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Mutations of G158 and their second-site revertants in the plasma membrane H^+-ATPase gene (*PMA1*) in *Saccharomyces cerevisiae*

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Abstract

A G158D mutation residing near the cytoplasmic end of transmembrane segment 2 of the H^+-ATPase from *Saccharomyces cerevisiae* appears to alter electrogenic proton transport by the proton pump (Perlin et al. (1988) J. Biol. Chem. 263, 18118–18122.) The mutation confers upon whole cells a pronounced growth sensitivity to low pH and a resistance to the antibiotic hygromycin B. The isolated enzyme retains high activity (70% of wild type) but is inefficient at pumping protons in a reconstituted vesicle system, suggesting that this enzyme may be partially uncoupled (Perlin et al. (1989) J. Biol. Chem. 264, 21857–21864.) In this study, the acid-sensitive growth phenotype of the *pma1*-D158 mutant was utilized to isolate second site suppressor mutations in an attempt to probe structural interactions involving amino acid 158. Site-directed mutagenesis of the G158 locus was also performed to explore its local environment. Nineteen independent revertants of *pma1*-G158D were selected as low pH-resistant colonies. Four were full phenotypic revertants showing both low pH resistance and hygromycin B sensitivity. Of three full revertants analyzed further, one restored the original glycine residue at position 158 while the other two carried compensatory mutations V336A or F830S, in transmembrane segments 4 and 7, respectively. Partial revertants, which could grow on low pH medium but still retained hygromycin B resistance, were identified in transmembrane segments 1 (V127A) and 2 (C148T, G156C), as well as in the cytoplasmic N-terminal domain (E110K) and in the cytoplasmic loop between transmembrane segments 2 and 3 (D170N, L275S). Relative to the G158D mutant, all revertants showed enhanced net proton transport in whole-cell medium acidification assays and/or improved ATP hydrolysis activity. Small polar amino acids (Asp and Ser) could be substituted for glycine at the 158 position to produce active, albeit somewhat defective, enzymes; larger hydrophobic residues (Leu and Val) produced more severe phenotypes. These results suggest that G158 is likely to reside in a tightly packed polar environment which interacts, either directly or indirectly, with transmembrane segments 1, 4 and 7. The revertant data are consistent with transmembrane segments 1 and 2 forming a conformationally sensitive helical hairpin structure.

Keywords: ATPase, H^+-; Suppressor mutation; Site-directed mutagenesis; Coupling; Yeast; (*S. cerevisiae*)

1. Introduction

The yeast plasma membrane H^+-ATPase, encoded by the *PMA1* gene, belongs to an evolutionarily highly conserved superfamily of P-type cation ATPases [1,2]. Very little is yet known about the detailed structure of these P-type ATPases or the way in which they are able to convert energy from the hydrolysis of ATP into the movement of cations across the plasma membrane. A combination of hydropathy analysis coupled to chemical and biochemical probing of exposed and buried residues has led to the current consensus that the superfamily of cation ATPases contain between 8 and 10 membrane spanning α helices, with the ambiguity lying in the C-terminal part of the structure [3–8] (Fig. 1). Very little of the protein is exposed to the extracellular environment. Both the N- and C-termini appear to be intracellular, along with a small and a very large intracellular domain that participate in the hydrolysis of ATP [9–11].

A number of individual residues have been shown to be important or essential for the function of these proteins through the use of site-directed mutagenesis [12]. However, this approach has generally been used to attack residues that might be a priori predicted to be important. An alternative approach has been to isolate randomly generated mutations in yeast that reduce the activity of the

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PMA1 gene product (since the protein is essential one cannot obtain viable 'knock out' mutations) [13,14]. In this way, we have found that changes as subtle as a mutation of alanine to either valine or glycine are sufficient to cause a marked loss of activity of the yeast H^+-ATPase [14,15]. However, neither of these approaches alone can elucidate how these residues are involved in affecting the conformational changes that are important for the transport of ions.

