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THE DCCD-BINDING POLYPEPTIDE ALONE IS INSUFFICIENT FOR PROTON TRANSLOCATION THROUGH F_0 IN MEMBRANES OF ESCHERICHIA COLI

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SUMMARY

In contrast to membrane vesicles of wild-type strains which become leaky to protons on removal of the F_1 ATPase, those of the mutant Escherichia coli N_{I44}, which lacks the F_1 ATPase, can maintain a proton gradient. A normal N,N'-dicyclohexylcarbodiimide (DCCD)-binding polypeptide is present in the F_0 portion of the ATPase complex of the mutant. However, the 19000 molecular weight component of F_0 is absent. We conclude that the latter polypeptide, in addition to the DCCD-binding polypeptide, is required for a functional proton channel in F_0.

INTRODUCTION

The membrane-bound ATPase complex of Escherichia coli plays an important role in energy metabolism being involved both in the formation of ATP by oxidative phosphorylation and in its utilization to energize various energy-requiring processes associated with the cell membrane. It is composed of an extrinsic protein of the membrane, F_1, which can hydrolyse ATP, and an intrinsic membrane protein, F_0, through which protons are translocated during the functioning of the ATPase complex (1-3). F_0 is composed of three polypeptides, a, b and c ("DCCD-binding polypeptide"), of molecular weights 24000, 19000 and 8400 (3). The stoichiometry of these polypeptides in F_0 is unknown although it is likely that polypeptide c exists as an oligomer (3). An aspartyl residue of polypeptide c reacts specifically with N,N'-dicyclohexylcarbodiimide (DCCD) to block proton translocation through F_0. Mutants in which this aspartyl residue has been replaced by a glycyl residue are defective in proton translocation (4,5). These results have led to the

Abbreviation: DCCD, N,N'-dicyclohexylcarbodiimide

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suggestion that the DCCD-binding polypeptide constitutes the proton channel through F_0. However, there is presently no evidence regarding the involvement or need for the other polypeptides in the proton channel. In the present paper we show that the presence of the DCCD-binding polypeptide alone is not sufficient to form a proton channel in F_0.

METHODS

Preparation of Everted Membrane Vesicles

E. coli WS1 (wild-type parent) and N_{I44} (ATPase-negative mutant) were generous gifts from Dr. D.L. Gutnick. These strains were grown on a minimal saltsglucose (0.4%) medium supplemented with 12 μM ferric citrate, histidine (50 mg/1), proline (50 mg/1), thiamine (1 mg/1) and casein amino acids (Difco; 1 g/1). The cells were grown with vigorous aeration to the late exponential phase and converted to washed everted membrane vesicles as previously described (6).

Stripping of F_1 ATPase from Membrane Vesicles

Removal of F_1 ATPase from E. coli WS1 was carried out by washing membrane vesicles with low ionic strength buffer as previously described (7). The following procedure was used to remove more tightly-bound polypeptides. Everted vesicles at a concentration of 10 mg protein/ml in a buffer containing 1 mM Tris-HCl, 0.5 mM EDTA, 0.1 mM dithiothreitol and 10% (v/v) glycerol, pH 7.5, were incubated for 30 min at 20°C with 2 M urea, 2 M guanidine hydrochloride, 2% (w/v) silicotungstic acid or TPCK-trypsin at a trypsin to protein ratio of 1:15. In the last case, the reaction was stopped by the addition of soyabean trypsin inhibitor at a ratio to trypsin of 3:5. The incubated mixture was centrifuged at 250000xg for 2 h. The sedimented vesicles were washed twice by suspension in the Tris buffer followed by resedimentation, and finally suspended for assay in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$. The pH of the vesicle suspension was readjusted to pH 7.5 with dilute KOH when silicotungstic acid was used.

DCCD-Binding Polypeptide

The labelling of membrane vesicles with [^{14}C]DCCD (Research Products International Corporation) and one-dimensional disc gel electrophoresis in the presence of 8 M urea were carried out as described by Fillingame (8). The DCCD-binding polypeptide was extracted and purified on CM-cellulose and Sephadex LH-60 by the procedure of Altendorf et al. (9).

