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Probing the cytoplasmic LOOP1 domain of the yeast plasma membrane H^+-ATPase by targeted factor Xa proteolysis

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Abstract

The cytoplasmic domain linking transmembrane segments 2 and 3 (LOOP1) of the yeast H^+-ATPase was probed by the introduction of unique factor Xa recognition sites. Three sites, I^{170}EGR, I^{254}EGR and I^{275}EGR, representing different structural regions of the LOOP1 domain, were engineered by site-specific mutagenesis of the *PMA1* gene. In each case, multiple amino acid substitutions were required to form the factor Xa sites, which enabled an analysis of clustered mutations. Both I^{170}EGR and I^{275}EGR-containing mutants grew at normal rates, but showed prominent growth resistance to hygromycin B and sensitivity to low external pH. The engineered I^{254}EGR site within the predicted β-strand region produced a recessive lethal phenotype, indicating that mutations G^{254}I and F^{257}R were not tolerated. Mutant I^{170}EGR- and I^{275}EGR-containing enzymes showed relatively normal K_m and V_{max} values, but they displayed a strong insensitivity to inhibition by vanadate. An I^{170}EGR/I^{275}EGR double mutant was more significantly perturbed showing a reduced V_{max} and pronounced vanadate insensitivity. The I^{170}EGR site within the putative α-helical stalk region was cleaved to a maximum of 10% by factor Xa under non-denaturing conditions resulting in a characteristic 81 kDa fragment, whereas the I^{275}EGR site, near the end of the β-strand region, showed about 30–35% cleavage with the appearance of a 70 kDa fragment. A I^{170}EGR/I^{275}EGR double mutant enzyme showed about 55–60% cleavage. The cleavage profile for the mutant enzymes was enhanced under denaturing conditions, but was unaffected by MgATP or MgATP plus vanadate. Cleavage at the I^{275}EGR position had no adverse effects on ATP hydrolysis or proton transport by the H^+-ATPase making it unlikely that this localized region of LOOP1 influences coupling. Overall, these results suggest that the local region encompassing I^{275}EGR is accessible to factor Xa, while the region around I^{170}EGR appears buried. Although there is no evidence for gross molecular motion at either site, the effects of multiple amino acid substitutions in these regions suggest that the LOOP1 domain is conformationally active, and that perturbations in this domain affect the distribution of conformational intermediates during steady-state catalysis.

Keywords: Proteolysis; Factor Xa; Plasma membrane; ATPase, H^+-; (Yeast)

1. Introduction

The plasma membrane H^+-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump that couples energy from ATP hydrolysis to the active extrusion of protons. It is an essential enzyme that plays a critical role in the maintenance of electrochemical proton gradients and the regulation of intracellular pH [1,2]. The H^+-ATPase belongs to the P-type ATPase family of ion-translocating ATPases, which are found in all plant, animal, fungal and bacterial cells, and actively transport ions such as H^+, K^+, Na^+, Ca^{2+} and Mg^{2+}. P-type enzymes form a characteristic aspartyl-phosphate intermediate and exist in two principal conformational states during catalysis [3,4]. Significant sequence similarity exists between the various P-type ATPase members, although the greatest degree of sequence homology lies within the ATP hydrolytic domain [5,6]. The topology of these enzymes is similar with the N- and C-termini residing in the cytosol [7–9], and the number of predicted transmembrane segments varying between 8 and 10 [10–15]. There is general agreement on the topology of the first four transmembrane segments, while less certainty exists with the C-terminal elements [16].

The mechanistic nature of energy coupling by P-type enzymes is not understood. The transmittal of energy from the binding and subsequent hydrolysis of ATP hydrolysis in a cytosolic catalytic domain to ion transport in a spa-

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tially distinct membrane domain is expected to involve long-range conformational interactions [4,17,18], and diverse studies involving higher eukaryotic enzymes support this notion [19–26]. Genetic evidence obtained for the yeast H^+-ATPase [27–29] also supports the concept of long-range conformational interactions. Furthermore, these studies helped define a region of the H^+-ATPase, encompassing transmembrane segments 1 (M1) and 2 (M2), that appears to be important to the coupling process. Transmembrane segments 1 and 2 are predicted to form a tightly-packed helical hairpin loop structure that is highly conformationally active [30]. Molecular dynamic simulations suggest that local perturbations in this region can be propagated over long distances which may be important for coupling to the catalytic ATP hydrolysis domain [30]. In addition, the inhibition of higher eucaryotic enzymes by therapeutic agents, which appear to interact with this region [20,26,31,32], supports the prediction that perturbations of local structure within M1 and M2 are communicated to the ATP hydrolysis domain.

How perturbations in M1 and M2 are conformationally linked to the catalytic ATP hydrolysis domain is not known. However, a likely structural candidate is the polar cytoplasmic loop domain (LOOP1) linking M2 and M3. This region is predicted to form both part of the stalk region and part of the catalytic ATP hydrolysis domain [16,33], along with the large central cytoplasmic loop domain, which links M4 and M5, and contains sites for ATP binding and phosphoryl transfer (D378). LOOP1 domains from all P-type enzymes contain several highly conserved regions [6] and share common structural motifs [34]. They are predicted to form α-helical stretches at the N- and C-terminal portions which contribute to the 'stalk' structure, and they also are suggested to form a β-strand domain that functions during catalysis [16,33]. The LOOP1 region was originally postulated to act as a transduction domain based largely on the observation that Ca^{2+} transport appeared uncoupled from ATP hydrolysis in the Ca^{2+}-ATPase following tryptic hydrolysis of R198 in the β-strand region of LOOP1 [35]. While more recent tryptic proteolysis [36,37] and genetic studies [38,39] of residues in this region suggest that the perturbed enzymes are fully coupled, this region does appear to undergo distinct conformational changes during catalysis, and these changes have been linked to cation movement in the Na^+,K^+-ATPase and Ca^{2+}-ATPase [40,41].

