

Functional analysis of conserved cysteine residues in the catalytic subunit of the yeast vacuolar H⁺-ATPase

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Received 7 March 1994

Abstract

The A subunit of the yeast vacuolar ATPase contains three highly conserved cysteines: Cys-261, Cys-284, and Cys-538. Cys-261 is located within the nucleotide-binding P-loop. Each of the conserved cysteines, and one nonconserved cysteine, Cys-254, were altered to serine by site-directed mutagenesis, and the effects on growth at pH 7.5 were determined. The Cys-254 → Ser, Cys-261 → Ser and the double mutants all grew at pH 7.5 and contained nitrate- and bafilomycin-sensitive ATPase activity. However, the ATPase activities of the Cys-261 → Ser and the double mutants were insensitive to the sulfhydryl group inhibitor, *N*-ethylmaleimide, demonstrating that Cys-261 is the site of inhibition by *N*-ethylmaleimide. Changing either Cys-284 or Cys-538 to serine prevented growth at pH 7.5. Cys-284 and Cys-538 thus appear to be essential cysteine residues which are required either for assembly or catalysis.

Key words: ATPase, vacuolar; ATPase, H⁺; Vacuole; Cysteine; *N*-Ethylmaleimide; Proton pump

1. Introduction

Eukaryotic vacuolar-type (V-type) H⁺-ATPases are closely related to the archaebacterial plasma membrane ATPases (A-ATPases) and more distantly related to the F-type H⁺-ATPases of mitochondria, chloroplasts and eubacteria [1–3]. The catalytic subunits of the three types of H⁺-ATPases share a number of highly conserved regions, including the GX₄GK(T/S) motif first identified by Walker et al. in a variety of nucleotide-binding proteins [4]. The V-type and A-type H⁺-ATPases can be distinguished from the F-ATPases by the presence of the ‘nonhomologous region’ in the former, which extends the length of the catalytic sub-

unit by about ninety amino acids [2,5]. The V-ATPases can be further distinguished from the F-type H⁺-ATPases by the presence of three highly conserved cysteine residues in the catalytic subunit. The first occurs next to the GKT portion of the nucleotide-binding motif. This position (Cys-261 in yeast) is occupied by a serine in the A-ATPases and a valine in the F-ATPases. The second conserved cysteine (Cys-284 in yeast) is located immediately downstream from the glycine-rich nucleotide-binding loop, adjacent to a region containing the homologues to the DCCD-binding glutamic acid residues of F-ATPase β -subunits [6]. The equivalent of Cys-284 is present in all eukaryotic and archaebacterial A subunits and is thus the most highly conserved of the three cysteine residues. This position is occupied by a valine in the F-ATPases. The third conserved cysteine occurs toward the carboxy-terminus (Cys-538 in yeast). The equivalent cysteine is present in all eukaryotic V-ATPases and in some archaebacterial ATPases. Archaebacteria lacking this cysteine have a serine at this position.

Little is known about the role of the conserved cysteines in regulating V-ATPase enzyme activity. Feng

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; V-ATPase, vacuolar H⁺-ATPase; A-ATPase, archaebacterial H⁺-ATPase; F-ATPase, F₀F₁-ATPase.

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and Forgac [7] provided evidence that Cys-254 of the bovine clathrin-coated vesicle ATPase A subunit (equivalent to Cys-261 in yeast) may be the site of *N*-ethylmaleimide (NEM) inhibition, although their labeling studies did not distinguish between Cys-254 and Cys-240. They also proposed that Cys-254 may regulate activity *in vivo* by forming an intramolecular disulfide bond to a second, unidentified cysteine [8]. However, it is not even known whether bovine Cys-254 is required for enzyme function. To determine whether the conserved cysteines of the A subunit are required for functional V-ATPase activity, we carried out the following mutations in yeast: Cys-261 → Ser, Cys-284 → Ser and Cys-538 → Ser. In addition, a mutation in a nonconserved cysteine (Cys-254 → Ser) and a double mutant (Cys-254/Cys-261 → Ser) were constructed. The results suggest that Cys-284 and Cys-538 are the only essential cysteines of the A subunit, and that Cys-261 is the inhibitory binding site for *N*-ethylmaleimide. The essentiality of Cys-284 for enzyme function may also have implications for the precise location of the splice sites during protein processing of the immature polypeptide [9,10].

