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Interactions of *Neurospora crassa* Plasma Membrane H^+-ATPase with *N*-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline†

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ABSTRACT: The carboxyl group activating reagent *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) interacts with the *Neurospora* plasma membrane H^+-ATPase in at least three different ways. This reagent irreversibly inhibits ATP hydrolysis with kinetics that are pseudo-first-order at several concentrations of EEDQ, and an appropriate transform of these data suggests that 1 mol of EEDQ inactivates 1 mol of the H^+-ATPase. Inhibition probably involves activation of an ATPase carboxyl group followed by a nucleophilic attack by a vicinal nucleophilic functional group in the ATPase polypeptide chain, resulting in an intramolecular cross-link. The enzyme is protected against EEDQ inhibition by MgATP in the presence of vanadate, a combination of ligands that has previously been shown to "lock" the H^+-ATPase in a conformation that presumably resembles the transition states of the enzyme phosphorylation and dephosphorylation reactions, but is not protected by the substrate analogue MgADP, which is consistent with the notion that one or both of the residues involved in the EEDQ-dependent inhibitory intramolecular cross-linking reaction normally participate in the transfer of the γ-phosphoryl group of ATP, or are near those that do. The ATPase is also labeled by the exogenous nucleophile [^{14}C]glycine ethyl ester in an EEDQ-dependent reaction, and the labeling is diminished in the presence of MgATP plus vanadate. However, peptide maps of [^{14}C]glycine ethyl ester labeled ATPase demonstrate that the labeling is not related to the EEDQ inhibition reaction in any simple way. In a third type of interaction, EEDQ mediates the specific cross-linking of ATPase monomers with some other membrane protein, possibly another ATPase monomer, leading to the formation of a product with an apparent molecular weight of about 260 000. This reaction occurs substantially more slowly than the inhibition reaction and is thus presumably not directly related either.

The principal ATP hydrolyzing enzyme in the plasma membrane of the filamentous fungus *Neurospora crassa* is an electrogenic proton pump (Scarborough, 1976, 1980) capable of generating a transmembrane electrical potential difference in excess of 200 mV (Slayman et al., 1973). The hydrolytic moiety of this enzyme has a molecular mass of about 105 000 daltons (Dame & Scarborough, 1980, Addison & Scarborough, 1981, Bowman et al., 1981), and recently, evidence has been provided indicating the strong likelihood that no subunits other than the hydrolytic moiety are involved in the catalysis of transport by this enzyme (Scarborough & Addison, 1984) and that monomers are efficient proton pumps (Goormaghtigh et al., 1986). The primary goal of this laboratory is an understanding of how this single polypeptide chain catalyzes electrogenic proton translocation at the expense of ATP hydrolysis. As part of our efforts in this regard, we have been investigating a variety of ATPase inhibitors as potential reagents for labeling the active site and other sites necessary for catalytic activity. With the knowledge that the β-carboxyl

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group of an aspartic acid residue in the 105 000-dalton hydrolytic moiety is phosphorylated and dephosphorylated once during each turn of the catalytic cycle (Dame & Scarborough, 1980, 1981) and is thus clearly one of the active site residues, we were drawn to investigate the effects of the carboxyl group activating reagent (Belleau & Malek, 1968; Dugas & Penney, 1981) and known transport ATPase inhibitor (Pougeois et al., 1978; Saccomani et al., 1981) *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ),[1] also known as Belleau's reagent. The multiple effects of this interesting reagent on the *Neurospora* plasma membrane H^+-ATPase are the subject of this paper.

Experimental Procedures

Treatment of Plasma Membrane H^+-ATPase with EEDQ. *Neurospora* plasma membrane vesicles isolated as described (Addison & Scarborough, 1982) (0.5 mg of protein) were treated with EEDQ in a reaction volume of 0.25 mL containing 40 mM Pipes (pH 6.2 with Tris) and any other additions at 18 °C. The various incubation times, EEDQ concentrations, and other additions are indicated in the figure legends. Unless stated otherwise, the mixtures were preincubated at 18 °C for 5 min before addition of the EEDQ. In the ligand protection experiments, the preincubation period was 10 min. The reactions were stopped by adding 1.25 mL of ice-cold 0.1 M MES (pH 7.5 with Tris), mixing, and placing the samples on ice. The vesicles were collected by centrifugation (1900*g*, 20 min, 4 °C) and were washed once by resuspending the pellets in 0.5 mL of ice-cold 10 mM MES (pH 6.8 with Tris) containing 1 μg of chymostatin/mL and repeating the centrifugation step. The membranes were then resuspended in 0.1 mL of ice-cold 10 mM MES (pH 6.8 with Tris). Control vesicles were treated as outlined above without EEDQ. The amounts of methanol, the solvent for EEDQ, and of ethanol, the solvent for glycine ethyl ester, that were added to the EEDQ reaction mixtures had no effect on the ATPase activity.

