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(U) Membrane Voltage Effects on Proton Transport by a Yeast H^+-ATPase

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The objective of this study is to examine structural mechanisms for proton transport and membrane voltage interactions by the plasma membrane H^+-ATPase from Saccharomyces cerevisiae. H^+-ATPase mutants (pma1), generated by random and site-directed mutagenesis techniques, have been isolated that cause deploarization of the cellular membrane potential. Three loci, one within a putative transmembrane domain (Gly158) and the other two (Ser368, Pro640) within putative membrane/cytoplasmic interface domains, were found to cause the most prominent effect on cellular membrane potential. All pma1 mutant enzymes were active in proton transport, although one mutant, Gly158-->Asp, appeared to be partially uncoupled from ATP hydrolysis. The locus causing the most severe effect on membrane potenial, Ser368, was subjected a detailed revertant and site-directed mutagenesis analysis. Amino acid substitutions of Ile, Val or

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Dr. Igor Vodyanoy	202 696-4956	ONR

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19. ABSTRACT (cont.)

Leu resulted in membrane potential depolarizations. Finally, to examine the effects of membrane voltage on mutant enzymes, a new procedure was developed to produce large and sustained membrane potentials in reconstituted proteoliposomes. In the next year, we plan to examine the role of membrane-embedded charged residues in proton transport and to study the voltage dependence of proton transport and ATP hydrolysis in existing and new mutant enzymes.

INTRODUCTION

The objective of this project is to define protein structure domains of the plasma membrane H^+-ATPase from yeast that are involved with proton transport and membrane voltage interactions. Our approach has been to isolate mutants of the H^+-ATPase (pma1) that alter one or more of the basic transport properties of the enzyme and then identify specific amino acid alterations.

The yeast H^+-ATPase is an electrogenic proton pump that plays a vital role in nutrient uptake and intracellular pH regulation, and the gene encoding this enzyme, PMA1, is essential for growth. The cellular importance of the H^+-ATPase requires that viable pma1 mutants can only arise from mutations resulting in partially active or conditionally inactive enzymes. In collaboration with Dr. James E. Haber (Brandeis University), we described a positive selection procedure, based on resistance to the aminoglycoside antibiotic hygromycin B, for isolating partially defective H^+-ATPases (McCusker, J.E., Perlin, D.S. and Haber, J.E. 1987 Mol. Cell. Biol. 7, 4082-4088). The original screen resulted in the isolation of 75 pma1 mutants and the first year of this project was spent characterizing the biochemical and transport properties of these mutant enzymes. The mutant H^+-ATPases showed diverse biochemical phenotypes (Km, Vmax, pH optima, inhibitor sensitivity, etc..). Yet, despite their biochemical differences, all of the mutant enzymes shared the common property of inducing strong depolarizations of cellular membrane potential (Perlin, D.S., Brown, C.L. and Haber, J.E. 1988 J. Biol. Chem. 263, 18118-18122). The pma1 mutants were viewed as important for understanding electrogenic proton transport by the H^+-ATPase and the past year was spent identifying their genetic defects, as well as their individual proton transport characteristics.

PROGRESS REPORT

1. Genetic defects of pma1 mutants. The most severely affected mutant alleles displaying membrane potential defects were cloned and sequenced. Single base-pair changes were found in pma1-105, pma1-147, pma1-141 and pma1-114 that resulted in amino acid substitutions of Ser368-->Phe, Pro640-->Leu, Ser368-->Phe and Gly158-->Asp, respectively. According to a recent proposed topographical model for the H^+-ATPase (Serrano, R. 1988 Biochim. Biophys. Acta 947, 1-28), Gly158 is expected to be buried within a transmembrane helical domain, while Ser368 and Pro640 lie within a large catalytic domain. Both residues are predicted to be close to the membrane/cytoplasmic interface. In the course of cloning and sequencing pma1 mutants, six amino acid substitutions, Pro74-->Leu, Val209-->Ile, Lys444-->Met, Ser479-->Phe, Ala480-->Val and Ala836-->Ser were identified in the Y55 wildtype background strain which had no apparent effect on enzyme function.

2. [illegible] in membrane potential depolarization. A mutation affecting Ser368 was found to cause one of the most severe phenotypes. To further examine the influence of this residue on steady-state membrane potential formation, a detailed revertant and site-directed mutagenesis approach was used to create numerous amino acid

substitutions. The results indicated that replacement of Ser368 with Phe, Val or Leu led to a marked depolarization of cellular membrane potential. Interestingly, Phe, Val and Leu substitutions resulted in a range of biochemical properties. The most prominent effect was seen by their sensitivity to the mechanistic inhibitor vanadate (Fig. 1). The Phe368 mutant is vanadate insesitive, the Val368 mutant enzyme is vanadate sensitive and the Leu368 mutant enzyme is intermediate in sensitivity. Wildtype enzyme, a second vanadate-insensitive mutant allele, Leu640, and a vanadate-sensitive mutant allele, Asp158 are included for comparison.