To further investigate structural properties of LOOP1 that are important for the function of this region, we introduced unique factor Xa recognition sites, which consist of four amino acids (IE(D)GR), into the LOOP1 domain of the yeast H^+-ATPase. These sites, located in putative α-helical and β-strand regions, allowed us to explore the solvent accessibility of structural domains of LOOP1, and to further examine the role of LOOP1 in catalytic function and/or coupling by assessing the affects of genetic and enzymatic perturbations on the H^+-ATPase.

2. Materials and methods

2.1. Yeast strains and cultures

All yeast strains utilized in this study are isogenic derivatives of Y55 (*HO gal3 MAL1 SUC1*) [42]. Wild type control strain *GW201* (*HO ade6-1 trp-5-1 arg4-1 leu2-1 lys1-1 ura3-1 pma1::ura3 / PMA1*) was obtained by selecting a wild type spore from strain SH122 (*HO ade6-1 trp5-1 leu2-1 lys1-1 ura3-1 pma1Δ::LEU2 / PMA1*) [27] that had been transformed with a 6.1 kb fragment of wild type DNA containing a *URA3*-marked *PMA1* gene [27]. All yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone and 2% dextrose, pH 5.7) at 22°C to mid-log phase ($A_{590} \sim 5$). Cell growth measurements were made in YEPD medium with constant shaking at 21°C in 200 μl aliquots of growth medium in 96 well microplates and monitored at A_{590} with a Spectra ELISA reader (Tecan SLT LabInstruments).

2.2. Site-directed mutagenesis

Site-directed *pma1* mutants were constructed essentially as described by Na et al. [28]. All *PMA1* mutations prepared in phagemid vector pGW201, which consists of a 6.1 kb *Hin*dIII fragment containing a *URA3*-marked *PMA1 gene* [27] subcloned into phagemid vector pGEM-3zf (Promega). Directed mutagenesis was performed as previously described [29] using primers 5′-CCACTGTTAAGAGAATTGAAGGTAGAATGGTTGTTACCGC for site I^{254}EGR, 5′-GGTAGAGCTGCTGCTATTGAAGGCAGAGCCGCTGGTGGTC for site I^{275}EGR and 5′-CCAAGCTGGTTCTATTGTCATTGAAGGTAGAAAGACTTTGGCTAAC ACTGC for site I^{173}EGR in the extension reaction. Isogenic *pma1* mutants were obtained by transplacing a 6.1 kb *Hin*dIII *pma1*-mutant and *URA3*-containing fragment into yeast strain SH122 (*HO ade6-1 trp5-1 leu2-1 lys1-1 ura3-1 pma1Δ::LEU2 / PMA1*) [27]. All *pma1* mutations transplaced into yeast were verified in viable Ura + spores, as described by [29].

2.3. Plasma membrane isolation and ATP hydrolysis assay

Plasma membranes were purified from wild type and *pma1* mutant strains by centrifugation on a sucrose step-gradient, as previously described [43]. ATP hydrolysis measurements were performed in microplate assay in a 100 μl volume containing 10 mM Mes/Tris pH 6.5, 25 mM NH_4Cl, 5 mM ATP, 5 mM $MgCl_2$ and 0.5–2 μg of membrane protein described, as by Monk et al. [44].

2.4. Factor Xa cleavage

A typical reaction consisted of 10 μl of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$, 25% glycerol, 6–10 μg purified membrane protein and 2 μg factor Xa

(New England BioLabs). The reaction medium was incubated at 12°C for 18 h. The level of cleavage was assessed by SDS gel electrophoresis.

2.5. Reconstitution and proton transport assays

Plasma membrane vesicles were first extracted with deoxycholate, as previously described by Seto-Young et al. [43]. The extracted membranes (250 μg) were combined with 10 mg sonicated asolectin in a 800 μl volume containing 10 mM Hepes-Tris, pH 7.0, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT , 45% (v/v) glycerol and 0.5% (w/v) deoxycholate (added dropwise). The mixture was allowed to sit at 4°C for 5 min, and then rapidly diluted into 25 ml of 10 mM Hepes-Tris, pH 7.0, 100 mM KCl and 1 mM DTT. The mixture was centrifuged at 45 000 rpm in a 50.2Ti rotor for 1 h. The pellet was resuspended in 400 μl of the dilution buffer and used fresh. Reconstituted vesicles treated with factor Xa were first washed by resuspension in 25 ml dilution buffer and then centrifuge, as above. The final pellet was resuspended in 150 μl of dilution buffer. Proton transport measurements were made according to the method of Perlin et al. [45]. A fluorescence quenching reaction volume consisted of 1 ml of 10 mM Hepes-Tris, pH 7.0, 50 mM KCl, 5 mM ATP and 1.5 μM acridine orange. The reaction was initiated by the addition of 5 mM $MgSO_4$. Fluorescence intensity was monitored on a Perkin-Elmer LS-5 spectrofluorometer.

