Modification of the Red Beet Plasma Membrane H\textsuperscript{+}-ATPase by Diethylpyrocarbonate\textsuperscript{1,2}

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ABSTRACT

Incubation of the red beet (Beta vulgaris L.) plasma membrane H\textsuperscript{+}-ATPase with micromolar concentrations of diethylpyrocarbonate (DEPC) resulted in inhibition of both ATP hydrolytic and proton pumping activity. Enzyme activity was restored when DEPC-modified protein was incubated with hydroxylamine, suggesting specific modification of histidine residues. Kinetic analyses of DEPC inhibition performed on both membrane-bound and solubilized enzyme preparations suggested the presence of at least one essential histidine moiety per active site. Inclusion of either ATP (substrate) or ADP (product and competitive inhibitor) in the modification medium reduced the amount of inhibition observed in the presence of DEPC. However, protection was not entirely effective in returning activity to noninhibited control values. These results suggest that the modified histidine does not reside directly in the ATP binding region of the enzyme, but is more likely involved in enzyme regulation through subtle conformational effects.

The H\textsuperscript{+}-ATPase associated with the plant plasma membrane transports H\textsuperscript{+} from the cytoplasm to the cell exterior, producing an inwardly directed proton electrochemical gradient (\(\Delta \mu \text{H}^{+}\)). Secondary solute transport occurs in response to this gradient which consists of a negative-interior electrical potential (\(\Delta \Psi \)) and an acid-exterior proton gradient (\(\Delta p\text{H} \)). In this fashion, the H\textsuperscript{+}-ATPase is proposed to have a vital role in providing the driving force for solute transport and to be involved in cellular processes including regulation of cytoplasmic pH; nutrient status and turgor driven responses such as stomatal movements and IAA-mediated growth responses (21).

The plant plasma membrane H\textsuperscript{+}-ATPase is representative of the E\textsubscript{1}E\textsubscript{2} class of transport enzyme (also called P-type). During ATP hydrolysis this enzyme forms a phosphorylated intermediate at an active site aspartyl residue and analysis of the phosphorylated protein by polyacrylamide gel electrophoresis indicates the presence of a 100 kilodalton catalytic subunit (3). Recently, the amino acid sequence of this protein has been deduced from cDNA clones produced from Arabidopsis thaliana (16) and Nicotiana plumbaginifolia (2). Hydropathy analysis of the deduced sequence (16) suggested eight probable transmembrane segments separated by three hydrophilic domains that extend into the cytoplasm. Within a large cytoplasmic domain are the aspartyl residue, which becomes phosphorylated, and a lysine, which in other E\textsubscript{1}E\textsubscript{2}-type ATPases binds the fluorescent probe fluorescein-5'-isothiocyanate. This latter residue is proposed to play an important role in binding the adenine portion of the ATP substrate to the enzyme (7). Chemical modification studies have indicated the presence of an essential arginine (13, 19) that may be involved in stabilizing binding of the anionic phosphate portion of the ATP substrate (31).

Results from a previous kinetic study on the plasma membrane H\textsuperscript{+}-ATPase from red beet suggested that histidine residues might also play a role in the catalytic cycle of the enzyme (6). When the rate of phosphoenzyme formation was determined as a function of assay pH, the generated curve resembled a pH titration curve for histidine, with an inflection point at approximately pH 6.5. This could be explained by the imidazole side chain of a histidine moiety having some role in the process of transferring the \(\gamma\)-phosphate from ATP to the aspartyl residue of the enzyme. Alternatively, this result could occur as the net effect of a number of residues acting in concert. In this study, the technique of specifically modifying histidine residues with the chemical reagent DEPC\textsuperscript{4} was used in order to determine if histidine moieties have an essential role within the mechanism of the red beet plasma membrane H\textsuperscript{+}-ATPase. Both membrane-bound and solubilized ATPase fractions were used in order to determine if there was a difference in accessibility or sensitivity of histidine residues to DEPC with these two enzyme preparations.

MATERIALS AND METHODS

Plant Material

Fresh red beets (Beta vulgaris L., cv Detroit Dark Red) were purchased commercially, detopped, and then stored in dark, moist conditions at 0 to 4°C for at least 10 d prior to use to ensure dormancy and tissue uniformity as previously described (11).