2. Materials and methods

2.1. Yeast strains

The *Saccharomyces cerevisiae* haploid strain used in this study was W303-1B (*MAT α* , *leu2 his3 ade2 trp1 ura3*). The null mutant (*LEU2::VMA1*) for the gene encoding the catalytic subunit, *VMA1* (*VAT1*), was generated as previously described [11].

2.2. Construction and analysis of cysteine mutants

The DNA fragment encoding subunit A was engineered to eliminate the 'spacer domain' as previously described [11]. The resulting gene, cloned into the *Bam*HI and *Nde*I sites of the shuttle vector YPN2 [12], complemented the null mutant *LEU2::VMA1*, and its product is identical to those described before [10,13]. Prior to site-directed mutagenesis, the first and third *Eco*RI sites in the reading frame were abolished by site-directed mutagenesis without changing the amino acid sequences. The mutations were generated by PCR using the overlap extension method as previously described [12]. Two separate PCR reactions were carried out, and the products were joined by a third PCR reaction using the two outside primers. The Cys-254 and Cys-261 → Ser mutations were introduced as follows. The outside primer at the 5' end was 5'-CGTTT-TTGAGAACTCGCTAATTTCAAG-3' and the outside primer at the 3' end was 5'-CTCTGGCTGCAAC-CGGCATGTTA-3'. The inside primers which intro-

duced the Cys-254 → Ser mutation were 5'-GGTG-GTACGACATCTATTCCAGGTGC-3' and 5'-GCAC-CTGGAATAGATGTCGTACCACC-3'. The inside primers which introduced the Cys-261 → Ser mutation were 5'-GGTGCTTTTGGTTCTGGTAAGACCGT-3' and 5'-ACGGTCTTACCAGAACCAAAAGCACC-3'. For both sets of inside primers, the former paired with the 5' outside primer and the latter paired with the 3' outside primer. The product of the final reaction using the two outside primers was digested with *Eco*RI and *Pvu*II, and the 0.32 kb DNA fragment was cloned into the unique *Eco*RI and *Pvu*II restriction sites of the control plasmid.

The Cys-284 → Ser mutation was generated similarly, except that the two outside primers were: 5'-TGAGCCAATTTCTTGTCTAAACC-3' and 5'-TAG-GACTATCAAGTGGCA-3' for the 5' and 3' ends, respectively. The two inside primers that introduced the mutation were: 5'-ATCTATGTCGGGTCCG-GAGAAAGAGGT-3' and 5'-ACCTCTTTCTCCG-GACCCGACATAGAT-3', the former pairing with the 5' primer and the latter pairing with the 3' primer. The final product was digested with *Eco*RI and *Bcl*I and cloned into the control plasmid cut with the same enzymes.

The double mutant cassette (Cys-254/Cys-261 → Ser) was obtained by a single PCR reaction using two large primers. The left primer was 5'-CACATCTATTC-CAGGTGCTTTTGGTTCTGGTAAGACCGTTATC-TCTCAATCTTTGTCCAAGTACTCG-3' and the right primer was 5'-AATTTCGAGTACTTGGAG-AAAGATTGAGAGATAACGGTCTTACCAGAAC-CAAAAGCACCTGGAATAGATGTGGTAC-3'. The product was digested with *Eco*RI and *Pvu*II and cloned into the unique *Eco*RI and *Pvu*II restriction sites of the control plasmid as above.

The Cys-538 → Ser mutation was generated by a modification of the overlap extension method which eliminated one of the PCR steps [14]. The first PCR reaction introduced the mutation into a 700 bp fragment. The left primer, 5'-GGCAGAAGTCTTGATG-GAATTCCCAG-3' contained the *Eco*RI site, while the right primer, 5'-CTTCCAAATTGGAGAGAAAG-C-3' introduced the Cys to Ser mutation at the indicated site. This fragment was used as the left primer for the second PCR reaction, using the following right primer: 5'-ATATCATATGCATACTAATCTCA-GGG-3', which included the *Nde*I restriction site. Because of the large size of the left primer, a longer annealing period was utilized in the PCR cycle, as follows: 1 min, 94°C (melting); 15 min, 45°C (annealing); 2 min, 72°C (extension); 30 cycles. The final product (1.1 kb) was digested with *Eco*RI and *Nde*I and ligated into the plasmid cut with *Eco*RI and *Nde*I.

All of the Cys → Ser mutations were confirmed by sequencing. No other mutations were detected in the

sequencing. No other mutations were detected in the final product.

The *E. coli* strain DH5 α was used for cloning and amplification of the plasmids. Transformation of yeast and selection of transformants on Trp⁻ minimal medium plates was performed as previously described [15]. The mutants were then tested for growth on YPD-agar plates buffered at either pH 5.5 or 7.5 with 50 mM Mops/Mes [15].

