The proton pump (H⁺-ATPase) found in the plasma membrane of the fungus *Neurospora crassa* is inactivated by dicyclohexylcarbodiimide (DCCD). Kinetic and labeling experiments have suggested that inactivation at 0 °C results from the covalent attachment of DCCD to a single site in the M₁ = 100,000 catalytic subunit (Sussman, M. R., and Slayman, C. W. (1983) *J. Biol. Chem.* 258, 1839–1843). In the present study, when [*¹⁴C]DCCD-labeled enzyme was treated with the cleavage reagent, N-bromosuccinimide, a single major radioactive peptide fragment migrating at about M₁ = 5,300 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was produced. The fragment was coupled to glass beads and partially sequenced by automated solid-phase Edman degradation at the amino terminus and at an internal tryptic cleavage site. By comparison to the DNA-derived amino acid sequence for the entire M₁ = 100,000 polypeptide (Hager, K., and Slayman, C. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 7693–7697), the fragment has been identified as arising by cleavage at tyrosine 100 and tryptophan 141. Covalently incorporated [*¹⁴C]DCCD was released at a position corresponding to glutamate 129. The DCCD-reactive glutamate is located in the middle of the first of eight predicted transmembrane sequences. When the sequence surrounding the DCCD site is compared to that surrounding the DCCD-reactive residue of two other proton pumps, the FₒF₁-ATPase and cytochrome c oxidase, no homology is apparent apart from an abundance of hydrophobic amino acids.

Despite recent success in elucidating the primary structure of several different enzymatic ion pumps, the molecular basis for protein-mediated energy transduction remains to be determined. The plasma membrane proton pump (H⁺-ATPase) found in fungi and plants converts chemical energy (ATP) into electrical energy, and investigations of its structure and function have been pursued utilizing electrophysiological (1), biochemical (2), and recombinant DNA (3, 4) techniques. These studies have shown that the enzyme belongs to a class of M₁ = 100,000 cation-translocating ATPases that includes the Na⁺,K⁺-ATPase and Ca²⁺-ATPase of animal cells and the K⁺-ATPase of *Escherichia coli*. For several of the ATPases, the complete amino acid sequence of the M₁ = 100,000 polypeptide has recently been deduced from the corresponding cloned gene (3–8). The results point to regions of primary sequence homology, especially in portions of the polypeptide that appear to be involved in ATP binding and hydrolysis. A further similarity has been revealed by hydrophathy analysis of the sequence data, which suggests that each of the M₁ = 100,000 polypeptides contains 8–10 hydrophobic segments (3–8). Presumably these segments serve to anchor the ATPase in the lipid bilayer, but they may also participate directly in ion translocation through the membrane. Interestingly, in view of the different ion specificities and stoichiometries of the various ATPases, the transmembrane segments are not especially homologous from one ATPase to the next. Thus, any reagent or modification procedure that allows the structure-function relationships of the transmembrane segments to be probed is likely to be particularly useful.

In the present study, we have found N,N' -dicyclohexylcarbodiimide (DCCD) to be one such reagent. It has been known for a number of years that DCCD reacts preferentially with a single glutamic or aspartic acid residue of the membranous portion of the FₒF₁-ATPases (9). The DCCD-sensitive carboxyl group is eliminated by conversion to a glycine or asparagine can no longer catalyze transmembrane proton transport (11). Similarly, the "proton channel" subunit of cytochrome c oxidase has been found to contain a DCCD-reactive essential glutamic acid (12). Thus, it was of interest several years ago to discover that the *Neurospora crassa* plasma membrane H⁺-ATPase is covalently modified and inhibited by DCCD (13). The purpose of the present study was to map the location of the DCCD-sensitive site within the M₁ = 100,000 ATPase polypeptide.

