

N,N'-Dicyclohexylcarbodiimide binds specifically to a single glutamyl residue of the proteolipid subunit of the mitochondrial adenosinetriphosphatases from *Neurospora crassa* and *Saccharomyces cerevisiae*

(inhibition of H⁺ translocation/amino acid sequence/automated solid-phase Edman degradation)

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ABSTRACT The *N,N'*-dicyclohexylcarbodiimide-binding proteolipid subunit of the mitochondrial adenosinetriphosphatases (ATP phosphohydrolase, EC 3.6.1.3) of *Neurospora crassa* and *Saccharomyces cerevisiae* were purified from mitochondria incubated with the radioactively labeled inhibitor. The specifically labeled subunit was cleaved with cyanogen bromide and *N*-bromosuccinimide, and the resultant fragments were separated by gel chromatography in the presence of 80% (vol/vol) formic acid. The *N,N'*-dicyclohexylcarbodiimide label was recovered in each organism exclusively in a 17-residue fragment. Further analysis by automated solid-phase Edman degradation revealed that the bound label was present at only one position, corresponding to a glutamyl residue. The *N,N'*-dicyclohexylcarbodiimide-modified glutamyl residue is the only identical acidic position in both proteins and occurs in the middle of a hydrophobic sequence of about 25 residues.

Evidence has been accumulated during the past few years that the ATPase complex (ATP phosphohydrolase, EC 3.6.1.3) of the mitochondrial inner membrane, and also the similar enzymes of the chloroplast thylakoid and bacterial plasma membrane, functions as a reversible ATP-driven H⁺ pump (for review see, e.g., ref. 1). Translocation of protons across the membrane is performed by the membrane-integrated part of the complex, *F*₀ (2, 3), which consists of at least three distinct hydrophobic polypeptides (4-6). Its major subunit is a probably hexameric proteolipid (7, 8) with a molecular weight around 8000. Recently, the proteolipids from lettuce chloroplasts (9) and yeast mitochondria (10) were reconstituted into phospholipid vesicles and then produced H⁺ leaks sensitive to ATPase inhibitors. Further evidence for the protonophoric activity of the proteolipid subunit was obtained with the membrane factor of a thermophilic ATPase complex that contained only the proteolipid and one further subunit (11).

N,N'-Dicyclohexylcarbodiimide (DCCD) inhibits the enzymatic activities of the whole ATPase complex (12) as well as H⁺ translocation mediated by the membrane factor (13, 14) or the reconstituted proteolipid subunit (9, 10). As shown with ¹⁴C-labeled DCCD, the inhibitor reacts covalently and quite specifically with the ATPase proteolipid (8, 9, 15, 16). Convincing evidence has been provided that the covalently bound DCCD is responsible for the inhibitory effect (8, 15, 16).

The present studies on the identification of the DCCD-modified amino acid residue of the ATPase-proteolipid became feasible after the amino acid sequences of the *Neurospora* and yeast polypeptide had been determined (ref. 17; unpublished data). In both organisms the DCCD was found to be bound to

a single glutamyl residue occurring in the middle of a hydrophobic segment of the polypeptide chain. Obviously, this residue is essential for the H⁺ translocation and energy-transducing properties of the ATPase complex. Its identification may help in the understanding of these processes on a molecular level.

METHODS AND MATERIALS

Growth and Labeling of Cells. Mitochondria labeled with [³H]leucine were prepared from *Neurospora crassa* and *Saccharomyces cerevisiae* as detailed (8). The specific radioactivities of the employed mitochondrial preparations were 400,000 (*Neurospora*) and 850,000 (yeast) cpm/mg of protein, respectively.

