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DIFFERENTIATION BETWEEN MUTANTS OF *ESCHERICHIA COLI* K_{12} DEFECTIVE IN OXIDATIVE PHOSPHORYLATION

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SUMMARY

Hybrid membrane particles from two mutants of *Escherichia coli* K_{12}, B_{V4} and K_{I1}, defective in oxidative phosphorylation, have been prepared, in which ATP-driven membrane energization is restored.

A soluble factor of mutant K_{I1} was found to have properties similar to parental crude coupling factor, ATPase (EC 3.6.1.3). Membrane particles of this mutant could not be reconstituted by parental coupling factor. Either parental coupling factor, or the soluble factor of mutant K_{I1} could reconstitute both respiration-driven and ATP-driven energization to membrane particles of mutant B_{V4} or to parental particles depleted of ATPase. Mutant B_{V4} was found to be devoid of coupling factor activity, while retaining the ability to hydrolyze ATP. Both mutants possess an ATPase with an altered binding to the membrane.

Mutant K_{I1} is impaired in respiration-driven amino acid transport, in contrast to mutant B_{V4}.

The three major subunits of parental *Escherichia coli* ATPase have been isolated and antibodies have been prepared against these subunits. Antibodies against the largest subunit (α component) or against the intact catalytic subunits ($\alpha+\beta$ components) inhibit both ATP-P_i exchange in the parent organism as well as ATP hydrolytic activity in parent and mutants. Antibodies against the two other subunits (β or γ components) also inhibit these two reactions, but were found to be less effective. Mutant N_{I44}, which lacks ATPase activity, shows no precipitin lines with anti-α, anti-β, anti-γ, or anti-($\alpha+\beta$) preparations. In contrast, mutants B_{V4} and K_{I1}, exhibit cross-reactivity with all of the antisera.

INTRODUCTION

The isolation and characterization of mutants of *Escherichia coli* defective

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; MES, 2-(*N*-morpholino)ethane sulfonic acid; Tricine, tris (hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMG, methyl-β-D-thiogalactoside.

in oxidative phosphorylation has led to the demonstration of a role for membrane ($Mg^{2+}+Ca^{2+}$)-ATPase in coupled ATP synthesis in this organism [1–8]. These mutants are of two types: (a) those which lack ATP hydrolytic activity; and (b) those in which the hydrolytic capacity of the enzyme is retained, but in which ATP hydrolysis cannot be coupled to the generation of a high energy state or intermediate, as measured by ATP-driven transhydrogenase [3, 4] or the ATP-driven quenching of a fluorescent dye ACMA [5].

Reconstitution of both respiratory driven as well as ATP-driven transhydrogenase to suitably depleted membranes of *E. coli* by a homogeneous preparation of ATPase has been reported [9], and such reconstitution has been described with soluble preparations from an *UncB* mutant with depleted membranes of *UncA* [6, 8]. Membranes from *UncB* could not be reconstituted. Similarly, Bragg and Hou [10] demonstrated the reconstitution of both respiratory and ATP-driven transhydrogenase in a mutant of *E. coli* ML, lacking ATPase activity; DL 54, using parental coupling factor.

The capacity of ATPase to reconstitute energy-dependent activities in depleted membranes apparently requires the presence of five subunits (α, β, γ, δ, ε) since purified preparations of ATPase lacking the δ subunit are inactive in either binding to membrane or in reconstitution [11–13]. In this regard, it has recently been demonstrated that the hydrolytic activity of the enzyme requires at most, a mixture of α and β subunits [12].

This report describes studies on the reconstitution of ATP-driven quenching of fluorescence of ACMA with mutants defective in oxidative phosphorylation which allow for the further subdivision of mutants retaining ATPase activity into two distinct biochemical classes. In addition, the isolation of antibodies prepared against three of the major denatured subunits of *E. coli* ATPase and their effects on energy-dependent reactions is also described.

