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Evidence for an essential histidine residue in the *Neurospora crassa* plasma membrane H^+-ATPase

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The *Neurospora crassa* plasma membrane H^+-ATPase is rapidly inactivated in the presence of diethyl pyrocarbonate (DEP). The reaction is pseudo-first-order showing time- and concentration-dependent inactivation with a second-order rate constant of 385–420 $M^{-1} \cdot min^{-1}$ at pH 6.9 and 25 °C. The difference spectrum of the native and modified enzyme has a maximum near 240 nm, characteristic of *N*-carbethoxyhistidine. No change in the absorbance of the inhibited ATPase at 278 nm or in the number of modifiable sulfhydryl groups is observed, indicating that the inhibition is not due to tyrosine or cysteine modification, and the inhibition is irreversible, ruling out serine residues. Furthermore, pretreatment of the ATPase with pyridoxal phosphate/$NaBH_4$ under the conditions of the DEP treatment does not inhibit the ATPase and does not alter the DEP inhibition kinetics, indicating that the inactivation by DEP is not due to amino group modification. The pH dependence of the inactivation reaction indicates that the essential residue has a pK_a near 7.5, and the activity lost as a result of H^+-ATPase modification by DEP is partially recovered after hydroxylamine treatment at 4 °C. Taken together, these results strongly indicate that the inactivation of the H^+-ATPase by DEP involves histidine modification. Analyses of the inhibition kinetics and the stoichiometry of modification indicate that among eight histidines modified per enzyme molecule, only one is essential for H^+-ATPase activity. Finally, ADP protects against inactivation by DEP, indicating that the essential residue modified may be located at or near the nucleotide binding site.

Introduction

The plasma membrane of the fungus, *Neurospora crassa*, contains an electrogenic, proton-translocating ATPase, the function of which is to generate a proton-motive force that energizes secondary active transport via a variety of porters [1–3]. Studies in this laboratory and others have suggested that this enzyme is both structurally and functionally related to the Na^+/K^+-, Ca^{2+}-, and H^+/K^+-ATPases of animal cell membranes [4]. In this laboratory, we are interested in understanding the molecular mechanism by which the *Neurospora* H^+-ATPase catalyzes proton translocation at the expense of ATP hydrolysis. As one important approach to this end, we are attempting to obtain information about the location of the active site in the H^+-ATPase molecule and the amino acid residues that are involved in substrate binding and catalysis. Previous studies using group-specific reagents suggested the existence of an essential cysteine residue [5] and an essential arginine residue [6] at or near the nucleotide binding site of the H^+-ATPase. The *Neurospora* H^+-ATPase was also investigated recently [7] using the carboxyl group-activating reagent *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ)$^+$. This reagent inactivates the H^+-ATPase by a mechanism probably involving the activation of a carboxyl group followed by a nucleophilic attack from a neighboring nucleophilic residue. The EEDQ inhibition reaction is prevented by Mg-ATP in the presence of vanadate, but not by Mg-ADP, which was suggested to implicate active site residues involved in transition state binding of the transferred phosphoryl group. In the present study, we have investigated and characterized the modification of histidine residues in

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Abbreviations: EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; DEP, diethyl pyrocarbonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FFI, bovine brain extract (Folch Fraction I); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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the *Neurospora* H^+-ATPase by DEP, which, in aqueous solution at neutral or slightly acidic pH, has been shown to modify histidine residues in proteins with considerable specificity [8]. The results of this study suggest the presence of an essential histidine residue at or near the enzyme active site.

Materials and Methods

Isolation of the H^+-ATPase

The *Neurospora* plasma membrane H^+-ATPase was prepared by the method of Smith and Scarborough [9]. The specific activity of the enzyme was 25 μmol P_i liberated/mg protein per min. SDS-polyacrylamide gel electrophoresis revealed a single Coomassie blue R250-staining band with an M_r of about 105 000. For spectral analyses, the enzyme was passed through a Sepharose CL4B-200 column (2.5 × 90 cm) equilibrated with 50 mM Hepes buffer, pH 6.9 with NaOH, containing 30% (w/v) glycerol (Buffer A).

