CHARACTERIZATION OF DICYCLOHEXYLCARBODIIMIDE BINDING SITE ON COUPLING FACTOR 1 OF MITOCHONDRIAL AND BACTERIAL MEMBRANE-BOUND ATPases

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1. Introduction

The H^+-linked ATPases (mitochondrial, chloroplastic, and bacterial ATPases) are made of two sectors, a membrane sector F_0 which is considered a proton pump, and an extrinsic sector (factor F_1) which possesses the catalytic site [1,2]. Until recently, it was thought that dicyclohexylcarbodiimide (DCCD), a carboxylic reagent, bound specifically to a hydrophobic small rel. mol. mass protein (8000 M_r) of the membrane sector (DCCD-binding protein) and concomitantly inactivated the entire ATPase complex (reviewed [3]). However, in [4,5] evidence was given that DCCD was also able to bind to and inactivate isolated factor 1 (F_1 in mitochondria and BF_1 in bacteria). Inactivation was much more rapid at acid pH than at alkaline pH, the half-maximum effect being obtained at pH ~7. Inactivation was accompanied by the covalent binding of DCCD to the β subunit of mitochondrial and *Escherichia coli* F_1. Evidence for binding of DCCD to a carboxyl group was provided by the interfering effect of a nucleophilic reagent, glycine ethyl ester, and by demonstration, in electrofocusing experiments, of the loss of a negative charge on the β subunit of *E. coli* F_1. Similar effects of DCCD on the soluble chloroplastic factor F_1 were reported [6].

That DCCD may bind to two sites on H^+-linked ATPases, i.e., the proteolipid of factor F_0 and the β subunit of factor F_1, is not yet fully accepted; interaction of DCCD with F_1 may be peculiar to the soluble F_1 and not occur with the membrane-bound form of F_1 [7,8]. The aim of this paper is to show that, under the appropriate conditions, DCCD added to submitochondrial particles or *E. coli* membranes binds to both the F_0 and F_1 sectors of the ATPase complex. The critical factor which controls the binding of DCCD to the F_1 sector is the pH of the medium; F_1 binds DCCD at acidic pH, but not at alkaline pH. At alkaline pH, the only protein of the ATPase complex to be labeled is the classical DCCD-binding protein of the F_0 sector, whereas at acidic pH both F_0 and F_1 are labeled.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Materials*

[^{14}C]DCCD (54.5 Ci/mol) was obtained from the Commissariat à l'Energie Atomique, Saclay. Labeled and unlabeled DCCD were used as methanolic solutions; appropriate controls were run with methanol alone with final methanol conc. <1%. *E. coli* BF_1-ATPase was purified as in [5]. Mg-ATP submitochondrial particles were obtained by sonication of beef heart mitochondria as in [9]. Beef heart mitochondrial F_1-ATPase was prepared and stored as an ammonium sulfate suspension [10].

2.2 *ATPase assay*

ATPase activity was measured at 30°C by determination of released P_i. The reaction medium (final vol. 0.5 ml) contained 40 mM Tris–HCl, 10 mM ATP, 5 mM $MgCl_2$, 20 μg pyruvate kinase and 2 mM phosphoenolpyruvate, final pH 8.0. The reaction was started by addition of an aliquot fraction of the enzyme preparation and stopped after 5 min by addi-

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tion of 0.2 ml trichloroacetic acid 50% (w/v). The released P_i was determined by the method in [11].

2.3. *Gel electrophoresis*

Electrophoresis in 12.5% polyacrylamide gels containing 0.1% SDS was done as in [12]. The gels stained with Coomassie blue were scanned in a Joyce Loebl densitometer, then sliced to measure radioactivity. Slices (1 mm thick) were digested by overnight incubation in 1 ml 15% H_2O_2 at 55–60°C and counted in 10 ml scintillation fluid. SDS slab gel electrophoresis was done in 10.3% polyacrylamide [12] supplemented with 0.5% linear polyacrylamide [13]. Autoradiography was done as in [14].

2.4. *Protein determination*

The protein concentration of membranes of *E. coli* or submitochondrial particles was determined by a biuret procedure. Soluble F_1-protein concentration was measured as in [15], using bovine serum albumin as standard.