It is evident that the conformational changes accompanying ATP hydrolysis and ion transport are significant. This is evident from a number of biochemical studies that have demonstrated that there are at least two major conformational states of the protein (E_1 and E_2) [16] associated with catalytic turnover by the P-type ATPases. Moreover, there is evidence that regions of the protein that are spatially distinct from each other are indeed tightly coupled. For example, in the mammalian Na^+,K^+-ATPase, the apparent interaction of ouabain with the extracellular region between membrane-spanning helices 1 and 2 causes a significant change in chemical reactivity of a cysteine residue located across the membrane in the catalytic region for ATP hydrolysis [17]. We have obtained similar evidence of conformational coupling between apparently distant sites in the yeast H^+-ATPase by a genetic approach involving the isolation of intragenic second-site suppressor mutations that relieve the low pH sensitivity of two different mutations [15,18]. In one case, a S368F mutation 10 amino acids from the site of ATP phosphorylation, D378, prevented cells from growing at pH 3.0 or in the presence of NH_4^+ [18]. Among eight second-site suppressing mutations analyzed, one (E367V) was in an adjacent amino acid and one (V289F) was located in the other, smaller predicted cytoplasmic loop, but all of the other 6 were located in putative transmembrane domains, especially in helices 1 and 2 (H1 and H2). In a complimentary study, we began with a mutation predicted to lie near the extracellular end of H1 (A135V) and recovered compensatory mutations in H2 and H4. But, most striking was that we recovered two other compensating mutations (S660C; S660F, F611L) in the putative ATP binding domain located in the large cytoplasmic loop [15].

In this communication, we report an analysis of second-site revertants of a G158D (*pma1*-114) mutation, which is predicted to lie at the base of H2 (Fig. 1), This mutation is interesting because it exhibits a marked inability to grow at low pH although it has approximately 70% wild-type ATPase activity, as determined in vitro [13,14,19]. Furthermore, the mutation appears to significantly reduce the cellular membrane potential, as shown both by its ability to induce hygromycin B growth resistance and diminish the uptake of TPP into whole cells, both parameters which require a significantly hyperpolarized membrane potential [19]. Cells carrying this mutation are unable to grow in the presence of 0.2 M acetate (pH 4.8), and grow very poorly in unbuffered medium at pH 2.5, where wild-type yeast cells grow well. Nineteen mutations able to grow under these conditions were isolated and analyzed, of which only one was a revertant of the original mutation back to wild type. Here we report the mapping and characterization of 11 of the 18 second-site alterations and show that they are located predominantly in regions within or close to transmembrane segments, especially in the first two membrane-spanning helices.

2. Materials and methods

2.1. Strains and growth conditions

The original *pma1*-114 (G158D) mutation was isolated in homothallic strain Y55 (*HO gal3 MAL1 SUC1*). Strain SA103 (*MAT***a** *ade6-1 ura3-1*) was constructed by transforming the cloned *pma1*-G158D mutation into diploid strain SH122 [18] and then recovering a haploid segregant [18]. Resistance to 300 μg/ml hygromycin B in YEPD medium was assayed as described previously [18].

2.2. Genetic methods

Standard genetic procedures have been described previously [13,15,18]. Revertants of SA103 able to grow on low pH medium (0.2 M acetate, pH 4.9 in YEPD) were selected on agar plates at 30°C after replica-plating UV-mutagenized colonies of cells from YEPD plates to the selective medium. A single revertant was chosen from each colony, to ensure their independence. Each revertant was crossed with haploid spores of strain SA1 (*HO ade6-1 trp5-1 his6-1 lys1-1 ura3-1*) to demonstrate that the revertants were intragenic. The *leu1-1* marker is very tightly linked to *PMA1*, so that intragenic revertants of *pma1*-G158D exhibit nearly complete linkage with this marker in tetrads dissected after sporulation.

2.3. DNA sequence analysis

DNA sequencing was carried out on PCR-amplified regions of the *PMA1* gene, using pairs of oligonucleotides as primers, and DNA sequencing of these fragments was performed, as previously described [18]. The regions sequenced include the first four membrane spanning helices and their extracellular regions as well as parts of the cytosolic N-terminal region and the small and large cytosolic regions (approximately amino acids 108 to 347). In addition, DNA sequence was obtained for the regions including the last four transmembrane domains and their interconnecting cytosolic and extracellular loops, plus most of the C-terminal region (approximately amino acids 660 to 905).