Isolation of Plasma Membrane Fractions and Detergent Solubilization of the H\textsuperscript{+}-ATPase

Plasma membrane fractions were obtained following the procedure of Giannini et al. (11) except that the homogeni-
zation buffer contained 0.2 mM PMSF. Plasma membrane H\textsuperscript{+}-ATPase was solubilized using the procedure of Briskin and Poole (4). This procedure involves extraction of the plasma membrane fraction with 0.1% (w/v) sodium deoxycholate to remove proteins other than the plasma membrane H\textsuperscript{+}-ATPase and then solubilization of the enzyme using 0.1% (w/v) Zwittergent 3-14. Both detergent treatments were carried out at a detergent to protein ratio of 1:1 (mg/mg).

**Modification of H\textsuperscript{+}-ATPase with DEPC**

Treatment of red beet plasma membrane H\textsuperscript{+}-ATPase preparations with DEPC was carried out for 20 min at 37°C in a reaction solution containing 30 mM BTP/Mes (pH 6.5), 50 mM KCl, 3 mM MgSO\textsubscript{4}, and various concentrations of DEPC (freshly prepared in absolute ethanol). Ethanol concentrations never exceeded 3% (v/v) in the incubation media and 0.3% (v/v) in the ATPase assay. At this level, no effect upon ATPase activity was observed. At specified times, aliquots were withdrawn and diluted into an ice-cold ATPase assay solution containing 30 mM imidazole to block unreacted DEPC. For time course studies, 10 mM L-histidine was also included to further inactivate any DEPC carried over from the incubation medium. Any deviations from this procedure are indicated in "Results and Discussion."

**Measurement of ATPase Activity**

ATPase activity was measured as described previously (11), in a reaction solution containing 3 mM ATP, 3 mM MgSO\textsubscript{4}, 50 mM KCl, and 30 mM imidazole/HCl (pH 6.5).

**Optical Measurement of Vesicle pH Gradient**

Acid-interior pH gradients were measured by the decrease of acridine orange absorbance at 490 nm as described by Giannini et al. (12). The standard assay (1 mL volume) contained various concentrations of freshly prepared DEPC in ethanol (ethanol concentration never exceeded 2%), 250 mM sorbitol, 25 mM BTP/Mes (pH 6.5), 100 mM KNO\textsubscript{3}, 3.75 mM MgSO\textsubscript{4}, 3.75 mM BTP/ATP, and 10 μM acridine orange. The reaction was started by the addition of ATP to vesicles equilibrated with dye and followed by absorbance decrease over time (at room temperature) using a Beckman DU-40 spectrophotometer fitted with a kinetics program.

**Protein Measurement**

Protein concentrations were determined as previously described (11) by the method of Bradford using BSA as a standard.

**RESULTS AND DISCUSSION**

DEPC was used to determine whether histidine plays an essential role in the mechanism of the plasma membrane H\textsuperscript{+}-ATPase. This reagent generally modifies the imidazole side chain of histidine at near neutral pH conditions (9, 24, 25). The reaction is covalent and irreversible under appropriate conditions. However, when subsequently treated with relatively high concentrations of hydroxylamine, this reagent can be discharged from the protein by deacylation of the DEPC-histidine moiety (24, 25). Determination of essential histidines with this reagent has been accomplished for a number of proteins isolated from bacterial and animal tissues, including actins (29), tyrosine phenyl lyase (20), lysyl oxidase (10), and an organic anion exchange protein isolated from renal membrane vesicles (32). Recently, DEPC was used to demonstrate the role of an essential histidine in the plasma membrane H\textsuperscript{+}-ATPase of the fungus Neurospora crassa (28). Our initial effort was directed towards optimizing the conditions for reaction of this reagent with the H\textsuperscript{+}-ATPase associated with the red beet plasma membrane.