3. Results

3.1. *Inactivation of* E. coli *BF_1 by DCCD*

DCCD is a hydrophobic molecule which exhibits much affinity for membranes; when [^{14}C]DCCD is added to *E. coli* membrane particles, half of the radioactivity binds to membranes at pH 6.5 and 65% at pH 8.0. Partition of DCCD in favour of membranes is therefore more marked at alkaline than at acid pH.

In the following experiment, *E. coli* membranes were incubated for 30 min at 30°C with increasing concentrations of DCCD at pH 6.5 and pH 8.0. After incubation, a sample of *E. coli* membranes was treated by chloroform [16] to release F_1 in a soluble form. ATPase activities of solubilized and membrane-bound F_1 were assayed as in section 2. As shown in fig.1, when preincubation with DCCD was at pH 6.5, activities of both solubilized and membrane-bound ATPase were inhibited. In contrast, when preincubation with DCCD was at pH 8.0, only the activity of membrane-bound F_1 was inhibited. Under the experi-

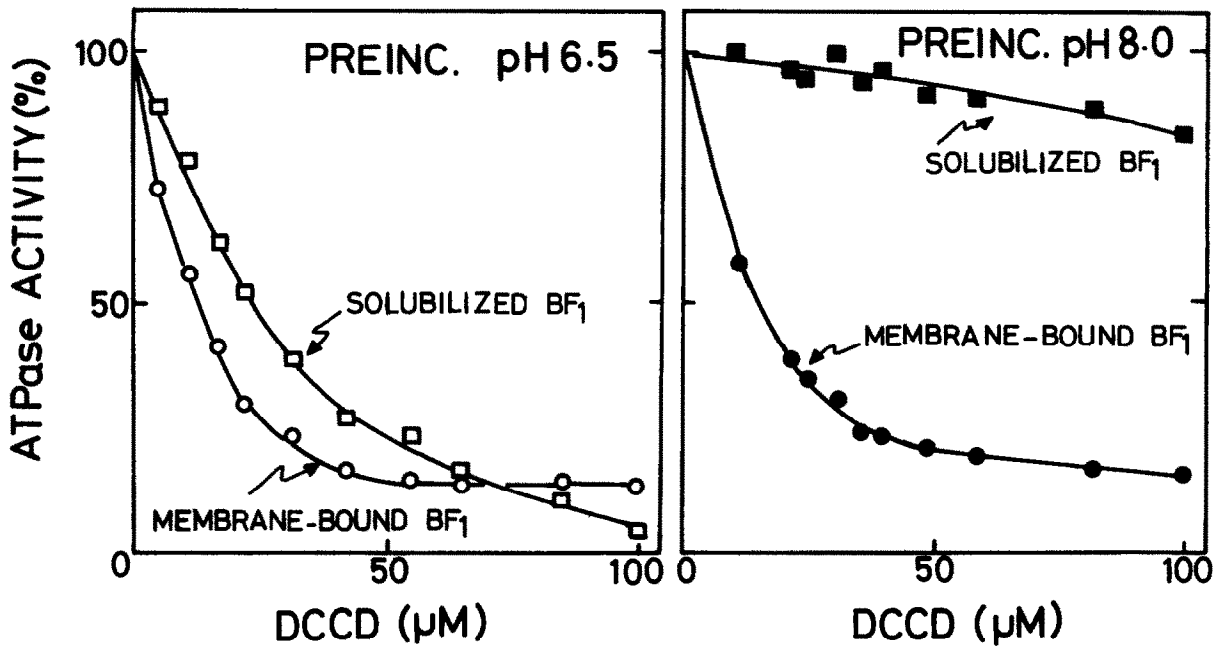

Fig.1. Inactivation of *E. coli* ATPase with DCCD. *E. coli* membranes (2.0 mg protein/ml) were preincubated for 30 min at 30°C in 50 mM MOPS, 250 mM sucrose (pH 6.5) or in 50 mM triethanolamine–HCl, 250 mM sucrose (pH 8.0) with increasing DCCD concentrations. At the end of the preincubation period, an aliquot was assayed for ATPase activity (membrane-bound ATPase). The remaining sample was supplemented with 2 mM ATP and 2 mM EDTA (final conc.) and BF_1-ATPase was released by chloroform treatment [16] and assayed for ATPase activity (solubilized ATPase). The specific activities were 0.8 μmol . min^{-1} . mg $protein^{-1}$ for membrane-bound ATPase and 3.0 and 3.7 μmol . min^{-1} . mg $protein^{-1}$ for solubilized ATPase extracted from membranes preincubated at pH 6.5 and pH 8.0, respectively.