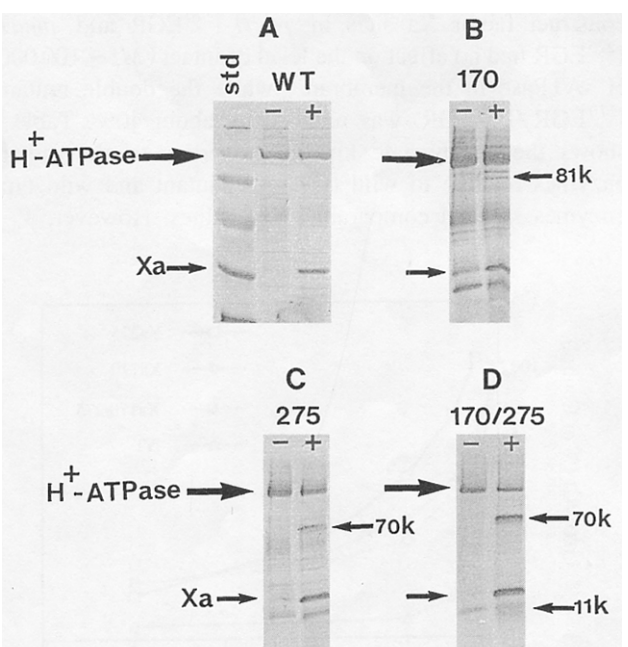

Fig. 1. Effect of factor Xa on wild type and IEGR-containing mutant H^+-ATPases. Sucrose gradient purified plasma membranes (10 μg) from wild type (A) and *pma1* mutant strains carrying mutations encoding I^{170}EGR (B), I^{275}EGR (C) and I^{170}EGR/I^{275}EGR (D) were incubated with factor Xa (2 μg) for 18 h at 12°C in a 10 μl volume consisting of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$ and 25% glycerol. The treated samples were run on 10% SDS polyacrylamide gels and stained with Coomassie blue. The intact H^+-ATPase (M_r ~ 100000) and factor Xa-induced fragments are indicated.

2.6. SDS gel electrophoresis, Western blotting and antibody purification

SDS gel electrophoresis of plasma membranes in 10% (w/v) pre-cast minigels (Bio-Rad) and semi-dry electroblotting of proteins was performed, as previously described [44]. Antibodies to the LOOP1 region were derived from a serum containing polyclonal whole anti-ATPase antibodies [29] by immuno-affinity purification utilizing a MalE fusion protein containing the LOOP1 region [44]. The antibodies were eluted with 100 mM glycine, pH 2.5, and then neutralized to pH 7.0 with Tris base.

2.7. Other procedures

Protein was determined by a modified Lowry method [45]. Yeast transformants were prepared by the lithium acetate treatment method using single-stranded carrier DNA [46], as described in Alkali-Cation Yeast Transformation Kit (BIO 101 Inc.). DNA sequencing of plasmid DNA was performed with the Sequenase system (United States Biochemicals).

3. Results

3.1. Introduction of factor Xa sites

The cytoplasmic domain of the yeast H^+-ATPase contains numerous potential cleavage sites for typical proteolytic agents, such as trypsin, chymotrypsin or other commonly used proteases, making it is difficult to obtain selective cleavage of the enzyme. Blood protease factor Xa requires a four amino acid recognition sequence consisting of I(E or D)GR for peptide bond cleavage, which limits the number of cleavage sites in most proteins. The yeast H^+-ATPase has no amino acid sequence corresponding to a factor Xa recognition site, and it is not expected that the enzyme would be cleaved by this reagent. Fig. 1A confirms that incubation of wild type enzyme in purified plasma membranes under conditions optimal for factor Xa cleavage had no apparent affect on the H^+-ATPase. Although, other membrane proteins were sensitive to incubation with factor Xa. Identical results were obtained for enzyme extracted with 0.1 or 0.5% deoxycholate prior to incubation with factor Xa (not shown). Unique factor Xa sites were introduced into the LOOP1 region by site-directed mutagenesis by converting D^{170}ELK^{173} to I^{170}EGR, L^{275}VNK^{278} to I^{275}EGR and G^{254}EGF^{257} to I^{254}EGR. The first two sites fall within the putative extended α-helical region comprising part of the 'stalk' and the last site

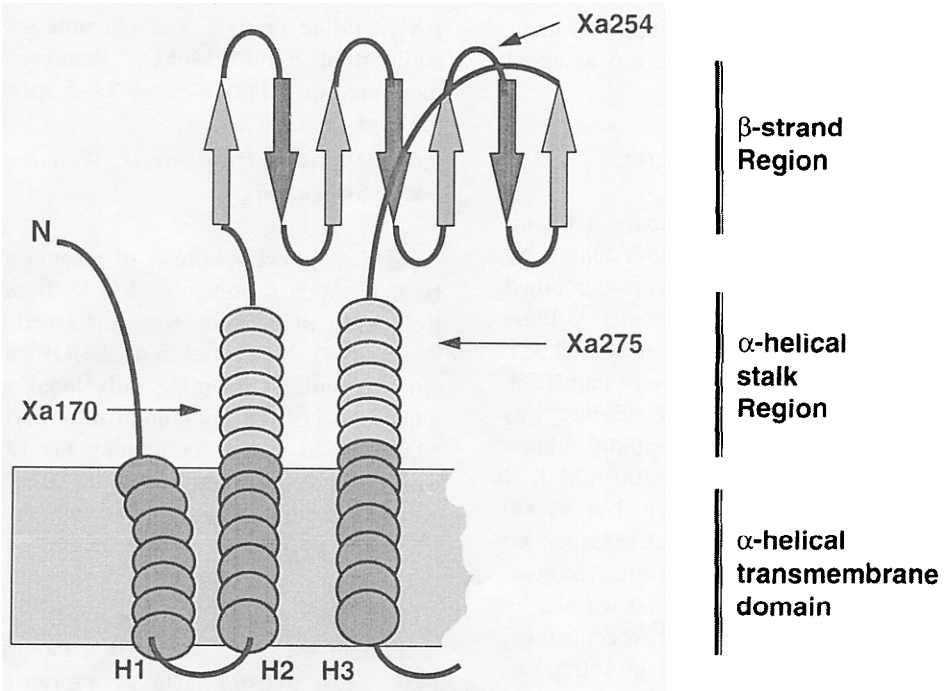

Fig. 2. Schematic diagram showing LOOP1 and associated trans-membrane domains. The LOOP1 domain of the H^+-ATPase is shown with predicted helical elements extending from transmembrane segments 2 and 3 leading into a predicted β-strand region. The relative positions of factor Xa sites introduced are designated by the arrows, as shown.

falls within the β-strand region, as predicted from secondary structure analysis [47] (Fig. 2).

