possibly due to the inability of the parental coupling factor to attach to the mutant membranes, a more drastic method for the preparation of depleted particles was employed. Cells were sonicated at high pH in ammonia, similar to the procedure for preparing A particles of mitochondria[21]. It could be shown that A428 particles were completely uncoupled, showing no quenching of ACMA fluorescence upon energization, but could be recoupled either by the addition of coupling factor or by low concentrations of DCCD. It should be remarked, however, that these ammonia-treated particles still contain more than 80% of the ATPase activity of P^+, in contrast to P^- particles which contain less than 10% of the ATPase activity of P^+. Mutant particles prepared in the same manner, however, displayed the same characteristics as the P^- particles described in Figs 4–6. Thus, partial coupling of succinate oxidation to quenching of ACMA fluorescence was observed, there was no reconstitution with the coupling factor from A428, and stimulation of the quenching of ACMA fluorescence by DCCD was observed in ammonia particles from N_{144}, K_{11} and A_{144}, but not in particles of B_{V4}.

Genetics of the mutants

The mutations in each of the four mutants are point mutations all of which have been located at 73.5 min on the *E. coli* chromosome. Each of the mutations reverts with a frequency of 1 in 10^8, and they are all co-transducible with the *ilv*, but not with *metE* markers.

DISCUSSION

The results presented in this paper show that at least three different mutants can be isolated with defects in the ATP-synthesizing complex. These differences can be distinguished by (1) using the fluorescence of an acridine dye as a measure of the energized state of the membrane by following quenching of fluorescence in both washed and non-washed membrane particles, with and without added coupling factor, or in the presence of low concentrations of an energy-transfer inhibitor such as DCCD, and (2) by comparing the ATPase activities of the various mutants. One class of mutants, represented by N_{144}, lacks the membrane Mg^{2+}-Ca^{2+}-ATPase activity. Two classes of mutants, B_{V4}, K_{11} and A_{144} contain ATPase, but quenching of ACMA fluorescence upon energization in the membranes of the latter two mutants can be stimulated by DCCD in contrast to the activity in particles of B_{V4}. Since the ATPase of all three mutants is resistant to concentrations of DCCD which inhibit parental ATPase, it is tempting to suggest that B_{V4} may be altered in the DCCD binding site, while A_{144} and K_{11} may be affected in some component linking ATPase to the membrane. Thus energization in A_{144} and K_{11} is enhanced by DCCD, while the inhibitor has no effect on B_{V4}.

The inability to find reconstitution between the parent coupling factor and washed membranes of any of the mutants may be due to the fact that the parent coupling factor is a tight complex of ATPase and some other component, possibly a DCCD sensitivity conferring protein (DSCP), which is unable to bind to membranes of the mutants from which DSCP is not readily solubilized. The DSCP may be analogous to the OSCP in mitochondria (*cf.* ref. 22) or to nectin in *Streptococcus faecalis*[23]. Evidence in favour of a DCCD-binding site in the membrane fraction of *S. faecalis* has been published previously[24].

The ability to distinguish between three different types of mutants suggests the possibility that three different genes have been affected. Nevertheless, all of the mutants map very closely together at 73.5 min on the *E. coli* chromosome, and the different phenotypes may reflect lesions in the same gene. The mutations, although point mutations, may have pleiotropic effects. For example, the point mutation in N_{144} not only leads to an inactivation of the catalytic ATPase activity, but apparently also affects the ability of the mutant protein complex to be solubilized from the membrane, since washed particles of N_{144} cannot bind parental coupling factor. In this regard, the mutants described in this paper differ from the ATPase-less mutant of Simoni and Shallenberger[7], since in the latter case, reconstitution of energy-dependent transhydrogenase was observed[10].

The observation that DCCD but not oligomycin is able to recouple oxidation and energy conservation is interesting in demonstrating that the restoration is a rather specific effect, possibly requiring a DCCD-binding site. The fact that Dio-9 cannot recouple washed particles although it inhibits the ATPase, suggests that this inhibitor may resemble aurovertin, an inhibitor of the mitochondrial F_1, which does not bind to the membrane but directly to the ATPase.

Work on the isolation and purification of the different components of the ATP-synthesizing complex both in parent and mutants, the role of oxidative phosphorylation in several energy-requiring processes such as active transport of solutes, and the correlation between genetic and biochemical data is in progress.

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