Studies on the Active Site of the *Neurospora crassa* Plasma Membrane H\(^+\)-ATPase with Periodate-oxidized Nucleotides*

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(Received for publication, February 1, 1989)

The *Neurospora crassa* plasma membrane H\(^+\)-ATPase is inactivated by the periodate-oxidized nucleotides, oATP, oADP, and oAMP, with oAMP the most effective. Inhibition of the ATPase is essentially irreversible, because Sephadex G-50 column chromatography of the oAMP-treated ATPase does not result in a reversal of the inhibition. Inhibition of the ATPase by oAMP is protected against the H\(^+\)-ATPase substrate ATP, the product ADP, and the competitive inhibitors TNP (2',3'-O-(2,4,6-trinitrocyclohexadienylidine)-ATP and TNP-ADP, suggesting that oAMP inhibition occurs at the nucleotide binding site of the enzyme. The rate of inactivation of the ATPase by oAMP is only slightly affected by EDTA, indicating that the oAMP interaction with the nucleotide binding site of the H\(^+\)-ATPase occurs in the absence of a divalent cation. The protection against oAMP inhibition by ADP is likewise unaffected by EDTA. The inhibition of the ATPase by oAMP is absolutely dependent on the presence of acidic phospholipids or acidic lysophospholipids known to be required for H\(^+\)-ATPase activity, suggesting that these lipids either aid in the formation of the nucleotide binding site or render it accessible. Incubation of the ATPase with Mg\(^{2+}\) plus vanadate, which locks the enzyme in a conformation resembling the transition state of the enzyme dephosphorylation reaction, completely protects against inhibition by oAMP, suggesting that in this transition state conformation the nucleotide site either does not exist, or is inaccessible to oAMP. Labeling studies with \(^{14}\)C oAMP indicate that the incorporation of 1 mol of oAMP is sufficient to cause complete inactivation of the ATPase.

The aspartyl phosphate intermediate family of transport ATPases includes the Na\(^+\)/K\(^+\)-ATPase of animal cell plasma membranes (1), the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum (2, 3), the K\(^+\)/H\(^+\)-ATPase of gastric mucosal membranes (4), the K\(^+\)-ATPase of the plasma membrane of *Escherichia coli* (5), and the H\(^+\)-ATPases of fungal (6, 7) and plant (8) plasma membranes. In the last decade, these enzymes have been the subject of intensive biochemical, kinetic, and structure-function studies that have yielded a wealth of information regarding their structure and mechanism of catalysis (1, 2, 6, 7). In the last few years, the genes for most of these enzymes have been cloned and sequenced (9-18), and deductions of the amino acid sequences of these enzymes based on the gene sequences have revealed that the overall sequence similarity is significant and that the predicted secondary structures of most of these enzymes are quite similar with respect to the positions of the putative transmembrane regions and those involved in ATP binding and phosphorylation (1). Such information has led to a prevalent notion in the transport field that these ATPases may have evolved from a common ancestor. For this and other reasons, it has been proposed that although there are variations with respect to the ions transported, the basic mechanisms of catalysis of these enzymes may be similar (19, 20). It is thus reasonable to expect that any biochemical motif observed in one of these ATPases may be conservatively applicable to other members of this family of transport enzymes.

With this in mind, this laboratory has been involved in elucidation of the mechanism of catalysis of the *Neurospora crassa* plasma membrane H\(^+\)-ATPase. Attempts toward such a goal are being made by both biophysical and chemical approaches to the elucidation of the structural organization of the ATPase. Circular dichroism studies have established the secondary structure characteristics of the H\(^+\)-ATPase and revealed marked similarity in secondary structure with the related Na\(^+\)/K\(^-\)- and Ca\(^{2+}\)-ATPases (21). These studies also indicated that during nucleotide binding and catalysis no major changes occur in the secondary structure of the enzyme, which has also been observed for the Ca\(^{2+}\)-ATPase (22-24) and the Na\(^+\)/K\(^-\)-ATPase (25). Another approach being undertaken is the identification of essential residues involved in substrate binding and catalysis in the H\(^+\)-ATPase by the well established methods of chemical modification of essential residues (26) and affinity labeling of the active site of the ATPase. To facilitate these efforts we have recently developed the protein chemistry methodology necessary for cleavage of the H\(^+\)-ATPase and the purification of peptides from all parts of the molecule, including the refractory hydrophobic sectors (27, 28). The availability of such methodology is crucial for the identification of key residues with a high degree of certainty.