2.3. Vacuole isolation and ATPase assays

Yeast cells were grown overnight in 12-l carboys at 30°C in 6 l of YPD buffered at pH 7.0 with 50 mM Mops-KOH. The pH of the medium at the end of the incubation did not fall below 6.5. Cells were collected by passage of the culture through a cream separator (Elecrom GL-1, Schluter, Fresno, CA) at 4°C. The rotor was modified by removing the internal set of plates and sealing off the port through which the denser phase of the suspension normally exits. This allowed the cells to pellet on the inside of the rotor. The flow rate was adjusted to allow passage of 12 l of culture in about 1.5 h. The cells were then washed into a beaker with distilled water and repelleted in a Sorvall GSA rotor at 3000 rpm for 10 min. All subsequent purification steps were carried out according to Uchida et al. [16].

ATPase activity was determined after a 30 min incubation by the release of free phosphate as previously described [17]. There was considerable variability in specific activity of the mutants from preparation to preparation; hence results are expressed as percent of the control specific activity. Protein was assayed according to the method of Lowry et al. [18].

3. Results

A sequence alignment showing the locations of the four cysteines mutated in this study is shown in Fig. 1.

Eukaryotes	2	2	2	5
	5	6	8	3
	4	1	4	8
S.c. 246- P	VQGGTT	IPGAFC	GKTVISQSLSKYSNSDAIIYVCG	GERGNE.... 535- DAI
N.c. 234- P	SVQGGT	VAIPGAFC	GKTVISQSVSKYSNSDVIVYVCG	GERGNE.... 524- DQF
D.c. 240- P	SVLGGT	VAIPGAFC	GKTVISQALSQYSNSDVTIVYVCG	GERGNE.... 531- DKF
Bv. 239- P	VQGGTT	IPGAFC	GKTVISQSLSKYSNSDVIIYVCG	GERGNE.... 529- DRF
Archaeobacteria				
H.s. 223- P	IAKGGT	AAIPG	FFSGKTVTQQLAKFADADIVYVCG	GERGNE.... 510- DRY
M.b. 216- P	VAKGGT	AAIPG	FFSGKTVTQQLAKWSDTEIVYVCG	GERGNE.... 503- DAY
S.a. 221- P	IAKGGT	AAIPG	FFSGKTVTQQLAKWSAAKVVIYVCG	GERGNE.... 510- DAF

Fig. 1. Sequence alignment of eukaryotic V-type and archaeobacterial A-type catalytic subunits showing the conserved cysteine residues. S.c., *Saccharomyces cerevisiae*; N.c., *Neurospora crassa*; D.c., *Daucus carota*; Bv., bovine; H.s., *Halobacterium saccharovum*; M.b., *Methanosarcina barkeri*; S.a., *Sulfolobus acidocaldarius*. The top numbers represent the yeast A subunit.

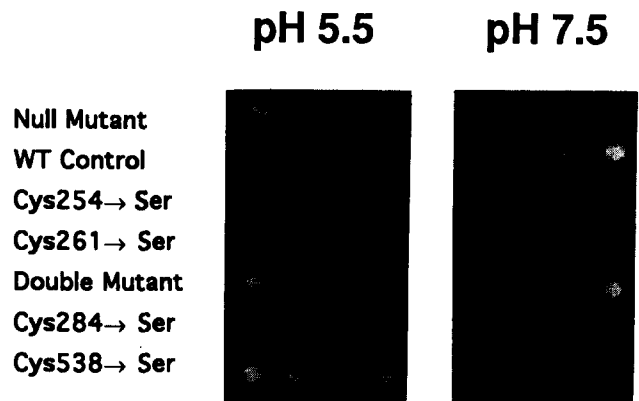


Fig. 2. Growth of the various V-ATPase mutants on agar at pH 5.5 and pH 7.5. The null mutant fails to grow at pH 7.5, and growth is restored after complementation with the wild-type plasmid (WT Control). Of the cysteine mutants, only Cys-284 → Ser and Cys → Ser were unable to grow at pH 7.5.

(Note that the numbering system for the yeast A subunit is based on the mature, spacer-deleted polypeptide [9,10].) Cys-254 (yeast) is nonconserved. Cys-261, Cys-284 and Cys-538 are conserved in all eukaryotic A subunits. Cys-284 is the only cysteine conserved in both eukaryotes and archaeobacteria, although the equivalent to Cys-583 was present in *Halobacterium*. Cys-247, which was not altered in this study, is conserved in some eukaryotes, but is missing from *Neurospora* and carrot.