MATERIALS AND METHODS

E. coli K_{12} strain A_{428} (F^-, pro^-, $lac_1{}^-$, $T_6{}^R$, $gal_2{}^-$, ara^-, his^-, mal^-, man^-, $B_1{}^-$, str^R) was used as a parent organism. The strains N_{144}, K_{11} and A_{144} were derived from strain A_{428} as described previously [2]. Mutant B_{V4} was isolated from parent A_{428} as follows: After mutagenesis of A_{428} by ultraviolet irradiation [2] cells were diluted (1 : 5) into nutrient broth, containing glucose (0.5 %) and yeast extract (0.5 %) and incubated overnight at 37 °C in a shaking water bath. Samples containing about 10^6 cells were passed through membrane filters (Sartorius: pore size, 0.45 μm, diameter 50 mm). The filters were washed with saline and placed on agar plates containing Davis minimal medium [14] supplemented with citrate, histidine (50 μg/ml), proline (50 μg/ml) and vitamin B (1 μg/ml) (supplemented Davis medium) with succinate (0.5 %) as a sole source of carbon and energy and penicillin (2000 units/ml). After 12 h incubation at 37 °C, the filters were transferred onto similar plates containing malate (0.5 %) instead of succinate, and after a similar incubation a third transfer was made to plates containing α-ketoglutarate (0.5 %) as a sole source of carbon and energy. Finally, the filters were transferred to plates containing glucose (but without penicillin) and incubated at 37 °C. After 2 days, colonies appeared on the filters. Before each transfer, the filters were placed on

unsupplemented Davis medium to remove adsorbed nutrients. The colonies which appeared on the filters were picked and replica plated as described [2]. Those colonies able to grow on glucose but unable to utilize Krebs cycle intermediates as sole sources of carbon and energy were grown and screened for the respiratory ability of their membrane particles. One of the colonies which had normal respiratory ability, mutant B_{V4}, was retained for further study.

Cells of all strains were grown on supplemented Davis medium, containing glucose as sole source of carbon and energy (unless stated otherwise in the legends of the tables). Membrane particles and supernatant fractions were prepared according to a slight modification of the method of Bragg and Hou [9] as outlined in Fig. 1. For ATP-P_i exchange measurements, membrane particles were prepared as follows: Cells, about 16 g wet weight were washed with 100 ml of medium containing 0.1 M $MgCl_2$ and 0.2 M KCl and suspended in 24 ml of a medium containing 0.32 M sucrose, 8 mM NaCl and 45 mM Tricine · NaOH (pH 8). The cells were broken in a French pressure cell at 20 000 lb/inch^2, and the unbroken cells and debris were removed by centrifugation at 10 000 $\times g$ for 10 min. The supernatant was centrifuged at 150 000 $\times g$ for 1 h and the pellet was homogenized in 4 ml

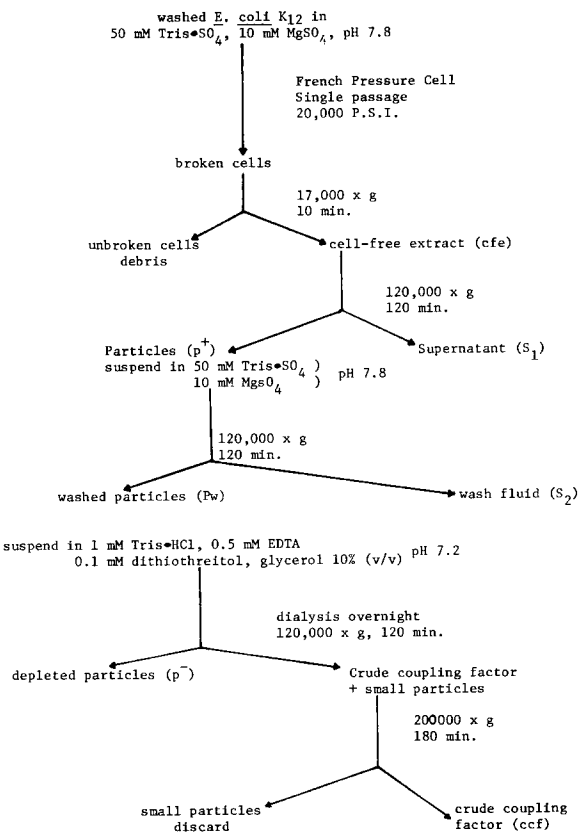

Fig. 1. Preparation of the various particulate and soluble fractions. All stages of the fractionation were carried out at 4 °C.

of buffer containing 0.32 M sucrose, 8 mM NaCl and 45 mM Tricine · NaOH (pH 8). The preparation was kept either at −70 or at −20 °C; repeated freezing and thawing did not affect its exchange activity.

ATPase activities were measured either by method I using [γ-^{32}P] ATP, as described previously [12] or by method II. In this case, inorganic phosphate released by ATP hydrolysis was assayed colorimetrically [15]. Amino acid transport [16] and energy-dependent quenching of ACMA fluorescence [5] were performed according to published methods. Cell protein was measured according to the procedure of Gornall et al. [17] and other protein determinations were performed according to the method of Lowry et al. [18].