SDS–Polyacrylamide Gel Electrophoresis. Plasma membrane vesicles treated as described in the individual experiments were pelleted by centrifugation (1900*g*, 20 min, 4 °C) and resuspended in disaggregation buffer [0.125 M Tris (pH 6.8 with H_3PO_4), 5% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 25% (v/v) glycerol, 2 mM EDTA, and 0.01% (w/v) bromophenol blue] to a concentration of 1.2 mg of protein/mL. The disaggregated samples were then analyzed by SDS–PAGE as described (Addison & Scarborough, 1982).

Fluorescein Isothiocyanate Labeling of Neurospora Plasma Membrane Vesicles. Plasma membrane vesicles (5 mg of protein) were dissolved in 5% (w/v) SDS, 4 mM EDTA, and 0.15 M Tris (pH 8.8 with H_3PO_4) to a concentration of 1 mg/mL. The solution was then incubated at 30 °C for 1 h with occasional mixing. To this was added, dropwise, 0.1 mL of a 10% (w/v) FITC solution in dimethyl sulfoxide–methanol (1:1 v/v) with vigorous mixing. The solution was then adjusted to 2% (w/v) dithiothreitol by the addition of the solid and incubated for an additional 30 min at 30 °C. Afterward, the solution was dialyzed against 2L of 0.15 M NH_4HCO_3, 0.2% (w/v) SDS, 5% (v/v) glycerol, 1 mM EDTA, and 1% (v/v) β-mercaptoethanol over a 24-h period with two changes. The contents of the dialysis bag were then frozen and lyophilized.

[1] Abbreviations: EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; FITC, fluorescein isothiocyanate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

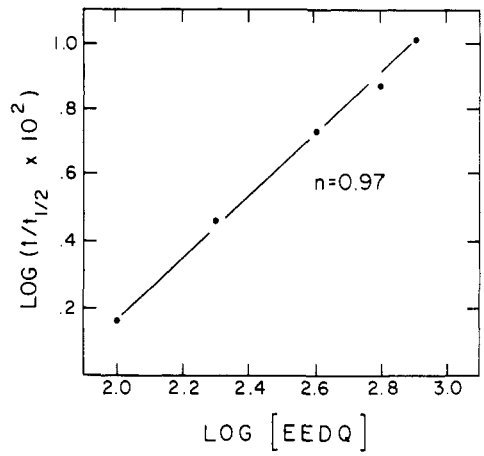

FIGURE 1: Determination of the order *n* of the inactivation reaction with respect to EEDQ concentration. Duplicate samples of plasma membrane vesicles were treated with EEDQ at concentrations of 100–800 μM for 10–40 min as described under Experimental Procedures. The data obtained were then plotted on semilog paper as the percentage of corresponding control samples incubated under identical conditions in the absence of EEDQ. Inactivation in the control samples was less than 15%. The half-time (in minutes) of inactivation at the various EEDQ concentrations was then determined from the linear plots obtained. The EEDQ concentrations are expressed in micromolar.

The product was then dissolved in 2.5 mL of water and applied to a Sephadex G-100 column (1.5 × 26 cm) and the column eluted with 30 mM NH_4HCO_3, containing 1 mM EDTA, 2 mM β-mercaptoethanol, and 2% (w/v) SDS. The void volume fractions were then pooled, frozen, and lyophilized. The lyophilized powder was then dissolved in SDS–PAGE disaggregation buffer (see above) to a protein concentration of 1.2 mg/mL and stored at −20 °C.

Other Procedures. ATPase activity was measured as described (Addison & Scarborough, 1982). The specific activities of the various membrane preparations were around 1.3 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Materials. The sources of most of the materials have been described previously (Dame & Scarborough, 1980; Addison & Scarborough, 1981, 1982). EEDQ (gold label) was from Aldrich. [1-^{14}C]Glycine ethyl ester (sp act. 52.5 mCi/mmol) was from New England Nuclear.

