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THE DCCD-BINDING POLYPEPTIDE IS CLOSE TO THE F_1 ATPase-BINDING SITE ON THE CYTOPLASMIC SURFACE OF THE CELL MEMBRANE OF ESCHERICHIA COLI

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Removal of the F_1 ATPase from membrane vesicles of Escherichia coli resulted in leakage of protons across the membrane through the F_0 portion of the ATPase complex. The leakage of protons was prevented by antiserum to the N,N'-dicyclohexylcarbodiimide (DCCD)-binding polypeptide in everted but not in "right-side out" membrane vesicles. The antiserum prevented the rebinding of F_1 ATPase to F_1-stripped everted membrane vesicles. It is concluded that in F_1-depleted vesicles the DCCD-binding polypeptide is exposed on the cytoplasmic surface of the cell membrane at or close to the binding site of the F_1 ATPase.

INTRODUCTION

The ATPase complexes of mitochondria, chloroplasts, chromatophores and bacterial cell membranes play an important role in the formation of ATP by oxidative and photophosphorylation. The complex 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). Evidence from reconstitution experiments (4, 5) or by the use of mutants (6, 7) suggests that a polypeptide of F_0 which is specifically modified by N, N^1-dicyclohexylcarbodiimide ("DCCD-binding polypeptide") is directly involved in proton translocation. This polypeptide should be transmembranous if it is the sole component of the proton translocation pathway through F_0. At present, there is no information regarding its orientation in the membrane or relationship to F_1. In the present paper we show that the DCCD-binding polypeptide is exposed at the cytoplasmic surface of the cell membrane of E. coli in close proximity to F_1.

Abbreviation: DCCD, N,N'-dicyclohexylcarbodiimide

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METHODS

Preparation of Everted Membrane Vesicles and F_1 ATPase

E. coli WS1 and ML308-225 (wild-type strains) were grown on a minimal salts-glucose medium as described previously (8, 9). The cells were grown with vigorous aeration to the late exponential phase and converted to washed everted membrane vesicles following disruption in a French press (8). F_1 ATPase was released from vesicles of strain ML308-225 and purified as before except that separation of the enzyme on a sucrose gradient was omitted (10). Everted vesicles of strain WS1 were stripped of F_1 APTase by treatment with 2M urea (9).

Preparation of DCCD-Binding Polypeptide and Antiserum

The DCCD-binding polypeptide was extracted from cells of strain ML308-225 and purified as described before (9). Antiserum to this polypeptide was obtained from New Zealand rabbits. The polypeptide (2 mg per ml 0.9% NaCl in 2% sodium dodecylsulfate) was emulsified with two volumes of complete Freund's adjuvant. The animals were injected subcutaneously at four week intervals with 0.3 mg protein. After five injections incomplete Freund's adjuvant was used. The serum used in these experiments was collected after the ninth injection. In some experiments the immunoglobulins were partially purified by fractionation with ammonium sulfate (11, 12). Their activity and specificity were checked by crossed immunoelectrophoresis. Antiserum to F_1 ATPase was donated by Dr. Helga Stan-Lotter.

Ascorbate Oxidation-Dependent Quenching of the Fluorescence of 9-Aminoacridine

Urea-stripped everted vesicles of strain WS1 (10 mg protein) in 50 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$ were incubated for 5 h at 4°C with different amounts of antiserum, preimmune serum, or bovine serum albumin in a final volume of 1 ml. Samples (0.1 ml) were assayed for the ability to quench the fluorescence of 9-aminoacridine with ascorbate (in the presence of phenazine methosulfate) as substrate as described previously (9).

Binding of F_1 ATPase to Everted Vesicles

1.67 ml urea-stripped everted vesicles of strain WS1 (15 mg/ml) in 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM $MgCl_2$ were incubated for 20 h at 4°C with different amounts of antiserum in a final volume of 4.42 ml. After a four-fold dilution in buffer the mixture was centrifuged at 250,000 x g for 2.5 h. The sedimented vesicles were washed once by suspension in buffer followed by resedimentation as before. The washed vesicles were suspended in buffer at 6.5 mg protein/ml. Different amounts of F_1 ATPase (7 mg protein/ml) were incubated at 4°C for 45 min with 2.5 mg vesicle protein in buffer. The mixture was then diluted 8 to 10-fold in buffer and the vesicles sedimented as before. The ATPase activity of the vesicles was assayed (10).