Miscellaneous Methods

Amino acid analyses were carried out as described by Fillingame (10) following acid hydrolysis for 24, 31, 42 and 60 h. The protein content of DCCD-binding polypeptide preparations was measured as in reference 8. Quenching of the fluorescence of 9-aminoacridine was assayed as previously (11). Two-dimensional isoelectric focusing-SDS polyacrylamide gel electrophoresis was carried out as in reference 12.

RESULTS AND DISCUSSION

Removal of F_1 from membranes of normal strains of E. coli results in the leakage of protons through F_0. Consequently, reactions, such as the quenching of the fluorescence of the dye 9-aminoacridine or the energy-dependent transhydrogenation of $NADP^+$ by NADH, which require the presence of a transmembrane proton gradient cannot occur. E. coli N_{144} was isolated by Kanner and Gutnick as an ATPase-deficient mutant (13). Subsequently, we found that membranes of this mutant although lacking F_1 were still able to carry out respiration-energized transhydrogenation (14). Thus, these membranes must be able to maintain a proton gradient even in the absence of F_1. Several explanations for this are possible. (i) Although intact F_1 is absent, a subunit of the enzyme could still be present and prevent the leakage of protons through F_0. Senior et al. (15) have described a mutant in which only β subunits are retained by the membrane without it becoming leaky. (ii) F_0 or the DCCD-binding polypeptide could be absent from the mutant membranes. (iii) A polypeptide of F_0, other than the DCCD-binding polypeptide, which is involved in the proton channel could be missing in the mutant. These possibilities were investigated.

Normal and mutant membrane vesicles were treated with 2 M urea, 2 M guanidine hydrochloride, 2% (w/v) silicotungstic acid, trypsin or sonicated at pH 9.5 (16) in order to remove any F_1 subunits retained by the membrane. The results shown in Fig. 1 for 2 M urea are typical for all of these treatments. Oxidation of ascorbate (in the presence of phenazine methosulfate to introduce electrons into the cytochrome region of the respiratory chain) by untreated ("washed") membranes resulted in the quenching of the fluorescence of 9-aminoacridine indicating that a proton gradient had been established across the vesicle membrane. When all of the oxygen in the cuvet had been consumed fluorescence was restored as protons reequilibrated across the membrane. In the case of the normal strain addition of ATP resulted in restoration of the gradient. Treatment of the membranes of the normal strain

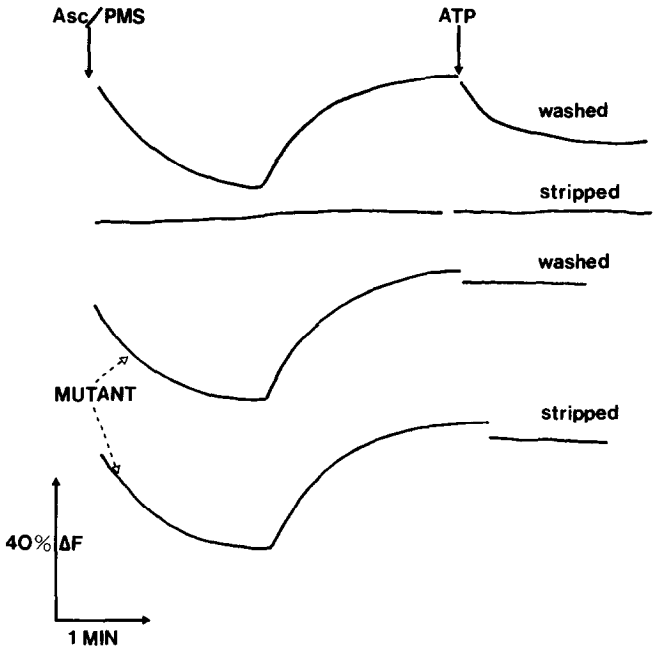

FIGURE 1. Effect of stripping everted membrane vesicles from parent (WS1) and mutant (N_{I44}) cells with 2 M urea on the ascorbate oxidation-dependent quenching of the fluorescence of 9-aminoacridine. The stripping and assay procedures are described in METHODS. Asc, ascorbate; PMS, phenazine methosulfate.

with urea ("stripping") resulted in removal of F_1 with loss of the ability to set up and maintain a proton gradient. By contrast, none of the stripping procedures destroyed the capacity of the mutant membranes to generate a proton gradient by oxidation of ascorbate. Thus, it is unlikely that retention of individual subunits on the membranes of the mutant is responsible for the relative impermeability of these membranes to protons.