Based on the low reactivity of water-soluble carbodiimides, we expected the DCCD-reactive residue of the fungal plasma membrane ATPase to be located in a hydrophobic environment (13). Unfortunately, hydrophobic peptide fragments have proven particularly difficult to isolate and purify using routine cleavage and high pressure liquid chromatography procedures. Thus, although several laboratories have published other methods for localization of DCCD-reactive sites (14–16), this has been achieved only in a protein which was the product of a cloned gene.

In the present study, we have found that DCCD selectively modifies and inhibits a subunit of the fungal plasma membrane ATPase which was shown previously to be particularly useful for structural probing (13). The subunit contains a single glutamic acid residue which is eliminated by DCCD treatment. The amino acid sequence of this subunit has been determined by automated solid-phase Edman degradation at several positions. By comparison to the DNA-derived amino acid sequence of the entire M₁ = 100,000 polypeptide (13), the DCCD-reactive glutamate residue is located in the middle of the first of eight predicted transmembrane sequences.

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lished protein sequence data for the \( M_r = 100,000 \) polypeptides of the Ca\(^{2+}\)-ATPase and Na\(^+\),K\(^+\)-ATPase (14, 15), none have reported sequences derived from Edman degradation of the hydrophobic transmembrane segments that make up about 30% of the total protein. Amino acid sequences for these regions have come exclusively from the cloned DNA. Our preliminary experiments indicated that standard procedures were not effective for isolating and sequencing a \([^{14}C]DCCD\)-labeled peptide from the \( M_r = 100,000 \) \( N.\ crassa \) plasma membrane ATPase. We decided instead to utilize a solid-phase protein sequencing strategy similar to that introduced by Sebald and co-workers for the DCCD-binding subunit of the Fal H\(^+\)-ATPase (16).

**EXPERIMENTAL PROCEDURES**

\([^{14}C]DCCD\) Labeling and Purification of \( N.\ crassa \) Plasma Membrane H\(^+\)-ATPase—Large-scale labeling experiments using \([^{14}C]DCCD\) were performed under conditions of maximal specificity (pH 7.5, 0 \(^\circ\)C) as described previously. 60 mg of \( N.\ crassa \) plasma membrane (specific activity, 6.0 \( \mu \)mol/min/mg of protein) were suspended in 30 ml of 1 mM EGTA (adjusted to pH 7.5 with Tris) in an ice bath. 200 \( \mu \)M \([^{14}C]DCCD\) (specific activity, 22–55 Ci/mol) was added from an ethanol stock at least 1,000-fold more concentrated. After 4 h at 0 \(^\circ\)C, the ATPase had typically been inactivated by 60%, whereas ethanol controls showed less than 10% loss of activity. After the addition of 300 \( \mu \)l of 10% (w/v) sodium deoxycholate, the membranes were collected by centrifugation at 200,000 \( \times \) g for 1 h. The detergent-washed membrane pellet was then resuspended in 3 ml of 10% (w/v) lithium dodecyl sulfate containing 5% (w/v) glycerol. Dissolution of the pellet in the detergent solution was achieved by use of a mechanically driven Teflon-glass homogenizer followed by a 5-min incubation at 30 \(^\circ\)C. The sample was then chilled and applied to a glass cylindrical column (115 \( \times \) 2.5 cm, inner diameter) containing Bio-Gel A-1.5m. The column was run at 4 \(^\circ\)C with a flow rate of about 10–20 ml/h, with 2 M (w/v) lithium dodecyl sulfate as running buffer. Fractions (2 ml) were collected and analyzed for protein according to protein described previously (17) and by SDS-PAGE. Fractions containing the \( M_r = 100,000 \) ATPase polypeptide were pooled, lyophilized, and resuspended in one-tenth of their original volume of water to give a final lithium dodecyl sulfate concentration of 1% (w/v). Typically, the pooled peak fractions contained 4–5 mg of protein, and on the basis of autoradiography and Coomassie stain after SDS-PAGE, the \( M_r = 100,000 \) ATPase polypeptide was at least 90% pure.