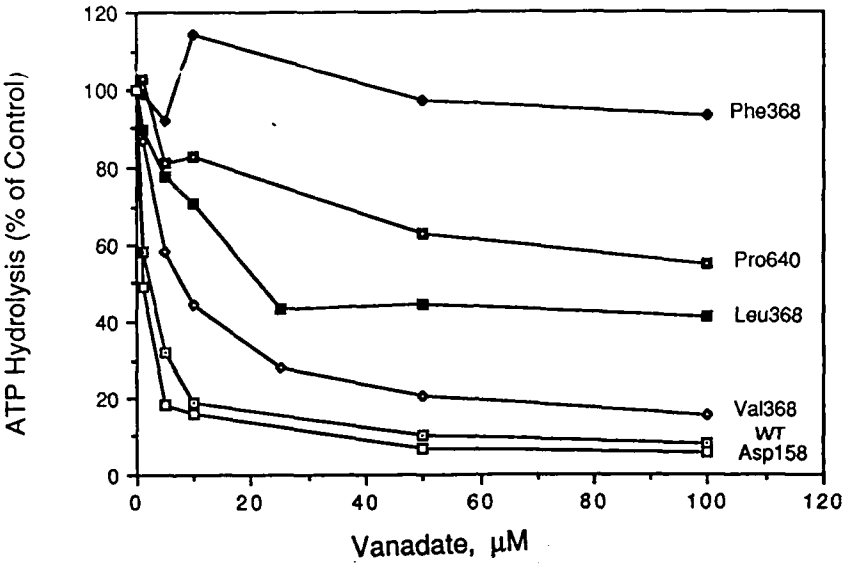

Fig. 1 Effect of vanadate on ATP hydrolysis by mutant enzymes.

3. Proton transport by mutant enzymes. Our initial assessment of proton transport by mutant enzymes relied on whole cell measurements of H^+-ATPase-dependent medium acidification. To more precisely define proton transport by the mutant enzymes, we developed a purification and reconstituion procedure that results in recovery of reconstituted enzyme at greater that 85% purity with nearly 100% of its initial actvity. When reconstituted, all mutant enzymes formed ATP-induced pH gradients, as determined by fluorescence quenching of the pH gradient probe acridine orange. Proton transport in K^+-loaded vesicles was found to be optimal in the presence of valinomycin which eliminated any transient membrane potential formation by allowing for compensating charge movement. When mutant enzymes, as illustrated for pma1-105 (Fig. 2A), were allowed to form transient membrane potentials in the absence of valinomycin, there was a pronounced decline in the apparent rate of proton transport relative to wildtype. The addition of valinomycin restored pH gradient formation to its optimal level. These effects are suggestive of an altered voltage sensitivity by the mutant enzyme. It was also observed that proton transport by pma1-114 mutant enzyme was significantly less than that of wildtype or other mutant enzymes with identical ATP turnover rates (Fig. 2B). The possiblity is raised that this represents a partially uncoupled mutant.

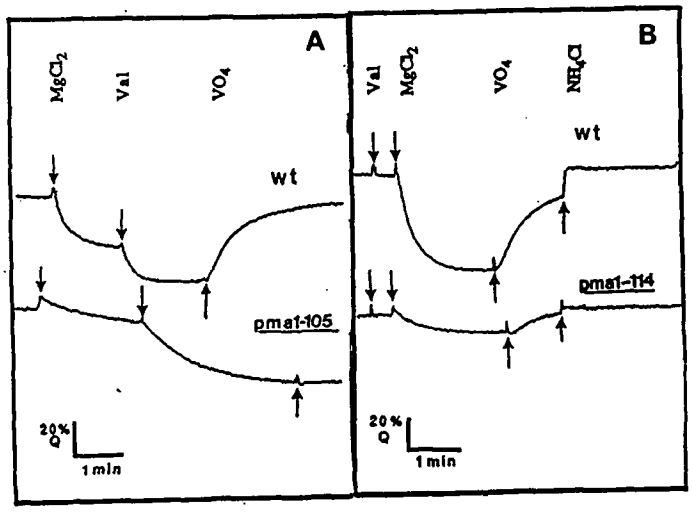

Fig. 2 Proton transport by reconstituted wildtype and pma1 mutant enzym[redacted]. The quenching of acridine orange fluorescence was used to assess interior acid pH gradient formation by reconstituted wildtype and mutant enzymes. The reaction medium contained 10mM HEPES-KOH, pH 6.8, 50mM KCl, 5mM ATP and 10μg reconstituted protein. Proteoliposomes were preloaded with 50mM KCl. ATP-linked proton transport was initiated following addition of 5mM $MgCl_2$. All other additions were as indicated (1μM valinomycin; 10μM vanadate; 10mM ammonium chloride).