Results

Characteristics of Inhibition of H^+-ATPase by EEDQ. An initial characterization of the effects of EEDQ on the *Neurospora* plasma membrane H^+-ATPase showed that this enzyme in isolated membranes is inhibited by EEDQ in a time- and concentration-dependent manner. Semilogarithmic plots of inhibition as a function of time were linear up to at least 80% inhibition at concentrations of 100–800 μM EEDQ (not shown). The inhibition is essentially irreversible as the membranes are washed free of EEDQ before the ATPase assays are carried out. In Figure 1, these data are transformed to a plot of the logarithm of the reciprocal of the half-times of inhibition vs. the logarithm of the EEDQ concentration. The slope of the resulting line is very near 1. Figure 2 shows that the EEDQ inactivation reaction is strongly pH dependent. Treatment of the membranes with EEDQ at a concentration of 400 μM of 30 min results in nearly complete inhibition of the H^+-ATPase near pH 6, and the inhibition is progressively less as the pH is raised. Under these conditions, half-maximal inhibition occurs around pH 7. From these results, pH 6.2

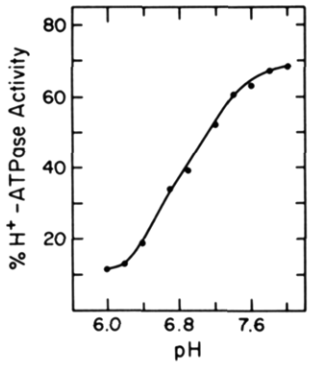

FIGURE 2: Effect of pH on inactivation of the H^+-ATPase by EEDQ. Plasma membrane vesicles (0.5 mg of protein) in a reaction volume of 0.25 mL containing 40 mM Pipes–Tris buffer at the indicated pH were preincubated at 18 °C for 5 min. Afterward, EEDQ was added to a final concentration of 400 μM, and the mixtures were then incubated for 30 min. The reactions were then stopped and the membranes washed and assayed for ATPase activity as described under Experimental Procedures. Individual points are the average values obtained from duplicate ATPase assays plotted as the percentage of the corresponding control samples incubated under identical conditions in the absence of EEDQ. Inactivation in the control samples varied between 0% at pH 7 and higher to near 30% at pH 6.[2]

was chosen for EEDQ treatment in the remainder of the experiments to be described. To minimize nonspecific inactivation of the H^+-ATPase, which is augmented at this pH,[2] the inhibition reactions were conducted at 18 °C. It is important to mention that replacement of the Pipes–Tris buffer used for most of the experiments presented with a Pipes–NaOH buffer had no effect on the EEDQ inhibition reaction (not shown), which means that Tris, a potential nucleophile, is not involved in the EEDQ inhibition reaction.

The effects of several ATPase ligands on ATPase inhibition by EEDQ were also investigated. In the presence of MgATP plus vanadate, a potent inhibitor of the H^+-ATPase (Bowman & Slayman, 1979) and a probable transition-state analogue of the aspartyl–phosphoryl–enzyme intermediate hydrolysis reaction (Addison & Scarborough, 1982; Macara, 1980), the rate of inactivation is markedly decreased. The pseudo-first-order rate constant for inactivation in the presence of 400 μM EEDQ is 0.035 min^{-1}, whereas in the presence of $MgSO_4$–ATP (6mM) and sodium vanadate (0.1 mM) it is 0.0034 min^{-1}. In the presence of 6 mM $MgSO_4$ plus 0.1 mM sodium vanadate, the inactivation rate constant has an intermediate value of 0.018 min^{-1}. Neither sodium vanadate alone (0.1 mM) nor $MgSO_4$–ADP (6 mM) afford significant protection against EEDQ inactivation. In the presence of these agents, the inactivation rate constant is 0.032 min^{-1}, nearly the same as that in the absence of any additions. $MgSO_4$ (6 mM) alone likewise has little effect.

