Effect of Membrane Voltage on the Plasma Membrane H\(^{+}\)-ATPase of *Saccharomyces cerevisiae*  

Donna Seto-Young and David S. Perlin

From the Department of Biochemistry, Public Health Research Institute, New York, New York 10016

A novel system for generating large interior positive membrane potentials in proteoliposomes was used to examine the effects of membrane voltage on reconstituted plasma membrane H\(^{+}\)-ATPase from *Saccharomyces cerevisiae*. The membrane potential-generating system was dependent upon the lipophilic electron carrier tetracyanoquinodimethane, located within the bilayer, to mediate electron flow from vesicle entrapped ascorbate to external K\(_2\)Fe(CN)\(_6\). Membrane potential formation was followed by the potential-dependent probe oxonol V and was found to rapidly reach a steady-state which lasted at least 90 s. A membrane potential of approximately 254 mV was determined under optimal conditions and ATP hydrolysis by wild-type H\(^{+}\)-ATPase was inhibited from 34 to 46% under these conditions. In contrast, membrane potential had little effect on *pma1-105* mutant enzyme suggesting that it is defective in electrogenic proton translocation. Applied membrane voltage was also found to alter the sensitivity of wild-type enzyme to vanadate at concentrations less than 50 μM. These data suggest a coupling between the charge-transfer and ATP hydrolysis domains and establish a solid basis for future probing of the electrogenic properties of the yeast H\(^{+}\)-ATPase.

The plasma membrane H\(^{+}\)-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump that is essential for nutrient uptake and intracellular pH regulation (1–4). The enzyme is a member of the P-type class of transport ATPases that includes members such as the fungal *Neurospera* H\(^{+}\)-ATPase and animal cell Ca\(^{2+}\)-ATPase, Na\(^{+}\),K\(^{+}\)-ATPase, and H\(^{+}\),K\(^{+}\)-ATPase (2, 5). The H\(^{+}\)-ATPase from *S. cerevisiae* is very closely related to the enzyme from *Neurospera*. It shows 74% direct amino acid homology (6, 7) with conservative replacements accounting for a majority of the dissimilar amino acids. When compared with the Na\(^{+}\),K\(^{+}\)-ATPase and Ca\(^{2+}\)-ATPase, the yeast H\(^{+}\)-ATPase shows about 25% overall homology, although a much higher degree of conservation is found in catalytic regions (2). The yeast H\(^{+}\)-ATPase consists of a single *Mr* = 100,000 subunit (8, 9). Like other members of its class, it forms a phosphorylated intermediate that exists in at least two forms, *E*\(_1\)P and *E*\(_2\)P (10, 11), and is sensitive to the inhibitor vanadate.

Most of our understanding about electrogenic proton transport by the fungal H\(^{+}\)-ATPase derives from direct electrochemical examination of whole cells from *Neurospera* by Slayman and colleagues (12). The *Neurospera* H\(^{+}\)-ATPase is a principal contributor to the overall cellular membrane potential which routinely exceeds −250 mV, interior negative. Whole-cell studies established detailed current-voltage relationships for the pump which, when analyzed using appropriate reaction-kinetic models, provided approximations for rate constants in the transport cycle (13, 14). Unlike *Neurospera*, yeast cells are relatively small and a direct electrophysiological investigation of proton pump current has not been possible. Instead, the electrogenic, of the yeast H\(^{+}\)-ATPase has been inferred from indirect measurements of membrane potential in whole cells (15), isolated plasma membrane vesicles (16), and reconstituted liposomes (17, 18). As yet, little is known about how membrane voltage affects the mechanistic properties of the yeast H\(^{+}\)-ATPase. In principal, it should be possible to analyze membrane voltage effects *in vitro* by examining purified enzyme reconstituted into either liposomes or planar bilayers. However, practical difficulties encountered in generating large and sustained membrane potentials (>100 mV) in proteoliposomes and problems of stable incorporation of transport enzymes into planar bilayers have hindered investigations of membrane voltage interactions.