Preparation of [¹⁴C]DCCD-Labeled Proteolipid Subunit. Mitochondria were incubated with [¹⁴C]DCCD (8.5 nmol/mg of protein) for 4 hr at pH 7.5 and 0°C (8). The ATPase proteolipid was purified from whole mitochondria by a selective extraction with chloroform/methanol (2:1, vol/vol) as described (8). The isolated proteins migrated as single bands during gel electrophoresis in the presence of dodecyl sulfate or phenol/formic acid (8). From 260 mg of *N. crassa* mitochondria, 1.5 mg of DCCD-binding protein (650,000 ³H cpm/mg of protein) containing 12 nmol of bound [¹⁴C]DCCD was obtained. From 60 mg of yeast mitochondria, 0.32 mg of DCCD-binding protein (1.6 × 10⁶ ³H cpm/mg of protein) containing 13.5 nmol of bound [¹⁴C]DCCD was obtained.

Separation of Cyanogen Bromide and *N*-Bromosuccinimide Fragments. The [¹⁴C]DCCD-labeled protein and 2 mg of the unmodified protein were dissolved in 0.4 ml of 1 M cyanogen bromide in 98% (vol/vol) formic acid. After addition of 0.1 ml of water, the mixture was incubated overnight at room temperature. The solvent was removed in a flash evaporator. The residue was dissolved in 0.4 ml of formic acid plus 0.1 ml water and then applied to a column (1500 × 8 mm) of Bio-Gel P-30 (minus 400 mesh) equilibrated with 80% formic acid. Fractions were collected every 30 min at a flow rate of 1.2 ml/hr.

The cyanogen bromide fragment B-3 from *N. crassa* (see Fig. 1) dissolved in 0.4 ml of formic acid was added to 1.6 mg of *N*-bromosuccinimide. After addition of 0.1 ml of water, the reaction was allowed to proceed for 4 hr at 37°C. The whole incubation mixture was applied to the Bio-Gel P-30 column described above, and separation was performed under the same conditions.

Automated Solid-Phase Edman Degradation. The 17-residue fragments N-2 (*N. crassa*) and B-3 (yeast) were covalently

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Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

bound to aminopropyl glass by the homoserine lactone coupling method and automated Edman degradation was performed as will be detailed elsewhere. Phenylthiohydantoin amino acids were identified by gas chromatography and mass spectroscopy. ^3H and ^{14}C radioactivities were measured in a Packard Tri-Carb liquid scintillation spectrometer with counting efficiencies of 45.7% for ^{14}C and 24.5% for ^3H radioactivity.

Materials. [^{14}C]DCCD was prepared from [^{14}C]urea (20 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) (8). [^3H]Leucine (55 Ci/mmol) and [^{14}C]urea were obtained from New England Nuclear. Bio-Gel P-30 (minus 400 mesh) was from Bio-Rad Laboratories. Only batches with the control number 106,044 proved to be satisfactory for chromatography in the presence of 80% formic acid.

RESULTS

Characteristics of the [^{14}C]DCCD-Labeled Proteolipid. Previous studies (8) had shown that the proteolipid subunit occurs in the ATPase complex as oligomer, most likely as hexamer. Incubation of mitochondria from *N. crassa* and yeast with about 10 nmol of [^{14}C]DCCD per mg of protein at 0°C for 4 hr resulted in an 80–90% inhibition of the ATPase activity and a reasonably specific labeling (80–100%) of the proteolipid subunit of the ATPase complex. The proteolipid purified from such mitochondria contained bound ^{14}C radioactivity, corresponding to 15–20 nmol of DCCD per mg of protein (see *Methods and Materials*). This low value indicates that only part of the protein has been labeled, because after a 1:1 reaction 125 nmol of DCCD should be bound per mg of the 8000-dalton protein (8). A separation of [^{14}C]DCCD-modified and free protein (16) was not attempted. Thus, the labeled species was present only in tracer amounts during the experiments described below. The proteolipid was obtained from cells grown in the presence of [^3H]leucine. During sequence analysis, the [^{14}C]DCCD label therefore could be related immediately to the internal [^3H]leucine label.