EEDQ-Dependent Labeling of H^+-ATPase. In addition to inhibiting the H^+-ATPase, EEDQ also mediates labeling of the enzyme by the exogenous nucleophile [^{14}C]glycine ethyl ester. Figure 3 demonstrates this phenomenon. In this experiment, plasma membrane vesicles were incubated with 1 mM [^{14}C]glycine ethyl ester in the absence of EEDQ or in the presence of EEDQ with or without the addition of MgATP plus vanadate, and the labeled membranes were then subjected to SDS–PAGE and analyzed by Coomassie blue staining and fluorography. Panel A shows the Coomassie blue stained gel. It can be seen that with the notable exception of the appearance of a high molecular weight band, which will be dealt with further below, EEDQ treatment in the presence or absence of MgATP plus vanadate has little effect on the membrane protein profile. The ATPase activity in the membranes used for lane 2 was 75% EEDQ inhibited, and the ATPase activity in the membranes used for lane 3 was 90% that of the control (not shown). It should be mentioned that 1 mM glycine ethyl ester has no effect on the kinetics of EEDQ inhibition. Panel B shows the [^{14}C]glycine ethyl ester labeling profile. There is no labeling of any protein in the absence of EEDQ, whereas in the presence of EEDQ many membrane proteins, including H^+-ATPase (arrow) and the EEDQ-induced high molecular weight band, become labeled. Interestingly, the amount of label present in both the ATPase and the high molecular weight band is reduced in the presence of MgATP plus vanadate, which is not the case for any other membrane protein.

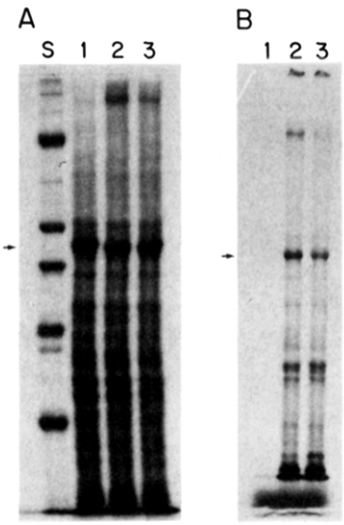

FIGURE 3: SDS–PAGE analysis of EEDQ-dependent [^{14}C]glycine ethyl ester labeling of the *Neurospora* plasma membranes. Three aliquots of a plasma membrane vesicles suspension (0.5 mg of protein each) in a reaction volume of 0.25 mL containing 40 mM Pipes (pH 6.2 with Tris) were preincubated at 18 °C for 10 min in the presence of the following additions: samples 1 and 2, 8 mM Na_2SO_4, 6 mM choline chloride, and 0.1 mM sodium vanadate; sample 3, 6 mM $MgSO_4$, 6 mM ATP, and 0.1 mM sodium vanadate. All samples contained 1 mM glycine ethyl ester and 10 μCi of [^{14}C]glycine ethyl ester. After the preincubation, EEDQ was added to a final concentration of 400 μM to samples 2 and 3. The reaction mixtures were then incubated for 40 min. The reactions were then stopped, and the membranes in each sample were washed 3 times and resuspended in buffer as described in the EEDQ treatment section of Experimental Procedures. The membranes were then assayed for ATPase activity and protein content, and subjected to SDS–PAGE as described under Experimental Procedures. The resulting gel was then analyzed by Coomassie blue staining and fluorography as described (Bonner & Laskey, 1974). Exposure time was 4 days with an intensifying screen at −70 °C. Panel A shows the Coomassie blue stained gel. (Lane S) Molecular weight standards myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin with M_r 200 000, 116 116, 97 114, 66 296, and 42 807, respectively; (lane 1) sample 1, 60 μg of protein; (lane 2) sample 2, 60 μg of protein; (lane 3) sample 3, 60 μg of protein. Panel B shows the results of the fluorography. (Lane 1) Sample 1; (lane 2) sample 2; (lane 3) sample 3. The arrows indicate the 105 000-dalton band of the H^+-ATPase.

In order to gain further insight as the products of EEDQ-dependent ATPase labeling by [^{14}C]glycine ethyl ester in the presence and absence of MgATP plus vanadate, the ATPase regions of SDS–PAGE gels of membranes labeled as described in the legend of Figure 3 for samples 2 and 3 were excised and subjected to *Staphylococcus aureus* V8 peptide mapping according to Cleveland et al. (1977), and the distribution of the label was subsequently analyzed by fluorography. Figure 4 shows the results of this experiment. It can be seen that virtually all of the ATPase fragments labeled in the absence

[2] R. Addison, unpublished observation.