The yeast H\(^{+}\)-ATPase is an extremely attractive enzyme to analyze charge-transfer reactions because its gene, *PMA1*, can be genetically manipulated by both classical and *in vitro* mutagenesis techniques (19–21), and numerous *pma1* mutants currently exist. One class of UV-induced *pma1* mutants, which was isolated on the basis of growth resistance to hygromycin B (HygB\(^{B}\)), is interesting because the mutants appear to be depolarized in cellular membrane potential (22). H\(^{+}\)-ATPases from the HygB\(^{B}\)-*pma1* mutants showed a variety of biochemical defects (23) and included mutants that were postulated to be defective in electrogenic proton transport (22, 23).

In an attempt to examine the response of wild-type and mutant H\(^{+}\)-ATPases to membrane voltage, we have developed a simple experimental system for generating large membrane potentials in liposomes reconstituted with purified enzyme. This system depends on electron flow from ascorbate to K\(_2\)Fe(CN)\(_6\) via the membrane-embedded electron carrier tetracyanoquinodimethane (TCNQ)\(^1\) to generate large interior positive membrane potentials and readily permits the catalytic properties of the H\(^{+}\)-ATPase to be examined in response to imposed voltage. In this study, we demonstrate that membrane voltage influences the rate of ATP hydrolysis by the H\(^{+}\)-ATPase and its sensitivity to vanadate. Furthermore, we provide evidence that the electrogenic properties of enzyme

\(^1\)The abbreviations used are: TCNQ, tetracyanoquinodimethane; MEGA-8, octanoyl-N-methylglycylamide; EGTA, (ethylenebis(oxyethylenenitri1)tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

(Received for publication, May 9, 1990)
from the HygB' mutant pma1-105 are altered relative to wild type.

EXPERIMENTAL PROCEDURES

RESULTS

Membrane Potential Formation in Vesicles—In an effort to analyze the effects of membrane voltage on H+-ATPase in vitro, a vesicle system was developed for sustained membrane potential generation. This system, based on the planar bilayer study of Tien and Lojewska (24), is described schematically in Fig. 1. Electron flow from ascorbate (inside liposomes) to K₃Fe(CN)₆ (outside liposomes) is mediated via the lipophilic electron carrier TCNQ. The addition of K₃Fe(CN)₆ to reconstituted TCNQ-containing liposomes preloaded with ascorbate and K⁺-gluconate resulted in the formation of a membrane potential (interior positive) that was readily followed by the potential-dependent probe oxonol V (Fig. 2). A typical experiment is illustrated in Fig. 2B. Upon addition of K₃Fe(CN)₆ to a vesicle suspension, a characteristic fluorescence quenching phase was observed, followed by a brief steady-state phase and then a slow recovery phase. Fluorescence quenching was fully collapsed by addition of the K⁺ ionophore valinomycin to K⁺-loaded vesicles (Fig. 2B) or the permeant anion SCN⁻ (Fig. 2C). Liposomes pretreated with valinomycin were unable to form a sustained membrane potential (Fig. 2A) and no membrane potential was formed when excess external ascorbate (>7.5 mM) was present to short circuit electron flow from inside to outside (not shown).

Nearly identical behavior was observed for liposomes and proteoliposomes reconstituted with protein at 20 pg/ml. When the amount of protein incorporated was increased 10-fold (200 µg/ml), there was no effect on the level of fluorescence quenching but the rate of fluorescence recovery increased by about 2-fold. Anions such as gluconate, Cl⁻ and NO₃⁻ at concentrations up to 100 mM had little effect on membrane potential formation or recovery from the steady state and were apparently weakly permeant in this vesicle system.