**Cleavage with N-Bromosuccinimide and Purification of the \([^{14}C]DCCD\)-labeled Peptide Fragment**—To 3 ml of \([^{14}C]DCCD\)-labeled \( M_r = 100,000 \) ATPase polypeptide was added 3 ml of solution containing 8 M urea, 0.368 M HCl, and 9 mg of N-bromosuccinimide. After 1 h at 37 \(^\circ\)C, 2 ml of gel sample buffer (8 M urea, 0.368 M HCl, 0.451 M pyridine, 5% (v/v) mercaptoethanol, and 5% (w/v) SDS) was added, and the sample was applied to two 20% (w/v) polyacrylamide gels, 1.5 mm \( \times \) 15 cm. Electrophoresis was performed using a system devised by Kye and Rodriguiez (18) for resolving low molecular weight peptide fragments of the Na\(^+\),K\(^+\)-ATPase. The running gel contained 20% (w/v) acrylamide, 1% (w/v) bisacrylamide, 0.323 M Tris-Cl, 0.1% (w/v) SDS, and 8 M urea; the stacking gel contained 6% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, 0.368 M HCl, 0.451 M pyridine, 0.1% (w/v) SDS, and 8 M urea; the upper buffer reservoir contained 0.213 M MES, 0.269 M pyridine, and 0.1% (w/v) SDS; and the lower buffer reservoir contained 0.32 M Tris, 0.32 M Tris-Cl. Electrophoresis was performed at 100 V at room temperature followed by staining with Coomassie Blue (0.05% w/v) in 25% (v/v) isopropl alcohol, 10% (v/v) acetic acid and destaining in the same solution lacking dye. Prior to elution, the gels were stored in 10% (v/v) acetic acid. The \([^{14}C]DCCD\)-labeled peptide fragment was identified by measuring the radioactivity incorporated in this region on a densitometer tracing of the gel. This was followed by slicing the lane of each gel into 1-mm slices and dissolving the gel pieces in glass scintillation vials containing 1 ml of 30% (w/v) hydrogen peroxide at 70 \(^\circ\)C overnight. Following digestion, the samples were cooled and counted in 20 ml of scintillation fluid. For sequencing, the peptide was purified by a combination of homogenized gel slices in 10% (w/v) formic acid. After shaking overnight at room temperature, the solid gel particles were centrifuged away and the supernatant evaporated under nitrogen at room temperature for several hours. The final dried sample was resuspended in a small volume (100–500 \( \mu \)l) of 88% (w/v) formic acid and coupled to diisothiocyanate- or ninhydrin-modified glass beads using standard procedures (19, 20).

**Protein Sequencing**—Solid-phase Edman degradations were performed on a Sequamat Mini 15 automated sequenator. Cycling times and conditions for PTH-derivative analysis were as described previously (21).

**Materials—**\( N.\ crassa \) was purchased from Research Products International. Dithiothreitol- and ninhydrin-modified glass beads and other solid-phase protein sequencing reagents were purchased from Sequamat, Inc., Watertown, MA 02172.

**RESULTS**

In a previous report (13) we correlated the covalent incorporation of \([^{14}C]DCCD\) into the \( M_r = 100,000 \) ATPase polypeptide at 0 \(^\circ\)C with the loss of ATPase activity. Based on the observation that inactivation followed pseudo-first order kinetics and that 100% inactivation occurred with less than 1 mol of \([^{14}C]DCCD\) bound/mol of ATPase, we suggested that DCCD inhibits the ATPase by reacting with a single amino acid residue. This idea has now been tested by protein cleavage and sequencing studies. Treatment of \([^{14}C]DCCD\)-labeled enzyme with N-bromosuccinimide, a reagent that cleaves at tyrosine, histidine, and tryptophan residues (19), produced a major fragment migrating with an apparent \( M_r = 5,300 \) on high resolution SDS-PAGE (18) (Fig. 1). The yield of the fragment was estimated by slicing a parallel lane of the gel and determining the radioactivity of each slice (see "Experimental Procedures"); in four experiments, the \( M_r = 5,300 \) fragment contained 31–63% of the total \( ^{14}C \) radioactivity, with an average value of 46%. Most of the radioactivity not present in the \( M_r = 5,300 \) peak appeared as a broad background of higher molecular weight material.