The absence of α, β, δ and ε subunits of F_1 on the membrane of the mutant was confirmed by comparing its polypeptide composition with that of the normal strain using two-dimensional isoelectric focusing-sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Fig. 2).

The presence of the DCCD-binding polypeptide in mutant membranes was investigated by labelling the membranes of normal and mutant cells with [^{14}C]DCCD followed by separation of the polypeptides by sodium dodecyl-

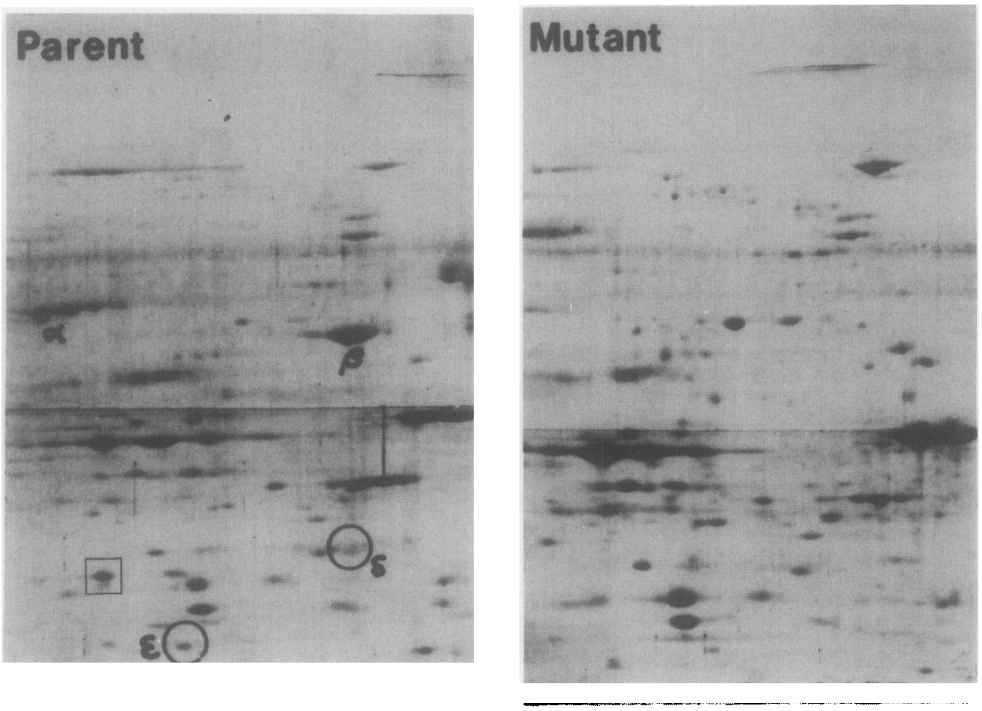

FIGURE 2. Two-dimensional isoelectric focusing (horizontal direction) dodecylsulfate (vertical direction) polyacrylamide gel electrophoresis of membranes of parent (WS1) and mutant (N_{I44}) cells. The second dimension is a discontinuous polyacrylamide gel consisting of a layer of 15% (w/v) polyacrylamide below a layer of 11% (w/v) polyacrylamide. The α, β, δ and ε subunits of the F_1 ATPase which are present only in membranes of the parent strain are indicated. The polypeptide outlined by the rectangle is absent in the mutant strain.

sulfate gel electrophoresis (Fig. 3, curves 1,2). Besides material at the top of the gel, two main peaks of radioactivity were observed both with normal and mutant membranes. The larger of these peaks coincided with a Coomassie-blue staining polypeptide (data not shown). A characteristic property of the DCCD-binding polypeptide of F_0 is its ability to be extracted by chloroformmethanol (2:1) (8-10). [^{14}C]-DCCD-labeled membrane vesicles were extracted with chloroform-methanol and the proteins precipitated with ether. Gel electrophoresis of this material from both normal and mutant membranes gave mainly a single polypeptide band of molecular weight 8000 (data not shown) coincident with the main peak of radioactivity (Fig. 3, curves 3,4).