Each of the four cysteines was altered to serine and the effect on growth at pH 5.5 and pH 7.5 was deter-

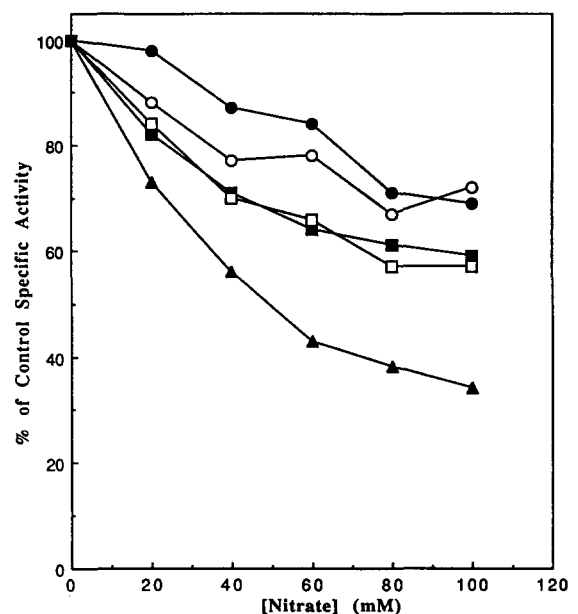


Fig. 3. Effect of nitrate on the V-ATPase activity of the A subunit cysteine mutants. \blacktriangle , wild-type strain (specific activity = 3.23 $\mu\text{mol}/\text{min}$ per mg); \blacksquare , wild-type plasmid control (0.629 $\mu\text{mol}/\text{min}$ per mg); \square , Cys-254 → Ser (0.991 $\mu\text{mol}/\text{min}$ per mg); \circ , Cys-261 → Ser (0.982 $\mu\text{mol}/\text{min}$ per mg); \bullet , Cys-254/Cys-261 → Ser (1.301 $\mu\text{mol}/\text{min}$ per mg).

mined. As shown in Fig. 2, the mutant with the disrupted A subunit gene (null mutant) grew at the permissive pH (5.5), but was unable to grow at pH 7.5. Following transformation with the plasmid containing the wild-type gene (wild-type control), growth at pH 7.5 was restored, demonstrating that a functional A subunit is required for growth at pH 7.5. Three of the mutants, Cys-254 → Ser, Cys-261 → Ser and the double mutants, grew at pH 7.5, characteristic of an active V-ATPase. In contrast, the Cys-284 → Ser and Cys-538 → Ser mutants failed to grow at pH 7.5, suggesting that these mutants are deficient in V-ATPase activity.

Vacuolar membranes were isolated from the Cys-254 → Ser, Cys-261 → Ser and the double mutants, and the ATPase activity was characterized. The results are expressed as the percent of the minus inhibitor control. As shown in Figs. 3 and 4, vacuolar membranes isolated from the wild-type yeast strain were inhibited nearly 70% by 100 mM nitrate and 80% by 400 nM bafilomycin. The 'wild-type control' (null mutant transformed with the wild-type gene) was inhibited 45% by the same concentrations of nitrate and bafilomycin, consistent with the fact that the starting specific activity of the plasmid control membranes averaged about 50% that of the wild-type yeast (see Figs. 1–3). The lower specific activity of the plasmid control could reflect either lower rates of expression of the plasmid-encoded gene or plasmid loss during the overnight culture at pH 7.0. The Cys-254 → Ser, Cys-261 → Ser and double mutants were all inhibited by nitrate and bafilomycin, although to varying degrees.