The periodate-oxidized nucleotides, oATP, oADP, and oAMP, have been used extensively for affinity labeling of a number of nucleotide binding proteins (29-37). These com-

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* This work was supported by United States Public Health Service-National Institutes of Health Grant GM 24784. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 N. A. Morjanaš and G. A. Scarborough, submitted for publication.

2 The abbreviations used are: oATP, periodate-oxidized ATP; oADP, periodate-oxidized ADP; oAMP, periodate-oxidized AMP; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-[N-morpholino]ethanesulfonic acid; TNP-ATP, 2',3'-O-(2,4,6-trinitrocyclohexadienylidine)-ATP; TNP-ADP, 2',3'-O-(2,4,6-trinitrocyclohexadienylidine)-ADP.
pounds have the advantage of being very similar to the parent nucleotides in that they contain an unperturbed purine ring and the appropriate number of phosphoryl groups. The only part that is modified is the ribose moiety, and many nucleotide binding proteins are believed to tolerate ribose modifications (38). In this paper, we provide evidence for a functional interaction of these compounds with the *Neurospora* plasma membrane H^+-ATPase which leads to irreversible inhibition and is protected by substrate and substrate analogs. We also demonstrate that nucleotide binding does not require the presence of a divalent cation, that acidic phospholipids are involved in nucleotide binding, that the nucleotide binding site is unavailable in the transition state of the enzyme dephosphorylation reaction, and that inhibition of the enzyme is achieved when 1 mol of nucleotide interacts with 1 mol of H^+-ATPase monomer. These studies shed significant new light on certain aspects of the catalytic mechanism of the H^+-ATPase and are an important prerequisite for future work aimed at the identification of key amino acid residues in the active site of this transport enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of most of the materials used have been described before (38). [³⁵S]AMP was obtained from ICN Radiochemicals. Periodate-oxidized nucleotides (oATP, oADP, and oAMP), L-α-lysophosphatidylglycerol, and L-α-lysophosphatidylcholine were purchased from Sigma. Glycerol was obtained from Fisher.

**Purification of the H^+-ATPase**—The *Neurospora* plasma membrane ATPase was isolated according to Smith and Scarborough (38, 39) with the minor modification that the cell lysis was achieved by 180 passes in a Pyrex glass homogenizer instead of 50. The glycerol gradient-isolated ATPase was centrifuged at 12,000 rpm (17,400 × g) for 20 min at 4 °C to remove the particulates that develop upon storage. The clear supernatant fluid containing the ATPase was then further purified by gel filtration (21) on a Sepharose CL-4B column (2.5 × 96 cm) equilibrated with buffer containing 30% (v/v) glycerol, 50 mM HEPES (pH 6.9 with NaOH), and 2 μg/ml chymostatin. The fractions containing the coincident ATPase and protein peaks were then pooled and concentrated to 0.5–1.0 mg of protein/ml using RCF- ConFilt hollow-fiber bundles (M, cutoff 6,000; Biorad Molecular Dynamics). The concentrate was then centrifuged at 12,000 rpm (17,400 × g) for 15 min at 4 °C to remove the particulates that develop during concentration. The clear solution of the essentially detergent-free ATPase was then divided into aliquots and stored at −20 °C. For unknown reasons, the efficiency of periodate-oxidized nucleotide inactivation of the H^+-ATPase increases with time after purification of the enzyme. Therefore, the purified H^+-ATPase preparation was usually stored at −20 °C, whereupon it did not solidify, for 10–14 days prior to use in the inactivation experiments. The specific activity of the ATPase preparation did not change during this period.

**Assay of ATPase Activity**—The ATPase activity was measured as described by Smith and Scarborough (38) with the exception that the Folch fraction 1 was added as a suspension in 0.3% (v/v) sodium deoxycholate. The specific activities of the enzyme preparation were in the range of 15–20 μmol of P, min”¹, mg”¹ protein.

