Conformational Changes of the *Neurospora* Plasma Membrane H⁺ATPase during Its Catalytic Cycle*

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Evidence is presented for the existence of at least 3 different conformational states of the *Neurospora* plasma membrane H⁺ATPase during its catalytic cycle. Incubation of isolated *Neurospora* plasma membrane vesicles with trypsin in the absence or presence of several different ATPase reaction cycle participants or analogues thereof gives rise to different ATPase degradation patterns depending upon the ligands employed, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses. In the absence of any ligand, the ATPase (Mᵦ ~ 105,000) is rapidly degraded by trypsin to small, undefined fragments with Mᵦ ≤ 10,000. In the presence of Mg⁺⁺ and vanadate, a presumed transition state analogue of the aspartyl-phosphoryl-enzyme hydrolysis reaction, trypsin rapidly degrades the ATPase to Mᵦ ~ 95,000 with further degradation to an Mᵦ ~ 88,000 form occurring much more slowly, suggesting that during the enzyme dephosphorylation reaction, the enzyme is in a conformational state significantly different than it is in when unliganded. In the presence of MgADP, a nonhydrolyzable competitive inhibitor of the ATPase, the enzyme is rapidly degraded to an Mᵦ ~ 88,000 form which is resistant to further degradation, suggesting that substrate binding is also associated with a conformational change. Finally, in the presence of Mg⁺⁺ plus another competitive, nonhydrolyzable ATP analogue, βγ-methylene ATP, the ATPase is rapidly degraded to an Mᵦ ~ 95,000 form similar to that which is produced in the presence of Mg⁺⁺ plus vanadate, and is then subsequently degraded to an Mᵦ ~ 88,900 form similar to that produced in the presence of MgADP. The difference between the responses seen in the presence of MgADP and βγ-methylene ATP is suggested to lie in the fact that, unlike ADP, βγ-methylene ATP can participate in the formation of the transition state of the enzyme phosphorylation reaction even though it cannot phosphorylate the enzyme. On the basis of these findings, an outline of the sequence of events that occur during the catalytic cycle of the ATPase is presented.

A substantial body of evidence has now accumulated which indicates that the plasma membrane of the eukaryotic microorganism *Neurospora crassa* contains an electrogenic, proton-translocating ATPase (1–3), the function of which is to generate a proton motive force that is used to drive the active transport of a variety of different ions and molecules via chemiosmotic coupling devices known as porters (4–6). A similar situation appears to exist in yeast (7–10). Biochemical studies of the fungal plasma membrane ATPase in this laboratory and several others have brought to light the realization that the properties of this enzyme are quite similar to those of the more thoroughly studied Na⁺/K⁺-, Ca⁺⁺-, and H⁺/K⁺- translocating ATPases of animal cell membranes. Specifically, all of these ATPases have a major polypeptide chain with an Mᵦ ~ 100,000 (11–18), all are inhibited by vanadate (19–21), all form a phosphoryl-enzyme intermediate that has been identified as β-aspartyl phosphate in at least 3 cases (22–24) and 3 of the 4 have been directly or indirectly shown to require a divalent cation in the enzyme dephosphorylation reaction (Refs. 25–27 and this communication). Such similarities have led several investigators to conclude that all of these ATPases may operate via a fundamentally similar mechanism (24, 28–30). Along these lines, we have recently proposed models which posit that the generation of protons at the active site is a fundamental feature of the molecular mechanisms of each of these enzymes (24, 30).