Estimation of Membrane Potential—The calibration of fluorescent potential-dependent probes has proved extremely difficult (25). In this study, the calibration of oxonol V fluorescence responses was complicated by the fact that steady-state potentials were relatively short-lived, and independent calibrations procedures, such as equilibrium ion distribution, could not be applied. We have used a variation of the red cell null point titration method (26) to circumvent these problems. In this assay, a K⁺ concentration gradient is imposed to prevent valinomycin-induced collapse of the membrane potential (oxonol V fluorescence recovery) at the steady state. It is assumed that at the null point there is no net charge movement and the K⁺ diffusion potential is equivalent to the K₃Fe(CN)₆-induced potential.

A near linear relationship was observed between oxonol V fluorescence quenching in the range between 10 and 45% quenching and measured membrane potential as determined from null point titration (Fig. 3). The level of measured membrane potential was consistent from one batch of vesicles to the next and was independent of the component variable (ascorbate, TCNQ, or K₃Fe(CN)₆) used to modulate potential. In addition, the calibration behavior of liposomes and reconstituted vesicles was indistinguishable. Under conditions of maximum membrane potential formation, the average potential was estimated to be 254 ± 3 mV (n = 9). This relatively high potential is consistent with related experiments on
FIG. 3. Relationship between oxonol V quenching and membrane potential. Liposomes prepared with 0.2 mg/ml TCNQ, 50 mM HEPES-NaOH, pH 7.2, 0.1 mM K+-gluconate, 100 mM Na+-gluconate, 100 mM ascorbate, and 1 mM DTT were incubated in a 2-ml reaction buffer containing K+- and Na+-gluconate buffer, pH 6.5, as above. K₃Fe(CN)₆-induced membrane potential formation was monitored by the oxonol V fluorescence quenching assay under the following conditions. Ascorbate in the medium was varied from 0.5 to 7.5 mM ( ), K₃Fe(CN)₆ used to initiate the reaction was varied from 2.5 to 625 mM ( ), liposomes were prepared with 0.05–0.2 mg/ml TCNQ ( ), liposomes were prepared with 0.05 to 0.2 mg/ml TCNQ, and 2 mM ascorbate was present in the medium (+). Null point titration of liposomes was used to estimate the magnitude of the membrane potential under each condition.

Liposomes and reconstituted vesicles were found to be nearly identical in null point assays which was expected from their virtually indistinguishable profiles in oxonol V fluorescence quenching assays (not shown). However, since prohibitively large amounts of purified protein (10–20 mg/experiment) would be required to produce enough reconstituted vesicles for routine null point determinations, it was necessary to use liposomes prepared under identical conditions to the reconstituted vesicles for routine measurements (see “Experimental Procedures”).

Effect of Membrane Potential on ATP Hydrolysis—The formation of a large membrane potential in reconstituted proteoliposomes permitted an examination of membrane voltage effects on ATP hydrolysis by the H⁺-ATPase. It was determined in control experiments that steady-state membrane potential formation started to recover after 90 s, and to avoid problems with decaying potentials ATP hydrolysis by reconstituted enzyme was measured over a 60-s time interval with a sensitive [γ-³²P]ATP hydrolysis assay. Table I shows that in three separate experiments K₃Fe(CN)₆-induced membrane potential formation produced maximum potentials of approximately 254 mV, as determined by null point titration. ATP hydrolysis under these conditions was inhibited by 34, 46, and 35%, respectively. In the absence of TCNQ, no membrane potential was formed, and the addition of K₃Fe(CN)₆ resulted in a slight enhancement of ATP hydrolysis. When the membrane potential was reduced from 254 to 75 mV by increasing the amount of external ascorbate from 0.5 to 5 mM, the extent of ATPase inhibition progressively declined.

To exclude the possibility that proton liberation from the oxidation of intravesicular ascorbate was responsible for the K₃Fe(CN)₆-induced inhibition of enzyme activity, NH₄Cl was included in the reaction assay to collapse any interior acid pH gradient (23). NH₄Cl produced a slight enhancement of inhibition which was consistent with its ability to slightly increase membrane potential (not shown). These results suggest that membrane potential is responsible for reducing ATP hydrolysis by the enzyme.