The introduction of the three factor Xa sites required amino acid substitutions in regions, which are believed to be important for progression of phosphorylated catalytic intermediates [48]. Mutant sequence I^{254}EGR was found to produce a recessive lethal phenotype in our conditional expression system indicating that these changes produced a severely defective enzyme. In contrast, both I^{170}EGR and I^{275}EGR resulted in viable enzymes displaying normal growth rates. Only the double site mutant containing both I^{170}EGR and I^{275}EGR resulted in a viable cell with a reduced growth rate approximating 60% of wild type. Typical of *pma1* mutants, more subtle effects of mutations were observed by examining growth responses to hygromycin B and low pH growth medium, which are linked to electrogenic proton transport and the regulation of cytoplasmic pH by the H^+-ATPase, respectively [49]. Fig. 3 shows that the growth of all *pma1* mutants was less sensitive to hygromycin B than wild type, with the I^{275}EGR-containing mutant showing the most resistance. A similar profile was observed for pH dependent growth, where all three *pma1*-mutants showed a diminished ability to grow at medium pH < 4.5 (Fig. 4). These data are consistent with fully functional, but kinetically inefficient mutant H^+-ATPases, as has been previously observed [45,49].

Plasma membranes were isolated from the three viable *pma1* mutant strains. SDS-PAGE and Western blot analysis was used to confirm that the mutations required to construct factor Xa sites in *pma1*-I^{170}EGR and *pma1*-I^{275}EGR had no affect on the level of intact (M_r ~ 100 000) H^+-ATPase in the membrane, while the double mutant, I^{170}EGR/I^{275}EGR, was reduced by about 40%. Table 1 shows the steady-state kinetic properties of the mutant enzymes relative to wild type. All mutant and wild type enzymes showed comparable K_m values. However, V_{max}

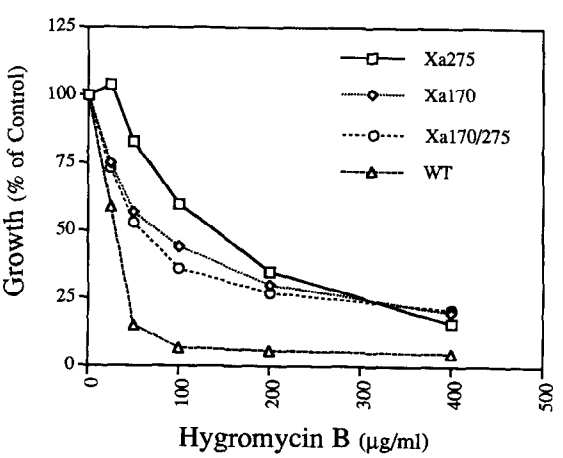

Fig. 3. Growth sensitivity of IEGR-containing *pma1* mutant to hygromycin B. The growth of wild type and *pma1* mutant strains containing factor Xa sites, I^{170}EGR, I^{275}EGR or I^{170}EGR/I^{275}EGR was assessed in YEPD medium containing increasing amounts of hygromycin B, as indicated. The amount of growth (A_{590}) in the absence of hygromycin B was taken as the control level.

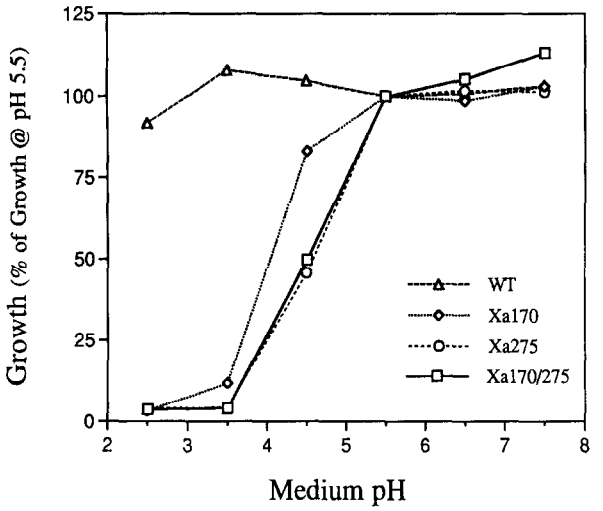

Fig. 4. Effect of medium pH on the growth of IEGR-containing *pma1* mutants. IEGR-containing *pma1* mutants were inoculated into YEPD medium adjusted to the indicated pH with either 1 M NaOH or 1 M HCl and the cells were grown for 24 h at 22°C. The level of growth at pH 5.5 was taken as the control value.