At present, the primary goal in this laboratory is an understanding of the molecular mechanism of proton translocation catalyzed by the *Neurospora* plasma membrane ATPase. As pointed out before (11, 24), it is likely that what is learned about this ATPase will contribute to our understanding of the molecular mechanism of ion-translocating ATPases in general. Pertinent to these ends, we have initiated studies designed to explore in detail the rather likely possibility that conformational changes in the ATPase play an important role in the overall mechanism of transport catalysis. In a previous communication (31), experimental results were presented which showed that inhibition of the *Neurospora* ATPase by the organic mercurial, para-chloromercuribenzoate is suppressed by ATP and that MgATP protects the ATPase against inactivation by trypsin. These findings suggested that the ATPase may undergo conformational changes during its catalytic cycle. It was subsequently found (11) that the ATPase inhibitor vanadate markedly augments the protection by MgATP against trypsin inactivation, and that MgATP plus vanadate markedly improves the yield of active ATPase solubilized by lyssolecithin (12). These findings strengthened the notion that significant conformational changes occur during the catalytic cycle of the *Neurospora* plasma membrane ATPase and raised some intriguing possibilities as to the mechanism of vanadate inhibition of this enzyme. In this communication, we describe in more detail the effects of vanadate and several other reaction cycle participants or analogues thereof on the rate and pattern of degradation of the *Neurospora* plasma membrane H⁺ATPase by trypsin as judged by SDS-PAGE of tryptic digests.
PAGE analyses. The results not only demonstrate that an enzyme conformational change occurs as a result of substrate binding, but, in addition, clearly point to a relationship between additional enzyme conformational changes and the formation of the transition states of the enzyme phosphorylation and dephosphorylation reactions.

EXPERIMENTAL PROCEDURES

Growth of Cells and Isolation of Plasma Membrane Vesicles—Cells of the N. crassa al strain were grown, and plasma membrane vesicles were isolated as previously described (12), except that the cells were grown under a stream of sterile air (flow rate of approximately 1.6 liters/min) which significantly increases cell and plasma membrane yields.

Trypsin Treatment—Plasma membrane vesicle pellets were resuspended in ice-cold 0.01 M MES (pH 6.8 with Tris) containing 1 µg/ml of chymostatin to a concentration of 7-10 mg of protein/ml. A portion of this suspension containing 0.2 mg of protein was then mixed on ice with 40 µl of 0.1 M MES (pH 6.8 with Tris) containing 1 µg/ml of chymostatin, 20 µl of 0.2 M solutions of the various nucleotides (pH 6.8 with Tris), and/or 20 µl of 0.2 M solutions of a divergent cation salt, in a total volume of 0.175 ml. The salt control contained 20 µl of 0.2 M phosphoric acid (pH 6.8 with Tris) and 27 µl of 0.2 M Na2SO4 in plasma membrane vesicle suspension. The reaction was terminated by adding 0.1 ml of soybean trypsin inhibitor solution (2 mg/ml in 0.01 M MES, pH 6.8 with Tris) and placing the resulting mixture on ice. The membranes were then pelleted by centrifugation (1800 g, 15 min, 4 °C), freed of the supernatant fluids, resuspended in 0.3 ml of ice-cold 0.01 M MES (pH 6.8 with Tris), and pelleted again under the same conditions.

After removing the supernatant fluids, the membrane pellets were resuspended in 70 µl of ice-cold 0.01 M MES (pH 6.8 with Tris), and aliquots were removed for ATPase assay, protein determination, and SDS-PAGE analysis. Zero time controls were obtained by adding a 0.1 ml of soybean trypsin inhibitor solution (2 mg/ml in 0.01 M MES, pH 6.8 with Tris) and placing the resulting mixture on ice. The membranes were then pelleted by centrifugation (1800 g, 15 min, 4 °C), freed of the supernatant fluids, resuspended in 0.3 ml of ice-cold 0.01 M MES (pH 6.8 with Tris), and pelleted again under the same conditions.

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tive inhibitors of the ATPase, MgADP, and Mg β,γ-methylene ATP, on the rate of tryptic inactivation of the ATPase are also shown in Fig. 1. ATPase activity is rapidly lost in the presence of MgADP, but in the presence of Mg β,γ-methylene ATP, there is a brief activation followed by a significantly slower rate of tryptic inactivation. Although the data are not shown, none of the above nucleotides very markedly affects the rate of tryptic degradation of the ATPase in the absence of a divalent cation.

The remarkable effect of vanadate in retarding the rate of tryptic inactivation of the ATPase in the presence of MgATP is further explored in Fig. 2. The vanadate effect does not require the presence of a nucleotide, but does require the presence of a divalent cation. Thus, the ATPase is transiently activated and effectively protected against tryptic inactivation in the presence of vanadate plus Mgβγ but is not significantly protected by Mgβγ alone. The results are quite similar when Ca2+ is used as the divalent cation; however, some protection by Ca2+ alone is observed. The hydrolysis of the chromogenic substrate N-3(carboxypropionyl)-L-phenylalanine-p-nitroanilide by trypsin is virtually unaffected by all of the above agents under experimental conditions identical with those in the above experiments which indicates that the ligand effects are indeed on the susceptibility of the ATPase to tryptic cleavage and not on the activity of the trypsin.