Membrane Potential Alters Vanadate-induced Inhibition—Vanadate inhibits P-type ATPases by acting as a terminal transition state analog (27, 28), and this inhibition may be voltage-sensitive (12). The formation of a large membrane potential by TCNQ-reconstituted H⁺-ATPase vesicles allowed experiments to test the effect of membrane voltage on the interaction of vanadate with the enzyme. In the presence of a membrane potential of approximately 254 mV determined by null point titration, the H⁺-ATPase was less sensitive to vanadate in the concentration range from 2.5 to 20 μM than in its absence (Fig. 4). At vanadate concentrations above 50 μM (Fig. 4, inset), the enzyme was equally sensitive in the presence or absence of the potential. To eliminate the possibility that K₃Fe(CN)₆ competes with vanadate for a phosphate-binding site, vesicles were prepared without TCNQ such that they could not form a membrane potential in the presence of K₃Fe(CN)₆. Under these conditions, K₃Fe(CN)₆ had no effect on vanadate-induced inhibition (not shown). Identical results were obtained when suboptimal amounts of TCNQ (0.02 mg/ml) were present. These results confirm that membrane potential was responsible for the protection from vanadate-induced inhibition.

Mutant Enzyme pm1-105 Is Unaffected by Membrane Voltage—The formation of large membrane potentials in vesicles reconstituted with H⁺-ATPase should greatly aid the analysis of mutant enzymes with possible defects in electrogenic proton transport. To illustrate the application of this system, wild-type enzyme and pm1-105 mutant enzyme, which has been suggested to be defective in electrogenic H⁺-transport (22, 23), were reconstituted with varying amounts of TCNQ. As the membrane potential increased from 0 to 254 mV, there was a progressive inhibition of ATP hydrolysis by wild-type enzyme from 0 to 46% (Fig. 5). In contrast, mutant enzyme pm1-105 showed little effect under the identical conditions. Parallel experiments showed that equivalent levels of mem-

\[ \text{H. Tien, personal communication.} \]
brane potential were generated in the calibrating liposomes and vesicles reconstituted with either wild-type or pmal-105 enzyme. The effect of membrane potential on H⁺-ATPase from pmal-105 suggests that a voltage-sensitive partial reaction(s) is altered in the mutant enzyme.

**DISCUSSION**

A simple system for generating large membrane potentials in reconstituted liposomes has been described and used to study the effects of membrane voltage on the plasma membrane H⁺-ATPase from yeast. A strong correlation was observed between the level of membrane potential formed and the rate of ATP hydrolysis (Table I, Fig. 5). Under conditions of maximal membrane potential formation, estimated to approach 254 mV (interior positive), ATP hydrolysis was reduced 46% (Table I).

The relatively high potential required for 50% inhibition is consistent with the reported current-voltage behavior of the closely related *Neurospora* H⁺-ATPase. In whole-cell electrophysiological experiments, the *Neurospora* H⁺-ATPase had a characteristic current-voltage profile that showed relative voltage insensitivity over a large range (approximately 100 mV) and an extrapolated reversal or stalling potential in excess of ~320 mV (12, 14). The current-voltage relationship also indicated that a membrane potential of approximately ~200 mV would be required to reduce current flow through the enzyme by 50%.

By analogy, it is expected that the yeast H⁺-ATPase would show a wide range of voltage insensitivity and would require a membrane potential in excess of 200 mV to reduce the *V*$_{\text{max}}$ for ATP hydrolysis by 50%. The data presented in this study (Table I, Fig. 5) support this expectation. The inhibitory effects of membrane potential on ATP hydrolysis or ³²P$_{\gamma}$-ATP exchange reactions have also been reported for other reconstituted H⁺-ATPases from *S. cerevisiae* (29) and *Schizosaccharomyces pombe* (17, 30). In these cases, membrane potentials were generated directly by the H⁺-ATPase, and voltage effects were reported as stimulations of ATP hydrolysis due to the addition of charge-carrying ionophores. The influence of membrane potential on rates of ATP hydrolysis is of physiological importance to yeast. If cells maintain a membrane potential in the range of ~200 mV, then the H⁺-ATPase should operate at a level significantly displaced from *V*$_{\text{max}}$, a factor that would facilitate rapid responses to perturbations in membrane potential or intracellular pH.