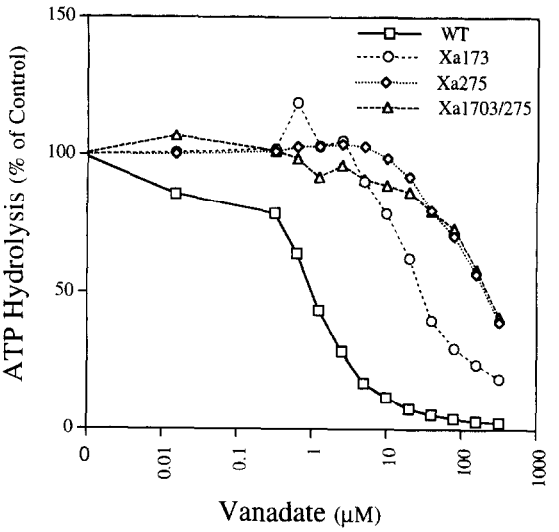

Fig. 5. Sensitivity of *pma1* mutant enzymes to vanadate. The hydrolysis of H^+-ATPases from wild type and IEGR-containing *pma1* mutants was determined as a function of increasing concentrations of vanadate, as indicated. A 100 μl reaction medium consisted of 10 mM Mes-Tris, pH 6.5, 5 mM ATP, 5 mM $MgSO_4$, 25 mM NH_4Cl and vanadate at 0–500 μM.

levels for I^{170}EGR and I^{275}EGR-containing enzymes approached wild type, while the double I^{170}EGR/I^{275}EGR mutant was reduced by about 30%, after correcting for the level of intact enzyme in the membrane. More prominent effects of the mutations were observed upon examining the mutant enzymes for vanadate sensitivity, which presumably reflects the phosphate binding domain of the catalytic domain. The I^{170}EGR mutant enzyme showed I_{50} values for vanadate inhibition which were 25-fold greater than wild type, whereas the I^{275}EGR and I^{170}EGR/I^{275}EGR mutant enzymes showed more than 200-fold decreased sensitivity (Fig. 5). The latter result was expected since the I^{275}EGR mutation occurs in a region that was previously shown to induce vanadate insensitivity [27,44].

The characterization data are consistent with the mutant enzymes being fully assembled and functional, which enables them to support cell growth. However, the mutations introduced are subtly perturbing, resulting in prominent responses to stress conditions (low pH) or inhibitors (hygromycin, vanadate).

Table 1
Kinetic properties of IEGR-containing pma1 mutants

Enzyme	V_{max} (μmol P_i / mg per min)	K_m (mM)	
		− factor Xa	+ factor Xa
Wild type	3.58 ± 0.03 [a]	1.56 ± 0.66	1.14 ± 0.49
I^{170}EGR	2.59 ± 0.10	1.35 ± 0.33	1.02 ± 0.10
I^{275}EGR	3.28 ± 0.40	1.10 ± 0.21	0.94 ± 0.25
I^{170}EGR/I^{275}EGR	1.51 ± 0.15 (2.52 ± 0.25) [b]	1.60 ± 0.51	0.98 ± 0.30

[a] $n = 4$.
[b] Adjusted for the amount of intact H^+-ATPase ($M_r \sim 100000$) relative to wild type.

3.2. Factor Xa cleavage of mutant enzymes

Fig. 1B–D shows the effects of treating purified plasma membranes containing either I^{170}EGR, I^{275}EGR or I^{170}EGR/^{275}EGR factor Xa binding sites with factor Xa at 12°C for 18 h. The prolonged incubation time was needed because factor Xa does not cleave with high efficiency. The 12°C temperature enabled the H^+-ATPase to be incubated for a prolonged time (~ 18 h) without suffering gross inactivation. Typically, wild type and mutant enzymes showed nearly 70% of their original specific activities after this treatment. All the mutant enzymes showed some degree of factor Xa cleavage with the appearance of appropriately sized peptide fragments in coomassie stained SDS gels (Fig. 1). Enzyme containing I^{170}EGR was cleaved to a maximum of about 10% with the appearance of a 81 kDa fragment (bottom band of doublet), while I^{275}EGR-containing enzyme showed about 30–35% cleavage and the double mutant I^{170}EGR/I^{275}EGR showed greater than 55% cleavage with the appearance of a characteristic 70 kDa fragment. Both major fragments, 81 and 70 kDa, cross-reacted with antibody directed at the LOOP1 region (see Section 2). In addition, a small amount of an 11 kDa fragment was detected with the double mutant, which was consistent with the fragment size expected following cleavage at sites 170 and 275.

The same overall level of cleavage was observed for deoxycholate-extracted mutant enzymes suggesting that a sided distribution of membrane vesicles did not account for partial proteolysis. It should also be noted that the level of cleavage for active enzymes was not due to the presence

of denatured enzyme, since cleavage did not correlate with the level of residually-inactivated enzyme after incubating at 12°C for 18 h. A linear relationship was not observed between the level of inactivated enzyme and the degree of factor Xa cleavage under standard incubation conditions. Factor Xa cleavage saturated after 16–18 h (at 12°C), irrespective of whether enzyme was inactivated or not. However, the overall level of cleavage by factor Xa could be increased 50% or more for the I^{275}EGR-containing mutants by increasing the incubation temperature to 41°C or by pre-treating the enzymes with 4 M or 8 M urea. Unfortunately, control enzymes prepared under these conditions were completely inactivated by this treatment.

These results suggest that the factor Xa site at position 170 appears highly occluded under normal conditions. In contrast, the site at position 275 is more accessible to the proteolytic enzyme under native-like conditions. The enhanced cleavage of the double mutant, I^{170}EGR/I^{275}EGR, suggests either that cleavage at one site then exposes the second site more readily to factor Xa or that the introduction of mutations at both positions alters the local structure sufficiently such as to expose each site more readily.