Table 1
Inactivation of beef heart mitochondrial ATPase with DCCD

	DCCD (μM)	ATPase activities (%)	
		Membrane bound	Solubilized
Preincubation at pH 6.5	0	100	100
	50	15	80
	100	13	56
	200	15	31
Preincubation at pH 8.0	0	100	100
	50	17	100
	100	8	100
	200	6	96

Experimental procedure was as described in the legend to fig.1. ATPase activity of membrane-bound ATPase was 1.7 μmol . min^{-1} . mg $protein^{-1}$ and pH 7.0 and pH 11.0 for solubilized ATPase extracted after preincubation at pH 6.5 or pH 8.0, respectively

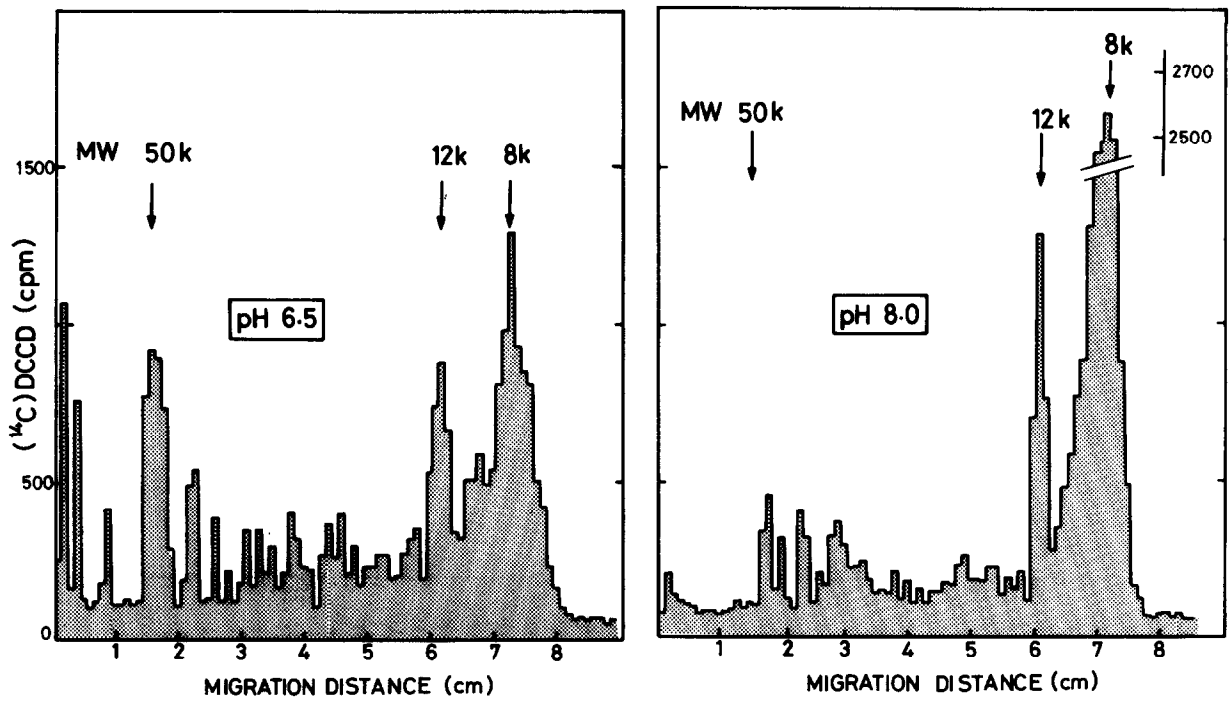

Fig.2. *E. coli* membranes (2.3 mg/ml) were incubated at 30°C in 50 mM MOPS (pH 6.5) or 50 mM triethanolamine–HCl (pH 8.0) with 40 μM [^{14}C]DCCD. The ATPase activity of the membranes was 0.5 μmol . min^{-1} . mg $protein^{-1}$ and after incubation for 30 min with DCCD, 82% and 72% inactivation values were reached at pH 6.5 and pH 8.0, respectively. Samples (200 μg protein) were depolymerized in 1% SDS (w/v) and electrophoresed on 12.5% polyacrylamide gels as in [12]. After staining with Coomassie blue 1 mm slices were dissolved in H_2O_2 and counted in 10 ml scintillation fluid.

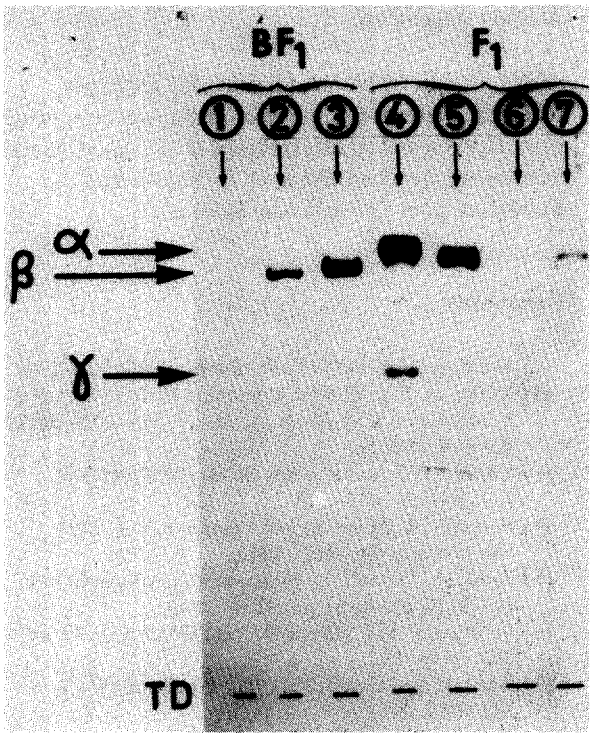

Fig.3. Slab gel electrophoresis in the presence of SDS of [^{14}C]DCCD-labeled ATPases from *E. coli* ATPase (BF_1) and mitochondrial ATPase (F_1), followed by autoradiography. F_1 released with chloroform from *E. coli* membranes incubated with [^{14}C]DCCD at pH 8.0, track 