Cyanogen Bromide and *N*-Bromosuccinimide Fragments. The DCCD-binding protein from *N. crassa* contains four methionines. Thus, five fragments are obtained after cleavage with cyanogen bromide. Fig. 1 shows the amino acid sequence of the whole protein as well as the alignment of the cyanogen bromide peptides. Separation by Bio-Gel P-30 chromatography in the presence of 80% formic acid (Fig. 2A) demonstrated that fragments B-1, B-2, B-4, and B-5 were completely free of [^{14}C]DCCD label. The bound inhibitor was found exclusively

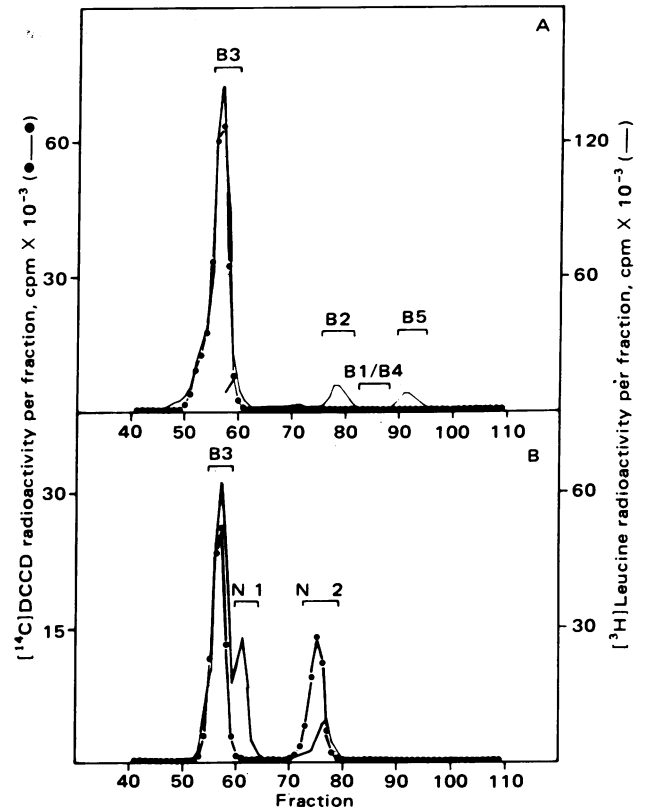


FIG. 2. Recovery of bound [^{14}C]DCCD label in the cyanogen bromide and *N*-bromosuccinimide fragments of the ATPase proteolipid from *N. crassa*. (A) The purified proteolipid, which had been labeled with [^3H]leucine and had reacted with [^{14}C]DCCD, was treated with cyanogen bromide. The resulting fragments were separated by Bio-Gel P-30 chromatography in the presence of 80% formic acid. (B) The isolated fragment B-3 was further incubated with *N*-bromosuccinimide. The split products were analyzed on the same column as the cyanogen bromide fragments. The fragments B-1 to B-5 as well as N-1 and N-2 correspond to the amino acid sequence as shown in Fig. 1. ●—●, [^{14}C]DCCD label; —, [^3H]leucine label.

in peptide B-3. This 55-residue peptide was further cleaved at its single tyrosine residue with *N*-bromosuccinimide. The DCCD label was recovered only in the 17-residue fragment N-2 (Fig. 2B), whereas the larger fragment N-1 was virtually free of bound inhibitor. A large amount of ^{14}C radioactivity was