Denatured ATPase subunits were prepared after sodium dodecyl sulfate electrophoresis of the enzyme by a modified procedure described for CF_1 [19]. 2 ml of purified bacterial coupling factor I (0.35 mg protein per ml) were incubated at room temperature for 2 h with 0.4 ml 10 % sodium dodecyl sulfate, 40 μl of mercaptoethanol and solid sucrose to give a final concentration of about 10 %. The solution was applied upon 24 sodium dodecyl sulfate gel tubes (8 % gel) and run for 5 h at 7 mA per tube. The gels were fixed, stained and destained as previously described [19] except that the staining period was reduced to 20 min. The bands were cut, lyophilized overnight and the protein was eluted by electrophoresis into dialysis bags as previously described [19].

Antisera against the denatured subunits α, β and γ and against native ($\alpha+\beta$) subunits [12] were prepared as described previously [19].

The ATP-P_i exchange reaction was performed in a medium containing in a final volume of 1 ml: 50 μmol of Tricine/MES (pH 7.0), 10 μmol of ATP, 5 μmol of sodium phosphate (pH 7.0), 5 μmol of $MgCl_2$, about 10^6 cpm of ^{32}P, 2.5 mg of bovine serum albumin and 170 μg membrane particles. After 10 min at 37 °C, the reaction was stopped with 0.2 ml trichloroacetic acid (30 %), and after centrifugation, [γ-^{32}P] ATP was determined in the supernatant [20].

Freund Bacto adjuvant (complete form) was purchased from Difco Laboratories and sodium dodecyl sulfate, acrylamide methylenebisacrylamide, ammonium persulfate and tetramethylethylene diamine were obtained from Bio-Rad. ACMA was a generous gift of Dr R. Kraayenhof. All other materials were of the highest purity commercially available.

RESULTS

We have recently described the isolation and characterization of several mutants of *E. coli* K_{12}, defective in oxidative phosphorylation, which possess DCCD-resistant ATPase activity [3, 5]. In order to determine whether these mutants, B_{V4}, K_{I1} and A_{I44}, can be differentiated into distinct biochemical classes, a technique of in vitro complementation has been developed. As shown in Fig. 2, hybrid particles can be prepared from a mixture of equal amounts of intact cells of mutants B_{V4} and K_{I1}, which exhibit ATP-driven quenching of ACMA fluorescence, although particles prepared from either of the mutants alone are completely devoid of this activity. Reconstitution of this ATP-dependent activity also occurs in hybrid particles prepared from mutants B_{V4} and A_{I44}, but not in hybrid particles of mutants A_{I44} and K_{I1}. The ATP-driven quenching in the hybrid particles is identical with

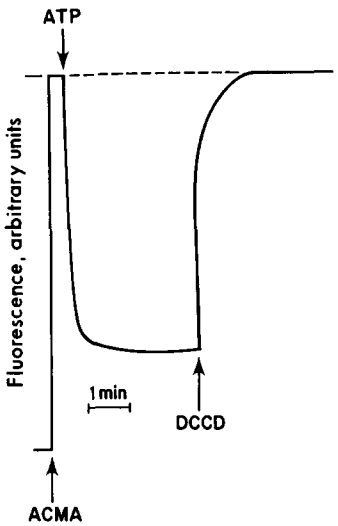

Fig. 2. Appearance of ATP-driven quenching of ACMA fluorescence in hybrid particles from class I (B_{V4}) and class II (K_{I1}) and A_{I44} mutants. Fluorescence quenching was measured as described in Materials and Methods. Intact cells of class I mutant (B_{V4}) were mixed with an equal amount (1 g wet weight each) of class II mutant cells prior to cell disruption and fractionation. The reaction was started by the addition of ATP (0.5 mM). At the arrow, DCCD (60 μM) was added. ——, p^+ (hybrid B_{V4} and K_{I1} 0.81 mg protein) or P^+ (hybrid B_{V4} and A_{I44}, 0.75 mg protein); - - -, p^+ of either B_{V4}, K_{I1} or A_{I44} alone.

that observed with parental particles in that it is sensitive to DCCD as well as to uncoupling agents. Although the activity in hybrid particles varies from preparation to preparation as does the activity in parental particles, the two preparations in this experiment showed about a 70 % quenching of ACMA fluorescence following the addition of ATP.