For sequencing studies, purified \( M_r = 5,300 \) fragment was obtained by large-scale N-bromosuccinimide cleavage and SDS-PAGE as shown in Fig. 1; followed by overnight elution from Coomassie-stained gel slices with 88% (v/v) formic acid. The peptide sample was covalently coupled via amino groups at the amino terminus and at N-terminals lysine residues using diisothiocyanate-modified glass beads. In the experiment of
Fig. 2, it could be estimated that approximately 0.5 nmol of peptide was coupled to the beads, based on the amount of $[^{14}C]$DCCD bound and on the previously determined stoichiometry of labeling (13). Automated solid-phase Edman degradation of the coupled peptide, followed by PTH-derivative analysis, demonstrated the presence of leucine, asparagine, and glutamine in cycles 2, 3, and 4, respectively. Approximately 0.1 nmol of leucine was observed in the second cycle, corresponding to a 20% yield of sequencing. Measurement of radioactivity released in the first 15 cycles showed no peak of $[^{14}C]$DCCD (results not shown). A computer search of the complete DNA-generated amino acid sequence for the Neurospora plasma membrane ATPase (4) revealed only a single location, residues 102-104, for the tripeptide -Leu-Asn-Gln-. As expected, this tripeptide is located 1 residue away from a potential cleavage point for N-bromosuccinimide on the carboxyl-terminal side of Tyr-100. Proceeding toward the carboxyl terminus in the DNA-derived amino acid sequence, Trp-141 is the next expected N-bromosuccinimide cleavage site. A DCCD-modified peptide starting with Gly-101 and ending with Trp-141 has a calculated $M_r = 4817$, quite close to the value determined by SDS-PAGE in the experiment of Fig. 1 ($M_r = 5300$).

Inspection of the amino acid sequence for the 41-residue fragment from Gly-101 and Trp-141 revealed six possible sites for DCCD modification, of which three were eliminated by radioactivity measurements performed during the DITC-coupled Edman degradation (see above). The remaining three were Glu-129, Glu-139, and Asp-140. To distinguish among them, $[^{14}C]$DCCD-labeled $M_r = 5300$ fragment was coupled via its carboxyl terminus to aminopropyl glass beads. Beads containing covalently attached peptide were then treated with trypsin, washed, and subjected to 25 cycles of automated Edman degradation. Analysis of PTH-derivatives released gave the sequence Phe-Leu-X-Phe-(Met/Val)-X-Pro-X-X-Phe, while measurement of radioactivity revealed a peak at cycle 14 (Fig. 3). The trypsinization result is consistent with the DNA-derived protein sequence which indicates a lysine at position 115, immediately preceding a phenylalanine. Overall, the partial sequence data and release of the $[^{14}C]$DCCD label unambiguously serve to locate the reactive amino acid as Glu-129 in the enzyme (Fig. 4). Although there were no peaks of radioactivity observed at either of the other two potential sites for DCCD modification (cycles 24 and 25),
radioactivity was observed as a gradually decreasing background beginning with cycle 1. Presumably this background is caused by noncovalently bound peptide that was eluted with heated anhydroxy trifluoroacetic acid and the other reagents used in each cycle of the degradation.