3.3. Effect of cleavage on enzyme function

To assess whether cleavage at factor Xa sites within LOOP1 had an affect on the catalytic properties of the enzyme, the mutant containing membranes were treated with factor Xa and aliquots were removed between 0 and 20 h for analysis for ATP hydrolysis. Since cleavage of *pma1* mutant enzyme in membranes is incomplete and ranges between 10 and 40%, it was expected that only the I^{275}EGR and I^{275}EGR/I^{170}EGR-containing mutants, which show approx. 25 and 45% maximum cleavage, would show sufficient cleavage to have an impact on ATP hydrolysis. Fig. 6 shows that the specific activity of wild type declined in a linear fashion over the 20 h incubation period and, as expected, there was no effect of factor Xa since no sites were available for cleavage. The I^{170}EGR-, I^{275}EGR- and I^{275}EGR/I^{170}EGR-containing mutants also showed a progressive inactivation over time but there was no effect on activity after factor Xa cleavage. The factor Xa-independent inactivation reflected a change in V_{max} for the enzymes. K_m (mM) values were essentially unchanged before or after factor Xa cleavage. In addition, there was no observable change in vanadate sensitivity (I_{50}) for the mutant enzymes following cleavage by factor Xa. These data suggest that the LOOP1 region participates in catalysis even when the peptide backbone is altered in regions (I^{275}EGR) believed to be important for phosphate binding and release.

The effect of factor Xa cleavage on ATP-coupled proton transport was assessed in H^+-ATPase-reconstituted vesicles by following the quenching of acridine orange fluorescence. Proton transport could only be assessed in the I^{275}EGR-containing mutant because it produced significant cleavage following treatment with factor Xa to allow a reasonable comparison between cut and uncut enzyme. The I^{170}EGR-containing enzyme showed less than 10% cleavage which was insufficient to detect a reasonable change. On the other hand, the double mutant, I^{170}EGR/I^{275}EGR produced greater levels of cleavage, but it had relatively low enzyme activities, which precluded analysis by the fluorescence quenching assay. Wild type and I^{275}EGR-containing mutant enzymes were recon-

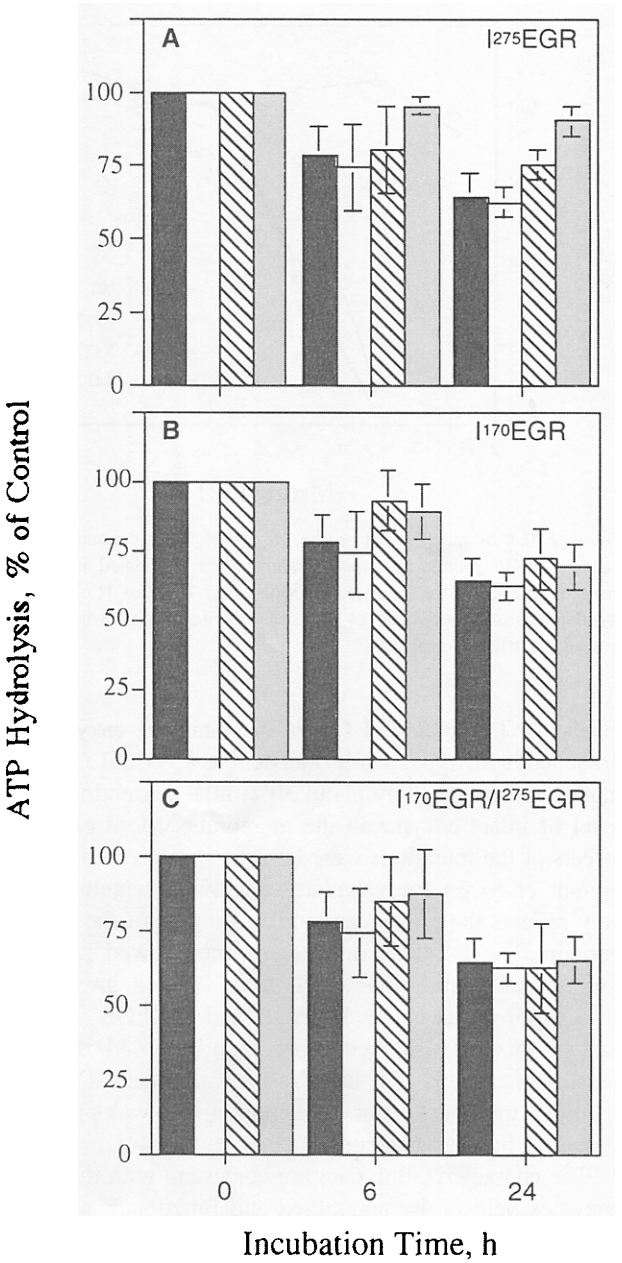

Fig. 6. Effect of factor Xa cleavage on inactivation of the H^+-ATPase. *pma1* mutant membranes, as indicated, were incubated with factor Xa under standard conditions. Aliquots were removed at 0, 6 and 24 h, and assayed for residual H^+-ATPase activity. In panels A, B and C, the wild type enzyme is represented by the open (cut) and solid (uncut) bars, whereas the mutant enzymes are represented by the striped (uncut) and gray (cut) bars.