**Synthesis of [³⁵S]oAMP**—The synthesis of the diazide derivative of AMP was carried out according to the method of Esterbrook-Smith (37) as modified by Evans et al. (35). The reaction was performed in the dark at 4 °C. To the [³⁵S]AMP (25 μCi, 53 μCi/μmol) was added a 200-fold excess of AMP in a volume of 0.9 ml, and the pH adjusted to 7.0 with NaOH. The reaction was initiated by the addition of sodium periodate (pH 7.0 with NaOH) in a 2:1 molar excess over the AMP and the reaction allowed to proceed for 1 h. The reaction was terminated by adding a 2-fold molar excess of ethane-1,2-diol over the periodate. The analogue was then purified from the reaction products by chromatography on a column of Sephadex G-10 (1.5 × 70 cm) equilibrated in distilled water, monitoring fractions at 259 nm. The iodine-containing fractions were determined as described (29), and the peak fractions at 259 nm that did not contain iodine were pooled, lyophilized, and stored at −80 °C. [³⁵S]oAMP concentration was calculated on the basis of an extinction coefficient of 14.5 cm”¹ mM”¹ for oAMP at 259 nm (37). Thin layer chromatography on cellulose plates run in tert-amyl alcohol/formic acid/water (3:2:1; v/v/v) showed that the purified [³⁵S]oAMP behaved the same as the pure nonradioactive commercial product. The final specific activity of the preparation was 605 cpm/nmol as determined by liquid scintillation counting in the scintillation mixture of Patterson and Greene (40).

**Oxidized Nucleotide Treatment of the H^+-ATPase**—Typical reaction mixtures contained 2.5–5.0 μM ATPase in buffer containing 30% (w/v) glycerol, 50 mM HEPES (pH 6.9 with NaOH), and 10 μl of bovine brain extract suspension (Folch fraction I, 5 mg/ml in 0.3% sodium deoxycholate) in a final volume of 100–200 μl at room temperature. The reactions were initiated by the addition of the oxidized nucleotide at the indicated concentration, and 5-μl aliquots were removed at various times and assayed for ATPase activity as described above. In experiments where the effect of lysophosphatidies was tested, the lipids were added at a final concentration of 250 μM, assuming molecular masses of 520 and 539 daltons for lysophosphatidyglycerol and lysophosphatidylcholine, respectively. In most of the experiments, control reactions were carried out in identical fashion without the addition of an oxidized nucleotide, and the inhibition data expressed as a percent of that control. There was essentially no loss of activity in such controls.

**Stoichiometry of Labeling of the H^+-ATPase with [³⁵S]oAMP**—Aliquots containing 100 μg of purified ATPase were incubated as described above in the presence of 5 mM [³⁵S]oAMP in a reaction volume of 200 μl, and the reactions were allowed to proceed at room temperature for 0–10 min. At different times, 5-μl aliquots were removed and assayed for activity while the remainder was mixed with 10 μl of 0.5 M NaBH₄ (0.1 N NaOH) and allowed to stand for 25 min on ice. The protein was then precipitated with trichloroacetic acid following incubation with deoxycholate according to the method of Bensadoun and Weinstein (41). The precipitates were washed by suspension in 4 ml of 6% trichloroacetic acid followed by centrifugation at 2,500 rpm for 30 min at room temperature two times. The washed precipitates were then dissolved in 0.1 ml of MES (pH 6.9 with Tris) containing 0.05% (w/v) SDS, and the radioactivity in the samples determined as described above.

**Protein Determination**—Protein was determined by the method of Lowry et al. (42) after precipitation of the protein by the deoxycholate-trichloroacetic acid method of Bensadoun and Weinstein (41) using bovine serum albumin as a standard.

**RESULTS**

Fig. 1 shows the results obtained when the purified H^+-ATPase was incubated with oATP, oADP, or oAMP for varying times and assayed for ATPase activity. All three periodate-oxidized nucleotides inactivate the H^+-ATPase in a time-dependent manner at a concentration of 5 mM. Oxidized AMP is the most effective analog tested, rapidly inactivating the enzyme in only a few minutes. Oxidized ATP and ADP were less effective. Increasing the concentration of oAMP in increments from 1 to 6 mM gave progressively increasing rates of inactivation, but semi-log plots of the inactivation time courses were nonlinear at all concentrations of oAMP tested (not shown), precluding the accurate esti-

**Figure 1.** Inactivation of the *Neurospora* plasma membrane H^+-ATPase by oxidized nucleotides. The enzyme was incubated with 5 mM oATP (○), 5 mM oADP (●), or 5 mM oAMP (▲), and samples were taken at different times and assayed for residual activity.
mation of inhibition rate constants. Although not shown, when the oAMP-inhibited ATPase was subjected to Sephadex G-50 column chromatography to remove the excess nucleotide the inhibition remained, indicating that the H+-ATPase inhibition reaction is essentially irreversible. When the native and oAMP-modified H+-ATPase were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under reducing conditions, no species larger than 100 kDa were observed, indicating that the inhibition is not due to intermolecular cross-linking of the H+-ATPase molecules.