It is generally believed that voltage interacts with the charge-transfer mechanism in the membrane-embedded portion of the enzyme (13, 31). In order for voltage to alter ATP hydrolysis, there must exist a strict coupling between ATP hydrolysis and the charge-translocation mechanism. Direct support for this coupling has been obtained by showing that proton fluxes catalyzed by reconstituted H⁺-ATPase from *S. pombe* were strongly dependent on the magnitude and polarity of imposed membrane potentials (32).

If membrane voltage effects are mediated via mechanical or conformational coupling between the proton translocation and catalytic domains, then voltage might be expected to alter the distribution of conformational intermediates present during catalysis. The protective effect of voltage on vanadate-induced inhibition of the H⁺-ATPase (Fig. 4) supports this notion. Vanadate is believed to inhibit P-type ATPases by binding to the site from which phosphate is released and then...
acting as a terminal transition state analog (27, 28). Protection from vanadate-induced inhibition suggests that voltage influences the conformation states required for vanadate binding.

In the Na+,K+-ATPase, it is believed that the translocation of Na+ is the major charge-carrying step in the pumping cycle, whereas the translocation of K+ is electrically silent (33–35). Numerous studies indicate that the conformational transition E2-P(3 Na+)→E1-P·3Na+ is voltage sensitive and may be at least partially rate limiting (36–38). In the yeast H+-ATPase, if H+ binds to the E1-P form of the enzyme and is released following transition to E2-P, then voltage should alter ATP hydrolysis by slowing the transition to E2-P and the subsequent release of H+. In this scheme, the enzyme should also be less sensitive to vanadate because there would be fewer E2 intermediates available for vanadate binding. Finally, voltage should increase the level of steady-state phosphorylated intermediate which is normally less than 10% of the total enzyme pool (10).

The apparent voltage insensitivity observed for enzyme from mutant strain pmal-105 (Fig. 5) is consistent with the generalized depolarization of cellular membrane potential observed for this strain (22). The pmal-105 enzyme, which is fully competent in ATP-driven proton transport, has a Vmax at pH 6.5 approximately 40% lower than wild-type enzyme and is highly insensitive to vanadate-induced inhibition (25). A Ser→Phe substitution near the site of phosphorylation (Asp59) is believed to confer mutant enzyme behavior by altering the availability of E2 intermediates (23). One explanation for voltage insensitivity of the pmal-105 enzyme may be that it already exists in a state limited by the distribution of catalytic intermediates and is therefore less susceptible to modulation by voltage.

Alternatively, electroneutral H+-transport by the pmal-105 enzyme may reflect an alteration in the translocation mechanism. For example, H+ translocation in one direction may be accompanied by compensating charge movement in the other direction. The gastric H+,K+-ATPase is electroneutral K' conductance for strain pmal-105 was shifted to lower Vmax (40). In the Na+,K+-ATPase, electroneutral Na+ transport observed in the absence of K+ has also been postulated to involve counter transport of H+ (38). Recently, Ramirez et al. (41) reported the existence of an ATP-activated, voltage-dependent K+ conductance in patch-clamped membranes from wild-type and pmal mutant strains of yeast. Interestingly, it was shown that activation of the K+ conductance for strain pmal-105 was shifted to lower voltages by ATP. It was suggested that the K+ conductance may be physically associated with the H+-ATPase and could represent an inefficient H+/K+ transporter or a closely linked voltage-regulated channel (41). H+-ATPase-mediated K+ transport has been demonstrated in vitro (16, 32), although K+ transport was usually less efficient than corresponding H+ transport. Since ATP hydrolysis and H+ transport by wild-type enzyme approach Vmax in the absence of K+, it does not appear likely that a K+ conductance pathway could be strictly coupled to H+ transport. However, a change in the properties of the K+ conductance pathway due to the pmal-105 mutation could permit sufficient K+ counterflow during the primary H+ transport to confer apparent electroneutrality.