Reaction of the H^+-ATPase with DEP

DEP solutions were freshly prepared in cold ethanol for each experiment. The concentration of the ethanolic solutions of DEP were determined spectrophotometrically by reaction with 10 mM imidazole-HCl, pH 7.5. An aliquot (5 μl) of the DEP solution was added to 3 ml of the 10 mM imidazole solution at room temperature and the DEP concentration was calculated from the increase in absorbance at 230 nm using an absorption coefficient of $3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [8,10]. Carbethoxylation of the ATPase was carried out by incubating the enzyme with DEP in Buffer A at 25°C. The final concentration of ethanol in the reaction mixtures never exceeded 1% (v/v). The modification reactions were started by the addition of DEP. The extent of modification was determined by measuring the residual ATPase activity in aliquots withdrawn from the reaction mixtures at various time intervals as described [9]. The number of histidines modified was calculated from the absorbance change at 240 nm using an absorption coefficient of 3200 $M^{-1} \cdot cm^{-1}$ [10]. The first-order rate constant of DEP decomposition was obtained by measuring the concentration of reagent in the actual enzyme modification mixtures. Samples were withdrawn at various time intervals, and the amount of DEP remaining was determined by reaction with imidazole as described above. The measured DEP decomposition rate constant under the conditions of the pH 6.9 ATPase treatments was $12.6 \cdot 10^{-2}\ min^{-1}$.

Estimation of modifiable sulfhydryl residues

The determination of modifiable sulfhydryl residues after DEP inactivation of the H^+-ATPase was carried out by the DTNB method of Ellman [11]. The DEP-treated enzyme was first passed through a Sephadex G50 column (0.5 × 10 cm) equilibrated with Buffer A to remove the excess DEP; untreated enzyme was processed similarly. The reactable sulfhydryl groups in the native and DEP-treated enzyme were then determined with DTNB in Buffer A containing 0.2% SDS.

Hydroxylamine treatment of DEP-inactivated H^+-ATPase

The DEP-modified enzyme was mixed with Buffer A containing hydroxylamine at a final concentration of 50 mM, 10 mM ATP, and 0.05% (w/v) FFI, and the resulting mixture was incubated at 4°C for 24–48 h. The pH of the mixture was 7.0. Enzyme without DEP treatment was treated with the same concentration of hydroxylamine under the same conditions and used as a control.

Spectroscopic studies

The UV spectra and the difference spectra of carbethoxylated and untreated enzyme were obtained in a Beckman DU-50 spectrophotometer. Fluorescence measurements were carried out in a Perkin-Elmer Hitachi MPF-2A recording spectrophotofluorometer.

Other methods

Protein was determined by the method of Lowry et al. [12], after precipitation of the protein by the deoxycholate-trichloroacetic acid procedure of Bensadoun and Weinstein [13]. Bovine serum albumin was used as a standard. SDS-PAGE was carried out essentially as described [14].

Materials

Diethyl pyrocarbonate, ATP, ADP, AMP, Hepes, FFI, pyridoxal phosphate, imidazole, and hydroxylamine monohydrochloride were purchased from Sigma. DTNB was obtained from Aldrich. Other chemicals were from sources previously described [4] or of the highest grade available from commercial sources.