Effect of Antiserum to DCCD-binding Polypeptide on Proton Permeability of "Right-Side Out" Membrane Vesicles

Strain DL-54 was grown to the late exponential phase on a minimal salts-glucose (0.8% w/v) medium and converted to spheroplasts by treatment with lysozyme in the presence of EDTA. K^+-loaded vesicles were prepared at pH 7.0 as described by Harold and coworkers (13, 14). The pH changes occurring following the addition of 3 µl valinomycin (5 mg/ml) to 0.85 ml K^+-loaded vesicles (1.5 mg protein) in 0.4 M sucrose - 10 mM $MgSO_4$ were measured with a glass pH electrode connected to a Fisher Accumet Model 325

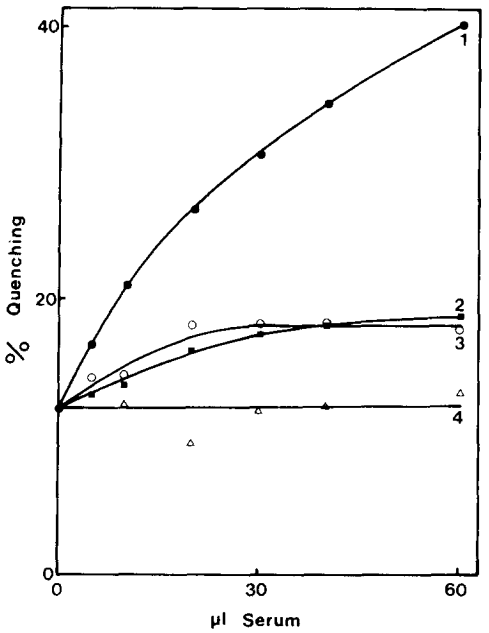

Figure 1. Effect of antiserum to DCCD-binding polypeptide on the ascorbate-oxidation-dependent quenching of fluorescence of 9-aminoacridine by urea-stripped everted membrane vesicles. The experiment was carried out as described in METHODS. 1, antiserum to DCCD-binding polypeptide; 2, preimmune serum; 3, antiserum to F_1 ATPase; 4, bovine serum albumin (37 mg/ml).

expanded scale pH meter. The output from the meter was amplified so that 0.2 pH units gave a full-scale deflection on the recorder. In some experiments the vesicles were preincubated with DCCD (15 µl in ethanol) or with ammonium sulfate purified antiserum to the DCCD-binding polypeptide for 45 min at 22°C.

RESULTS AND DISCUSSION

Removal of F_1 from membrane vesicles 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, which require the presence of a transmembrane pH gradient cannot occur. Fluorescence quenching can be observed if the leakage of protons through F_0 is blocked by the readdition of F_1 or by reaction with DCCD (1-3). Figure 1 shows the effect on fluorescence quenching of various additions to urea-stripped everted vesicles. Bovine serum albumin, antiserum to F_1 ATPase and preimmune serum have, at the most, only a slight effect on the residual fluorescence quenching of the F_1-depleted vesicles. By contrast

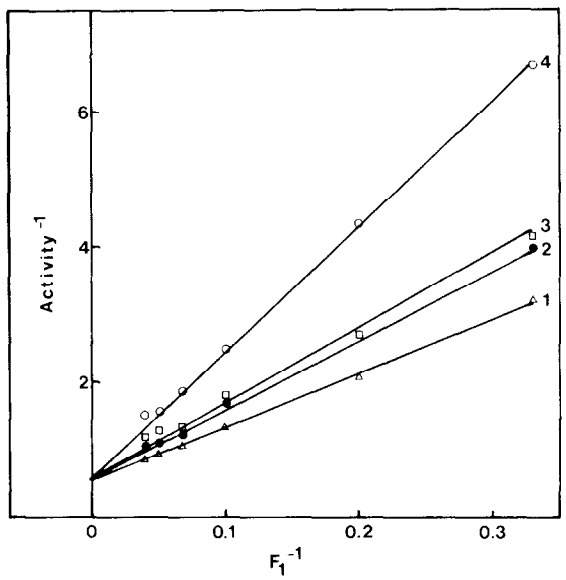

Figure 2. Effect of antiserum to DCCD-binding polypeptide on the binding of F_1 ATPase to urea-stripped everted membrane vesicles. The experiment was carried out as described in METHODS. Binding is expressed in units/min of ATPase activity bound/mg membrane protein. The amount of F_1 ATPase added is given as µl of enzyme at a concentration of 7 mg protein/ml. 1-4, 0, 50, 75 and 110 µl of antiserum to DCCD-binding polypeptide.

antiserum to the DCCD-binding polypeptide markedly stimulates fluorescence quenching indicating that reaction of the antiserum with this polypeptide blocks the leakage of protons through F_0. If the data of Fig. 1 are examined as a Lineweaver-Burk plot a value can be calculated for the maximum quenching to be expected at saturating levels of antiserum. This value (62% quenching) is in good agreement with that observed following the addition of saturating levels of F_1 ATPase (69% quenching).