Fig. 2A shows the effects of the H+-ATPase substrate, ATP, and product, ADP, on the oAMP inhibition reaction. Inactivation of the ATPase by oAMP is completely protected against by including ATP (10 mM) or ADP (5 mM) during the inactivation reactions. Fig. 2B shows the effects of TNP-ATP and TNP-ADP, which are competitive inhibitors of the H+-ATPase with apparent $K_i$ values of approximately 65 and 250 $\mu$M, respectively, on the oAMP inhibition reaction. The inclusion of 0.5 mM TNP-ATP completely protects against the inactivation, and 0.5 mM TNP-ADP protects the enzyme partially. The addition of the TNP-nucleotides at the concentrations indicated without oAMP did not affect subsequent assays of the enzyme activity.

The experiments presented thus far were carried out in the absence of added Mg$^{2+}$, suggesting that a divalent cation is not required for the oxidized nucleotide inhibition reaction. However, the presence of traces of Mg$^{2+}$ or other divalent cations as contaminants in the various reagents used in these experiments is possible. Therefore, to clarify this important issue, the effect of EDTA on oAMP inactivation of the H+-ATPase was investigated. Fig. 3 shows the effect of EDTA on inactivation of the ATPase by oAMP and protection against inactivation by ADP. In the presence of 2 mM EDTA, the inactivation rate observed with oAMP is only slightly reduced, and the addition of 5 mM ADP completely protects against this inactivation, indicating that neither the oAMP inhibition nor protection by ADP require a divalent cation.

We have demonstrated before that the H+-ATPase in detergent-washed membranes requires acidic phospholipids for activity (43), and more recent experience with the purified H+-ATPase indicates an absolute requirement for acidic phospholipids for activity. Moreover, we have recently reported that the acidic phospholipid requirement can be satisfied by acidic lyso phospholipids, including lyso phosphatidylglycerol (21). For this reason, the effects of several pertinent lipids on the oAMP inhibition reaction were investigated. The results (Fig. 4), show that in the absence of acidic phospholipids from bovine brain (Folch fraction I), which is normally used to satisfy the acidic phospholipid requirement of the H+-ATPase in our ATPase assays, oAMP does not inhibit the ATPase. The addition of Folch fraction I (0.05%, w/v) induces a rapid inactivation of ATPase activity. The addition of Folch fraction I alone in such an experiment does not inhibit the

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2 A. P. Bidwai and G. A. Scarborough, unpublished observations.
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**FIG. 5. Effect of MgSO₄ and sodium vanadate alone and in combination on oAMP inactivation of the H⁺-ATPase.** The enzyme was incubated with 5 mM oAMP alone (●), or in the presence of 1 mM MgSO₄ (○), 0.1 mM Na₂VO₄ (△), or 1 mM MgSO₄ plus 0.1 mM Na₂VO₄ (□) for 3 min. At that time, the reactions were brought to 0.025% (w/v) Folch fraction I. The Folch fraction I concentration was decreased by 50% compared to the other experiments described, in order to prevent precipitation of the lipids by MgSO₄.

**FIG. 6. Stoichiometry of labeling of the H⁺-ATPase by [¹⁴C]oAMP.** The ATPase was incubated for varying times with 5 mM [¹⁴C]oAMP (600 cpm/nmol), and the residual activity and ATPase labeling were determined as described under “Experimental Procedures.”

ATPase in subsequent assay, indicating that the combination of oAMP and acidic phospholipids is required for inactivation (not shown). When Folch fraction I is replaced by lysophosphatidylglycerol, which also supports ATPase activity, inactivation is observed (Fig. 4). On the other hand, the addition of lysophosphatidyicholine, a lysolipid that does not support ATPase activity, to the incubation mixture does not result in any oAMP inactivation of the enzyme during the time course of these experiments.

As has been explained previously, the combination of the H⁺-ATPase ligands, Mg⁺⁺ plus vanadate, is thought to lock the enzyme in a state resembling the transition state of the enzyme dephosphorylation reaction (44). It was therefore of interest to determine whether or not oAMP is capable of inactivating the H⁺-ATPase when it is locked in this transition state. Fig. 5 shows the effect of Mg⁺⁺ and vanadate, alone and in combination, on oAMP inactivation of the H⁺-ATPase. The addition of MgSO₄ (1 mM), or Na₂VO₄ (100 μM) alone do not significantly affect the rate of oAMP inactivation of the enzyme. However, in the presence of Mg⁺⁺ and vanadate together, oAMP is unable to inhibit the enzyme.