On the basis of these experiments, mutants B_{V4}, K_{I1} and A_{I44} can be subdivided into class I-B_{V4}, and class II-K_{I1} and A_{I44}. Thus far, we have been unable to differentiate between K_{I1} and A_{I44} by any physiological or biochemical test. Hybrid particles, prepared from any of these mutants and mutant N_{I44}, which lacks ATPase activity [2, 21], failed to exhibit ATP-driven quenching of ACMA fluorescence. Complementation between the various mutants is also obtained when the appropriate mutant cells are disrupted separately and subsequently mixed. Upon incubation with supernatant [9] of class II mutants, membrane particles (p^+) of class I mutant B_{V4}, display similar ATP-driven quenching as do the hybrid particles prepared by disrupting the cells together (Fig. 3). In contrast, supernatant of class I mutant B_{V4} cannot reconstitute particles (p^+) of class II mutants (not shown).

As reported previously [5], crude parental coupling factor can restore both respiration-driven and ATP-driven quenching of ACMA fluorescence to particles of parent A_{428} depleted of ATPase (p^-). In addition, purified *E. coli* ATPase can restore both respiration-driven and ATP-driven transhydrogenase activity to such depleted particles [9, 11]. As illustrated in Fig. 4, both respiration-driven and ATP-driven quenching of ACMA fluorescence can be restored to depleted particles (p^-)

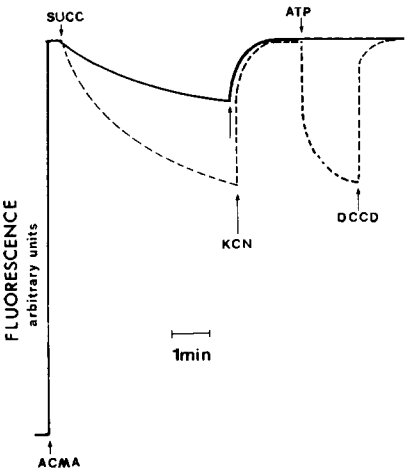

Fig. 3. Reconstitution of respiration-driven and ATP-driven quenching of ACMA fluorescence in depleted (P^-) or non-depleted (P^+) particles of class I mutant B_{V4} by crude coupling factor from A_{428} or class II mutants. The reaction conditions were identical to those described in the legend to Fig. 2. ——, P^+ B_{V4} (0.5 mg protein); - - -, P^+ B_{V4} (0.5 mg protein), together with crude coupling factor either from parent A_{428} or from class II mutants (K_{I1} or A_{I44}).

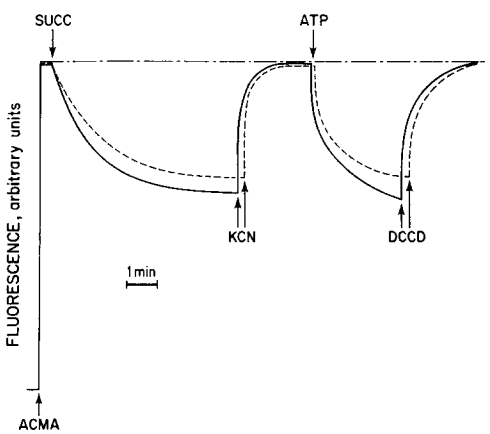

Fig. 4. Reconstitution of respiration-driven and ATP-driven quenching of ACMA fluorescence in depleted particles (P^-) of strain A_{428} by crude coupling factor from A_{428} or class II mutants. Fluorescence quenching was measured as described in Materials and Methods. Fraction "ccf" (Fig. 1) was used as a source of parental coupling factor whereas fraction S_1 was the source of coupling factor from class II mutants. Membrane particles and crude coupling factor from parent A_{428}, 0.6 mg (spec. act. 2 μmol P_i released/min per mg protein at 37 °C) or from class II mutants K_{I1} or A_{I44}, 1.0 mg (spec. act. 1 μmol P_i released/min per mg protein at 37 °C) were preincubated for 5 min. The reaction was started by the addition of 5 mM succinate. KCN (3 mM), ATP (0.5 mM) and DCCD (60 μM) were added at the arrows respectively. ——, P^+ A_{428} (0.75 mg protein); – · – · – · –, P^- A_{428} (0.6 mg protein); - - -, P^- A_{428} (0.31 mg protein) together with crude coupling factor either from parent A_{428} or from class II mutants (K_{I1} or A_{I44}).

of the parent organism by the supernatant fraction (s_1) of class II mutants. Thus, this fraction from the mutants K_{I1} or A_{I44} behaves in this regard, in a similar manner to the crude coupling factor of the parent. On the other hand, none of the soluble fractions of class I mutant B_{V4} was able to restore either respiration-driven or ATP-driven energization to depleted particles of the parent.