The effect of the antiserum to the DCCD-binding polypeptide on the binding of F_1 was examined as follows. Stripped everted vesicles were incubated with different amounts of antiserum. The vesicles were sedimented by centrifugation and then washed to remove unbound antiserum. Various amounts of F_1 were added to the suspension of the treated vesicles and the extent of binding of F_1 measured by the increase in ATPase activity of the vesicles. Stripped vesicles had no ATPase activity. The extent of binding of F_1 as a function of the amount of F_1 added to treated and untreated vesicles is shown as a Lineweaver-Burk plot in Fig. 2. The lines

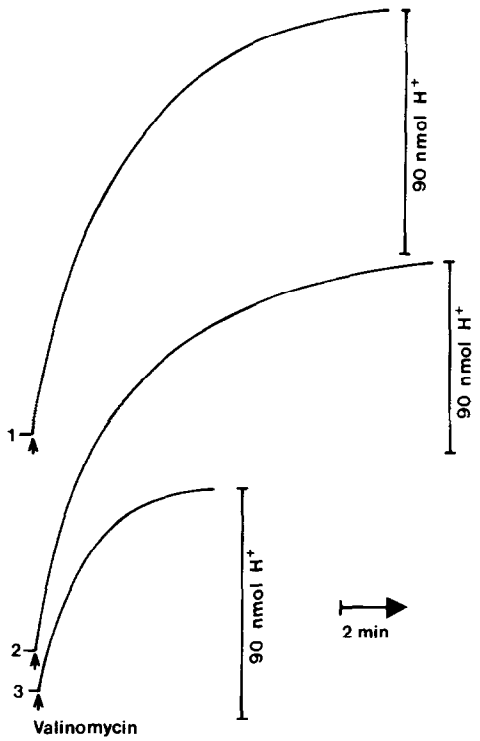

Figure 3. Effect of DCCD and of antiserum to DCCD-binding polypeptide on the proton permeability of "right-side out" membrane vesicles of strain DL-54. The experiment was carried out as described in METHODS. 1, no additions; 2, 50 µl antiserum to DCCD-binding polypeptide; 3, 880 µM DCCD.

intersecting close to the ordinate suggest that the antiserum to the DCCD-binding polypeptide interferes with the binding of F_1 in a near competitive manner.

The experiments described above have detected the exposure of the DCCD-binding polypeptide at the cytoplasmic surface of F_1-depleted membrane vesicles. The extent of exposure of this polypeptide at the external surface of the cell membrane was examined as follows. The F_1 ATPase-defective mutant DL-54 readily loses F_1 from its binding sites on F_0. Consequently, vesicles of DL-54 leak protons through F_0 (14, 15). Addition of valinomycin to K^+-loaded "right-side out" vesicles results in an efflux of K^+ concomitantly with a compensatory influx of protons provided that a pathway for protons is available. This pathway is provided in DL-54 by the F_1-depleted F_0 proteins (14, 15). The influx of protons can be detected as a drop in the pH of the medium external to the vesicles.

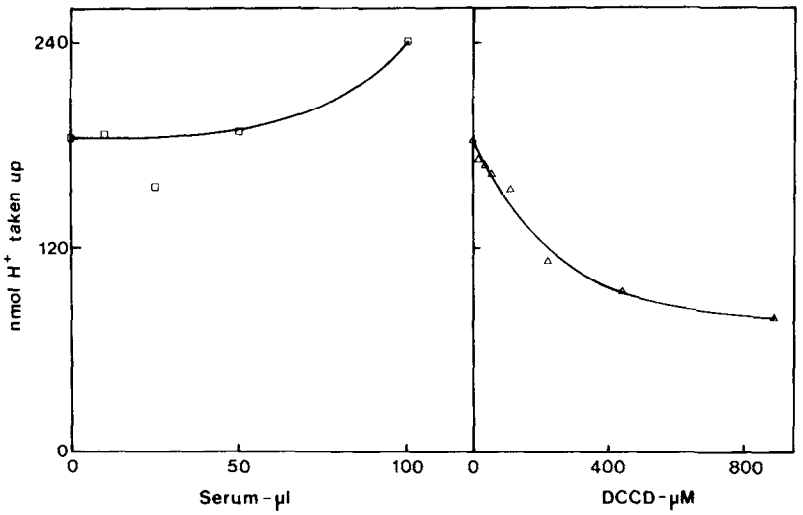

Figure 4. Effect of DCCD and of antiserum to DCCD-binding polypeptide on the proton permeability of "right-side out" membrane vesicles of strain DL-54. The experiment was carried out as in Figure 3.

A typical result is shown in Fig. 3. Addition of DCCD to the vesicles blocks the movement of protons through F_0. However, antiserum to the DCCD-binding polypeptide has no effect on proton movement (Fig. 4). The absence of effect of the antiserum may be due to several causes: (a) the DCCD-binding polypeptide may not be exposed on the external surface of the cell membrane or be inaccessible to the antibody, (b) the portions of the DCCD-binding polypeptide exposed on the external surface of the cell membrane may be so weakly antigenic that our antiserum does not contain antibodies to these regions.

In summary, our results indicate that in F_1-depleted vesicles the DCCD-binding polypeptide is exposed on the cytoplasmic surface of the cell membrane. Antibody to this polypeptide will block the rebinding of F_1 in a near competitive manner suggesting that F_1 interacts with or is close to the DCCD-binding polypeptide. We have not been able to determine unambiguously if the DCCD-binding polypeptide is exposed on the external surface of the membrane, that is, if it is transmembranous. However, our negative results are consistent with the finding that a 19,000 molecular weight polypeptide is also involved in the proton pathway through F_0 (9, 16).

ACKNOWLEDGMENT

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