Results

Inactivation of the H^+-ATPase by DEP

Incubation of the H^+-ATPase with DEP at pH 6.9 and 25°C results in a time- and concentration-dependent loss of enzyme activity, but semilog plots of the residual enzyme activity vs. time at various concentrations of DEP do not yield straight lines. However, when such data are corrected for the decomposition of DEP during the reactions (see Materials and Methods) by the method of Gomi and Fujioka [15], straight line plots are obtained (Fig. 1). A plot of the pseudo-first-order rate constants for inactivation (k_{obs}) obtained from such plots vs. the DEP concentration is linear (Fig. 2A), indicating, as described by Gomi and Fujioka [15], and Church et al. [16], that a reversible complex between the

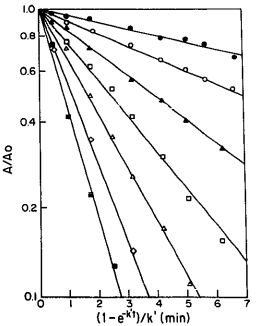

Fig. 1. Inactivation of the H^+-ATPase by DEP. The H^+-ATPase (1.3 μM) was treated with 0.125 (●), 0.25 (○), 0.50 (▲), 0.75 (□), 1.0 (△), 1.5 (◇), and 2.0 (■) mM DEP as described in Materials and Methods. At various time intervals, aliquots were withdrawn and assayed for the residual H^+-ATPase activity, and the residual activities (A/A_0) at the various times (t) were then plotted vs. $(1-e^{-k't})/k'$, where k' is the first-order rate constant of DEP decomposition, to correct for the decomposition of DEP during the reactions as described by Gomi and Fujioka [15].

enzyme and DEP is not formed prior to the inactivation process, and that the DEP inhibition probably proceeds as follows:

$$E + n\ DEP \rightarrow E\text{-}DEP_n$$

where E stands for the H^+-ATPase. The second-order rate constant for H^+-ATPase inactivation by DEP, calculated from the data of Fig. 2A, is 385 $M^{-1} \cdot min^{-1}$. A double-logarithmic plot of the pseudo-first-order rate constants for inactivation vs. DEP concentration [17] has a slope, or apparent reaction order, of 1.1 (Fig. 2B).

Properties of the DEP-derivatized H^+-ATPase

Fig. 3A shows ultraviolet absorption spectra of the native and DEP-inhibited H^+-ATPase, and Fig. 3B shows difference spectra between the native and modified enzyme at several time points during DEP treatment. The difference spectra exhibit maxima near 240 nm and show very little difference around 278 nm.

The number of sulfhydryl groups modifiable by DTNB was also estimated for the untreated ATPase and ATPase inhibited by DEP to 7% residual activity, as described in Materials and Methods. The values obtained for the untreated and DEP-treated ATPase were 2.6 and 2.9 mol/mol, respectively (data not shown).

Fig. 4 shows the effect of the amino group-reactive reagent combination pyridoxal phosphate/$NaBH_4$ on the H^+-ATPase activity and subsequent rate of inactivation of the H^+-ATPase by DEP. The pyridoxal phosphate/$NaBH_4$ treatment was carried out under the conditions of the DEP inhibition reaction to derivatize any amino groups that might be reactive under these conditions. It can be seen that the pyridoxal phosphate/$NaBH_4$ treatment has no effect on the H^+-ATPase activity under these conditions. Moreover, the rate of inactivation of the pyridoxal phosphate treated/$NaBH_4$-reduced enzyme by 1 mM DEP is quite similar to that obtained with the native H^+-ATPase (cf. Figs. 1 and 2). This experiment bears importantly on the identity of the essential residue modified by DEP, and will be elaborated upon below.

Additional experiments indicated that the H^+-ATPase inhibition by DEP is not reversed by dilution, that H^+-ATPase inactivated by DEP to about 5% residual activity has the same intrinsic fluorescence as the native enzyme at 340 nm when excited at 280 nm (data not shown), and that the native and DEP-modified H^+-ATPase also have the same elution volume when subjected to column chromatography on Sepharose CL-4B (data not shown).