The respiration-driven energization of p^+ particles of class II mutants is always extremely low [5] and neither respiration-driven nor ATP-driven energization in p^+ or p^- of class II mutants can be reconstituted by any of the supernatant fractions of parent A_{428} or class I mutant B_{V4}. Furthermore, as depicted in Fig. 5, whole cells from class II mutant K_{I1} exhibit a very low rate and extent of active transport of the amino acid proline as compared with the parent organism. This is in agreement with cell-free studies of energy-dependent quenching of fluorescence of the dye ACMA [5]. The rate and extent of proline and TMG transport of mutants N_{I44} and B_{V4} was shown to be similar to rate and extent of uptake of these solutes by the parent organism. In contrast to the parent organism, this transport in mutants N_{I44} and B_{V4} was completely inhibited by KCN [16]. The residual transport in class II mutants was also completely abolished by 2 mM KCN (Fig. 5).

Membrane particles (p^+) of mutants both of class I and class II exhibit ATPase activity, but in contrast to p^+ particles of the parent A_{428}, this activity is resistant to the energy transfer inhibitor DCCD [3, 5]. The sensitivity of ATPase to DCCD is restricted to the membrane-associated enzyme [5, 13]. It is of interest, therefore, that the distribution of ATPase activity between particulate and supernatant fractions is quite different in mutant B_{V4} as compared with parent A_{428} (Table I). Under the

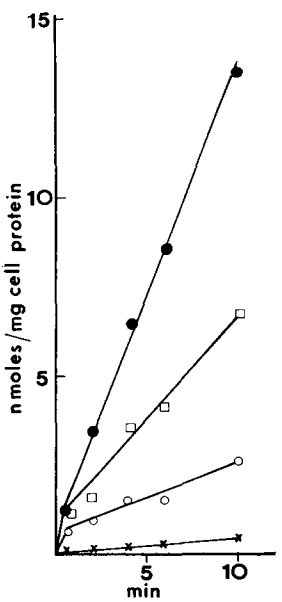

Fig. 5. Aerobic uptake of [^{3}H]proline by intact cells of the parent A_{428} and class I mutant K_{I1}. The experiment was conducted as previously described [16]. The final concentrations of proline (10 Ci/mol) were 10^{-5} M. ●–●, A_{428}; □–□, A_{428}+2 mM KCN; ○–○, K_{I1}; ×–×, K_{I1}+2 mM KCN.

TABLE I

DISTRIBUTION OF ATPase ACTIVITY BETWEEN PARTICULATE AND "SOLUBLE" FRACTIONS OF STRAINS A_{428} (PARENT) AND B_{v4} (MUTANT)

Membrane particles (P^+) and supernatant (s_1) were prepared from 2 g intact cells (wet weight) of strains A_{428} and B_{v4}: as illustrated in Fig. 1. ATPase was assayed according to method II as described in Materials and Methods.

Strain	Fraction	ATPase (units)	Protein (mg)	Specific activity (units/mg protein)
A_{428}	P^+	40	34	1.18
	S_1	10	78	0.13
	$P^+ + S_1$	50	112	
B_{v4}	P^+	20	34	0.59
	S_1	150	100	1.5
	$P^+ + S_1$	170	134	

conditions described, about 80 % of the parental ATPase activity is recovered in the particulate fraction (p^+), whereas in this experiment, nearly 90 % of the total ATPase activity of mutant B_{v4} is found in the supernatant (s_1). In addition, the total amount of ATPase activity in this mutant is more than 3-fold that of the total parental ATPase activity. Similar results were obtained for the class II mutants K_{11} and A_{144} (not shown), both with respect to distribution and total amount of ATPase activity. Even though the absolute amounts of ATPase activity are variable from day to day, both classes of mutants always have higher levels of ATPase activity than the parent, varying between 1.5 - and 5-fold.

