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# ASSEMBLY AND REGULATION OF THE YEAST VACUOLAR H$^+$-ATPase

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**Summary**

**The yeast vacuolar H$^+$-ATPase (V-ATPase) consists of a complex of peripheral subunits containing the ATP binding sites, termed the $V_1$ sector, attached to a complex of membrane subunits containing the proton pore, termed the $V_o$ sector. Interaction between the $V_1$ and $V_o$ sectors is essential for ATP-driven proton transport, and this interaction is manipulated *in vivo* as a means of regulating V-ATPase activity. When yeast (*Saccharomyces cerevisiae*) cells are deprived of glucose for as little as 5 min, up to 75 % of the assembled V-ATPase complexes are disassembled into cytoplasmic $V_1$ sectors and membrane-bound $V_o$ sectors. Remarkably, this disassembly is completely reversible. Restoration of glucose to the growth medium results in quantitative reassembly of the disassembled complexes in as little as 5 min, even in the absence of any new protein synthesis. Cells also appear to regulate the extent of $V_1V_o$ assembly on a long-term basis. Yeast cells grown for extended periods in a poor carbon source contain a high proportion of free $V_1$ and $V_o$ sectors, and these sectors remain poised for reassembly when growth conditions improve. Parallel experiments on the *Manduca sexta* V-ATPase suggest that reversible disassembly may be a general regulatory mechanism for V-ATPases. These results imply that V-ATPases are surprisingly dynamic structures, and their unique 'regulated instability' raises a number of interesting physiological and structural questions. How are extracellular conditions such as carbon source communicated to V-ATPase complexes present on intracellular membranes? How are such major structural changes in the V-ATPase generated and how are $V_1$ sectors 'silenced' *in vivo* to prevent unproductive hydrolysis of cytoplasmic ATP by the dissociated enzyme? We are addressing these questions using a combination of genetic and biochemical approaches.**

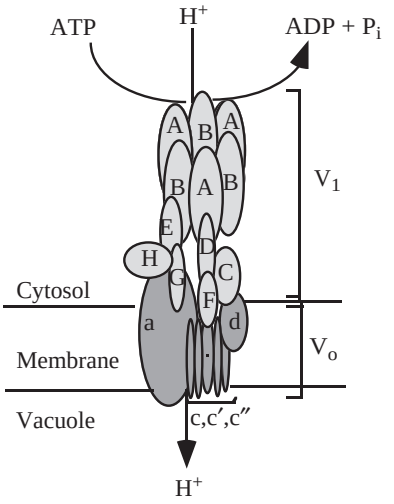
Key words: V-ATPase, regulation, assembly, yeast, acidification, carbon source, *Saccharomyces cerevisiae*.

## Structural organization of the yeast vacuolar H$^+$-ATPase

The yeast vacuolar proton-translocating ATPase (V-ATPase) is structurally very similar to the V-ATPases of other fungi, plants and animals (for reviews, see Stevens and Forgac, 1997; Forgac, 1999; Nelson and Harvey, 1999). In all these systems, the V-ATPase is composed of a complex of peripheral membrane proteins containing the ATP binding sites attached to a complex of integral membrane proteins that makes up the proton pore. A structural model of the yeast V-ATPase is shown in Fig. 1. The yeast 69 kDa A subunit, which appears to be present in three copies per complex, contains the catalytic sites for ATP hydrolysis. The 60 kDa B subunit is also present in multiple copies and may contain regulatory sites for nucleotide binding. Three structurally related proteolipid subunits (c, c′ and c″) form all or part of the proton pore. These five subunits appear to constitute the core of the catalytic machinery of V-ATPases (for reviews, see Stevens and Forgac, 1997; Forgac, 1999). The other eight subunits are also essential for activity of the enzyme, since genetic deletion of any of these subunits in yeast results in a set of growth defects characteristic of loss of V-ATPase activity (the Vma$^-$ phenotype), and each of the subunits is part of the final assembled V-ATPase structure (Stevens and Forgac, 1997). Their individual functions, however, are not clear. Some of these subunits must be involved in the conformational coupling of ATP hydrolysis and proton transport. Others may be essential for regulation of V-ATPases.