**Optimization of Conditions for Modification of the H\textsuperscript{+}-ATPase by DEPC**

Since DEPC has not been used previously in studies involving the H\textsuperscript{+}-ATPase associated with the higher plant plasma membrane, the reaction between the enzyme and DEPC was examined under various conditions. Miles (27) reported that Tris buffer accelerated the decomposition of DEPC and it is also possible for Tris to react with this reagent as a nucleophile (1). In either case, Tris buffer could potentially reduce the effective DEPC concentration during incubation with the enzyme preparation. Likewise, boric acid buffer is known to stabilize the reaction between butanedione and arginine residues (13). However, when red beet plasma membrane H\textsuperscript{+}-ATPase was treated with DEPC in any of the three buffer systems tested, very little difference was observed for enzyme inhibition (Table I). Although a slight increase in inhibition was observed in the presence of BTP/Mes buffer, this was the result of increased activity of controls incubated without DEPC. BTP/Mes buffer was used for DEPC-enzyme incubation in order to maximize relative activities and eliminate any possibility of a Tris-DEPC reaction. Magnesium and potassium were included in the incubation medium since they are necessary for optimal ATPase functioning and did not decrease inhibition of the enzyme by DEPC.

| Table I. Effects of ATPase Reaction Ligands and Various Buffers at pH 6.5 on Inhibition of Plasma Membrane H\textsuperscript{+}-ATPase by 5 mM DEPC |
|---------------------------------------------|-----------------|-----------------|-----------------|
| Incubation took place at 0°C for 20 min in the indicated buffer systems. |                   |                  |                  |
| Additions       | ATPase          |                |                |
|                 | Tris/Mes        | BTP/Mes        | Boric acid/Mes  |
|                 | μmol Pi mg\textsuperscript{-1} h\textsuperscript{-1} |                  |                  |
| DEPC, +Mg, +KCl | 24.4           | 27.8           | 25.7           |
| +DEPC, –Mg, –KCl| 5.8 (24)\textsuperscript{a} | 4.7 (17)      | 6.8 (27)       |
| +DEPC, –Mg, –KCl| 4.6 (19)        | 4.3 (16)       | 4.6 (18)       |
| +DEPC, –Mg, +KCl| 5.0 (21)        | 3.6 (13)       | 5.2 (20)       |
| +DEPC, +Mg, +KCl| 4.8 (20)        | –              | 4.9 (19)       |

\textsuperscript{a} When added, ligand concentrations were 3 mM MgSO\textsubscript{4} and 50 mM KCl. \textsuperscript{b} Numbers in parentheses represent percent control incubated with 3 mM MgSO\textsubscript{4} and 50 mM KCl in the absence of DEPC.
have varied from 0°C (20, 29) to room temperature (22–25°C) (10, 32). Berger (1) reported that temperatures exceeding 60°C caused DEPC to decompose while low temperature greatly enhanced the stability of this reagent. When both membrane-bound (Fig. 1A) and detergent-solubilized (Fig. 1B) ATPase preparations were incubated with DEPC at temperatures ranging from 0°C to 37°C, inhibition relative to a control sample (incubated in the absence of inhibitor) increased as the temperature was increased. As incubation with DEPC at 37°C resulted in a maximal degree of inhibition for both the plasma membrane and solubilized preparations, this reaction temperature was utilized in further experiments (except where noted).

The effect of pH on DEPC reaction with plasma membrane and solubilized ATPase preparations is shown in Figure 2. Inhibition by 2.0 mM DEPC for membrane-bound (Fig. 2A) and 1.0 mM for solubilized (Fig. 2B) ATPase was nearly complete at all pH values tested following a 20 min incubation period at 37°C. Enzyme activity in the absence of DEPC was inhibited when enzyme was preincubated at pH values below 6.5. To ensure specificity of DEPC for histidine moieties and maintain high activity in unmodified control samples, all further incubations were performed at pH 6.5. This would also serve to stabilize the N-carbethoxy-histidine residue which is reported to be most stable at near neutral pH values (26, 27). The effect of pH on the rate of inhibition by DEPC was also investigated using a solubilized enzyme preparation and a lower DEPC concentration. Under these conditions, measurement of the time course of inhibition could be made and the rate of inhibition exhibited a pseudo-first order kinetic relationship. As observed in Figure 3, when the enzyme was incubated with 800 μM DEPC at pH values ranging from 5.5 to 9.0, a series of straight lines were observed when log percent residual activity was plotted as a function of time. When apparent first order rate constants were calculated and then plotted as a function of pH, a tripartite pattern of rate change was observed (Fig. 3B). Between pH 5.5 and 6.5 the rate of inhibition was relatively unchanged while a linear increase in rate was observed between pH 7.0 and 8. At pH values above 8.0 a dramatic increase in inhibition rate was observed with the increase in pH. This is similar to what was observed by Morjana and Scarborough (28) in their investigation on the role of histidine in the N. crassa plasma membrane H+-ATPase. This effect could be due to complex factors, reflecting
Inhibition of ATPase activity by DEPC treatment.