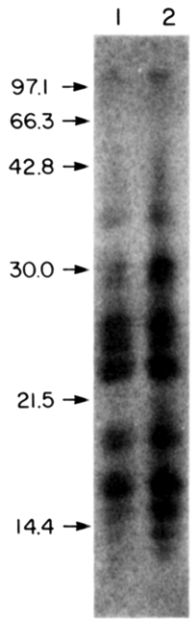

FIGURE 4: Peptide maps of [^{14}C]glycine ethyl ester labeled H^+-ATPase. Two aliquots of a plasma membrane vesicles suspension (0.5 mg of protein each) in a reaction volume of 0.25 mL containing 40 mM Pipes (pH 6.2 with Tris) were treated with 400 μM EEDQ for 40 min at 18 °C in the presence of 1 mM glycine ethyl ester and 10 μCi of [^{14}C]glycine ethyl ester and the following additions: (sample 1) 8 mM Na_2SO_4, 6 mM choline chloride, and 0.1 mM sodium vanadate; (sample 2) 6 mM $MgSO_4$, 6 mM ATP (pH 6.2 with Tris), and 0.1 mM sodium vanadate. The mixtures were preincubated at 18 °C for 10 min before addition of EEDQ. After the EEDQ treatment, the reactions were stopped, and the vesicles were washed, assayed, and subjected to SDS–PAGE as described in the legend for Figure 3. Inhibition and protection of the ATPase activity were similar to those in the experiment of Figure 3. Several wells of the gel contained FITC-labeled plasma membranes prepared as described under Experimental Procedures. After electrophoresis, the gel was removed from the plates and viewed under ultraviolet light. The position of the FITC-labeled 105 000-dalton band in neighboring wells was used to locate the 105 000-dalton regions of samples 1 and 2. The 105 000-dalton regions were then excised and treated with *Staphylococcus aureus* V8 protease (0.5 μg/gel slice) and subjected to SDS–PAGE as described (Cleveland et al., 1977). Two gel slices containing the 105 000-dalton region were added per well. The resulting gel was then subjected to fluorography (Bonner & Laskey, 1974) for 3 weeks with an intensifying screen at −70 °C. (Lane 1) Peptide map of the 105 000-dalton band from sample 2; (lane 2) peptide map of the 105 000-dalton band from sample 1. Arrows point to the position of migration of six polypeptide standards. The molecular mass of the standards (in kilodaltons) is indicated beside the corresponding arrows. The standard polypeptides used, from top to bottom, were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

of MgATP plus vanadate are also labeled in the presence of MgATP plus vanadate, each at a somewhat reduced intensity. A similar experiment utilizing cyanogen bromide as the fragmenting agent yielded similar results (not shown).

An EEDQ-Dependent Intermolecular Membrane Protein Cross-Linking Reaction. As mentioned above, another effect of EEDQ treatment of the isolated *Neurospora* plasma membranes, also apparent in Figure 3, is the appearance of an additional high molecular weight band with an apparent molecular weight of about 260 000, indicating that EEDQ mediates a specific intermolecular membrane protein cross-linking reaction. To gain additional insight into the possible participants in the cross-linking reaction, the experiment described in Figure 5 was carried out. In this experiment, plasma membranes were not treated or treated with trypsin in the presence of MgATP plus vanadate, which produces a truncated (ca. 95 000-dalton) form of the H^+-ATPase (Addison & Scarborough, 1982), and the resulting membranes were then incubated in the presence of EEDQ and analyzed by SDS–PAGE. Lanes 2 and 3 show that the H^+-ATPase, but little else, undergoes a reduction in molecular weight as a result of the trypsin treatment, and lanes 4 and 5 show that the cross-linking product undergoes an analogous reduction in molecular weight to a form with a molecular weight of about 250 000.