1 and pH 6.5, track 2. Purified *E. coli* F_1 labeled with [^{14}C]DCCD, track 3. Purified mitochondrial F_1 labeled with [^{14}C]DCCD, track 5. F_1 released with chloroform from beef heart submitochondrial particles incubated with [^{14}C]DCCD at pH 8.0, track 6 and at pH 6.5, track 7. Track 4 corresponds to the migration of mitochondrial F_1 labeled with *N*-ethyl[^{14}C]maleimide [21]; only the α and γ subunits are labeled. Autoradiography was done as in [14]. The position of the tracking dye, bromophenol blue (TD) is indicated on the autoradiograph.

mental conditions detailed in fig.1, and at the pH of maximal efficiency of inhibition (pH 8.0 for membrane-bound F_1, pH 6.5 for solubilized F_1), half-inactivation of ATPase required 25 μM DCCD for solubilized F_1 and 15 μM DCCD for membrane-bound F_1.

Preincubation of beef heart submitochondrial particles with DCCD at pH 6.5 or pH 8.0 provided similar data to those mentioned with *E. coli* F_1; F_1 extracted from submitochondrial particles which had been incubated with DCCD at acidic pH was inactivated whereas F_1 extracted from particles incubated at alkaline pH remained fully active (table 1).

As shown in [4,5], inactivation of purified mitochondrial and *E. coli* F_1 markedly depended on pH; inactivation was more efficient at acidic than at alkaline pH. A similar pH dependence is revealed here, where DCCD was first reacted with membranes, then F_1 released and assayed for ATPase activity.