Table I also shows that although most of the ATPase activity in the mutants is found in the supernatant, membrane particles (p^+) of these mutants still contain

TABLE II

SOLUBILIZATION OF MEMBRANE-BOUND ATPase FROM MUTANT B_{v4}

Membrane particles (P^+) were prepared from 4 g cells (wet weight) of strains A_{428} (parent) and B_{v4} (mutant) as described in Materials and Methods. These particles were suspended each in 24 ml 50 mM Tris · SO_4^{2-}, pH 7.8, containing 10 mM $MgSO_4$, and were centrifuged to yield washed particles (P_w) and wash fluid (S_2) as outlined in Fig. 1. ATPase was measured using method II as described in Materials and Methods

Strain	Fraction	ATPase (units)	Protein (mg)	Specific activity (units/mg protein)
A_{428}	P^+	66	60	1.1
	P_w	61	46	1.33
	S_2	3	10	0.3
B_{v4}	P^+	60	64	0.94
	P_w	12	50	0.24
	S_2	48	16	3.0

considerable levels of ATPase activity. Usually, ATPase is stripped from *E. coli* membranes following washing in the absence of magnesium at low ionic strength [9]. The results in Table II demonstrate that some 80 % of the ATPase activity of membrane particles of mutant B_{V4} can be easily removed in the presence of magnesium (10 mM) without lowering the ionic strength. Similar results are also obtained for class II mutants (not shown). These results, taken together with the resistance of the mutant ATPases to DCCD clearly indicate that the genetic lesions in class I and class II mutants result in an altered association of the ATPase with the membranes.

It has recently been shown that a mixture of α and β subunits is sufficient for ATP hydrolysis, and that an antibody prepared against this mixture inhibits the activity [12]. As illustrated in Table III, the membrane ATPase activity of parent A_{428} and mutants B_{V4} (class I) and K_{I1} (class II) is also inhibited by anti-$(\alpha+\beta)$. Although most of the ATPase activity of class I and class II mutants is not isolated in its membrane-bound form (Table I) the ATPase activity of the supernatants of the mutants is also inhibited by the antibody (not shown).

Antibodies have also been obtained against the denatured subunits α, β, and γ prepared in a fashion similar to that reported previously for coupling factor 1 from chloroplasts [19]. The results in Fig. 6 illustrate the immunodiffusion profiles with membrane particles (p^+) from A_{428} (parent), N_{I44}, B_{V4}, and A_{I44} treated with 0.5 % sodium dodecyl sulfate (to solubilize the subunits) with anti-α, anti-β, anti-γ, and anti-$(\alpha+\beta)$. It can be seen that mutants B_{V4} and A_{I44} exhibit patterns similar to those obtained with the parental membranes. In striking contrast, however, p^+ particles from N_{I44} which contain no ATPase activity, show no precipitin lines with any of the antibodies. In addition, no precipitin lines could be detected in the supernatant fraction obtained from mutant N_{I44}.

The effects of the various antisera on the energy-dependent ATP-P_i exchange reaction can be observed in Table IV. Anti-$(\alpha+\beta)$ and anti-α, which inhibit ATPase

TABLE III

INHIBITION OF MEMBRANE ATPase OF STRAINS A_{428} (PARENT), B_{V4}, AND K_{I1} BY ANTI-$(\alpha+\beta)$

ATPase was assayed according to method II as described in Materials and Methods. Antisera were added just prior to the initiation of the reaction.

Particles	Additions*	ATPase (units/mg membrane protein)
A_{428} (62 μg)	None	0.91
	anti-$(\alpha+\beta)$	0.14
	control serum	0.95
B_{V4} (67 μg)	None	0.84
	anti-$(\alpha+\beta)$	0.10
	control serum	0.84
K_{I1} (49 μg)	None	0.59
	anti-$(\alpha+\beta)$	0.06
	control serum	0.63

* Either 5 μl antiserum or 5 μl control serum was added.

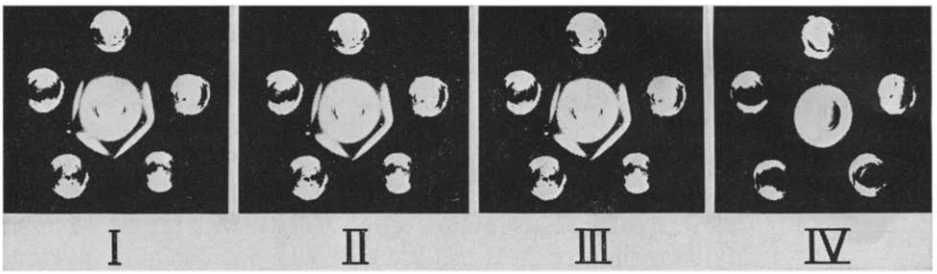

Fig. 6. Interactions between sodium dodecyl sulfate-treated particles (P^+) and antibodies against bacterial coupling factor I subunits. In immunodiffusion experiments the plates were prepared as described [20]. Membrane particles containing sodium dodecyl sulfate (0.5 %) were placed in the center well (I, A_{428}; II, A_{144}; III, B_{V4}; IV, N_{144}). Antisera were added to the peripheral wells: top, control sera; upper right, anti-α; lower right, anti-β; lower left, anti-$(\alpha+\beta)$; upper left, anti-γ.