The changes in the ATPase molecule during the trypsin treatments are shown in Fig. 3. Entire gels are not shown for reasons of space economy and to focus attention on the ATPase. As we have shown before, only a few other membrane proteins are affected by trypsin under these experimental conditions and none of these displays differential trypsin sensitivity (11). In the absence of any ligand (Series A), trypsin rapidly degrades the ATPase as judged by its rapid disappearance from the M1 ~ 105,000 area of the gel. Although not shown, the fragments produced are either released from the membranes or are smaller than 10,000 daltons, because no identifiable new bands can be found in trypsin-treated mem-

FIG. 2. Effects of divalent cations with or without vanadate on tryptic inactivation of the H’ATPase. Plasma membrane vesicles were treated with trypsin as described under "Experimental Procedures" in the presence of the following additions: □ -□ 27 mM Na2SO4; ○ -○ 20 mM MgSO4; △ -△ 20 mM CaCl2; ● -● 20 mM MgSO4, 0.1 mM Na2VO4; ■ -■ 20 mM CaCl2, 0.1 mM Na2VO4. Individual points are the average values obtained from duplicate ATPase assays plotted as the percentage of the corresponding zero time controls.

Comparision of the data presented in Figs. 1 and 2 with the tryptic degradation patterns shown in Fig. 3 provides a reasonable explanation of the changes in ATPase activity that occur as a result of the treatment of the membranes with trypsin in the presence or absence of the various ligands. When the ATPase is degraded to small pieces, the activity is abolished. However, when the ATPase is present predominantly as the M1 ~ 95,000 form (e.g. at 1 min of trypsin treatment in the presence of MgATP plus vanadate or Mgβγ plus vanadate), the ATPase activity of the membranes is 50-60% higher than it is in the control membranes. And when the ATPase is present predominantly in the M1 ~ 88,000 form (e.g. at 6-12 min of trypsin treatment in the presence of MgADP), the activity of the membranes is much lower. The most reasonable interpretation of these results is that the M1 ~ 95,000 form is roughly 50% more active than the M1 ~ 105,000 form, while the M1 ~ 88,000 form is virtually inactive.

The next point to be considered is whether or not the results presented here constitute sufficient evidence that the H’ATPase does indeed undergo conformational changes during its catalytic cycle. In this regard, the only reasonable alternative explanation for the results we have obtained is that the trypsin-sensitive sites are physically shielded from trypsin by the binding of the various ligands. Because the side
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Time of Trypsin Treatment (Minutes)

![SDS-PAGE analyses of the effects of trypsin on the H\(^{+}\)ATPase in the presence or absence of several ATPase ligands.](image)

Each horizontal series, designated A–J, shows the ATPase area of Coomassie blue-stained SDS-polyacrylamide gel analyses of membranes obtained in the experiments of Figs. 1 and 2. In each case, chains of the amino acids responsible for the specificity of trypsin, i.e., lysine and arginine, are cationic at pH 6.8 and the protective nucleotides and vanadate are anionic at this pH, this possibility must be taken seriously. However, it is difficult to put forward such interactions as the sole explanation of the protective effects of the nucleotides and vanadate in view of the absolute requirement for a divalent cation. The ability of Ca\(^{2+}\) alone to partially protect the ATPase against trypsin degradation (Figs. 2 and 3) supports this conclusion. Furthermore, it is also worthwhile in this regard to mention again our recent observation (12) that vanadate markedly enhances the ability of MgATP to protect the ATPase against nonproteolysis-related inactivation during solubilization by lysolecithin, which suggests that MgATP plus vanadate help to hold the enzyme in a conformation that is more stable than the unliganded one. It is also pertinent to point out that the related enzymes, the Na\(^{+}/K^{+}\)- and Ca\(^{2+}\)-translocating ATPases of animal cells have both been shown to undergo substrate binding-dependent conformational changes by criteria other than sensitivity to trypsin degradation (e.g., Refs. 35–48). And finally, it should also be mentioned that the occurrence of conformational changes upon the interactions of enzymes with their substrates is rapidly becoming recognized as the rule rather than the exception (49–53).