3.2. *Labeling of mitochondrial and bacterial ATPases by [^{14}C]DCCD*

The labeling patterns of *E. coli* membranes after incubation with [^{14}C]DCCD and subsequent inactivation at pH 6.5 and pH 8.0 are shown in fig.2. Radioactivity accumulated in several regions of the gel as in [17]. Under our experimental conditions, dicyclohexyl urea, the hydration product of DCCD, was eliminated from the gel during the staining step. The distribution of the bound radioactivity was different with incubation at pH 6.5 or 8.0. After incubation at pH 8.0, the bound radioactivity was concentrated in two discrete peaks of small M_r (8000, 12 000) (fig.2). After incubation at pH 6.5, the bound radioactivity was again found in peaks corresponding to the same rel. mol. mass, but in addition, a significant percentage of radioactivity was now located in a peak of M_r 50 000 (fig.2).

In keeping with classical data showing that the DCCD-binding protein of the membrane sector of H^+-linked ATPase has M_r 8000 [3], it may be inferred that incubation of DCCD with *E. coli* membranes at pH 6.5 or 8.0 led to the labeling of this small M_r protein. The supplementary labeling of the 50 000 M_r peptide after preincubation at pH 6.5 may be tentatively ascribed to the β subunit of *E. coli* F_1; identification of the β subunit was made easier after selective release with chloroform of F_1 from *E. coli* membranes prelabeled with [^{14}C]DCCD and analysis of the bound radioactivity by SDS–polyacrylamide gel electrophoresis (fig.3). Radioactivity was located only in the β subunit of *E. coli* F_1.

A labeling experiment with [^{14}C]DCCD similar to the above for *E. coli* membranes was conducted with beef heart submitochondrial particles at pH 6.5 and 8. The solubilized F_1 after preincubation of particles with [^{14}C]DCCD at pH 6.5 or 8.0 was subjected to SDS–polyacrylamide gel electrophoresis. Similarly to *E. coli* F_1, mitochondrial F_1 was labeled on the β subunit after preincubation of particles at pH 6.5, but not after preincubation at pH 8.0.

4. Discussion

DCCD, a potent inhibitor of H^+-linked ATPases, was postulated to be characterized by selective binding to a carboxyl residue of a proteolipid located in the membrane sector of those ATPases [3]. However, the soluble F_1 portion of H^+-ATPases, could also bind DCCD and be subsequently inactivated [4–6,18–20]. Our data show unambiguously that DCCD binds not only to isolated F_1, but also to membrane-bound F_1 in mitochondrial or *E. coli* membranes, provided the pH of incubation of the membranes with DCCD is slightly acidic [20]. Due to its hydrophobic nature, it is presumed that DCCD is distributed between the hydrophobic core of bacterial or mitochondrial membranes and the aqueous medium. The data indicate that the partition in favor of membranes is further favored by alkaline pH. That DCCD reacts only at acidic pH with the F_1 portion of membrane-bound ATPases or with solubilized F_1 could reflect the requirement for protonation of the reactive carboxylic group.

In summary, DCCD at acidic pH can bind to two different sites on both *E. coli* membrane particles and beef heart submitochondrial particles. One site is the classical DCCD binding protein which is part of the proton channel of the F_0 sector of the ATPase complex, the other site is the β subunit of coupling factor 1 in the ATPase complex. Binding of DCCD to F_1 and the subsequent inactivation of F_1 are negligible at alkaline pH. The reported absence of DCCD labeling of the F_1 portion of the ATPase complex [7,8] can be explained by the fact that incubation of membranes with DCCD in those experiments was carried out at alkaline pH.

DCCD is only 50% as efficient on F_1 at pH 6.5 than on F_0 at pH 8.0, indicating that the inactivation efficiencies of DCCD with respect to the two sites of *E. coli* and mitochondria ATPases under conditions of optimal pH are quite similar. The preferential attack of F_0 in bacterial or mitochondrial membrane-bound ATPase may be explained by the trapping of DCCD by membranes, due to the hydrophobic character of DCCD. Thus, by lateral diffusion in the membrane plane, DCCD reaches and reacts first with the F_0 sector of the membrane-bound ATPase.

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