2.4. In vitro mutagenesis of G158

Site-directed mutagenesis of the G158 site was carried out as follows: An oligonucleotide spanning the G158

codon was synthesized in which the nucleotide sequence of the three nucleotides comprising the G158 codon were represented as an equal mixture of all four deoxynucleotides. The 'dirty' oligonucleotide was then hybridized to single-stranded plasmid DNA containing the wild-type sequence and treated as previously described [15] to create mutations at this site. Individual plasmid transformants were sequenced to determine what changes had occurred in the nucleotide sequence of the G158 codon. The 700 bp region between *Eco*RV and *Bst*EII surrounding the mutation was sequenced to ensure there were no other changes and then the *Eco*RV-*Bst*EII fragment of a purified plasmid was inserted in place of the wild-type sequence in plasmid pSN104, which contains the entire *Hin*dIII fragment including *PMA1* and also an insertion of *URA3* in the 3′ noncoding region [18]. The plasmids carrying the in vitro-generated mutation were cleaved with *Hin*dIII and transplaced into diploid strain SH122 (*pma1Δ::LEU2/PMA1*) to select Ura$^+$ Leu$^-$ transformants in which the mutation was introduced in place of the deleted *pma1Δ::LEU2* region [18]. These diploids were sporulated and dissected in order to obtain haploid-viable segregants carrying the site-directed mutation. Some mutants proved to be either recessive lethal or dominant lethal. Dominant lethal mutants were discovered as cases in which the site-directed mutation was invariably lost by gene conversion with the wild-type gene in the diploid after transformation [19].

2.5. Biochemical analysis

Plasma membrane-enriched fractions were prepared in duplicate from revertant strains grown in 1-liter batches and associated ATPase assays were carried out, as previously described [14,18]. Vanadate-sensitive ATP hydrolysis was also determined, as previously described [14]. An indirect assessment of whole cell proton transport was performed by following glucose-induced medium acidification, as previous described [20].

3. Results

Yeast cells carrying the *pma1*-G158D mutation are unable to grow under acid-loading conditions, such as on YEPD plates containing 0.2 M acetate (pH 4.8) [13,20]. To select intragenic revertants of the *pma1*-G158D that could grow under these conditions, we UV-irradiated fresh replicas of colonies of cells on YEPD plates and, after overnight growth in the dark, replica plated these colonies on to YEPD plates containing 0.2 M acetate (pH 4.8). Nineteen independent revertants of *pma1*-G158D that could grow on this medium were selected. These colonies were purified by streaking for single colonies. They were then classified as phenotypically full revertants if they exhibited wild-type sensitivity to 300 μg/ml hygromycin B or as partial revertants if they were still resistant to this concentration of the inhibitor. This same distinction has been used in previous studies of other intragenic revertants of *pma1* mutations [15,18]. Four of the mutants were full revertants.

To establish that the revertants were likely to be intragenic, each was crossed to a *leu1-1* strain, SA1, and the resulting diploids sporulated for tetrad analysis. Because *leu1-1* is very tightly linked to *PMA1*, intragenic revertants would be expected to give two Leu$^+$ segregants that were always revertant and two Leu$^-$ segregants that were always truly wild type. If the reversion depended on mutations of another, unlinked gene, then half of the Leu$^+$ segregants should once again show the original *pma1*-G158D phenotype. In all cases, the revertants appeared to be intragenic (data not shown).

Full revertants might arise by restoring G158D back to G158 or a similar amino acid. Partial revertants could either be less favorable changes at the original site or bona fide second-site revertants. To learn how many of the revertants had changed the original codon back to wild-type or pseudowild-type amino acids, the region of DNA surrounding the G158D mutation was amplified by PCR and sequenced (see Materials and methods). One of the four phenotypically wild-type revertants had indeed lost the G158D mutation and was again G158. The three other full revertants and all of the 15 partial revertants retained the original mutation; these must perforce contain another mutation.

The compensatory mutations in two of the three full revertants were located by sequencing the entire gene, as described previously [18]. In both cases, a single mutation was found. One revertant SA12 carried a V336A mutation, while another harbored F830S (Fig. 1). As in our previous studies [15,18], we found that sites very distant in the