- **Figure 3.** Effect of pH on the rate of ATPase inactivation by 800 μM DEPC for the solubilized enzyme preparation. Treatment of the solubilized plasma membrane H⁺-ATPase with 800 μM DEPC was conducted over time at 37°C and at the indicated pH. Values for ATP hydrolysis at zero time were based upon a -DEPC control sample assayed immediately. Rates calculated from regression analyses of inhibition data observed over 8 min (A) were then plotted as a function of pH (B).

- **Kinetics of DEPC Inhibition of Plasma Membrane ATPase Activity**

  When membrane-bound red beet plasma membrane H⁺-ATPase preparations were incubated for 20 min with DEPC at various concentrations in the range from 0 to 20 mM, it was evident that this reagent was a potent inhibitor of phosphohydrolase activity, even when incubation took place at 0°C (Fig. 4). At reagent concentrations exceeding 2 mM, a sharp decrease in the activity of DEPC-modified enzyme was observed, although inhibitor concentrations as low as 600 μM were effective in inhibiting enzyme activity to less than half of the control value. With higher concentrations of DEPC (i.e., 5, 10, and 20 mM), the enzyme was inhibited by 90 to 98% and no significant increase in the degree of inhibition was shown as the DEPC concentration exceeded 5 mM. DEPC concentrations in the low millimolar range have been reported for inactivation of enzymes such as the organic anion exchanger isolated from renal tissue (32) and lysyl oxidase catalase from calf aorta (10), but concentrations below 1 mM have been effective when used with *Escherichia coli* phenol lyase (20) and pig dopa decarboxylase (8). The plant plasma membrane H⁺-ATPase appears to fall into this latter category since it is effectively inhibited by low DEPC concentrations. As was observed in Figure 1, the inhibition percentage was increased when incubation with DEPC took place at 37°C, where activity in the presence of 2.5 mM DEPC was inhibited 97 to 99% in both the membrane-bound and solubilized preparations.

  When 0 to 800 μM DEPC was used to modify the red beet plasma membrane H⁺-ATPase, a pseudo-first-order kinetic relationship for inhibition was observed for both the membrane-bound (Fig. 5A) and solubilized (Fig. 5B) preparations of enzyme. Incubation of the enzyme at 37°C for up to 8 min yielded a series of straight lines when data were plotted as log residual activity (percent control) versus time. Use of irreversible modifiers (such as DEPC under slightly acid conditions) and modifier concentrations in excess of the enzyme concentration allows for calculation of the reaction order number, n, according to the equation of Levy et al. (22):

  \[
  \log k_{\text{obs}} = \log k + n \log [\text{inhibitor}]
  \]

  - **Figure 4.** Effect of DEPC concentration on inhibition of membrane-bound ATPase. Treatment with the indicated concentration of DEPC was conducted for 20 min, at 0°C in the presence of 30 mM BTP/Mes (pH 6.5), 50 mM KCl, and 3 mM MgSO₄. Aliquots were withdrawn and then assayed for ATPase activity. The data are presented as a percent of the control activity for samples treated identically except in the absence of DEPC.
where $k$ equals the true inhibition rate constant and $k_{o bs}$ equals the apparent first-order rate constant estimated from slope values (multiplied by 2.3) of inhibition time course plots such as those shown in Figure 5. Plotting the log of these apparent first-order rate constants as a function of log inhibitor concentrations yields a straight line with a slope value equal to the reaction order number, $n$. According to Levy et al. (22), the reaction order number calculated through use of this equation estimates the number of molecules of inhibitor bound per molecule of enzyme. As shown in Figure 6, both enzyme preparations had $n$ values close to one when analyzed in this fashion. This suggests that at least one histidine residue actively participates in the ATPase reaction. However, care must be taken with interpretation of these data, as it is possible that more than one essential histidine, with similar reaction characteristics, might be taking part in the enzyme catalytic cycle (33). Therefore, it is not possible to conclude that only one histidine is essential for the ATPase reaction, but rather that one or more histidine residues might be essential for catalytic activity.