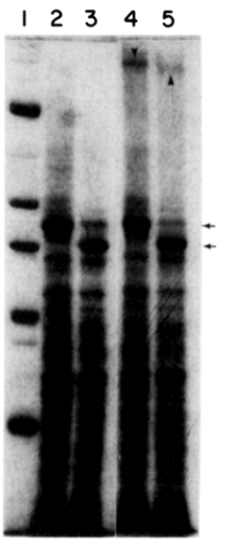

FIGURE 5: SDS–PAGE analysis of the results of EEDQ treatment of membranes containing the 105 000-dalton and 95 000-dalton forms of the H^+-ATPase. Plasma membrane vesicles were untreated or treated with trypsin for 2 min in the presence of MgATP and vanadate as described (Addison & Scarborough, 1982) to generate the 95 000-dalton form of the H^+-ATPase. Control and trypsin-treated vesicles were then incubated at 18 °C for 50 min in the presence or absence of 600 μM EEDQ, washed, and subjected to SDS–PAGE as described under Experimental Procedures. The resulting gel was then stained with Coomassie blue as described (Addison & Scarborough, 1981). (Lane 1) Molecular weight standards as in Figure 3; (lane 2) control vesicles; (lane 3) trypsin-treated, MgATP/vanadate-protected vesicles; (lane 4) control vesicles treated with EEDQ; (lane 5) trypsin-treated, MgATP/vanadate-protected vesicles treated with EEDQ. In all cases, 60 μg of vesicle protein was applied per well. The lower pair of arrows point to the 105 000- and 95 000-dalton forms of H^+-ATPase. The downward pointing arrow in lane 4 indicates the high molecular weight band induced by EEDQ in control membranes, and the upward pointing arrow in lane 5 indicates the high molecular weight band formed upon EEDQ treatment of trypsin-treated, MgATP/vanadate-protected membranes.

Discussion

The initial inhibition data obtained in these experiments showed that the *Neurospora* plasma membrane H^+-ATPase is irreversibly inhibited by EEDQ in a process that displays pseudo-first-order kinetics over a range of EEDQ concentrations. As explained by Levy et al. (1963) and by Keech and Farrant (1968), when such data are plotted as the logarithm of the reciprocal of the inhibition half-time at each concentration of inhibitor vs. the logarithm of the inhibitor concentration, the slope of the resulting line indicates the order of the reaction with respect to inhibitor concentration or the apparent number of molecules of inhibitor reacting with the enzyme to produce the inhibition. The slope of 1 of the plot shown in Figure 1 thus suggests that EEDQ inhibition of the H^+-ATPase involves the reaction of one molecule of EEDQ with one molecule of the H^+-ATPase.

The molecular nature of the inhibition reaction is of considerable interest, as it is pertinent to the catalytic mechanism of the H^+-ATPase. EEDQ is a known coupling agent for the chemical synthesis of peptide bonds (Belleau & Malek, 1968;

Dugas & Penney, 1981). It acts by forming highly reactive mixed carbonic anhydrides with carboxyl groups, which in turn react with an amino function resulting in the formation of a peptide bond (Dugas & Penney, 1981). It is also a known inhibitor of certain enzymes and receptors, and the rather specific carboxyl group reactivity and minimal side reactions of EEDQ have prompted several investigators to implicate essential carboxyl groups in the mechanism of enzymes and receptors that it inhibits (Pougeois et al., 1978; Saccomani et al., 1981; Belleau et al., 1969). Furthermore, because the mixed carbonic anhydrides formed from the reaction of EEDQ with carboxyl groups are quite labile, but the receptor and enzyme inhibitions induced by EEDQ are usually essentially irreversible, it has also been generally concluded that EEDQ induces derivatization of the essential carboxyl group, via the mixed anhydride intermediate, by some nucleophile in the vicinity in a manner much like that seen in peptide bond synthesis (Pougeois et al., 1978; Saccomani et al., 1981; Belleau et al., 1969). If the inhibition takes place when no exogenous nucleophile other than water is present, the derivatization presumably involves an endogenous adjacent nucleophile with the resultant production of an intramolecular cross-link. On the basis of this information, it is most likely that the single-site irreversible inhibition of the *Neurospora* plasma membrane H^+-ATPase by EEDQ, which occurs without the addition of exogenous nucleophile and is unaffected by the presence of the exogenous nucleophile glycine ethyl ester at 1 mM, involves the production of an intramolecular cross-link between an ATPase carboxyl group and an adjacent nucleophile in the polypeptide chain.