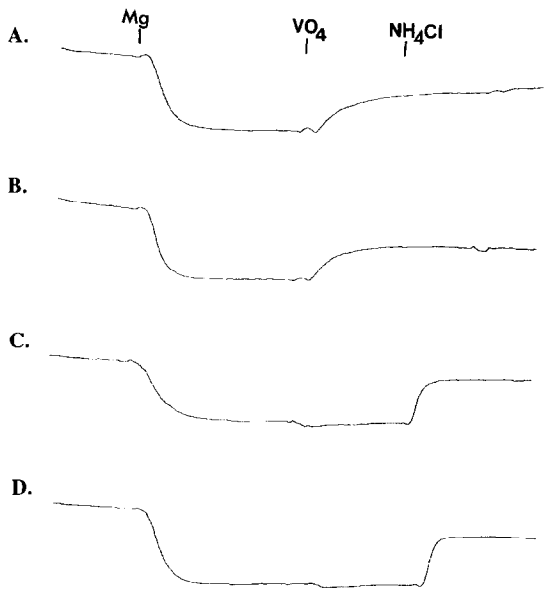

Fig. 7. Effect of factor Xa cleavage on proton transport. Highly purified H^+-ATPases from wild type (A,B) and I^{275}EGR-containing *pma1* strains (C,D) were separately reconstituted in liposomes (see Section 2). The liposomes were assayed for ATP mediated proton transport by the acridine orange fluorescence quenching assay (see Section 2). Reconstituted vesicles were partitioned into two sets, with one set left untreated (A,C), and the other set treated with factor Xa (B,D) under standard conditions. The treated vesicles were then assayed for ATP mediated proton transport. The reaction was initiated by the addition of a final concentration of 5 mM $MgSO_4$. Vanadate (100 μM) and NH_4Cl (10 mM) were added at a steady state to collapse the pH gradients. The latter was only added to the I^{275}EGR-containing mutant enzymes.

stituted in liposomes and incubated in the presence or absence of factor Xa, under standard cleavage conditions (see Section 2). SDS gel electrophoresis was used to confirm that the I^{275}EGR-containing enzyme was cleaved suitably by the factor Xa incubation, and that the wild type enzyme was unaffected (not shown). It should be noted that cleavage was somewhat less (~ 20–25% of total) than that observed with plasma membrane vesicles but this was expected from an approximate 50–50 orientation of reconstituted enzyme within the liposome bilayer. Only everted enzymes, those with their active site regions outside, would be transport active and accessible to factor Xa. Taking the sidedness factor into account, it is estimated that the effective cleavage percentage approached 40–50%. Fig. 7 shows that ATP-driven proton transport was identical for untreated (panel A) and factor Xa-treated wild type enzyme (panel B). A similar result was found for untreated (panel C) and treated (panel D) I^{275}EGR-containing mutant enzyme. In fact, the initial rate of proton transport was slightly greater for the factor Xa treated mutant enzyme, which is consistent with it showing somewhat less inactivation then untreated enzyme (Fig. 6). The addition of 50 μM vanadate to wild type enzyme reversed the pH gradient, as the pump was shut down and protons equilibrated across the membrane (Fig. 7A,B). In contrast, vanadate at this concentration had no effect on pH gradient formation by I^{275}EGR-containing enzyme, as expected from its vanadate insensitivity of ATP hydrolysis (Fig. 5). These results indicate that cleavage of the protein backbone at amino acid position 275 does not uncouple proton transport from ATP hydrolysis.

4. Discussion

Selective proteolysis has been an effective tool for the investigation of conformationally-linked molecular motion in P-type transport enzymes [40,41,50]. In general, limited proteolysis is used to restrict the site(s) of proteolytic cleavage. Since proteolytic enzymes are by themselves rather large, there is an underlying assumption that for a site to be recognized and cleaved, it must be freely accessible to the proteolytic agent. The loss or gain of a recognition site upon binding a ligand or upon binding an inhibitor, which freezes the enzyme in a specific conformation, has been interpreted to represent conformationally-induced molecular motion of the local protein structure containing the recognition site [41]. This assumes that the conformational probe does not interfere sterically with the protease-protein complex. We have used the yeast H^+-ATPase to selectively introduce unique factor Xa proteolytic sites at defined loci in the cytoplasmic LOOP1 region linking transmembrane segments 2 and 3. This approach permits proteolysis at a defined site in the enzyme without concerns from other potential cleavage sites due to the fact that the H^+-ATPase has no endogenous factor Xa sites. The ability to selectively introduce unique proteolysis sites is important for the H^+-ATPase because limited proteolysis resulting in selective cleavage at sites other than the N- or C-termini has been difficult to achieve; it frequently results in multiple sites being rapidly cleaved [51].

The factor Xa sites were introduced by modifying existing amino acids within the LOOP1 region by site directed mutagenesis of the *PMA1* gene to create the recognition sequence IEGR. Three sites, I^{170}EGR, I^{254}EGR and I^{275}EGR, were engineered to examine functional and topological properties of the LOOP1 domain. The LOOP1 domain displays structural motifs that are strongly preserved in all P-type ion motive ATPases [34]. It is believed to have α-helical character at its N- and C-terminal ends, extending to and from transmembrane segments 3 and 2, respectively, where it forms part of the 'stalk' region of the cytoplasmic domain [16,52]. The remainder of the sequence is predicted to form a β-strand domain that interacts with the catalytic ATP binding/phosphorylation domain within the large central cytoplasmic domain. The I^{170}EGR site is predicted to lie within the α-helical region of the stalk, and within 2–3 turns of the helix extended from the bilayer [53]. This site is poorly cut with Factor Xa under native conditions, rarely exceeding about 10% cleavage, as determined by the reduction of the intact 100 kDa

protein and the appearance of an 81 kDa cleavage product (Fig. 1). ATP hydrolysis had no affect on the level of cleavage at this site suggesting that it has restricted motion during catalysis. The I^{170}EGR site must be poorly solvent exposed or sterically constrained, which limits access of the factor Xa enzyme. If the LOOP1 helices comprise 2 of the 5 helical elements, which are believed to make up the stalk [54], then the organization of this region, perhaps as a bundled helical structure, might limit molecular motion within the structure. The fact that the multiple amino acid substitutions D^{170}I, L^{172}G and K^{173}R required to form the factor Xa site had little effect on the overall activity of the enzyme suggests that backbone structure and not individual side groups are most important in this region.