Structural and sequence similarities between the V-ATPases and F-ATPases have been examined extensively (for reviews, see Margolles-Clark et al., 1999; Forgac, 1999). In both enzyme classes, nucleotide-dependent conformational changes are communicated between the peripheral complex and the proton pore *via* a number of smaller subunits that form a stalk. There are readily identifiable sequence similarities between the A, B and proteolipid subunits of the V-ATPases, and the β, α and c subunits of the F-ATPases, suggesting a fundamental similarity between the catalytic mechanisms of the two enzymes. Structural and functional parallels between the other subunits of the two enzyme classes have proved to be more elusive (Margolles-Clark et al., 1999).

For the purposes of this review, it is also important to emphasize certain differences between the V- and F-ATPases.




| | Gene product | MW (kDa) | Letter designation |
|---|---|---|---|
| $V_1$ subunits | Vma1p | 69 | A |
| | Vma2p | 60 | B |
| | Vma13p | 54 | H |
| | Vma5p | 42 | C |
| | Vma8p | 32 | D |
| | Vma4p | 27 | E |
| | Vma7p | 14 | F |
| | Vma10p | 13 | G |
| $V_o$ subunits | Vph1p | 100 | a |
| | Stv1p | 100 | -- |
| | Vma6p | 36 | d |
| | Vma16p | 21 | c″ |
| | Vma11p | 17 | c′ |
| | Vma3p | 17 | c |

Fig. 1. Structural model of the yeast V-ATPase. Subunits of the $V_1$ sector are shown in light gray and subunits of the $V_o$ sector are shown in dark gray. The arrangement of the A and B subunits is based on the structure of the mitochondrial $F_1$-ATPase (Abrahams et al., 1994). The enzyme is shown as having two stalks, consistent with current views of $F_1$-ATPase structure and the electron microscope structure of a single $Na^+$-pumping V-ATPase (Boekema et al., 1997), but assignment of subunits C–H to specific positions in the two stalks is still speculative (see Margolles-Clark et al., 1999). MW, molecular mass (kDa).

At least in eukaryotic cells, V-ATPases appear to act solely as ATP-driven proton pumps, whereas F-ATPases act almost exclusively as ATP synthases *in vivo*. The cellular localization of the V-ATPases, which places the $V_1$ sector at the cytoplasmic face of the membrane, makes these enzymes much more accessible to a wide range of potential cellular regulators than the F-ATPases, which are localized to the mitochondrial interior in eukaryotic cells. *In vitro*, the separated membrane and peripheral sectors of the F-ATPases retain their functions: the peripheral $F_1$ sector from most systems is a potent $Mg^{2+}$-dependent ATPase, and the integral membrane $F_o$ sector acts as a proton pore (Penefsky and Cross, 1991; Schneider and Altendorf, 1987). In contrast, the $V_1$ sector shows very little ATPase activity, except under rather non-physiological conditions (high concentrations of $Ca^{2+}$, the presence of organic solvents or reconstituted combinations of subunits not necessarily found *in vivo*), and the $V_o$ sector does not appear to form an open proton pore (Graf et al., 1996; Xie, 1996; Zhang et al., 1992). Finally, there is evidence that the $V_1$ and $V_o$ sectors may be more structurally independent *in vivo* than the $F_1$ and $F_o$ sectors. As described below, many cells contain populations of free $V_1$ and free $V_o$ sectors, and yeast mutants lacking subunits of the enzyme can assemble either $V_1$ or $V_o$ sectors as stable complexes (Doherty and Kane, 1993).