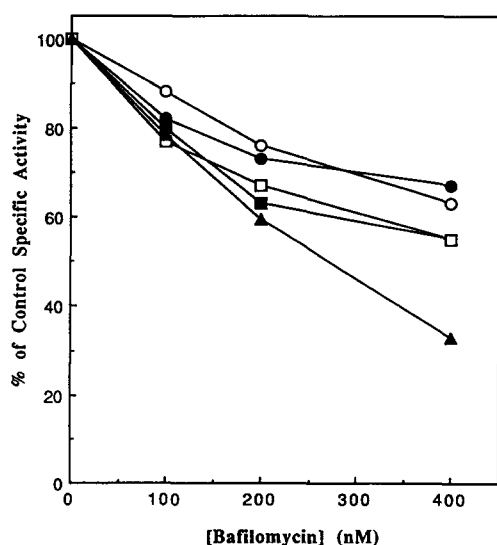


Fig. 4. Effect of bafilomycin on the V-ATPase activity of the A subunit cysteine mutants. ▲, wild-type strain (specific activity = 2.54 $\mu\text{mol}/\text{min per mg}$); ■, wild-type plasmid control (1.328 $\mu\text{mol}/\text{min per mg}$); □, Cys-254 → Ser (0.877 $\mu\text{mol}/\text{min per mg}$); ○, Cys-261 → Ser (0.834 $\mu\text{mol}/\text{min per mg}$); ●, Cys-254/Cys-261 → Ser (0.690 $\mu\text{mol}/\text{min per mg}$).

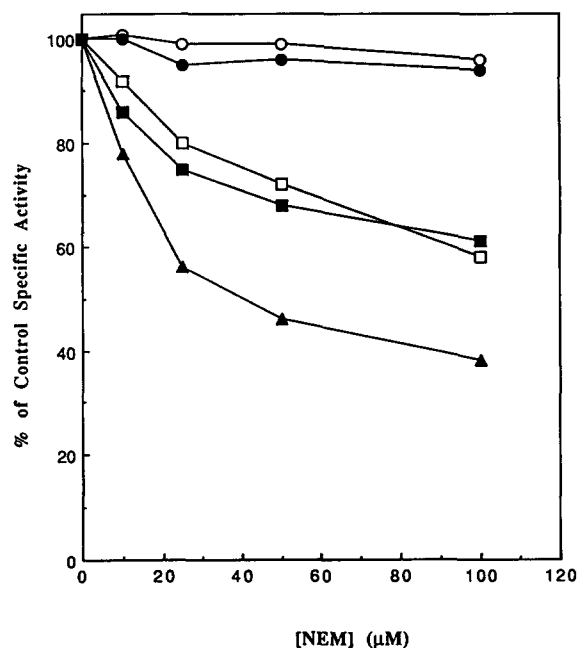


Fig. 5. Effect of NEM on the ATPase activity of the A subunit cysteine mutants. ▲, wild-type strain (specific activity = 2.350 $\mu\text{mol}/\text{min per mg}$); ■, wild-type plasmid control (1.835 $\mu\text{mol}/\text{min per mg}$); □, Cys-254 → Ser (1.560 $\mu\text{mol}/\text{min per mg}$); ○, Cys-261 → Ser (0.765 $\mu\text{mol}/\text{min per mg}$); ●, Cys-254/Cys-261 → Ser (1.050 $\mu\text{mol}/\text{min per mg}$).

The effect of NEM on ATPase activity is shown in Fig. 5. The wild-type activity was inhibited about 60% by 100 μM NEM, whereas the wild-type plasmid control was inhibited by about 40%. The Cys-254 → Ser mutant showed the same sensitivity to NEM as the wild-type control. In contrast, the Cys-261 → Ser mutant and the double mutant were inhibited less than 5% by 100 μM NEM. Based on the amount of nitrate- or bafilomycin-sensitive activity present, the expected level of inhibition by NEM would be at least 25%. Thus the Cys-261 → Ser mutant is insensitive to NEM.

4. Discussion

The presence of a highly conserved cysteine in the catalytic site has long been a puzzling feature of the V-ATPases. V-type and F-type H^+ -ATPases are thought to have similar catalytic mechanisms [19,20], yet the F-ATPase β -subunits have no conserved cysteines. In yeast, Cys-261 is located adjacent to the 'GKTV' motif of the P-loop. We have shown that neither Cys-261 nor Cys-254, a nonconserved cysteine, is required for enzyme activity. Mutants in which the cysteines were altered to serine grew nearly as well as the wild-type control on pH 7.5 agar, indicative of an active ATPase [13,15]. In addition, the vacuolar membranes of the mutants contained both nitrate- and bafilomycin-sensitive V-ATPase activity.

In contrast to Cys-261, both Cys-284 and Cys-538 were found to be essential for growth at pH 7.5. It is perhaps significant that Cys-284 is the only cysteine in the catalytic subunit that is conserved in both eukaryotes and archaeobacteria. Cys-538 is less well conserved since it is not present in *Sulfolobus acidocaldarius* and *Methanosarcina barkeri* (see Fig. 1). Although it is tempting to speculate that Cys-284 may form an intramolecular disulfide bond with Cys-538, such a disulfide bond could not form in either *Sulfolobus* or *Methanosarcina*. Possible alternative functions for Cys-284 include metal binding or intermolecular disulfide bonds. Mutations near the equivalent site of the F-ATPase β -subunit have been shown to bring about altered metal dependence as well as defects in assembly [6,21].