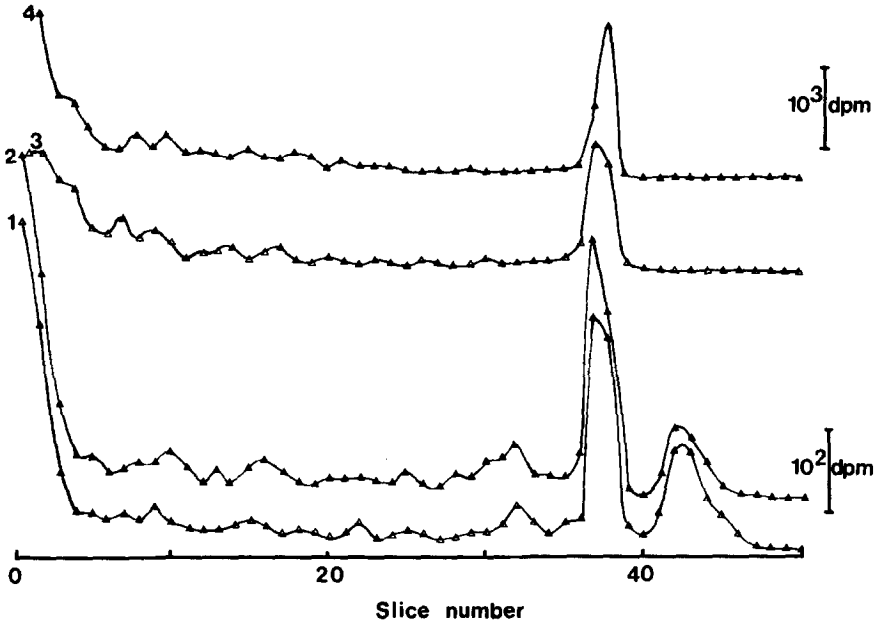

FIGURE 3. One-dimensional dodecylsulfate gel electrophoresis of [^{14}C]DCCD-labelled membranes (1,2) and of ether-precipitated proteins of chloroform-methanol extracts of labelled membranes (3,4) from parent (WS1)(2,4) and mutant (N_{I44})(1,3) cells. The preparation of the samples, the running and slicing of the gels, and the determination of radioactivity were carried out as described by Fillingame (8).

This indicates that the DCCD-binding polypeptide of F_0 occurs in the mutant and that the aspartyl residue which reacts with DCCD, and is involved in proton translocation (4,5), is present.

The DCCD-binding polypeptides from normal and mutant cells were extracted and purified to homogeneity by the method of Altendorf et al. (9). Their amino acid compositions are similar suggesting that the polypeptides from the two strains are identical (Table 1).

Careful examination of the gels shown in Fig. 2 reveals that a major polypeptide of molecular weight 19000 (shown in the square) is absent in the mutant. (An adjacent minor polypeptide of identical molecular weight is also missing in the mutant. This adjacent spot may be caused by carbamylation of the major polypeptide by the cyanate formed from the urea present in the samples). It is likely that this polypeptide is polypeptide b of F_0 since

TABLE 1. Amino acid composition of DCCD-binding polypeptides of E. coli WS1 and N_{I44}

Amino acid	mol amino acid/mol polypeptide*	
	WS1	N_{I44}
Asp	4.80	4.67
Thr	1.03	1.20
Ser	0	0.10
Glu	4.47	4.53
Pro	2.13	2.64
Gly	10.23	10.40
Ala	13,20	13.24
Cys	0	0
Val	5.90	5.84
Met	7.09	6.61
Ile	7.82	7.88
Leu	12.39	12.50
Tyr	1.30	1.23
Phe	4.41	4.68
His	0	0
Lys	1.26	1.23
Arg	2.14	2.15

* Mean of four determinations.

it migrates in this position on the gels of this type (17). Unfortunately, polypeptide a does not enter these polyacrylamide gels. Thus, we were unable to determine if it is present in the mutant. We are presently examining this point.

We conclude from the results above that the DCCD-binding protein alone is unable to form a proton channel through the membrane. It is likely that polypeptide b is also involved in the formation of the proton channel of F_0. However, we do not know if it forms part of the channel or acts indirectly by influencing the conformation of the DCCD-binding polypeptide. This problem is under investigation.

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