Fig. 6 shows the combined results of several experiments in which the H⁺-ATPase was inhibited to varying degrees by [¹⁴C]oAMP by incubating for increasing time periods and then assessed for the extent of labeling, as described under “Experimental Procedures.” The results indicate that the extent of incorporation of label parallels the extent of inactivation, with complete inactivation being obtained when approximately 1 mol of nucleotide has been incorporated per mol of 100,000-dalton monomer.

**DISCUSSION**

The results presented demonstrate interactions of the di-aldehyde analogs oATP, oADP, and oAMP with the *Neurospora* plasma membrane H⁺-ATPase. The interaction of these nucleotides with the ATPase results in a time- and concentration-dependent, irreversible inactivation of the ATPase. Semi-log plots of the time courses of the oAMP inactivation reactions were nonlinear at all concentrations of the nucleotide tested, precluding the accurate estimation of inhibition rate constants. The reasons for this nonlinearity are presently unknown. It is not due to the breakdown of the oAMP during the inactivation process, as the addition of acidic phospholipids, which are required for the inhibition reaction, at increasing times after the addition of oAMP resulted in identical rates of inactivation (data not shown). The nonlinearity is also not due to diminution of the oAMP concentration as a result of interaction with amino groups of the added acidic phospholipids, as the characteristics of the nonlinearity did not vary as a function of the acidic phospholipid concentration. Furthermore, the nonlinearity is also seen when lysophosphatidylglycerol, which would not be expected to interact with oAMP, is used to satisfy the acidic phospholipid requirement. It is possible that the nonlinearity is related to the aging process required for the H⁺-ATPase to become efficiently inhibited by oxidized nucleotides (see "Experimental Procedures"). Or, because the H⁺-ATPase is a hexamer as isolated (45), it is possible that the nonlinearity is the result of monomer-monomer interactions in the hexamers. Or finally, the nonlinearity may be an intrinsic characteristic of the interaction of oxidized nucleotides with certain enzymes, as similar nonlinear inactivation kinetics have been observed for phosphofructokinase (32), and the F₆F₁-ATPase (34). In any case, as the rest of the results presented in this communication clearly show, the atypical kinetics of the oAMP inhibition reaction do not preclude the usefulness of oAMP as a specific, irreversible inhibitor of the H⁺-ATPase.

Regarding the nature of the inhibitor reaction, periodate-oxidized nucleotides are normally thought to interact with the amino group of lysine residues in enzymes, resulting in the formation of a Schiff base that requires NaBH₄ reduction in order to obtain irreversible inhibition. However, Sephadex column chromatography experiments indicate that oAMP inhibition of the *Neurospora* H⁺-ATPase is irreversible without reduction by NaBH₄. Similar irreversible inactivation by oxidized nucleotides has been observed for the beef heart mitochondrial ATPase (34), the Na⁺-ATPase from Acholeplasma laidlawii (31), and phosphofructokinase (32) and has been suggested to be due to the formation of morpholino-type adducts between the oxidized nucleotide and the enzyme (32).

Oxidized AMP inactivated the ATPase with the highest rate of the nucleotides tried, followed by oADP and then oATP. This would appear to indicate that the affinity of the enzyme for the nucleotides is in the order oAMP > oADP > oATP. However, ADP is a fairly strong competitive inhibitor of the ATPase with an apparent *Kₐ* of about 200 μM (44), whereas AMP is a relatively poor competitive inhibitor of the ATPase with an apparent *Kₐ* of about 20 mM.³ It is thus interesting that oAMP should have such a high apparent affinity for the H⁺-ATPase compared to oADP. A reasonable explanation for such a result is the suggestion put forward for

³ Typically, the half-maximal inhibition observed with oAMP is about 0.4 mM.
the inactivation of the mitochondrial F_{0}/F_{1}-ATPase by αAMP, that due to cleavage of the ribose, the molecule can undergo some degree of flexing and thus take on the molecular dimensions of ADP (34). It is also possible that the nucleotides interact with the ATPase with different affinities in the presence and absence of a divalent cation.