In summary, a new vesicle system was described for analyzing membrane voltage effects on the H+-ATPase from yeast plasma membranes. It was demonstrated that wild-type enzyme is electrically silent since membrane voltage interacts with the enzyme to alter ATP hydrolysis and its sensitivity to inhibition by vanadate. These data have been interpreted in terms of a redistribution of catalytic intermediates that results from applied voltage. Furthermore, it was demonstrated that the enzyme from strain pmal-105 is relatively insensitive to voltage and that electroneutrality might involve counter ion flow. Finally, we believe this report provides a basis for the future probing of pmal mutant enzymes with membrane voltage. This approach should greatly enhance our understanding of electrogenic ion transport by the H+-ATPase and related enzymes.

Acknowledgments—We would like to thank J. E. Haber and S. L. Harris for helpful discussions and B. C. Monk for critical reading of the manuscript.

REFERENCES

1. Goffeau, A., and Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197–222
2. Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28
3. Serrano, R. (1986) Curr. Top. Cell. Reg. 23, 87–127
4. Portillo, F., and Serrano, R. (1988) Eur. J. Biochem. 166, 501–507
5. Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986) Nature 319, 689–693
6. Hager, K. M., Mandaia, S. M., Davenport, J. W., Speicher, D. W., Benz, E. J., and Slayman, C. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7693–7697
7. Addison, R. (1986) J. Biol. Chem. 261, 14866–14901
8. Dufour, J.-P., and Goffeau, A. (1978) J. Biol. Chem. 253, 7026–7032
9. Malpartida, F., and Serrano, R. (1980) FEBS Lett. 111, 69–72
10. Malpartida, F., and Serrano, R. (1981) Eur. J. Biochem. 116, 413–417
11. Bashford, C. L., and Smith, J. C. (1979) J. Biol. Chem. 254, 7361–7368
12. Slayman, C. W. (1987) J. Bioenerg. Biomembr. 19, 1–20
13. Hansen, U.-P., Gradmann, D., Sanders, D., and Slayman, C. L. (1981) J. Membr. Biol. 63, 165–190
14. Gradmann, D., Hansen, U.-P., and Slayman, C. L. (1982) Curr. Top. Membr. Biol. Trans. 18, 257–276
15. Borst-Pauwels, G. W. F. H. (1981) Biochim. Biophys. Acta 650, 88–127
16. Calahorra, M., Ramirez, J., Clemente, M., and Peña, A. (1987) Biochim. Biophys. Acta 899, 229–238
17. Dufour, J.-P., Goffeau, A., and Tsong, T. Y. (1982) J. Biol. Chem. 257, 9695–9701
18. Villalobo, A., Boutry, M., and Goffeau, A. (1981) J. Biol. Chem. 256, 12081–12087
19. Cid, A., and Serrano, R. (1988) J. Biol. Chem. 263, 14134–14139
20. Portillo, F., and Serrano, R. (1988) EMBO J. 7, 1793–1798
21. McCusker, J. H., Perlin, D. S., and Haber, J. E. (1988) Mol. Cell. Biol. 7, 4082–4086
22. Perlin, D. S., Brown, C. L., and Haber, J. E. (1988) J. Biol. Chem. 263, 18118–18122
23. Perlin, D. S., Harris, S. L., Seto-Young, D., and Haber, J. E. (1989) J. Biol. Chem. 264, 21857–21864
24. Tian, H. T., and Lojewska, Z. K. (1984) Biochim. Biophys. Acta 789, 229–238
25. Bashford, C. L., and Smith, J. C. (1979) Methods Enzymol. 55, 589–596
26. Hoffman, S. C., and Laris, P. C. (1974). J. Physiol. 239, 519–552
27. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) J. Biol. Chem. 253, 7361–7368
28. Pick, U. (1982) J. Biol. Chem. 257, 6101–6109
29. Malpartida, F., and Serrano, R. (1981) J. Biol. Chem. 256, 4175–4177
30. Villalobo, A. (1982) J. Biol. Chem. 257, 1824–1828
31. Läuger, P. (1987) Physiol. Rev. 67, 1296–1331
32. Villalobo, A. (1984) Can. J. Biochem. Cell Biol. 62, 865–877
33. DeWeer, P., Gadaby, D. C., and Rakowski, R. F. (1988) Annu. Rev. Physiol. 50, 225–241
34. Gadaby, D. C., Nakao, M., and Bahninski, A. (1989) Mol. Cell. Biochem. 89, 141–149
35. Bahninski, A., Nakao, M., and Gadaby, D. C. (1988) Proc. Natl.
Voltage Effects on H+-ATPase