Assuming that the results presented here and elsewhere (12, 31) do indicate that the H\(^{+}\)ATPase undergoes conformational changes during its catalytic cycle, we can now discuss the individual ligand effects as they may relate to the events that occur during the catalytic sequence of the H\(^{+}\)ATPase. The effects of vanadate provide important clues in this regard. It is generally agreed that enzyme-catalyzed phosphoryl transfers usually proceed via in-line SN2-type nucleophilic displacement reactions which go through pentacoordinated intermediaes or transition states that have trigonal bipyramidal geometry (54–58). The powerful inhibitory effects of orthovanadate on numerous phosphotransferase reactions at relatively low concentrations (59) and its ability to form stable pentacovalent trigonal bipyramidal complexes (60) have led to the general opinion that vanadate acts as a transition state analogue for the enzymes it inhibits (59). For enzymes which involve covalent phosphoryl enzyme intermediates, there must be two transition states, one for enzyme phosphorylation and one for enzyme dephosphorylation, and for one enzyme of this type, the Na\(^{+}/K^{+}\)-translocating ATPase of animal cell plasma membranes, Carty et al. (61) have recently provided reasonable evidence that vanadate inhibits by acting as a transition state analogue for the enzymes it inhibits (59). Because the Neurospora plasma membrane H\(^{+}\)ATPase is similar to the (Na\(^{+}/K^{+}\))ATPase, and even more importantly, because the effects of vanadate on the resistance of the Neurospora ATPase to trypsin can be seen in the absence of any nucleotide, it is most reasonable to conclude that vanadate also inhibits this enzyme by acting as a transition state analogue of the enzyme dephosphorylation reaction.

the well at the extreme left contained control (untreated) membranes followed by membranes treated with trypsin for the indicated times in the presence of the following additions: A, 20 mM Tri-phosphate, 27 mM Na\(_2\)SO\(_4\); B, 20 mM MgSO\(_4\), 20 mM Tris-ATP; C, 20 mM MgSO\(_4\), 20 mM Tris-ATP, 0.1 mM Na\(_2\)VO\(_4\); D, 20 mM MgSO\(_4\), 20 mM Tris-ADP, 20 mM Mg\(_2\)SO\(_4\), 20 mM Tris/Na\(_2\)VO\(_4\); F, 20 mM MgSO\(_4\), 0.1 mM Na\(_2\)VO\(_4\); G, 20 mM MgSO\(_4\); H, 20 mM Tris-phosphate, 27 mM Na\(_2\)SO\(_4\), 0.1 mM Na\(_2\)VO\(_4\); I, 20 mM CaCl\(_2\); J, 20 mM CaCl\(_2\), 0.1 mM Na\(_2\)VO\(_4\). The top, middle, and bottom arrows beside each series point to the M, ~105,000, M, ~95,000, and M, ~88,000 regions of the gels, respectively. Certain series appear lighter than others because of variable destaining times.
As pointed out by Pauling (62) and elaborated upon by others (63–65), enzymes should have their greatest affinity for the transition states of the reactions that they catalyze, from which it follows that the complex between an enzyme and an analogue of the transition state of the reaction it catalyzes should be quite stable. Extending these considerations to the results obtained here, it appears that the H' ATPase which, in the unliganded form, is extremely sensitive to degradation by trypsin, coalesces with Mg$^{2+}$ (or Ca$^{2+}$) and vanadate to form a stable complex, of relatively low free energy, with a polypeptide chain conformation different from that of the unliganded enzyme. When the enzyme is in this conformation, only a piece of about 10,000 daltons can be removed by trypsin. Extrapolating to the physiological ligands, it can be concluded that Mg$^{2+}$ is required for the enzyme dephosphorylation reaction and that during the enzyme dephosphorylation reaction, the H' ATPase is in a specific conformation which changes back to the unliganded conformation after hydrolysis of the phosphoryl-enzyme intermediate and the release of products.