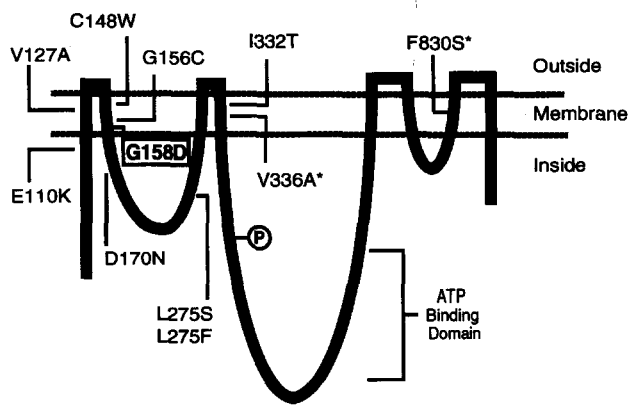

Fig. 1. Schematic diagram showing putative membrane topology of the yeast plasma membrane H$^+$-ATPase, as previously defined [18]. The position of the G158D mutation is indicated in a box and its associated second-site suppressor mutations are indicated. The two mutations that are starred are full revertants, with fully wild type growth phenotypes. The other mutations are low pH-resistant, but still hygromycin B-resistant. The position of the D378, which is phosphorylated during the catalytic cycle, is indicated by a circle containing a P.

primary structure can act to compensate for the original lesion.

The second site mutations of the partial revertants were then sought by PCR amplification and DNA sequencing of segments of the gene. The first region to be amplified was an approx. 700 bp region from *Eco*RV to *Bst*EII that includes the first two putative membrane-spanning helices, H1 and H2, as well as G158D. Six of the 15 revertants contained a second mutation in this region, including two independent changes of G156C (Fig. 1). The location of the other partial revertant mutations were then sought by sequencing other regions of the *PMA1* gene. As indicated in Materials and methods, all of the eight putative membrane-spanning regions were sequenced along with their interconnecting cytosolic and extracellular loops. Only a small part of the beginning and end of the large cytoplasmic domain was sequenced. In all, we located 9 out of 15 second-site mutations. No tertiary mutations were detected but since the entire gene was not sequenced, this possibility cannot be totally eliminated. The positions of the secondary mutations are noted on Fig. 1. It is noteworthy that the second-site reverting mutations include both sites presumed to be in membrane-spanning domains and sites in segments presumed to lie in the cytosol. The other second-site changes are likely to be in the N terminus (amino acids 1–108) or in the large cytosolic domain (approximately amino acids 350–660). The significance of the locations of these second-site mutations will be elaborated further in the Discussion.

3.1. Biochemical and physiological characterization of second-site revertants of pma1-G158D

The revertant enzymes were also characterized biochemically and physiologically. First, most of the revertants were analyzed for their in vitro ability to hydrolyze ATP (see Materials and methods). The results of this analysis are presented in Table 1. One of the two full revertants, G158D V336A, regained complete wild-type activity, but the second, G158D F830S, was only slightly higher than the original G158D mutant. We believe that the second revertant may be effectively wild type in its behavior because of an improvement in its ability to transport protons (see below).

Among the partial revertants, some regained nearly all of the wild-type level of ATP hydrolysis, although they gave evidence of a reduced cellular membrane potential by their continued resistance to hygromycin B (e.g. G158D E110K). Other partial revertants showed no significant change in the level of ATPase activity compared to the original mutation (e.g. G158D L275F and G158D L275S). The remaining partial revertants showed ATP hydrolysis activity intermediate between the mutant and wild type. Again, the effectiveness of the revertants in restoring efficient growth under acid-loading conditions is not simply a function ATP hydrolysis per se, but also involves the efficiency with which ATP hydrolysis is coupled to proton transport.

Table 1
ATPase activity of second-site revertants of G158

PMA1 genotype	ATP hydrolysis		VO_4 inhibition
	activity [a]	% of control	(K_i, μM)
G158	1.97±0.27	100	0.4
G158D	1.34±0.03	68	0.4
Full revertants			
SA12 G158D V336A	2.14±0.05	109	1.6
SA23 G158D F830S	1.51±0.11	77	0.8
Partial revertants			
SA30 G158D E110K	2.05±0.22	97	0.5
SA7 G158D V127A	1.75±0.18	89	0.7
SA26 G158D C148T	1.71±0.12	87	1.6
SA13 G158D G156C	1.74±0.06	88	0.7
SA26 G158D G156C			
SA25 G158D D170N	1.51±0.05	77	3.1
SA32 G158D L275S	1.31±0.09	66	4.0
SA11 G158D L275F	1.16±0.01	59	20.0
SA34 G158D I332T	1.87±0.12	95	0.7

[a] Activity expressed as μmol P_i/mg per min.