The pH vs. EEDQ inhibition profile shown in Figure 2 raises an important issue regarding the chemistry of the EEDQ inhibition reaction and the molecular milieu of the inhibitory EEDQ-reactive carboxyl group. On the basis of similar profiles obtained for the mitochondrial F_1F_0 H^+-ATPase/ATP synthase and the gastric mucosal H^+/K^+-ATPase, Pougeois et al. (1978) and Saccomani et al. (1981) concluded that the pK_a of the inhibitory EEDQ-reactive carboxyl group in each of these enzymes is abnormally high, near pH 7, possibly by virtue of its location in a hydrophobic environment. This is a provocative suggestion, particularly in view of the role of these enzymes as transmembrane proton pumps. However, it is based upon the assumption that the EEDQ-reactive carboxyl group must be protonated for reaction, which, in our view, is an assumption of questionable validity. There appears to be no documented reason for assuming that the reactive carboxyl group must be protonated in order for the mixed anhydride formation to occur, and in fact, the reaction pathway outlined by Dugas and Penney (1981) strongly suggests that the carboxylate anion is the reactive species. For this reason, we consider it unlikely that the acid augmentation of the EEDQ inhibition reaction is indicative of a hydrophobic environment of the inhibitory EEDQ-reactive carboxyl group for any of these enzymes. Rather, such augmentation is more likely related to the known acid enhancement of the ring ethoxy group removal with the resultant formation of the quinolinium cation, which is the species that reacts with the carboxylate anion with subsequent production of the mixed carbonic anhydride (Dugas & Penney, 1981).

The identities of the inhibitory EEDQ-reactive carboxyl group and the adjacent nucleophile are obviously central to the molecular nature of the EEDQ inhibition reaction, and the ligand protection experiments provide some important information in this regard. We have previously shown that the ATPase in isolated plasma membranes is extremely sensitive to degradation by trypsin and that the pattern of tryptic degradation is markedly influenced by the presence of specific ATPase ligands (Addison & Scarborough, 1982). It is inappropriate to recount the argument here, but the final conclusions of these experiments were that, when saturated with MgADP, the ATPase is locked in its substrate binding conformation and, when saturated with vanadate, which requires Mg^{2+} and is enhanced by ATP, it is locked in a conformation resembling the transition state of the phosphoryl-transfer reaction. And, the most obvious predictable difference between the state of the enzyme with MgADP bound and a transition-state analogue bound is that in the former the phosphorylatable aspartate β-carboxyl group and any other enzyme residues that normally interact with the transferred phosphoryl group in the transition state are more or less free and in the latter they are bonded. With this information, the ligand protection results take on a particularly straightforward interpretation. The phosphorylatable aspartate carboxyl group or a carboxyl group near it in the active site is the inhibitory EEDQ-reactive carboxyl group, and in the presence of MgATP plus vanadate, the ATPase is "locked" in the transition-state configuration with the active site carboxyl group and/or the adjacent nucleophile bonded to or at least occluded by vanadate and/or Mg^{2+}, which precludes the EEDQ-induced cross-linking reaction, affording strong protection. Mg^{2+} plus vanadate alone afford similar protection when the ligands are bound, but the ATPase is unliganded a greater percentage of the time, which allows a somewhat more rapid rate of EEDQ inhibition. Analogous results were obtained in the tryptic cleavage experiments (Addison & Scarborough, 1982). And finally, when the enzyme is unliganded or even when it is saturated with MgADP, the inhibitory EEDQ-reactive carboxyl group and adjacent nucleophile are free, and inhibition proceeds at its maximum rate under both conditions, even though the enzyme is in significantly different conformational states. Alternative possibilites such as occlusion of EEDQ access to an essential carboxyl group other than an active site carboxyl group or occlusion of the access of the adjacent nucleophile to an activated non active site essential carboxyl group in the transition-state conformation but not in the substrate binding conformation are also conceivable and potentially of equal future interest. However, the marked difference in the effects of MgADP and MgATP plus vanadate on EEDQ inhibition is most simply explained if one or both of the residues that are cross-linked by EEDQ are involved in transition-state binding of the transferred phosphoryl group or are at least near the residues that are so involved. From the gene sequence we now know that a lysine residue is positioned next to the phosphorylatable aspartate in the amino acid sequence of the ATPase,[2] and we consider this Asp–Lys pair to be the prime candidate for the site of the EEDQ inhibition reaction.