### Reversible disassembly of V-ATPases

Despite the evidence that structural coupling between the $V_1$ and $V_o$ sectors is essential for significant activity *in vivo*, there is extensive evidence that many different cell types contain pools of free $V_1$ and $V_o$ sectors along with fully assembled V-ATPase complexes (Doherty and Kane, 1993; Myers and Forgac, 1993; Sumner et al., 1995; Tomashek et al., 1996; Peng et al., 1999). We and others (Doherty and Kane, 1993; Myers and Forgac, 1993; Tomashek et al., 1996) suggested that the free $V_1$ and $V_o$ sectors might be assembly intermediates, but subsequent studies suggested that the major pathway for V-ATPase assembly does not involve a combination of pre-formed $V_1$ and $V_o$ sectors (Kane et al., 1999). In the light of all these results, we considered the possibility that the V-ATPase was not a static structure after biosynthesis but rather a dynamic one, with free $V_1$ and $V_o$ complexes existing in a dynamic equilibrium with fully assembled $V_1V_o$ complexes.

To test this model, we investigated whether fully assembled V-ATPase complexes could be disassembled in response to changes in extracellular conditions (Kane, 1995). V-ATPase complexes were biosynthetically labelled and allowed to assemble in medium containing relatively high concentrations of glucose, the cells' preferred carbon source. Under these conditions, 60–70 % of the $V_o$ sectors were assembled with $V_1$ sectors, and 30–40 % were free, not bound with $V_1$ sectors. When cells were deprived of glucose, even for as little as 5 min, this ratio shifted dramatically. Following glucose deprivation, only 15–20 % of the $V_o$ sectors were assembled into complexes with $V_1$ sectors, and the remainder of the complexes had disassembled into free $V_1$ and $V_o$ sectors. This disassembly was fully reversible; readdition of glucose, even for 5 min, gave a quantitative reassembly of the disassembled complexes to the original levels. This result is represented in Fig. 2.

Several features of this process were notable. Both disassembly and reassembly of the V-ATPase occur in the presence of 100 μg $ml^{-1}$ cycloheximide, indicating that neither process depends on new protein synthesis (Kane, 1995). At least one subunit, the 42 kDa C subunit of the $V_1$ sector, was lost from both sectors during disassembly and reassembled upon glucose readdition. Disassembly was not an artifact of shifting to medium lacking any carbon source; cells disassembled a similar proportion of V-ATPase complexes when shifted from glucose to a number of poorer carbon sources (Kane, 1995). Disassembly and reassembly of the V-

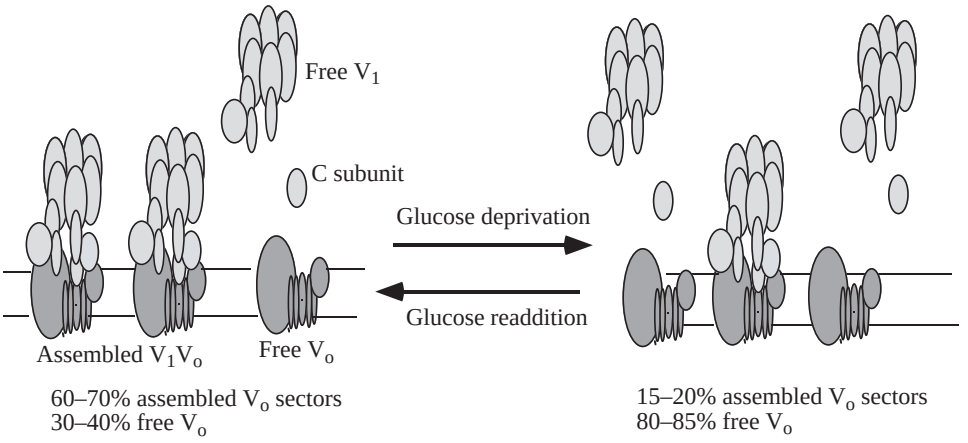



Fig. 2. Reversible disassembly of the yeast V-ATPase. The distribution of $V_o$ subunits between the fully assembled V-ATPase and the disassembled $V_o$ and $V_1$ sectors is altered in response to changes in carbon source as shown. Disassembly of the enzyme (in response to glucose deprivation) results in release of the C subunit from both the $V_1$ and $V_o$ sectors as described in the text. $V_1$ subunits are shown in light gray and $V_o$ subunits are in dark gray.