Another measure of the regain of function was to assess the ability of the revertant cells to acidify the medium [20]. Glucose-induced medium acidification provides a qualitative measure of the whole cell proton pumping properties of the H^+-ATPase. In these experiments, glucose-starved cultures were tested for their ability to acidify the medium after the re-addition of glucose. The effect of the G158D mutation on medium acidification was subtle but significant and has been reported previously [14,20]. All of the revertants were found to acidify the medium at rates exceeding the G158D mutant and comparable to wild-type rates (not shown.)

Finally, plasma membrane-bound H^+-ATPases from wild-type, primary mutant and revertant cells were assayed for vanadate sensitive ATP hydrolysis. Previously, we showed that alterations of *PMA1* at sites quite distant from the active site could alter vanadate sensitivity, as well as ATP hydrolysis activity. Among the revertants tested, G158D L275F showed the most notable increase in K_i (Table 1), although several revertant enzymes were somewhat more resistant. The K_i for G158D L275S was also significantly higher than wild type but much lower than the L275F mutation.

3.2. Site-directed mutations of G158

To learn more about the specific role of G158 in the function of PMA1, we also created site-directed mutations at amino acid position 158 [15,18]. A variety of changes at this position were created using 'dirty' oligonucleotide-directed mutagenesis (see Materials and methods). The mutant sequences were then introduced into a complete *PMA1* gene that was marked in the 3′ non-coding region

Table 2
Site-directed mutations at position 158

Site-directed mutant	Growth rate	Hygromycin B resistance	Growth at pH 2.5	ATP hydrolysis activity [a]	% [b]	Vanadate inhibition (K_i, μM)
G158	normal	−	+	1.97±0.27	100	0.4
G158D	normal	+	+	1.34±0.03	68	0.5
G158S	slow	±	+	1.53±0.16	78	0.2
G158V	very slow	+ +	+	0.88±0.08	45	1.9
G158L	very slow	+	−	0.36±0.09	18	0.7
G158E	normal	+ +	±	n.d. [c]		n.d.

[a] ATP hydrolysis is expressed as μmol P^i/mg per min.
[b] These values represent the percent of wild-type activity (G158).
[c] n.d., not determined.

by the *URA3* selectable gene and introduced into diploid strains of yeast (see Materials and methods). Haploid segregants carrying the mutations were then recovered for further study (Table 2). In two cases, the mutations could not be recovered in viable segregants. G158N is apparently recessive lethal, as it yields two out of four viable spores, both of which are Ura − and therefore not linked to the mutant (data not shown). The mutation G158P is apparently dominant lethal, as the mutation is invariably lost by gene conversion with the wild-type *PMA1* sequence in the diploid *pma1*-G158P/*PMA1* transformants (not shown). Such dominant lethal mutations of *PMA1* have been described previously [18,19].

Four different mutations were viable but none of them were fully wild-type. *pma1*-G158S grows similar to wild-type cells and is weakly hygromycin B-resistant. *pma1*-G158E is quite similar in its overall behavior to G158D, except that it was resistant to higher levels of hygromycin B (450 μg/ml) and was somewhat more low-pH sensitive, as monitored by residual growth on 0.2 M acetate, pH 4.8 plates. The two other mutations, G158V and G158L, were both very slow-growing, producing colonies of less than half the diameter of wild-type cells, and hygromycin B-resistant.

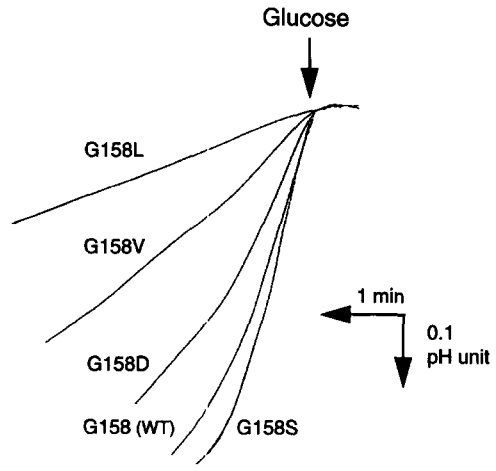

Fig. 2. Glucose-induced medium acidification of *pma1* mutants carrying substitutions at position 158. *pma1* mutants G158D, S, L and V were analyzed along with wild type (G158) for glucose-induced medium acidification, as described in Materials and methods. Glucose (2%) was added to starved cells to initiate medium acidification in cells equilibrated in weak buffer at pH 4.5.