An important criterion for the establishment of a compound as an affinity analog is the ability of substrates, products, and competitive inhibitors to protect against inactivation by that compound. The results of the experiments shown in Fig. 2 indicate that αAMP inhibition of the H^{+}-ATPase is effectively protected against by substrate and product levels of ATP and ADP, and even lower levels of the relatively high affinity competitive inhibitors, TNP-ATP and TNP-ADP. Whereas we have previously presented strong evidence for the induction of substantial H^{+}-ATPase conformational changes by MgATP, MgADP, and Mg^{2+} plus vanadate (44), little evidence has been obtained indicating H^{+}-ATPase conformational changes induced by nucleotides in the absence of a divalent cation. Therefore, since protection against αAMP inhibition by ATP and ADP clearly occurs in the absence of a divalent cation, the possibility that ATP and ADP protect the H^{+}-ATPase against αAMP inhibition by inducing long range H^{+}-ATPase conformational changes is considered less likely than direct competition between αAMP and ATP, ADP, and the TNP-nucleotides, for the H^{+}-ATPase nucleotide binding site. Thus, the results of the ATP, ADP, and TNP-nucleotide protection experiments strongly support the idea that αAMP inhibits the H^{+}-ATPase by an irreversible interaction at its nucleotide binding site. Obviously, the fact that αAMP is a nucleotide analog gives additional strong support to this idea.

We have previously shown that 100-kDa monomers of the H^{+}-ATPase are fully functional (46), and, in our hands, plots of the H^{+}-ATPase activity versus the concentration of MgATP show no sigmoidicity, indicating that the H^{+}-ATPase monomers probably contain a single nucleotide binding site. Moreover, as will be further discussed below, the H^{+}-ATPase labeling studies with [32P]αAMP strongly support this contention. If so, assuming that αAMP inhibition does occur by interaction at this nucleotide binding site as reasoned above, the facts that αAMP inhibition and ATP, ADP, and TNP-nucleotide protection against αAMP inhibition occur in the absence of a divalent cation is evidence that nucleotide binding to the H^{+}-ATPase can take place in the absence of a divalent cation. Thus the implications from kinetic experiments that the "true substrate" of the ATPase is MgATP (7, 47) should not be taken to indicate that nucleotide binding cannot occur in the absence of a divalent cation. We have recently reached the same conclusion on the basis of nucleotide protection against inhibition of the H^{+}-ATPase by diethylypyrocarbonate, and convincing evidence for the interaction of nucleotides with the nucleotide binding site in the absence of a divalent cation has also been presented for both the Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase (48–50). Pertinent in this regard, electron paramagnetic resonance studies on the Na^{+}/K^{+}-ATPase by Grisham and coworkers (51–54) indicate that the spectra of the enzyme-Mn^{2+}, enzyme-ATP, and enzyme-Mn^{2+}-ATP complexes are significantly different than those obtained for Mn^{2+}-ATP. For enzymes that use Mn^{2+}-ATP as the substrate and form enzyme-ATP-Mn^{2+} bridge complexes, the EPR spectrum of the ternary complex is usually similar to that of the binary complex between Mn^{2+} and ATP (51).

Although it has been clear for some time now that the H^{+}-ATPase absolutely requires acidic phospholipids or acidic lysophospholipids for activity (21, 43), the precise role of these lipids and the stage of the catalytic cycle in which they are important has been totally unknown. The acidic phospholipid/lysophospholipid requirement for inhibition of the H^{+}-ATPase by αAMP (Fig. 4) sheds significant light on the interesting question. Because αAMP binding to the H^{+}-ATPase nucleotide binding site appears to be the only prerequisite for inhibition of the ATPase, the fact that the acidic phospholipids or lysophospholipids are required for αAMP inhibition can be interpreted to mean that the nucleotide binding site is not available in the absence of acidic phospholipids or lysophospholipids. One explanation of this phenomenon could be that the complete nucleotide binding site exists but is occluded in the absence of acidic phospholipids or lysophospholipids, as for example in an interdomain cleft that requires the specific lipids for opening and exposure of the nucleotide binding site. Such interdomain movements are more likely than major structural rearrangements in the ATPase molecule, as we have recently shown by circular dichroism measurements that significant changes in secondary structure do not occur upon activation of the H^{+}-ATPase by the acidic lysophosphatide, lysophosphatidylglycerol (21). Alternatively, it may be that the binding site, per se, is incomplete until the acidic lipid has bound. In any case, these results tend to define nucleotide binding as a step in the catalytic cycle of the Neurospora H^{+}-ATPase that requires acidic phospholipids or lysophospholipids. Acidic phospholipid requirements have also been described for the Na^{+}/K^{+}-ATPase (55), the plasma membrane H^{+}-ATPase from Schizosaccharomyces cerevisiae (56), and the Ca^{2+}-ATPase of erythrocytes (57). It is possible that nucleotide binding to these enzymes is the step requiring acidic phospholipids as well.