The comparative effects of MgADP and Mg $\beta$$\gamma$-methylene ATP on tryptic degradation of the ATPase also provide important information about the catalytic sequence. Both of these ligands are nonhydrolyzable competitive inhibitors of the ATP hydrolysis reaction, yet their effects on tryptic degradation are significantly different. In the presence of MgADP, the ATPase is rapidly degraded to an $M_r \approx 88,000$ form with very little intermediate appearance of the $M_r \approx 95,000$ form. However, in the presence of Mg $\beta$$\gamma$-methylene ATP, the pattern of enzyme degradation is much like that seen with Mg$^{2+}$ plus vanadate, i.e. rapid production of the $M_r \approx 95,000$ form and slower degradation of this form to an $M_r \approx 88,000$ form. A comparison of the structures of ADP and $\beta$$\gamma$-methylene ATP provides an explanation for this apparent paradox. The most obvious difference between the two molecules is the presence in $\beta$$\gamma$-methylene ATP, of a $\gamma$-phosphorus atom, and it is the $\gamma$-phosphorus atom that undergoes nucleophilic attack by the enzyme-bound $\beta$-carboxyl group of aspartate during the formation of the transition state of the enzyme phosphorylation reaction. Thus, we propose that the most significant difference between the two nonhydrolyzable, competitive inhibitors of the H' ATPase, MgADP, and Mg $\beta$$\gamma$-methylene ATP, is that the latter, because of the presence of a $\gamma$-phosphoryl moiety, can participate in the formation of the transition state of the enzyme phosphorylation reaction, even though it cannot complete the transphosphorylation reaction due to the stability of the methylene bridge.

From the above considerations, an outline of the events which take place during the catalytic sequence of the Neurospora H' ATPase can be formulated. Before substrate binding, the ATPase is in a conformation that is rapidly degraded by trypsin to small pieces. Upon binding of Mg$^{2+}$ and ATP, the enzyme undergoes a conformational change to a form that can be degraded by trypsin to $M_r \approx 88,000$. This is the form which predominates when the enzyme is saturated with MgADP. However, when a $\gamma$-phosphate group is present in the substrate and the transition state for enzyme phosphorylation can form, the ATPase undergoes further conformational change to a form which is degraded by trypsin to $M_r \approx 95,000$. When the substrate is Mg $\beta$$\gamma$-methylene ATP, an equilibrium is struck between the binding conformation of the enzyme (degradable to $M_r \approx 88,000$) and the transition state conformation (degradable to $M_r \approx 95,000$) with the latter predominating. Thus, the $M_r \approx 95,000$ form predominates in the early stages of the tryptic degradation, but it steadily gives way to the $M_r \approx 88,000$ form. During the normal reaction cycle, i.e. when the substrate is MgATP, the transition state next breaks down with the resultant formation of the aspartyl-phosphoryl-enzyme intermediate. The events that occur next in the reaction cycle are uncertain, but at some point ADP is released and the hydrolytic water molecule approaches the aspartyl phosphate phosphorus atom prior to the formation of the second transition state. Whether or not the Mg$^{2+}$ stays with the enzyme is unclear, but evidence in support of such an idea has been presented for both the Na$^+$/K$^+$- and Ca$^{2+}$-translocating ATPases (25, 27). Additional conformational changes almost certainly occur as the second transition state is approached, and it seems quite likely that these changes have as their counterparts the traditional $E_i \approx P$ to $E_T P$ conformational changes of the Na$^+$/K$^+$- and Ca$^{2+}$-translocating ATPases (66, 67). Upon the formation of the transition state of the phosphoryl-enzyme hydrolysis reaction, we again have information as to the nature of the conformation of the enzyme. This is the conformation that is so markedly stabilized by binding with Mg$^{2+}$ and vanadate. It is apparently quite similar to the first transition state conformation because the tryptic degradation profiles seen in the presence of Mg $\beta$$\gamma$-methylene-ATP and Mg$^{2+}$ plus vanadate are similar. The reaction sequence concludes with the decomposition of the second transition state and the production of enzyme-bound P, Mg$^{2+}$ and P; then they bind and the ATPase returns to its unliganded conformation, whereupon a new catalytic cycle begins.

In conclusion, the results presented in this communication provide evidence for conformational changes in the Neurospora plasma membrane H' ATPase related to substrate binding and the formation of the transition states of the enzyme phosphorylation and dephosphorylation reactions. Notably absent is any mention of the possible nature of the ion translocating events. This question will not be discussed here because none of the data presented bears directly upon it. However, explicit notions as to how these results may relate to the mechanisms of ion translocation catalyzed by all of the ATPases in the aspartyl-phosphoryl-enzyme intermediate family will be presented in a forthcoming communication (68).

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