Feng and Forgac [7] used fluorescein-maleimide to identify a V8 proteinase fragment of the bovine coated vesicle ATPase that contained both Cys-240 and Cys-254. The authors argued on evolutionary grounds that Cys-254 (equivalent to Cys-261 in yeast) was the probable site of NEM-binding and inhibition in the bovine coated vesicle ATPase, although the labeled cysteine was never identified directly. In support of their conclusion, Futai et al. [22] recently reported that the Val¹⁵³ \rightarrow Cys mutation in the *E. coli* β -subunit (equivalent to yeast Cys-261 and bovine Cys-254 of the A subunit) makes the F-ATPase activity sensitive to NEM. Here we provide direct evidence that Cys-261 of the yeast V-ATPase is the site of NEM-inhibition, since changing Cys-261 to Ser eliminated NEM-inhibition of the yeast V-ATPase, while the Cys-254 \rightarrow Ser mutant retained NEM-sensitivity. Cys-254 in yeast, like Cys-240 in bovine, is a nonconserved cysteine located upstream of the inhibitory cysteine.

Could Cys-261, which is conserved in all eukaryotes, play a role in regulating enzyme activity *in vivo*? Feng and Forgac [8] have shown that the coated vesicle V-ATPase can be reversibly inactivated by oxidation. The authors proposed that Cys-254 (Cys-261 in yeast) can form an intramolecular disulfide bond with another cysteine upon oxidation. Cys-538 is located in a region with no known function in catalysis. However, it occupies a similar position to His⁴²⁷ of the bovine mitochondrial β -subunit, which binds the nucleotide analog FSBA at pH 6; hence it may occur within a noncatalytic site [21,23]. It is thus possible that Cys-538 occupies a position in the noncatalytic site analogous to Cys-261 in the catalytic site. Inasmuch as these two sites are thought to be physically close [6], they may become cross-linked by an intramolecular disulfide bond between the two cysteines. Hence, Cys-538 may be the unidentified partner in a disulfide bond with Cys-261 during inactivation.

Alternatively, disulfide bonds may form between the A and B subunits. Based on the alignment of the

P-loop regions by Bowman et al. [24], ACys-250 of *Neurospora* (equivalent to ACys-261 in yeast) aligns with BCys-178 of *Neurospora* (equivalent to BCys-188 in yeast). BCys-178 is the most highly conserved cysteine in the eukaryotic B subunit. If the P-loop regions of the A and B subunits interact, intramolecular disulfide bonds may form between them. It seems likely that both inter- and intramolecular disulfide bonds participate in V-ATPase assembly and regulation. Further site-directed mutagenesis studies, as well as more detailed biochemical and X-ray crystallographic analyses [25,26], are needed to determine the sites and roles of disulfide bonding in the V-ATPases.

Finally, the finding that Cys-284 is required for growth at pH 7.5 has implications for studies on protein splicing. The wild-type yeast A subunit gene is unique in that it encodes a 120 kDa precursor polypeptide [9,10]. During maturation, a 50 kDa 'spacer domain' is post-translationally excised [9]. The spacer region is bracketed by two cysteine residues, Cys-284 and Cys-738. Following splicing, a single cysteine remains at the splice site, Cys-284 of the mature subunit. Since direct amino acid sequencing showed that the cysteine of the excised spacer region is located at the amino terminus, Cooper et al. [27] concluded that Cys-284 of the mature A subunit must be derived from Cys-738 of the unprocessed precursor. However, Hirata and Anraku [28] reported that a Cys-738 \rightarrow Ser mutant, although partially blocked in splicing, contained a small amount ($\sim 20\%$) of fully active bafilomycin-sensitive V-ATPase activity. This would imply either that Cys-284 of the mature A subunit is non-essential, or that Cys-738 is not the source of Cys-284 of the processed subunit. In view of this apparent discrepancy, further studies of the Cys-738 \rightarrow Ser mutant are warranted.

Acknowledgements

This research was supported in part by Grant DE-FGO3-84ER13245 from the U.S. Department of Energy to L.T. The authors are indebted to Dr. Tom Stevens of the University of Oregon for generously providing the original plasmid containing the spacer-deleted A subunit gene which was utilized in preliminary experiments. We also wish to thank Drs. B.J. Bowman, E.J. Bowman, and J.P. Gogarten for helpful discussions.

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