The marked protection of the H^{+}-ATPase against αAMP inactivation by Mg^{2+} plus vanadate (Fig. 5) also bears in an important way on the catalytic mechanism of the H^{+}-ATPase. We have previously presented a reasonable argument, based on the effects of a variety of H^{+}-ATPase ligands on the sensitivity of the enzyme to degradation by trypsin, that the combination of H^{+}-ATPase ligands, Mg^{2+} plus vanadate, locks the molecule in a conformation resembling the transition state of the enzyme dephosphorylation reaction (44). Because the experiments of Fig. 5 were carried out in the presence of acidic phospholipids, it is likely that the nucleotide binding site was functional in these experiments. Therefore, the most straightforward interpretation of the profound Mg^{2+} plus vanadate protection of the H^{+}-ATPase against inactivation by αAMP is that the nucleotide binding site is unavailable when the H^{+}-ATPase is in the transition state of the enzyme dephosphorylation reaction. Whereas conformational rearrangements in the H^{+}-ATPase molecule that totally disrupt the nucleotide binding site at this stage of the catalytic cycle cannot be ruled out, we prefer a more biochemically orthodox interpretation in which the nucleotide binding site is simply occluded at this step in the cycle by virtue of its being buried in a crevice between two or more domains of the molecule, by analogy with numerous other phospholipid transfer enzymes for which the structures are known (58). In support of this notion, the circular dichroism studies mentioned above also showed that major changes in the secondary structure composition of the H^{+}-ATPase do not occur when the acidic lysophosphatide-activated enzyme proceeds from its unliganded conformation to the conformation it assumes during the enzyme dephosphorylation reaction (21). The idea that the H^{+}-ATPase may undergo hinge-bending interdomain movements during the catalytic cycle was also mentioned in that paper and has been elaborated upon elsewhere (19, 20). It should be mentioned that the data are also consistent with 11794 Active Site of the Neurospora Plasma Membrane H^{+}-ATPase
a simple competitive block of oAMP binding to the active site by Mg\(^{2+}\) plus vanadate.

Finally, our results on the stoichiometry of labeling of the H\(^+\)-ATPase indicating the attachment of 1 mol of oAMP/mol of H\(^+\)-ATPase monomer (Fig. 6) are important for at least two reasons. First, this outcome supports further the contention presented earlier that monomers of the H\(^+\)-ATPase have a single nucleotide binding site. Second, H\(^+\)-ATPase labeling with a stoichiometry of one is the simplest possible starting point for identification of the residue(s) in the H\(^+\)-ATPase nucleotide binding site that are modified by oAMP. This, and our recently developed methodology for quantitatively fragmenting the H\(^+\)-ATPase and isolating the peptides produced (27, 28) augur that information regarding the identity of one or more residues in the H\(^+\)-ATPase nucleotide binding site may become available in the near future.