Effect of pH on H^+-ATPase inactivation by DEP

The pH-dependence of DEP inactivation of the H^+-ATPase was investigated in 50 mM Hepes/30% glycerol (w/v) buffer between 6 and 8.5. The results of these

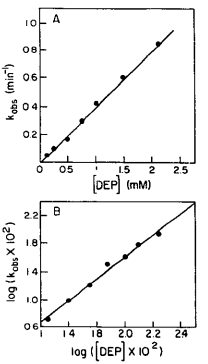

Fig. 2. Pseudo-first-order rate constants for H^+-ATPase inhibition by DEP as a function of the concentration of DEP. (A) Rate constants for DEP inhibition of the H^+-ATPase (k_{obs}) at several DEP concentrations were calculated from the data of Fig. 1 according to Gomi and Fujioka [15], and plotted as a function of the DEP concentration. (B) Log-log plot of the same data.

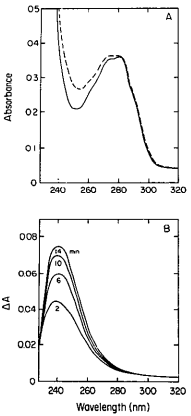

Fig. 3. (A) Ultraviolet spectra of the H^+-ATPase before and after inactivation by DEP. The H^+-ATPase (2 μM) was treated with 1 mM DEP for 14 min as described in Materials and Methods or not treated, and the ultraviolet spectra of the two samples were then measured as described in Materials and Methods. ———, untreated enzyme; — — —, enzyme treated with DEP. (B) Difference spectra of similar samples recorded after 2, 6, 10, and 14 min of DEP treatment.

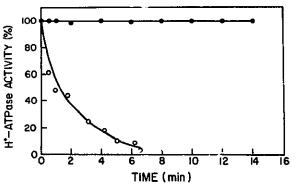

Fig. 4. Effect of pyridoxal phosphate/$NaBH_4$ on H^+-ATPase activity and effect of DEP on H^+-ATPase pretreated with pyridoxal phosphate/$NaBH_4$. ●———●, the H^+-ATPase (1.3 μM) was incubated with pyridoxal phosphate (1 mM) in Buffer A at room temperature in the dark. At various time intervals, 20-μl aliquots were removed, adjusted to 5 mM $NaBH_4$, incubated on ice for 15 min, and assayed for H^+-ATPase activity as described in Materials and Methods. ○———○, the H^+-ATPase (1.3 μM) was incubated with pyridoxal phosphate as described above for 14 min in a final volume of 0.3 ml, after which 5 mM $NaBH_4$ was added and the incubation continued for 15 min on ice. The mixture was then passed over a Sephadex G-50 column (0.5×10 cm) to remove the excess reagents. The pyridoxal phosphate/$NaBH_4$-treated H^+-ATPase was then assayed for DEP inhibition (1 mM) as described in Materials and Methods. In this case, the time points are corrected for the decomposition of DEP according to the expression $t = (1 - e^{-k't})/k'$ with the symbols defined as in the legend of Fig. 1 [15].

experiments are plotted in Fig. 5. In this pH range, the pseudo-first-order rate constants of inactivation vary with increasing pH in a tripartite manner. Between pH 6 and 6.5, there is only a minimal increase in the inhibition rate constant. Above pH 6.5, the inactivation rate increases steadily up to about pH 7.75. Then, between pH 8 and 8.5 there is a pronounced increase in the inhibition rate constants. A plot of the logarithm of the pseudo-first-order rate constants for H^+-ATPase inactivation vs. pH in the range between 6.5 and 8 is shown in the inset of Fig. 5. The log-log plot is almost linear between 6.5 and 7.4, and then levels off abruptly at around pH 7.5, indicating a pKa of about 7.5 for the functional group with which DEP reacts to produce ATPase inhibition at pH values below 8.

Reversibility of DEP-inhibition by hydroxylamine

H^+-ATPase that has been inactivated by DEP is partially reactivated when treated with hydroxylamine. Approximately 40% of the original ATPase activity is regained by treatment of H^+-ATPase inactivated to 25% residual activity with 50 mM hydroxylamine for 48 h as described in Materials and Methods (data not shown). Treatment with a higher concentration of hydroxylamine (0.5 M) caused a considerable loss of ATPase activity in the control experiment making it difficult to explore the reversibility at higher concentrations of hydroxylamine.