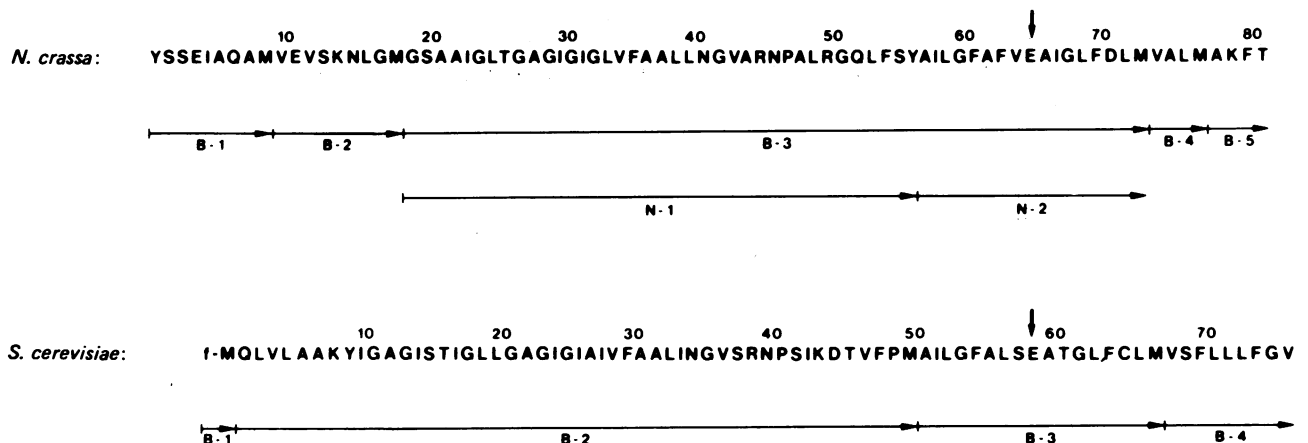


FIG. 1. Amino acid sequences of the DCCD-binding ATPase proteolipids from *N. crassa* and *S. cerevisiae*. The amino acid sequences were determined as described elsewhere (17; unpublished). The amino acid residues are represented in the one-letter code as defined in ref. 18. The *Neurospora* polypeptide was cleaved by cyanogen bromide into five fragments (B-1 to B-5). Fragment B-3 was further split into peptides N-1 and N-2 by *N*-bromosuccinimide. From the yeast polypeptide four cyanogen bromide fragments (B-1 to B-4) were obtained.

present at the position of the original fragment B-3, which had been cleaved by *N*-bromosuccinimide to only 40%.

The DCCD-binding protein from yeast contains three methionines, and consequently four cyanogen-bromide fragments are obtained (Fig. 1). Separation of [¹⁴C]DCCD-labeled peptides indicated that no ¹⁴C radioactivity was bound to fragments B-1, B-2, and B-4 (Fig. 3). The [¹⁴C]DCCD label was recovered in two fractions, both consisting of the 17-residue peptide B-3. The fraction B-3a corresponds to the monomer, the fraction B-3b to a dimeric peptide that most likely originates by the formation of a disulfide bridge (unpublished data). Some residual ¹⁴C radioactivity occurred at the position of the un-cleaved proteolipid. The trailing of [¹⁴C]DCCD and [³H]leucine label in fractions B-3a and B-3b indicated the presence of fragments composed of B-3 and B-4, due to incomplete cleavage.

During analysis of the peptides from both *Neurospora* and yeast, the [¹⁴C]DCCD-labeled material behaved like the 17-residue peptides N-2 (*Neurospora*) or B-3 (yeast). It has to be noted, however, that after gel chromatography the ¹⁴C label was found to be slightly displaced to higher molecular weight compared to the [³H]leucine label (Fig. 2 A and B, Fig. 3). The bound DCCD will add about 200 daltons in the modified peptide species, a molecular mass equivalent to about two additional amino acid residues. This difference becomes apparent due to the double-labeling technique and due to the high resolution of the gel chromatographic system applied.