4. An in vitro assay for assessing membrane voltage effects. In an effort to analyze the effects of membrane voltage on mutant enzymes more precisely, an in vitro system was developed which allows large and sustained membrane potentials to be generated in liposomes. In this assay system, which was developed at the suggestion of Dr. H. Ti Tien (Michigan State University), electron flow from ascorbate (inside liposomes) to ferricyanide (outside liposomes) is mediated via the electron carrier TCNQ. With this procedure, we are able to generate relatively large (>150mV) and sustained interior positive membrane potentials. Fig. 3A illustrates that membrane potential formation is readily followed by the potential-dependent probe Oxonol V. In this example, membrane potential formation is initiated by the addition of ferricyanide to proteoliposomes containing ascorbate, K^+-gluconate and TCNQ. The membrane potential decays with time and can be fully collapsed by the addition of valinomycin. By varying the lipid composition, the decay kinetics can be altered considerably (Fig. 3B). In the presence of 80% E. coli lipids and 20% phosphatidylserine the rate of decay in reconstituted vesicles is sufficiently slow to allow measurements of ATP hydrolysis. Preliminary data has shown a 50% decline in ATP hydrolysis during maximum potential formation.

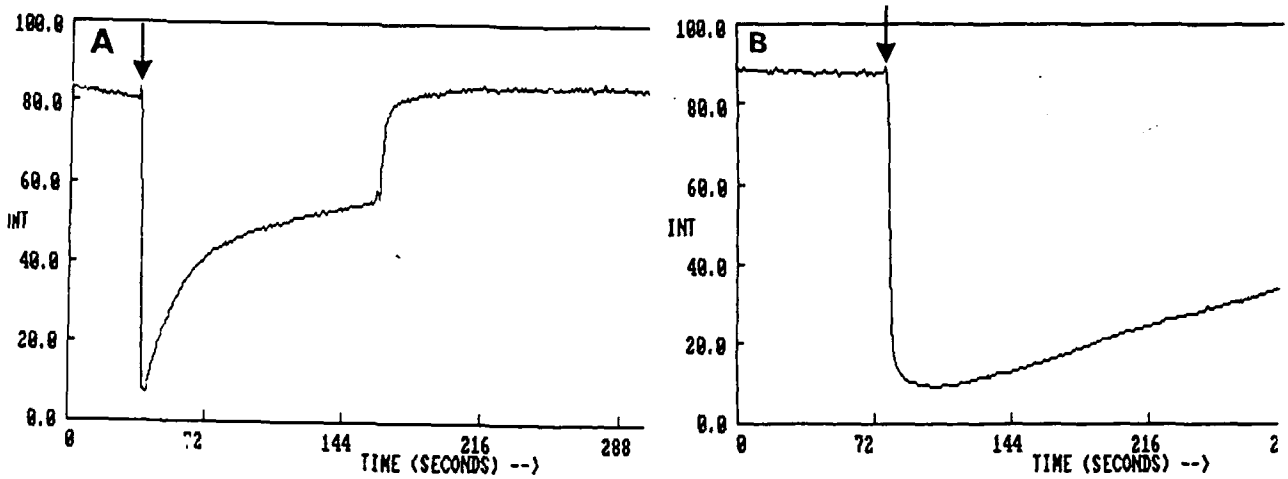

Fig. 3. Induced membrane potential formation in proteoliposomes. The quenching of oxonol V fluorescence was used to assess interior positive membrane potenial formation. Proteoliposomes (10ug) pre-equlibrated with ascorbate, K+ and TCNQ were suspended in medium containing 10mM HEPES-KOH, pH 7.0, 100mM K-gluconate and 1uM oxonol V. Ferricyanide was added to initiated membrane potential formation. Proteoliposomes were prepared with 20% asolectin, 70% E. coli lipids and 10% PS (panel A) or 80% E. coli lipids and 20% PS (panel B).

5. **Future goals.** In an effort to probe the proton translocation domain(s) more directly, site-directed and localized random mutagenesis will be used to target charged residues presumed to lie within the membrane bilayer. Mutant enzymes generated by this approach will be evaluated for basic biochemical and proton transport properties. The new mutant enzymes, as well as existing mutant enzymes, will also be examined for the effects of membrane voltage following purification and reconstitution in the newly developed ascorbate/TCNQ/ferricyanide assay.

PUBLICATIONS

Perlin, D.S., Brown, C.L. and Haber, J.E. 1988 Membrane potential defect in hygromycin B-resistant pma1 mutants of Saccharomyces cerevisiae. J. Biol. Chem. 263, 18118-18122

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