Inactivation of the H^+-ATPase by DEP as a function of the number of histidine residues modified

Fig. 6 shows the relationship between DEP inhibition of H^+-ATPase activity and the extent of histidine modification, as judged from difference spectra at 240 nm,

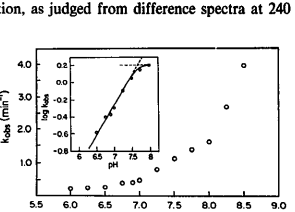

Fig. 5. pH dependence of the pseudo-first-order rate constants for inactivation of the H^+-ATPase by DEP. The H^+-ATPase (1.3 μM) was incubated with 1 mM DEP in 50 mM Hepes buffer, pH 6–8.5 with NaOH, in the presence of 30% (w/v) glycerol at 25°C. The value of the first-order rate constant for DEP decomposition (k') was determined at each pH and the pseudo-first-order rate constants for inactivation (k_{obs}) were obtained from plots similar to those shown in Fig. 1. Inset: Plot of the log of the k_{obs} vs. pH between pH 6.5 and 8.

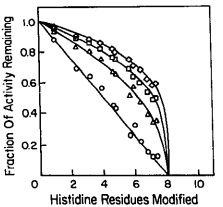

Fig. 6. Relationship between the residual H^+-ATPase activity and the number of histidine residues modified by DEP. The H^+-ATPase (2 μM) in 0.3 ml of Buffer A was modified with 1 mM DEP at 25 °C as described in Materials and Methods and the increase in absorbance at 240 nm was measured. At various time intervals, aliquots (10 μl) of the reaction mixture were withdrawn and assayed for H^+-ATPase activity, and the number of histidine residues modified was calculated on the basis of the absorbance at 240 nm as described in Experimental Procedures. The data are presented in the form of a Tsou plot [18] for $i = 1$ (○), $i = 2$ (△), $i = 3$ (□), and $i = 4$ (◇).

plotted according to the method of Tsou [18]. Clearly, a straight line is seen only when an i value of 1 is used.

Protection against DEP inactivation by H^+-ATPase ligands

The effect of several H^+-ATPase ligands on the ATPase inhibition by DEP is shown in Table I. Treatment of the enzyme in the presence of 5 mM Mg-ADP results in a considerable protection against inactivation. 5 mM ADP without Mg^{2+} is almost as effective. On the other hand, little or no protection is observed in the presence of 15 mM ATP, 15 mM $MgSO_4$-ATP plus 0.1 mM sodium vanadate, 5 mM $MgSO_4$ plus 0.1 mM sodium vanadate, 5 mM $MgSO_4$-AMP, 5 mM AMP, or 5 mM $MgSO_4$ alone. It should be noted that the presence of acidic phospholipids (FFI), which is essential for H^+-ATPase activity, is also essential in order to obtain the protection by ligands against inhibition by DEP.

TABLE I

The effects of several H^+-ATPase ligands on the rate of DEP inhibition

The H^+-ATPase (1.3 μM) was preincubated with the indicated ligands for 5 min in Buffer A containing 0.05% FFI, after which the inhibition reactions were started by the addition of DEP (1 mM). The reaction mixtures were incubated at 25 °C, and 20 μl aliquots were removed at various time intervals and assayed for H^+-ATPase activity. The inhibition rate constants (k_{obs}) were then determined from plots of the data obtained, generated as described in the legend of Fig. 1. Data are the means of at least two separate experiments.