Solid-Phase Edman Degradation. The DCCD-labeled fragments N-2 and B-3 of the *Neurospora* and yeast protein, respectively, represent the same segment of the polypeptide chain. Thirteen of the 17 residues are identical in the two organisms (Fig. 1). Each of the [¹⁴C]DCCD-labeled peptides was covalently coupled to aminopropyl glass via the COOH-terminal homoserine lactone and then submitted to 18 cycles of automated Edman degradation (unpublished data). The phenylthiohydantoin amino acids released at each sequencer cycle

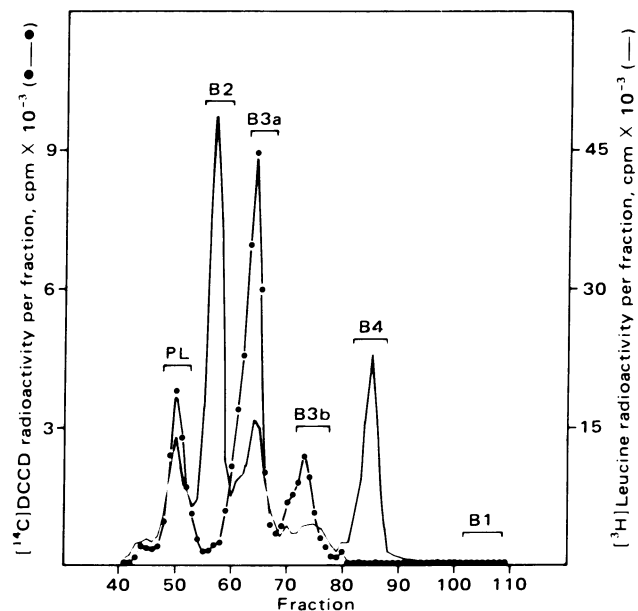


FIG. 3. Recovery of bound [¹⁴C]DCCD label in the cyanogen bromide fragments of the ATPase proteolipid from *S. cerevisiae*. The purified proteolipid, which had been labeled with [³H]leucine and had reacted with [¹⁴C]DCCD, was cleaved with cyanogen bromide. The resulting fragments were separated by Bio-Gel P-30 chromatography in the presence of 80% formic acid. B-1 to B-3 designates the fragments shown in Fig. 1. ●—●, [¹⁴C]DCCD label; —, [³H]leucine label; PL, un-cleaved proteolipid.

were examined for [¹⁴C]DCCD and [³H]leucine label, as indicated in Fig. 4. The cleaved off ³H radioactivity was found at the positions of the leucine residues as expected. A repetitive yield of 94% was calculated in both sequencer runs. Some radioactivity was recovered in cycle 1 (less than 10%) due to the elution of noncovalently bound peptide. With both peptides, the [¹⁴C]DCCD label was removed at step 9. The lower radioactivity at steps 10 and 11 was due to the overlap from the previous cycle. ¹⁴C radioactivity released at other cycles was negligible (less than 1%). Together with the [¹⁴C]DCCD label a glutamyl residue was identified, which corresponds to position 65 of the *Neurospora* protein and to the identical position 59 of the yeast protein.

Quantitative Evaluation. The results on the peptide separations and the sequence analysis shown in the previous paragraphs demonstrate the selective binding of the DCCD to one position of the polypeptide chain, which is identical in the cells