Acad. Sci. U. S. A. 845, 3412-3416

Sturmer, W., Apell, H.-J., Wuddel, I., and Lauger, P. (1989) J. Membr. Biol. 110, 67-86

Rephaeli, A., Richards, D., and Karlsh, S. J. D. (1986) J. Biol. Chem. 261, 6248-6254

Goldshleger, R., Shahak, Y., and Karlsh, S. J. D. (1990) J. Membr. Biol. 113, 139-164

Lorentzon, P., Sachs, G., and Wallmark, B. (1988) J. Biol. Chem. 263, 10705-10710

Dixon, D. A., and Haynes, D. H. (1989) J. Membr. Biol. 112, 169-183

Ramirez, J. A., Vaca, V., McCusker, J. H., Haber, J. E., Mortimer, R. K., Owen, W. G., and Lecar, H. (1989) Proc. Natl. Sci. U. S. A. 86, 7866-7870

36. Perlin, D. S., and Brown, C. L. (1987) J. Biol. Chem. 262, 6788-6792

37. Perlin, D. S., Kasamo, K., Brooker, R. J., and Slayman, C. W. (1984) J. Biol. Chem. 259, 7884-7892

38. Kaplan, R. S., and Pedersen, P. L. (1986) Anal. Biochem. 150, 97-104

Supplementary Material To:
Effect of Membrane Voltage on the Plasma Membrane H+-ATPase

Sanna Seto-Young, and David S. Perlin

EXPERIMENTAL PROCEDURES

Materials
Mega-ATP and oxygencase derivatives were obtained from Reicherser Munkem. [3-3H]GTP was from New England Nuclear. Crotone phosphatase was obtained as a 10x Fraction III extract from Sigma. Ammonium-merthoxy phospholipids and phospholipids were obtained from Avantis. Lipids. Teramycin was from Collaborative. Goat IgG was from Miles Proteins. KC1 was obtained from Kelco and all other reagents were from Sigma.

Preparation of plasma membranes
A microosomal fraction isolated by the method of Perlin and Brown (14) was further purified by a deoxycholate step gradient. The microsomes were suspended at 10 mg/mal in 10 mM Tris HC1, pH 7.5, 10% (v/v) glycerol, 1 mM EDTA, 0.5% PEG-6000, and 5 µl of the suspension was carefully layered onto a 4 ml step gradient consisting of 5 ml of 55, 7.5 ml of 10%, and 5 ml of 20% (w/v) sucrose in a buffer containing 10 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mM EDTA. The gradient was centrifuged for 60 min in an SW 40 rotor.

Purification and reconstitution of H+-ATPase
The purity of the crude plasma membranes was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The membranes were solubilized in a 2:1 ratio of buffer containing 100 mM KC1, 50% glycerol, 10% sucrose, and 1 mM EDTA. The membranes were then centrifuged for 60 min at 4°C. The supernatant was collected and assayed for H+-ATPase activity.

Membrane permeability determination
To determine the effect of membrane voltage on the plasma membrane H+-ATPase, the assays were performed in a 1:1 ratio of buffer containing 100 mM KC1, 50% glycerol, 10% sucrose, and 1 mM EDTA. The membranes were then centrifuged for 60 min at 4°C. The supernatant was collected and assayed for H+-ATPase activity.