The I^{254}EGR and I^{275}EGR sites are expected to lie toward the C-terminal end of the β-strand region. The I^{254}EGR position approximates the region where trypsin is known to cleave the LOOP1 domain in the Ca^{2+}-ATPase [35,55]. This controversial tryptic sensitive region was initially implicated as a potential site of uncoupling [35], although more recent studies do not support this conclusion [35,36]. However, the strongly basic stretch of amino acids in the Ca^{2+}-ATPase where tryptic cleavage actually occurs is absent from the yeast H^+-ATPase [6]. The introduction of IEGR at position 254 was sufficiently perturbing to the enzyme so as to be lethal for cell growth. It is not clear whether lethality resulted from the introduction of a bulkier Ile at G254, a positively-charged Arg at F257, or both. Interestingly, a S^{248}C(A or V) mutation in this region has little effect on the overall catalysis by the enzyme (Bandell, Wang and Perlin, unpublished data), although a K250T mutation rendered the *S. pombe* enzyme highly vanadate insensitive [56]. Finally, cleavage of the H^+-ATPase at the nearby I^{275}EGR site had no effect on ATP-mediated proton transport (Fig. 7), indicating that this region is not likely to be involved directly in coupling via a transmittal of conformational energy through its backbone structure and transmembrane segment 3.

The I^{275}EGR site is predicted to lie near the end of the β-strand region. It is considerably more accessible to factor Xa, showing 35% or more cleavage of the 100 kDa protein with the appearance of a characteristic 70 kDa fragment (Fig. 1). When both I^{275}EGR and I^{170}EGR are present, the level of factor Xa-induced cleavage increases to more than 55% with the appearance of the characteristic 70 kDa fragment, and a minor 11 kDa fragment representing most of the LOOP1 domain (amino acids 170–275) (Fig. 1). The position of the I^{275}EGR site is clearly more accessible to factor Xa, but as with the I^{170}EGR site, it does not show altered reactivity in the presence of MgATP or MgATP + vanadate. Thus, it appears that this region does not undergo extensive molecular motion. The apparent lack of flexibility is somewhat surprising because this portion of the LOOP1 domain has been suggested to comprise part of the phosphate binding domain, based largely on mutations which confer vanadate insensitivity [45,56–59] and show altered phosphate interactions [60]. Our finding that the introduction of mutations L^{275}I, V^{276}E, N^{277}G and K^{278}R, which comprise the I^{275}EGR factor Xa site, confer prominent vanadate insensitivity to the enzyme (Fig. 5) is consistent with these previous findings. It is noteworthy that cleavage of the backbone at position 275, within the region suggested to be important for phosphate binding, had little effect on the overall ATP hydrolysis by the enzyme (Fig. 6). We were somewhat surprised that the I^{170}EGR site, which is expected to lie in a completely separate structural region, also confers strong vanadate insensitivity (Fig. 5). This finding suggests that subtle structural perturbations in the LOOP1 domain may alter the distribution of conformational intermediates leading to vanadate insensitivity, as has been previously proposed [27,45]. The effects of these mutations on the steady-state distribution of conformational intermediates leading to vanadate insensitivity, and reduced catalytic activity for the I^{275}EGR/I^{170}EGR double mutant are consistent with the effects of LOOP1 mutations on the Ca^{2+}-ATPase [48].

Perturbations in the LOOP1 domain are believed to affect catalytic turnover of the enzyme. Portillo and Serrano [61] first reported that phosphoenzyme levels in the yeast H^+-ATPase during steady-state ATP hydrolysis were enhanced by an E^{233}N mutation, suggesting a defect in the hydrolytic step in this enzyme. A comparable mutation in the Ca^{2+}-ATPase did not support this suggestion, rather the mutation was found to cause a defect in the E_1P-E_2P transition [48]. Clarke et al. [62] also suggested that an alteration of the E_1P-E_2P transition was responsible for catalytic defects observed with mutations in the LOOP1 β-strand domain. Thus, mutations in the LOOP1 domain may alter the progression of catalysis by blocking the interconversion of catalytic intermediates. Conformational transitions in the LOOP1 domain appear to be an important part of the overall catalytic cycle for the Na^+,K^+-ATPase [41]. It has also been found that the formation of E_1P exposes the tryptic cleavage site at R198 (M2) in the Ca^{2+}-ATPase, whereas the site is largely protected in E_2P [63]. Finally, proteolytic cleavage of a V8-sensitive site, E231, on the Ca^{2+}-ATPase prevents conformational changes necessary for calcium transport after phosphorylation [40], and this finding is similar to the decline in calcium transport observed with a site-directed mutation N^{111}A [64].

It is noteworthy that Goffeau and colleagues [65] successfully expressed the large central catalytic domain as a fusion product that could bind nucleotide analogs. However, co-expression of the LOOP1 domain did not appear to alter the profile of nucleotide analog binding, nor did it induce hydrolysis of ATP when present. These experiments suggest that LOOP1 is not required for nucleotide binding, but its role in catalysis remains unclear.

Finally, the conformational properties of the LOOP1 domain appear to be most prominent in the putative β-strand region. The results in this study suggest that the

regions flanking the β-strand region do not undergo significant molecular motion, since the accessibility of factor Xa sites are unaltered during catalysis. Nonetheless, the introduction of multiple mutations outside the projected β-strand region induces conformational perturbations, which appear to alter the distribution of catalytic intermediates resulting in vanadate insensitivity. Factor Xa-induced cleavage of the protein backbone at the 275 site had essentially no effect on ATP hydrolysis or ATP-mediated proton transport suggesting that this localized region of the LOOP1 structure plays a minor role in coupled catalysis.

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