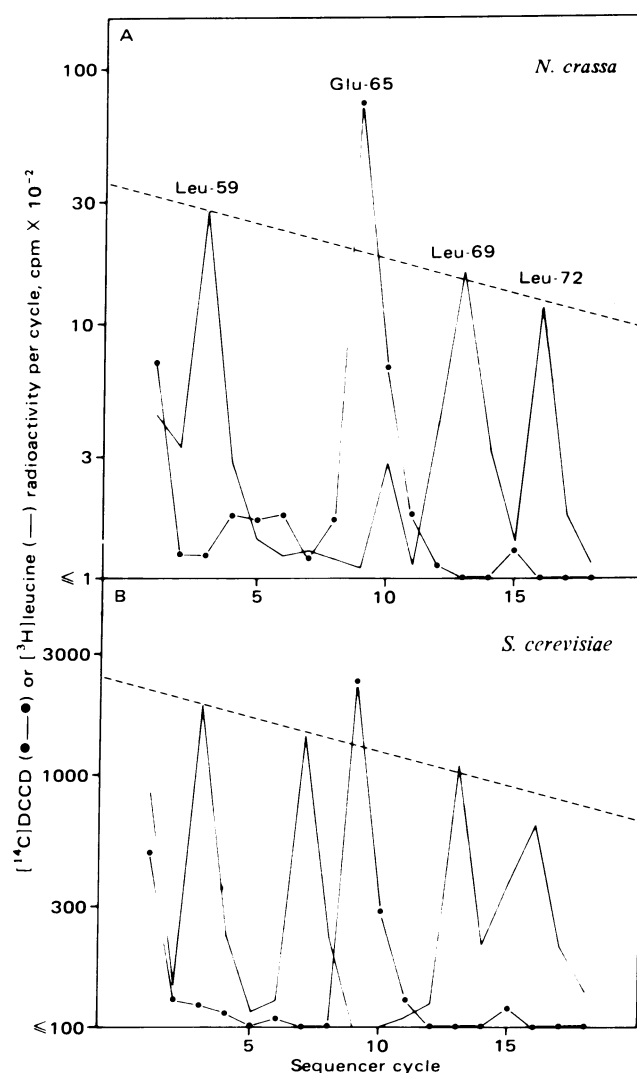


FIG. 4. Identification of the DCCD-binding residue by sequence analysis. (A) The [¹⁴C]DCCD-modified fragment N-2 from *N. crassa* was obtained from the experiment described in Fig. 2. It was submitted to 18 cycles of automated solid-phase Edman degradation. (B) The [¹⁴C]DCCD-modified fragment B-3a from *S. cerevisiae* (see Fig. 3) was submitted to 18 cycles of automated solid-phase Edman degradation. Corresponding results were obtained with fragment B-3b. The residues released during each sequencer cycle were analyzed for [¹⁴C]DCCD (●—●) and [³H]leucine (—) radioactivity. The slope of the broken line corresponds to a repetitive yield of 94% per cycle.

Table 1. Enrichment of [¹⁴C]DCCD label in fractions analyzed during identification of the DCCD-binding residue

Fraction	¹⁴ C/ ³ H ratio*	
	Measured	Calculated†
<i>N. crassa</i>		
Proteolipid	0.36	= 0.36 (11)
Fragment B-3	0.44	0.44 (9)
Fragment N-2	1.2	1.3 (3)
Sequencer cycle 9	3.84‡	3.96 (1)
<i>S. cerevisiae</i>		
Proteolipid	0.16	= 0.16 (12)
Fragment B-3	0.46	0.48 (4)
Sequencer cycle 9	1.8‡	1.92 (1)

* The ¹⁴C/³H ratios were calculated from the [¹⁴C]DCCD and [³H]-leucine radioactivities as determined in the experiments presented in Figs. 2, 3, and 4.

† The ¹⁴C/³H ratios of the individual fractions were calculated on the basis of the assumption that the [³H]leucine radioactivities decrease proportionally to the number of leucine residues present in each fraction (numbers in parentheses).

‡ The [¹⁴C]DCCD radioactivity recovered in sequencer cycle 9 (see Fig. 4) was related to the ³H radioactivity of a single leucine residue, taking into account a repetitive yield of 94% per cycle.

of the two species. The specific binding of DCCD is further stressed by a quantitative evaluation of these double-labeling experiments. On the basis of the data obtained during the sequence studies (Fig. 4), the [¹⁴C]DCCD radioactivity can be related to the ³H radioactivity of a single leucine residue (Table 1). The ¹⁴C/³H ratios measured in the original protein and the various fragments correspond reasonably to the number of leucine residues present in these fractions. This indicates that the amount of [¹⁴C]DCCD label, which was recovered with the glutamyl residue during the sequencer run, accounts quantitatively for the amount of label originally present in the whole protein. Apparently, the reaction product between DCCD and the glutamyl side chain is stable enough to survive under the conditions of peptide cleavage and separation (80% formic acid) as well as of Edman degradation (trifluoroacetic acid). The [¹⁴C]DCCD label was removed, however, when the whole protein was hydrolyzed at 105°C in 5.7 M HCl.