Membrane Permeability Determination
A full factorial design was used to study the effects of membrane permeability on the plasma membrane H+-ATPase. The permeability of the membrane was determined by the method of Perlin et al. (23). Plasma membranes purified by sucrose density gradient centrifugation were suspended in 1 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mg/ml of bovine serum albumin. The suspension was centrifuged at 15,000 x g for 30 min. The supernatant was collected and assayed for H+-ATPase activity.

Membrane Permeability Determination
A full factorial design was used to study the effects of membrane permeability on the plasma membrane H+-ATPase. The permeability of the membrane was determined by the method of Perlin et al. (23). Plasma membranes purified by sucrose density gradient centrifugation were suspended in 1 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mg/ml of bovine serum albumin. The suspension was centrifuged at 15,000 x g for 30 min. The supernatant was collected and assayed for H+-ATPase activity.

Membrane Permeability Determination
A full factorial design was used to study the effects of membrane permeability on the plasma membrane H+-ATPase. The permeability of the membrane was determined by the method of Perlin et al. (23). Plasma membranes purified by sucrose density gradient centrifugation were suspended in 1 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mg/ml of bovine serum albumin. The suspension was centrifuged at 15,000 x g for 30 min. The supernatant was collected and assayed for H+-ATPase activity.

Membrane Permeability Determination
A full factorial design was used to study the effects of membrane permeability on the plasma membrane H+-ATPase. The permeability of the membrane was determined by the method of Perlin et al. (23). Plasma membranes purified by sucrose density gradient centrifugation were suspended in 1 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mg/ml of bovine serum albumin. The suspension was centrifuged at 15,000 x g for 30 min. The supernatant was collected and assayed for H+-ATPase activity.

Membrane Permeability Determination
A full factorial design was used to study the effects of membrane permeability on the plasma membrane H+-ATPase. The permeability of the membrane was determined by the method of Perlin et al. (23). Plasma membranes purified by sucrose density gradient centrifugation were suspended in 1 mM Hepes HC1, pH 7.5, 10% glycerol, and 5 mg/ml of bovine serum albumin. The suspension was centrifuged at 15,000 x g for 30 min. The supernatant was collected and assayed for H+-ATPase activity.
Voltage Effects on H+-ATPase

Calibration of membrane potential by null point titration. Membrane potential formation was followed using the oxonol-V fluorescence quenching assay, as described under "Experimental Procedures" and was estimated by determining the K+ concentration gradient necessary to limit valinomycin-induced recovery of oxonol-V fluorescence at the steady-state. Liposomes prepared with TCNQ at 0.2 mg/ml were preloaded with 0.1 mM K+ gluconate buffer and added to a 2 ml oxonol-V fluorescence quenching medium containing K+ at 0.1, 10, 100, 500, 1250, 2000 mM (curves 1-7, respectively). K$_{3}$Fe(CN)$_{6}$ (5 mM) was added to initiate the reaction and valinomycin (0.1 µM) was added at the steady-state.

Fig. 6. Gelsmassie-stained SDS gel of reconstituted H+-ATPase. The H+-ATPase was purified and reconstituted as described under "Experimental Procedures" and 15 µg of reconstituted protein was applied to the SDS gel. The M$_{r}$ of PAGE standards are as indicated.

Fig. 7. Fluorescence quenching as a function of lipid/oxonol V concentrations. Reconstituted TCNQ liposomes were prepared as described under "Experimental Procedure." The optimal lipid/oxonol ratio for K$_{3}$Fe(CN)$_{6}$-induced quenching of oxonol-V fluorescence was determined by either varying the amount of liposomes (25-150 µg) with a fixed oxonol-V concentration (1 µM) (— - —) or by maintaining a fixed liposome amount (250 µg) and varying the oxonol-V concentration (0.5 to 20 µM) (— — —).