TABLE IV

EFFECTS OF ANTISERA AGAINST SUBUNITS OF BACTERIAL COUPLING FACTOR I ON THE ATP-P_i EXCHANGE AND THE ATPase REACTIONS IN MEMBRANE PARTICLES OF STRAIN A_{428}

ATPase and ATP-P_i exchange were measured as described in Materials and Methods. For the ATPase reaction method I was followed. The reaction mixture for ATPase under ATP-P_i exchange conditions were identical as described for the ATP-P_i reaction, except that $^{32}P_i$ was replaced by [γ-^{32}P]ATP (about 50 000 cpm per reaction). Subsequently the liberation of $^{32}P_i$ was determined [21]. 170 μg particle protein was used in each reaction. A_{428} in these experiments was grown on a medium containing Tryptone broth (0.8 %), yeast extract (0.5 %) and NaCl (0.8 %). Specific activity expressed as nmol P_i/mg protein per min.

Addition*	ATP-P; exchange (spec. act.)	ATPase (pH 7) under exchange conditions (spec. act.)	ATPase (pH 8) (spec. act.)
None	23.5	947	1650
Control serum	26	986	1525
Anti-$(\alpha+\beta)$	2.2	172	155
Anti-α	2.6	334	377
Anti-β	14.3	794	1100
Anti-γ	15.1	965	1280
DCCD	2.0	212	348
FCCP	2.5	278	1505

* 50 μl of each serum was used. DCCD and FCCP were added at concentrations of 60 and 50 μM, respectively.

activity in these preparations also inhibit the ATP-P_i exchange activity. In addition, anti-β and anti-γ cause a small but reproducible inhibition of both ATP hydrolysis as well as the exchange activity. It is of interest that the uncoupler FCCP inhibits the ATPase activity only under conditions of optimal ATP-P_i exchange, but not under conditions in which the hydrolytic activity is optimal. We have previously reported that anti-$(\alpha+\beta)$ inhibits the ATP-driven quenching of ACMA fluorescence [12]. This reaction is also inhibited 90 % by anti-α, but at much higher antibody concentrations.

DISCUSSION

By use of an in vitro complementation assay, it has been possible to differentiate between mutants defective in oxidative phosphorylation which retain ATPase activity: class I (B_{V4}), possessing reconstitutable membranes, but defective in a soluble factor; and class II (A_{I44}, K_{I1}) defective in the membranes themselves, but possessing a soluble factor capable of reconstituting particles of class I mutants or stripped particles from the parent. Since the ATPase activity in both classes is resistant to DCCD, it appears as if at least two different polypeptides are involved in the conferral of DCCD sensitivity on the membrane ATPase of *E. coli*. It is of interest to note that in mammalian mitochondria several different factors have been shown to be involved in the conferral of DCCD or oligomycin sensitivity on mitochondrial coupling factor I [25, 26]. Similar results have been demonstrated with the membrane-ATPase complex of *Streptococcus faecalis* [27].

The supernatant fraction (s_1) of class II mutants can reconstitute both respiration-driven and ATP-driven quenching in depleted particles of the parent organism and in depleted and non-depleted particles of class I mutant B_{V4}. This supernatant fraction of class II mutants also exhibits a potent ATPase activity. This fraction therefore exhibits properties similar to the crude coupling factor of the parent organism. The active principle of crude parental coupling factor has been demonstrated to be the enzyme ATPase, consisting of five different subunits, including the δ subunit [9, 11, 13, 22]. Moreover, membrane particles of class II mutants cannot be reconstituted by crude coupling factor of the parent. It therefore seems that class II mutants possess a functional coupling factor presumably consisting of five subunits. The presence of the three large subunits α, β and γ in class II mutants has been verified using antibodies prepared against the respective denatured subunits of the parent. Class II mutants appear therefore, to be defective in the hydrophobic part of the ATPase complex, i.e. in the membrane itself. In this regard, class II mutants (K_{I1} and A_{I44}) resemble the *UncB* mutant described by Cox et al. [8]. The ability of crude coupling factor of the parent, on the other hand, to reconstitute membrane particles of class I mutants, appears to indicate that in this class of mutants, the ATPase enzyme is defective. Indeed, none of the supernatant fractions of class I mutant B_{V4} was able to reconstitute particles of the parent organism depleted from the ATPase enzyme. In this regard, mutant B_{V4} resembles mutants isolated in other laboratories [28, 29], the non-depleted particles of which can be reconstituted by parental coupling factor [10, 29]. Mutant B_{V4} can be distinguished from mutants used in the previous cases in that B_{V4} possesses ATPase activity.