REFERENCES
1. Jørgensen, P. L. (1982) Biochim. Biophys. Acta 694, 27-68
2. Inesi, G. (1985) Annu. Rev. Physiol. 47, 573-601
3. Tanford, C. (1983) Annu. Rev. Biochem. 52, 379-409
4. Sachs, G., Wallmark, B., Saccomani, G., Rabon, E., Stewart, H. B., DiBona, D. R., and Berglindh, T. (1982) Curr. Top. Membr. Trans. 16, 135-159
5. Hesse, J. E., Weizcorek, L., Attendorf, K., Reicin, A. S., Dorus, E., and Epstein, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4746-4750
6. Serrano, R. (1988) Biochim. Biophys. Acta 947, 1-28
7. Goffau, A., and Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197-223
8. Sze, H. (1985) Annu. Rev. Plant. Physiol. 36, 175-208
9. Schull, G. E., Greeb, J., and Lingrel, J. B. (1986) Biochemistry 25, 8125-8132
10. Schull, G. E., Lane, L. K., and Lingrel, J. B. (1986) Nature 321, 429-431
11. Schall, G. E., Schwartz, A., and Lingrel, J. B. (1986) Nature 316, 691-698
12. MacLennan, D. H., Brandl, C. J., Korczak, B., and Green, N. M. (1985) Nature 316, 696-700
13. Brandl, C. J., Green, N. M., Korczak, B., and MacLennan, D. H. (1986) Cell 44, 597-607
14. Schull, G. E., and Lingrel, J. B. (1986) J. Biol. Chem. 261, 16788-16791
15. Solier, M., Mathews, S., and Fürst, P. (1987) J. Biol. Chem. 262, 7338-7342
16. Serrano, R., Kieland-Brandt, M. C., and Fink, G. R. (1986) Nature 319, 689-693
17. Addison, R. (1986) J. Biol. Chem. 261, 14986-14901
18. Hager, K. M., Mandia, S. M., Davenport, J. W., Speicher, D. W., Benz, E. J., and Slayman, C. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7683-7687
19. Scarborough, G. A. (1985) Microbiol. Rev. 49, 214-231
20. Scarborough, G. A. (1982) Ann. N. Y. Acad. Sci. 402, 99-115
21. Hennessey, J. P., Jr., and Scarborough, G. A. (1987) J. Biol. Chem. 262, 3123-3130
22. le Maire, M., Jørgensen, K. E., Reigaard-Petersen, H., and Tanford, C. (1976) Biochemistry 15, 5805-5812
23. Dean, W. L., and Tanford, C. (1978) Biochemistry 17, 1683-1690
24. Andersen, J. P., le Maire, M., and Møller, J. V. (1980) Biochem. Biophys. Acta 603, 84-100
25. Hastings, D. F., Reynolds, J. A., and Tanford, C. (1986) Biochim. Biophys. Acta 860, 566-569
26. Addison, R., and Scarborough, G. A. (1986) Biochemistry 25, 4071-4076
27. Rao, U. S., Hennessey, J. P., Jr., and Scarborough, G. A. (1988) Anal. Biochem. 173, 251-264
28. Hennessey, J. P., Jr., and Scarborough, G. A. (1989) Anal. Biochem. 176, 284-289
29. Hinrichs, M. A., and Eyzaguire, J. (1982) Biochim. Biophys. Acta 704, 177-185
30. Lowe, P. N., and Beechey, R. B. (1982) Biochemistry 21, 4073-4082
31. George, R., and MeElhaney, R. N. (1985) Biochim. Biophys. Acta 813, 161-166
32. Gregory, M. R., and Kaiser, E. T. (1979) Arch. Biochem. Biophys. 196, 199-208
33. Bragg, P. D., and Hou, C. (1980) Biochim. Biophys. Res. Commun. 95, 952-957
34. de Melo, D. F., Satre, M., and Vignais, P. V. (1984) FEBS Lett. 168, 123-128
35. Evans, C. T., Goss, N. H., and Wood, H. G. (1980) Biochemistry 19, 5809-5814
36. Coleman, R. F. (1983) Annu. Rev. Biochem. 52, 67-91
37. Easterbrook-Smith, S. B., Wallace, J. C., and Keech, D. B. (1976) Eur. J. Biochem. 62, 125-130
38. Smith, R., and Scarborough, G. A. (1984) Anal. Biochem. 138, 156-163
39. Scarborough, G. A. (1988) Methods Enzymol. 157, 574-579
40. Patterson, M. S., and Greene, R. C. (1968) Anal. Chem. 37, 854-857
41. Bensadoun, A., and Weinstein, D. (1976) Anal. Chem. 70, 241-250
42. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) 193, 265-275
43. Scarborough, G. A. (1977) Arch. Biochem. Biophys. 180, 384-393
44. Addison, R., and Scarborough, G. A. (1982) J. Biol. Chem. 257, 10421-10426
45. Chadwick, C. C., Goormaghtigh, E., and Scarborough, G. A. (1987) Arch. Biochem. Biophys. 252, 348-356
46. Goormaghtigh, E., Chadwick, C., and Scarborough, G. A. (1986) J. Biol. Chem. 261, 7466-7471
47. Brooker, R. J., and Slayman, C. W. (1983) J. Biol. Chem. 258, 8833-8838
48. Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2346-2356
49. Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2357-2366
50. Matsuo, K., and Kinosita, K., Jr. (1984) J. Biol. Chem. 259, 2961-2970
51. Connor, S. E., and Grisham, C. M. (1979) Biochemistry 18, 2315-2323
52. Gantzer, M. L., and Grisham, C. M. (1979) Arch. Biochem. Biophys. 198, 268-279
53. Grisham, C. M., and Mildvan, A. S. (1975) J. Supramol. Struct. 3, 304-313
54. Gupta, R. K., and Mildvan, A. S. (1977) J. Biol. Chem. 252, 5967-5976
55. Krasnov, H. K., and Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 282, 277-292
56. Serrano, R., Montesinos, C., and Sanchez, J. (1988) Plant Science 56, 117-122
57. Nelson, D. R., and Hanahan, D. J. (1985) Arch. Biochem. Biophys. 236, 720-730
58. Schütz, G. E., and Schirmer, R. H. (1979) Principles of Protein Structure, 2nd Ed., Springer-Verlag, New York