**Effect of DEPC on the Proton Pumping Activity of the Plasma Membrane ATPase**

The effect of increasing concentrations of DEPC on the proton pumping activity associated with the red beet plasma membrane H+-ATPase in sealed plasma membrane vesicles was tested. For these initial assays, the enzyme was not preincubated with DEPC and DEPC was simply included in the transport reaction mixture. As shown in Figure 7, proton pumping activity was progressively inhibited in the presence of 0 to 400 μM DEPC. At DEPC concentrations above 400 μM inhibition was relatively constant, generally around 60%. However, when plasma membrane vesicles were preincubated (10 min at 22°C with 400 or 800 μM DEPC), inhibition of proton transport was increased to 89% and 99%, respectively. As preincubation with DEPC represented reaction conditions closer to those used for examining effects upon ATP hydrolytic activity (i.e. Fig. 4), this would suggest a greater sensitivity of proton pumping activity to DEPC.

As the measured proton pumping activity associated with the red beet plasma membrane vesicles can be a reflection of both the rate of H+ influx driven by the ATPase and the rate of passive H+ efflux from the vesicles ("leakage"), effects of...
Consistent with the involvement of histidyl moieties in enzyme activity following hydroxylamine treatment, it is generally considered to be specific for derivatization of histidine and is used to confirm histidine essentiality (24, 25).

When red beet plasma membrane ATPase preparations previously inhibited by DEPC were subsequently incubated with increasing amounts of hydroxylamine for 1 h at room temperature, a corresponding increase in the percentage of enzyme activity relative to controls occurred (Table II). When enzyme modified with 800 µM DEPC was treated with 0.9 mM hydroxylamine, inhibition was reduced from 72% to 20%, consistent with the involvement of histidyl moieties in enzyme activity. Although hydroxylamine treatment did result in an elevation of control activities (no DEPC treatment), inhibition data are expressed on a percent inhibition basis relative to such controls. This effect may be due to stabilization of the enzyme during incubation since inclusion of 0 to 6 mM hydroxylamine (maximum possible after repeated washing) did not change absorbance readings of control tubes (not shown). While it is possible that complete restoration of enzyme activity might be accomplished with a longer exposure to NH₄OH, this could not be tested as the ATPase loses activity over prolonged incubation periods at room temperature.

Protection against DEPC Inhibition by Nucleoside Phosphates

If the modified histidine residue is present in the active site of the plasma membrane H⁺-ATPase, incubation with DEPC in the presence of saturating amounts of substrate ATP, or the initial reaction product (and competitive inhibitor) ADP,

Reversal of DEPC Modification by Hydroxylamine

Although DEPC has been shown to be a relatively specific modifier of histidine residues, some investigations have indicated possible reaction with other residues under certain conditions (24, 27). Treatment of the DEPC-modified enzyme with hydroxylamine can result in deacylation of derivatized histidine moieties and provide a restoration of previously inhibited enzyme activity (24, 25, 27). This ability to restore

**Figure 7.** Effect of increasing concentrations of DEPC on proton pumping activity of plasma membrane vesicles. DEPC was included directly in the assay which took place in a buffer containing 250 mM sorbitol, 25 mM BTP/Mes (pH 6.5), 100 mM KNO₃, 3.75 mM MgSO₄, 10 µM acridine orange, and 3.75 mM ATP (BTP salt, pH 6.5), and the rate of proton movement was determined by observing the change in absorbance at 490 nm of acridine orange over a 4 min period at room temperature.

DEPC on overall proton pumping activity could be due to this reagent modifying either one or both of these parameters. To examine DEPC effects upon passive H⁺ efflux from the vesicles, proton pumping was allowed to proceed for 5 min to establish a pH gradient, and then either DEPC, gramicidin D, EDTA, or orthovanadate were added. As shown in Figure 8, when gramicidin D (channel forming ionophore) was added, the pH gradient was collapsed as shown by the rapid reversal of acridine orange absorbance decrease. In contrast, treatments which stop the plasma membrane H⁺-ATPase such as the addition of EDTA to chelate Mg²⁺ or addition of the inhibitor orthovanadate result in a more gradual decrease in the pH gradient over time. Clearly, the addition of 800 µM DEPC results in a gradual decrease in the pH gradient similar to these treatments which stop the H⁺-pump. Thus, it would appear that DEPC effects upon proton pumping activity are related to modification of the H⁺-ATPase rather than increasing passive H⁺ conductance. The greater sensitivity of proton pumping to DEPC as compared to ATP hydrolytic activity may reflect some effect on the process of energy coupling to H⁺ transport or that histidine moieties may have an important role in the process of H⁺ translocation across the membrane.