Apart from the differences between the two mutant classes mentioned above, whole cells of class II mutants are defective in respiration-driven amino acid transport. In addition, Van Thienen and Postma [30] demonstrated that membrane vesicles of mutant K_{I1} were defective in the transport of serine. The location of class II mutations on the *E. coli* chromosome [2], their DCCD-resistant ATPase activity [3], as well as their complementation by another mutant defective in the DCCD-sensitive ATPase complex, demonstrate that the mutation in class II mutants primarily affects this complex. The resulting defect in respiration-driven amino acid transport as well as in respiration-driven energization [5] indicates the existence of a structural effect of the hydrophobic part of the DCCD-sensitive membrane-ATPase complex in

E. coli. Evidence for a similar structural effect of the ATPase enzyme, consisting of five subunits, has been presented in the past [5, 9, 13, 22, 31, 32]. With respect to respiration-driven amino acid transport, class II mutants resemble "etc" mutants [33] although in the latter case, the defect is presumably located in the γ subunit of ATPase [13].

A common property of class I and class II mutants is their altered binding of ATPase to the membrane. Not only is most of the ATPase activity in these mutants found in the supernatant, but the membrane-associated activity is readily solubilized from membranes of these mutants in the presence of magnesium without lowering the ionic strength. Soluble ATPase of *E. coli* has been demonstrated to be DCCD resistant [5, 22]. It seems likely therefore, that when membranes of class I or class II mutants are tested for ATPase activity, the enzyme is in the detached state and therefore not sensitive to DCCD. It is of particular interest that both class I and class II mutants have a higher total ATPase activity than the parent organism. This may indicate a relationship between the binding of ATPase to the membrane and its synthesis and/or activation. Recently, the isolation of a mutant of *E. coli* with derepressed ATPase activity has been described [34].

Immunodiffusion experiments of membranes or supernatant fractions of mutant N_{I44}, which lacks ATPase activity, against antibodies prepared either against intact ($\alpha+\beta$) subunits or against denatured α, β, or γ subunits, do not reveal any cross-reacting material in this mutant. However, the structural role of the ATPase complex is not abolished in this mutant since it retains respiratory driven activities and normal transport [5, 21]. The possibility that membranes of this mutant are altered in such a fashion that sodium dodecyl sulfate is unable to solubilize the enzyme from the mutant membranes cannot be excluded, but other possibilities are valid as well.

Another probe for the ATP-driven energization of *E. coli* membranes, namely the ATP-P_i exchange, has been developed. This reaction is uncoupler sensitive and is inhibited by the energy transfer inhibitor DCCD. Antibodies prepared against either the two catalytic subunits $\alpha+\beta$ or against the denatured α subunit both inhibit ATPase in the parent and in the class I and class II mutants. These antibodies also inhibit the parental ATP-P_i exchange reaction, whereas anti-β and anti-γ had a smaller effect. It is of interest to note that energy-linked reactions involving the ATPase complex in chloroplasts were inhibited both by anti-α and anti-γ [19]

In chloroplasts, uncouplers have been shown to inhibit the light-triggered ATPase reaction [35]. It has been proposed that in this case constant energization of the membrane is needed for ATPase activity. Although in *E. coli* the presence of a naturally occurring inhibitor has not yet been fully demonstrated, Carreira et al. [36] have described the activation by trypsin of membrane ATPase from *E. coli*, and recently van Nieuwenhuis et al. [37] reported the isolation of a factor by urea treatment of *E. coli* membranes, which brings about an inhibition of the trypsin-activated membranes. It is possible that under exchange conditions at pH 7 a similar mechanism is operative as in chloroplasts. This effect is abolished at pH 8.0.

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