Fig. 5 illustrates the amino acid sequence surrounding the DCCD-reactive glutamic acid residue of the N. crassa plasma membrane ATPase aligned with similar sequences from several other enzymes. Although direct labeling studies have not been performed with plasma membrane ATPase from the yeast Saccharomyces cerevisiae, it seems likely that Glu-129 is responsible for the known DCCD sensitivity of this enzyme (3) because it is located in a region of the polypeptide that shows nearly 100% homology between the two fungal species. The relationship between the DCCD sites of the fungal plasma membrane ATPase, F_{p}, F_{h}^-H^+-ATPase (9 species), and cytochrome oxidase (7 species) is much less pronounced. Little specific homology is present although there is a clear preference for hydrophobic residues on either side of the DCCD-reactive residue in all 19 cases that have been examined.

**DISCUSSION**

The results described in this paper serve to identify Glu-129 as the major site at which DCCD reacts with the N. crassa plasma membrane H^+-ATPase. Identification was achieved by a solid-phase protein sequencing strategy combined with knowledge of the complete protein sequence deduced from the cloned DNA (4). When the M_t = 100,000 ATPase polypeptide was cleaved with N-bromosuccinimide, an M_t = 5,300 fragment containing the [14C]DCCD-labeled residue was obtained with about 50% yield. It is possible that much of the remaining plasma membrane H^+-ATPase was cleaved with N-bromosuccinimide, an M_t = 5,300 fragment containing the [14C]DCCD-labeled residue was obtained with about 50% yield. It is possible that much of the remaining incorporated [14C]DCCD is also located at Glu-129 but does not migrate with the M_t = 5,300 fragment on SDS-PAGE either because of incomplete cleavage or aggregation of the fragment. Incomplete cleavage has been reported with N-bromosuccinimide for other peptides (19), and the two minor radioactive bands we observed at about M_t = 19,000 (possibly Gly-101 to His-240) and M_t = 11,000 (possibly Asp-59 to Trp-141) are consistent with this interpretation. Aggregation is also a common problem with hydrophobic peptides; it is noteworthy that, when the eluted M_t = 5,300 fragment was rerun on SDS-PAGE, it formed a background of high molecular weight material similar to that observed in Fig. 1. Radioactivity not coincident with the M_t = 5,300 fragment may also represent [14C]DCCD attached to glutamic and aspartic acid residues at other locations in the enzyme. In spite of the fact that labeling conditions were carefully chosen to be as selective as possible (high pH and low temperature, see Ref. 21), it is reasonable to expect a small amount of nonspecific labeling since the ATPase contains a total of 114 aspartic and glutamic acid residues.

On the basis of a hydrophobicity profile calculated from the primary sequence, it has been suggested that the N. crassa plasma membrane ATPase traverses the membrane 8 times (4). According to this analysis, Glu-129 is located in the middle of the first transmembrane segment. Its position in the protein is thus similar to that of the DCCD-reactive residue in the postulated proton channel portions of the F_{p}, F_{h}^-H^+-ATPase and cytochrome c oxidase. Furthermore, although there is no detailed homology in the amino acid sequences, there does appear to be a strict requirement for several hydrophobic residues on either side of the DCCD-reactive residue in all three enzymes. The similarity both in hydrophobic environment and high reactivity to DCCD suggests that Glu-129 in the plasma membrane H^+-ATPase may perform a proton-translocating function, as has been proposed for the two other enzymes.

Some support for this notion has come from an independent line of electrophysiological experiments. In the case of the giant alga Chara corallina, Kishimoto et al. (22) used current-voltage analysis to follow the effect of DCCD on the plasma membrane H^+ pump. They observed that the major effect was nearly complete inhibition of pump current, accompanied by a smaller reduction in reversal potential, and suggested that DCCD was acting to decrease the conductance of a proton channel. In preliminary experiments with N. crassa, Sanders and Sussman obtained similar results. Because of the complexity of the reaction cycle of the H^+ pump (23), however, the effects of DCCD will need to be explored as a function of pH in order to test the notion that proton translocation through the membrane is being specifically blocked. At the same time, mutagenesis of the recently cloned gene for N. crassa pump should provide additional information about the role of Glu-129 in the reaction cycle.

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