**Figure 8.** Effects of various reagents upon the rate of proton pumping observed in red beet plasma membrane vesicles. The dye acridine orange was allowed to equilibrate across the vesicle membrane in a buffer containing 250 mM sorbitol, 25 mM BTP/Mes (pH 6.5), 100 mM KNO₃, 3.75 mM MgSO₄, and then 3.75 mM ATP was added to initiate the proton-pumping reaction. After 5 min, either gramicidin D, EDTA, sodium vanadate, or DEPC was added and quickly mixed. The reaction was then allowed to proceed for a subsequent 5 min. All absorbance changes were monitored by use of a Beckman DU-40 spectrophotometer fitted with a kinetics program.
should greatly decrease the amount of inhibition of ATPase activity produced by DEPC treatment. When solubilized ATPase was incubated with increasing micromolar concentrations of DEPC for 30 min at 37°C in the presence of either 10 mM ATP or ADP, activity was protected but not returned to the control level (Fig. 9). Increased inhibition was observed when the inhibitor concentration was increased from 0 to 500 μM and this was correlated with a decreasing amount of protection against DEPC modification by ATP or ADP. When incubation was performed in the presence of 500 μM DEPC, enzyme activity was inhibited approximately 95% and the inclusion of either ATP or ADP caused an increase in activity. However, the enzyme was still inhibited to approximately 65% of the control level.

At lower concentrations of inhibitor, activity in the presence of nucleotide returned to a level closer to that of the control but there was less effect of DEPC on subsequent ATPase activity at these lower DEPC concentrations. Increasing the magnesium concentration to 10 mM did not increase protection by nucleotides (data not shown), consistent with previous observations that nucleotide binding to the enzyme can occur in the absence of magnesium (5, 13). In all cases, it appeared as if ATP allowed the inhibited enzyme to recover approximately 30% over inhibited activity and ADP was slightly more effective as a protectant since activity was increased by approximately 40%. These results suggest that the modified histidine moiety might not represent a residue (or residues) that directly participates in the binding of substrate to enzyme. These results are somewhat different from those of Morjana and Scarborough (28) who reported that the addition of ADP, but not ATP, decreased the rate of DEPC inhibition in the H⁺-ATPase located in the plasma membrane of N. crassa. These authors concluded that the modified histidine probably played a part in the binding of nucleotide to the active site, but they did not rule out longer range conformational changes (28).

Table II. Effect of Hydroxylamine on ATPase Activity in Presence and Absence of 800 μM DEPC

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* Activities in parentheses represent relative activities based on percent of control ATPase treated in the same fashion, but in the absence of DEPC.

In conclusion, the results of this study indicate that the H⁺-ATPase associated with the plasma membrane of red beet storage tissue was sensitive to micromolar concentrations of the histidine-specific reagent DEPC. This would confirm the earlier proposal for a role of histidine in the mechanism of the plasma membrane H⁺-ATPase based upon transient state kinetic studies on the enzyme (6). That the characteristics of DEPC inhibition were similar for the enzyme in the membrane-bound and solubilized form would suggest equal accessibility and/or sensitivity of histidine residues to this reagent for both preparations. Recently, DEPC has been used to investigate the role of histidine in animal membrane transport proteins and has allowed the identification of essential histidine residues associated with a number of transport systems such as the band 3 protein from erythrocyte ghosts (18), the Na⁺/H⁺ exchanger found in renal tissue (15) and the renal H⁺/organic cation antiport system, where histidine was found to play a regulatory role in transport (17). Preliminary results in this laboratory indicate that DEPC may block the transformation of the E₁P state of the enzyme to the E₀P state (14) as observed on acid pH dodecylsulfate PAGE (23, 30). Thus, DEPC modification may serve as a useful tool for the further study of mechanistic aspects of this enzyme. In the future, ¹³C-DEPC could also be used to probe for the sequence surrounding the modified histidyl residue on ATPases in the E-P class isolated from various sources in order to determine if this might be a structurally and mechanistically common theme.

LITERATURE CITED