Additions	k_{obs} (min^{-1})
None	0.42
5 mM $MgSO_4$ + 5 mM ADP	0.11
5 mM ADP	0.15
15 mM ATP	0.39
15 mM $MgSO_4$ + 15 mM ATP + 0.1 mM Na_3VO_4	0.34
5 mM $MgSO_4$ + 0.1 mM Na_3VO_4	0.36
5 mM $MgSO_4$ + 5 mM AMP	0.34
5 mM AMP	0.39
5 mM $MgSO_4$	0.38

Discussion

The results shown in Fig. 1 indicate that DEP inhibits the *Neurospora* plasma membrane H^+-ATPase with kinetics that are pseudo-first-order with respect to time, and the results shown in Fig. 2 indicate that the reaction order with respect to DEP concentration is close to unity, suggesting that inhibition of the ATPase by DEP results from modification of a single essential residue. The second order rate constant for inactivation of 385–420 $M^{-1} \cdot min^{-1}$ measured in these studies is in the range found for proteins containing catalytically essential histidine residues [10,19].

DEP reacts in a highly selective manner with histidine residues in a number of proteins, but it also reacts with other amino acid side chains including those of tyrosine, serine, cysteine, and lysine [8,10]. O-Carbethoxylation of tyrosine residues can be detected by a decrease in absorbance at 278 nm. In the studies described in this communication, tyrosine residues were presumably not modified, because no decrease in absorbance at 278 nm was observed in the difference spectra between untreated ATPase and ATPase treated with DEP at pH 6.9 (Fig. 3). The possible modification of serine residues can also be ruled out since modification of serine residues by DEP is readily reversible in neutral aqueous solution [10] but the H^+-ATPase inhibition is not. The possibility of cysteine residue modification by DEP can be ruled out since there is no difference in the number of DTNB-modifiable cysteine residues in the native and DEP-inhibited ATPase molecules. Lysine modification by DEP, which is a distinct possibility [20], is unlikely to be responsible for the H^+-ATPase inhibition when the DEP treatment is carried out at pH 6.9, since pretreatment of the H^+-ATPase with pyridoxal phosphate/$NaBH_4$ at pH 6.9, which should modify any lysine residues reactive at this pH, does not inhibit the H^+-ATPase and has no effect on the rate of subsequent DEP inhibition (Fig. 4). It should be noted that DEP modification of an essential lysine residue in the H^+-ATPase is a strong possibility when the treatment is carried out at pH values above 8, as indicated by the marked increase in the inhibition rate constants in this pH range. However, for the purposes of this communication, the pyridoxal phosphate-$NaBH_4$ experiment of Fig. 4 argues strongly that DEP does not modify an essential lysine residue when the treatment is carried out at pH 6.9. This information, taken together

with the fact that the difference spectra of Fig. 3 exhibit maxima at about 240 nm, characteristic of *N*-carbethoxyhistidine, strongly suggests that the essential residue modified by DEP at pH 6.9 is a histidine. This interpretation is further strongly supported by the apparent pK_a of the inhibition reaction near neutrality, commonly seen with essential histidine residues [19]. The partial reversal of DEP inhibition by hydroxylamine is additional evidence that the inhibitory residue modified is histidine [10,21]. The inability to completely reactivate the DEP-inhibited H^+-ATPase with hydroxylamine may indicate histidine dicarbethoxylation [10]. Finally, the facts that DEP-treatment does not significantly alter the intrinsic fluorescence spectrum of the H^+-ATPase nor its behavior upon Sephadex CI-4B column chromatography indicate that the histidine modification does not induce major conformational rearrangements in the H^+-ATPase molecule.