Three of these site-directed changes were then analyzed biochemically and physiologically, as described above. Consistent with its nearly wild-type behavior, *pma1*-G158S has about 80% wild-type activity (Table 2). In contrast, the slow-growing *pma1*-G158V and the even slower-growing *pma1*-G158L mutants have quite low ATPase activity. The site-directed mutants also show some alteration of vanadate sensitivity (Table 2). There is a general correlation between the size of the residue at this site and vanadate resistance, but G158V is more resistant than G158L at intermediate concentrations of the inhibitor.

The effects on proton-pumping of the site-directed mutants also reflected their overall mutant phenotypes. G158S was comparable to wild type, while G158V and G158L were much more severely affected than the original mutant, G158D (Fig. 2).

4. Discussion

In several previous papers we have used the reversion of mutations in the *PMA1* gene to explore interacting domains of the protein [15,18]. Those papers showed that there was coupling between regions presumed to be in the cytosol and involved in ATP binding and catalysis, and other domains presumed to be in membrane-spanning parts of the protein. The results in this paper are generally similar, in that a G158D mutation that presumably lies at the base of the second transmembrane helix 2 can be partially or completely suppressed by second-site mutations within the enzyme. Some of the suppressing mutations lie at a great distance in the primary structure (e.g. F830S), but presumably these two sites are conformationally coupled, either by a change that is transmitted directly between the second and seventh membrane spanning regions or more indirectly, through a series of interactions among transmembrane helices [21]. It is interesting, however, that, with this one exception, all of the other second site changes that are located in membrane-spanning regions (six in all, since one was isolated twice) are located in the first four membrane-spanning domains. Given that all of the mutants were sequenced over all eight putative transmembrane segments, this result is not a consequence of any bias in the regions chosen for DNA sequencing.

Second, many of the compensatory changes are in the region comprising transmembrane helices 1 and 2 (H1 and H2). These helices are predicted to form a helical hairpin structure in which these antiparallel alpha helices are joined by a short extracellular turn [21] with the G158D mutation predicted to lie at the C-terminal end of H2, near the cytosolic surface of the membrane bilayer. Recently we described the effects of mutations on the close packing

arrangement of amino acids near the extracellular side of H1 and H2 that were consistent with this hairpin model [21]. Furthermore we have used molecular dynamic simulations to describe the potential for localized coupling in this region by showing how perturbations in local structure due to mutations in H1 could be propagated to H2, and how suppressor mutations in H2 could compensate for mutations in H1 and permit the adoption of an overall more wild-type structure [21]. The suppressor analysis in this study provides additional evidence of H1–H2 interactions, as the G158D mutation in H2 can be partially or fully suppressed by mutations either in H2 (C148T and G156C) or in H1 (V127A). Two other compensatory changes are found in region assigned to the cytosolic interface near H1 (E110K) and H2 (D170N).

It should be noted that among the 55 original spontaneous hygromycin B-resistant *pma1* mutations that were isolated, *pma1*-G158D was identified as particularly interesting. Despite having a reasonably high level of ATPase activity measured in vitro, cells carrying this mutation were more sensitive to lowered pH than most of the mutants studied [13,14]. Moreover, cells carrying *pma1*-G158D exhibited a very low membrane potential by the criterion of TPP uptake [20]. These considerations led us to the possibility that mutations at this site were partially de-coupled between ATP hydrolysis and proton transport. In support of this idea is that one of the full revertants G158D V336A still has the same lowered ATPase activity of G158D, but is no longer sensitive to acid-loading. Thus it may be possible to restore more wild-type function to this mutation in two ways: (1) by improving the coupling between ATP hydrolysis and proton transport or (2) by increasing ATPase activity back to wild-type levels even though coupling is still faulty. Examples of the latter case might be the changes L275F and L275S.

In its own right, G158 appears to be an important residue. Two of the five site-directed mutations we constructed are lethal. The substitution of proline for glycine might be expected to radically disrupt the structure by inducing a kink in the helical structure of transmembrane segment 2. However, the lethal substitution of asparagine for glycine is more puzzling in view of the fact that aspartate at this position is well tolerated. The severe, but viable, phenotypes of G158V and G158L argue that large, bulky hydrophobic amino acids are not well tolerated in this region. This result would suggest a steric constraint due tight packing of the helices in this region. Since both serine and aspartate yield active enzymes at the 158 position, it is also apparent that this region must be somewhat polar, despite its predicted position in the bilayer. Obviously, we must await a definitive three-dimensional structure for this protein before we can begin to assess the actual residues that form interacting networks.

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