DISCUSSION

The present results demonstrate that the ATPase inhibitor DCCD binds covalently and selectively to a single glutamyl residue of the proteolipid subunit of the mitochondrial ATPase complexes from *N. crassa* and *S. cerevisiae*. The identification of the DCCD-binding residue became possible because the amino acid sequence of this highly hydrophobic polypeptide recently has been elucidated in both microorganisms (ref. 17; unpublished data), and because the reaction product between the glutamyl side chain and [¹⁴C]DCCD is stable under the acidic conditions occurring during peptide separation and Edman degradation.

The chemical nature of the reaction product between DCCD and the glutamyl side chain is still unknown. In the present studies only tracer amounts of the [¹⁴C]DCCD-modified polypeptide were used, because the DCCD-modified and the free species could not be separated in *N. crassa* and yeast. With the *Escherichia coli* proteolipid, however, the DCCD-modified species could be eluted from DEAE-cellulose at lower salt concentrations than the unmodified species (16, 19). This was interpreted to indicate that a negative charge of the proteolipid was lost upon reaction with DCCD. Thus the carboxyl group of the acidic side chain most likely has formed a stable *N*-acylisourea. This stable product can originate from an unstable *O*-acylisourea by intramolecular rearrangement (20, 21).

A specific role of the DCCD-binding glutamyl residue may be anticipated when the amino acid sequences of the ATPase proteolipids from different organisms are compared. The *Neurospora* and yeast proteins show a high degree of homology especially in the case of two hydrophobic segments of about 25 residues each. The only conserved acidic residue is the glutamic acid that binds the DCCD. Recently, the DCCD-binding ATPase proteolipid from *E. coli* has been analyzed and found to be homologous to the mitochondrial protein (17). In the bacterial protein the DCCD is bound to an aspartyl residue occurring at the same position as the glutamyl residue of the mitochondrial proteins. Apparently an acidic group in this position is essential for the function or structure of the protein.

The DCCD-reactive acidic residue is located amidst a hydrophobic sequence of about 25 residues, whereas most of the other polar residues are clustered either at the ends or in a polar 12-residue segment in the middle of the polypeptide chain. Thus, the acidic residue possibly resides within the hydrophobic part of the membrane. This assumption is supported by the observation of Beechey and coworkers that the activity of the ATPase complex is inhibited by hydrophobic carbodiimides but not by water-soluble ones (22). The velocity of proton conductance measured with the membrane factor *TF*₀ of the ATPase complex from the thermophilic bacterium PS-3 shows a pH profile identical with a titration curve for monoprotic acid of pK_a = 6.76 (4). It appears to be possible that this postulated proton-binding site in *TF*₀ corresponds to the DCCD-binding acidic residue of the proteolipid.

Several models have been proposed to explain on a molecular level the translocation of protons across biological membranes. Such a process occurs not only in the energy-transducing ATPase complex but also in bacteriorhodopsin (23) and possibly in the respiration-linked proton pumps (24). One type of mechanism includes a net of hydrogen bonds provided by polar amino acid side chains of the protonophoric protein or by water lining the interior of a hydrophilic pore (25, 26). Another type of model, developed by Boyer (27), involves the migration of a negatively charged amino acid side chain, or the alternating exposure of such a group to different sides of the membrane. Obviously, the latter mechanism depends on conformational changes of the protonophoric protein. At present, too little information is available to decide whether the modification of the acidic residue by DCCD prevents the functional conformation of the protonophoric protein or whether this residue is directly involved in the translocation of protons.

Note Added in Proof. Recently it was found that the DCCD-binding aspartyl residue is substituted by a glycine in the proteolipid from an ATPase mutant of *E. coli* whose membrane factor is unable to conduct protons (28).

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