The difference spectra of Fig. 3B show that H^+-ATPase histidine modification increases as a function of time, and from such spectral data, it can be estimated that approximately 8 histidine residues of the H^+-ATPase are derivatized in a 14 minute incubation. This raises the distinct possibility that the modification of more than one H^+-ATPase histidine residue is involved in the DEP inhibition reaction. However, as shown in Fig. 2B, a plot of the inhibition kinetics data according the method of Levy et al. [17], suggests a minimal inhibition stoichiometry of 1. Moreover, the statistical approach of Tsou [18] strongly supports this notion. Thus, since the eight readily modifiable histidine residues in the H^+-ATPase react with DEP at essentially the same rate (from the timed difference spectra), according to the relationship

$$a^{1/i} = (p - m)/p$$

where a is the residual activity when m residues are modified, p is the total number of residues modified by the inhibitory reagent, and i is the number of residues essential for enzyme activity [18], the number of essential residues is that value of i that gives a straight line when $a^{1/i}$ is plotted against m. As shown in Fig. 6, only when a value of i equal to 1 is used, is a straight line obtained, indicating that H^+-ATPase inactivation by DEP is due to the modification of one critical histidine residue.

The effects of certain H^+-ATPase ligands on inhibition of the H^+-ATPase by DEP (Table I) provide important information as to the site of the critical histidine modification and the H^+-ATPase catalytic mechanism. As has previously been explained [7,22], the combination of H^+-ATPase ligands, Mg^{2+} plus vanadate, and the combination, Mg^{2+} plus vanadate plus ATP, are thought to 'lock' the H^+-ATPase in a conformation resembling the transition state of the enzyme dephosphorylation reaction. The fact that the DEP inhibition reaction is essentially unaffected by the presence of these ligands suggests that the critical histidine residue is equally available in the unliganded ATPase and the ATPase locked in the above mentioned transition state conformation, consistent with the notion that the critical histidine residue is not liganded to the transferred phosphoryl group during the catalytic cycle. On the other hand, the marked effects of Mg-ADP, and particularly ADP alone, strongly suggest that the critical histidine is intimately involved in the H^+-ATPase nucleotide binding site, although longer range, conformational effects cannot be totally excluded. The failure of ATP and AMP to significantly affect the DEP inhibition rate, is probably due to the substantially lower affinity of the H^+-ATPase for these nucleotides [22, and the data not shown].

Finally, the results presented in Table I bear in an additional way on the H^+-ATPase catalytic mechanism. We have previously shown that the H^+-ATPase is fully functional as a monomer [4], and under most conditions, plots of the H^+-ATPase reaction rate vs. the Mg-ATP concentration obey the Michaelis-Menten equation. It is thus quite likely that H^+-ATPase monomers contain a single nucleotide binding site. Although it is generally assumed that the 'true substrate' for this nucleotide binding site of the H^+-ATPase is the nucleotide-divalent cation complex [23], the marked protection against DEP inhibition by ADP alone clearly indicates that ADP is able to bind productively to the H^+-ATPase nucleotide binding site in the absence of any divalent cation. Therefore, whereas it may well be true that the H^+-ATPase most often encounters the nucleotide-divalent cation complex in a typical ATPase reaction mixture, it does not follow that the H^+-ATPase has no affinity for nucleotides in the absence of a divalent cation. Divalent cation-independent nucleotide binding to the active site of the Na^+/K^+-ATPase has also been clearly demonstrated by Moczydlowski and Fortes [24], and similar results have been obtained for the Ca^{2+}-ATPase of the sarcoplasmic reticulum [25], although the results of these latter studies were interpreted somewhat differently than the results of Moczydlowski and Fortes.

In conclusion, in this communication we have provided strong evidence for the existence of an essential histidine residue in or near the nucleotide-binding site of the *Neurospora* plasma membrane H^+-ATPase. The next important and interesting step will be to identify the modified histidine. Now that highly effective methodology for manipulating the protein chemistry of the H^+-ATPase has been worked out [26], it may be possible to identify the relatively unstable [10] carbethoxyhistidine residue by the 'neutral pH' approach of Hegyi et al. [27] or by an indirect approach such as that described by Tenu et al. [28]. Alternatively, if an effective expression system for the H^+-ATPase can be worked

out, it may be possible to identify the critical histidine residue by recombinant DNA methodology.

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