It has been suggested (Saccomani et al., 1981) that the inclusion of a radioactive exogenous nucleophile such as [^{14}C]glycine ethyl ester in the medium during EEDQ treatment might give rise to labeling of the carboxyl group involved in the inhibition reaction, and it was with this suggestion in mind that the [^{14}C]glycine ethyl ester labeling experiments of the type shown in Figure 3 were initiated. The initial results of such experiments were encouraging in that EEDQ-dependent labeling of the H^+-ATPase by [^{14}C]glycine ethyl ester, part of which was decreased in the presence of MgATP plus vanadate, was readily demonstrable (Figure 3). Unfortunately, subsequent experiments such as that shown in Figure 4 strongly suggest that EEDQ-dependent labeling of the H^+-ATPase by

[^{14}C]glycine ethyl ester is not related to the inhibition reaction in any simple way. Proteolytic fragmentation of the H^+-ATPase labeled by [^{14}C]glycine ethyl ester under the influence of EEDQ in the presence or absence of MgATP plus vanadate followed by analysis of the labeled cleavage products by SDS–PAGE and fluorography clearly shows that many different peptides are labeled and that the labeling of none of these is specifically reduced in the presence of MgATP plus vanadate. And, very similar results were obtained when cyanogen bromide was used to fragment the ATPase. Although the interpretation of the protease fragmentation experiment is complicated by the possibility of fragment redundancy, this is much less likely to be the case with the cyanogen bromide fragmentation experiment. These results thus suggest that numerous carboxyl groups are labeled by [^{14}C]glycine ethyl ester in EEDQ-dependent reactions and that the labeling of most or all of these is partially attenuated in the presence of MgATP plus vanadate. We therefore conclude, at least for the *Neurospora* plasma membrane H^+-ATPase, that exogenous nucleophiles are probably not useful as specific probes for the inhibitory EEDQ-reactive carboxyl group, at least not under the conditions tried thus far.

The facts that the formation of the EEDQ-dependent intermolecular membrane protein cross-linking product and the extent of its EEDQ-dependent labeling by [^{14}C]glycine ethyl ester are both attenuated in the presence of the ATPase ligands, MgATP plus vanadate (Figure 3), are consistent with the notion that the ATPase is involved in the EEDQ-dependent cross-linking reaction. The experiment shown in Figure 5 provides strong additional support for this notion. The fact that EEDQ treatment of membranes containing a nicked form of the H^+-ATPase gives rise to the formation of a somewhat smaller form of the crosslinking product is difficult to interpret in any other way, and it is therefore clear that the H^+-ATPase is part of the cross-linking product. However, whether the cross-linking product represents an ATPase homodimer or whether it represents one ATPase monomer cross-linked to some other specific membrane protein, perhaps one involved in the regulation of the H^+-ATPase, is not quite so certain. The apparent molecular mass of about 260 000 daltons is too high for a homodimer, suggesting that the cross-linking product may be a heterodimer. On the other hand, aparent molecular weights in SDS–PAGE gels can be considerably inaccurate, and no obvious ca. 155 000-dalton membrane protein can be seen to disappear at the expense of the formation of the cross-linking product, which could be put forth in favor of an ATPase homodimer as the cross-linking product. Obviously, additional experimentation will be needed in order to resolve this interesting issue. Finally, it should be mentioned that the data presented in Figure 3 demonstrate that the ATPase inhibition reaction and the ATPase cross-linking reaction are not the same. At a time when the H^+-ATPase is inhibited by 75%, the amount of ATPase that has been cross-linked and thus removed from the 105 000-dalton region of the gel is very small, as evidenced by the virtually equal intensities of the ATPase bands in lanes 1 and 2 of Figure 3A.

In summary, the results presented in this paper demonstrate that the carboxyl group activating reagent EEDQ interacts with the *Neurospora* plasma membrane H^+-ATPase in at least three different ways. It inactivates the ATPase, probably by mediating the formation of an intramolecular cross-link between a reactive carboxyl group and an adjacent nucleophile in or near the enzyme active site. EEDQ also mediates the incorporation of exogenous glycine ethyl ester into numerous other sites on the ATPase, presumably via peptide linkage with carboxyl groups, and it also mediates a cross-linking reaction between ATPase monomers or between the H^+-ATPase and some other membrane protein. Future work on the precise molecular nature of these sites of EEDQ interaction could contribute significantly to our understanding of the structure and catalytic mechanism of this important biological pump and possibly its regulation as well.

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