ATPase appear to occur on both short-term and long-term time scales. As cells deplete glucose in the medium, they gradually accumulate free $V_1$ and $V_o$ sectors, suggesting an ongoing disassembly of the complexes over time (Parra and Kane, 1998). Cells grown overnight in a poor carbon source also contain a higher proportion of disassembled $V_1$ and $V_o$ sectors, suggesting that they have readjusted the 'assembly setpoint' of the complex as a reflection of the carbon source. The free $V_1$ and $V_o$ sectors present after overnight incubation in a poor carbon source remain competent for reassembly; addition of glucose to these cells stimulates additional assembly of $V_1$ with $V_o$ within 5–15 min (Kane, 1995). Finally, an independent series of experiments in *Manduca sexta* suggested that disassembly and reassembly were not unique to the yeast V-ATPase and, instead, may be a common feature of V-ATPases. V-ATPases in midgut epithelial cells of the *M. sexta* midgut were shown to disassemble during molting or starvation and to reassemble upon refeeding in a process very similar to that described in yeast cells (Graf et al., 1996; Sumner et al., 1995).

The disassembly and reassembly of the $V_1$ and $V_o$ sectors therefore appears to be a general regulatory mechanism for V-ATPases. ATP-driven proton transport is down-regulated by disassembly of the V-ATPase and, as described above, the disassembled sectors also appear to be inactive. Reassembly of the enzyme is accompanied by reactivation of ATP hydrolysis and proton transport. These changes in enzyme activity make physiological sense in the context of the conditions triggering assembly and disassembly. Disassembly of the enzyme upon glucose deprivation could help conserve cytosolic ATP stores, and this may be particularly important in cells such as the *M. sexta* midgut, where V-ATPases are major cellular proteins that can consume a significant percentage of total cell ATP (Graf et al., 1996). Reassembly of existing, free $V_1$ and $V_o$ sectors upon readdition of glucose could rapidly generate a renewed proton-pumping capacity that might help cells to handle the reduction in cytosolic pH that accompanies the resumption of active metabolism (Purwin et al., 1986). A variety of biochemical and physiological questions about disassembly and reassembly of V-ATPases remain, however. How are glucose concentrations outside the yeast cell rapidly sensed by V-ATPases on internal membranes? How are such drastic structural changes in the V-ATPase generated? How are the ATP hydrolysis and proton pore activities of the $V_1$ and $V_o$ sectors silenced upon disassembly? How are the final levels of assembled and disassembled V-ATPases determined under different conditions, and why do both assembly and disassembly appear to be incomplete under most conditions? We have begun to address these questions in the experiments described below.

## Cellular signals for disassembly and reassembly of the yeast V-ATPase

Changes in carbon source generate a wide variety of transcriptional and post-translational changes in yeast cells generally directed towards efficient utilization of the available nutrients (for reviews, see Johnston and Carlson, 1991; Thevelein and Hohmann, 1995). A number of glucose signalling pathways have been fully or partially characterized, and yeast mutants defective in these pathways have been identified. As an initial step towards determining how glucose deprivation might trigger disassembly, we investigated the effects of mutations in several known glucose signalling pathways on the assembly state of the V-ATPase and its response to changes in carbon source (Parra and Kane, 1998). Mutants defective in several of the major glucose signalling pathways in yeast, including the ras–cyclic AMP pathway, the main glucose repression/derepression pathway, and the protein kinase C pathway, continued to assemble the V-ATPase to levels similar to those of wild-type cells in the presence of glucose and to disassemble and reassemble the enzyme in response to glucose deprivation and readdition (Parra and Kane, 1998). The results suggest that these well-characterized glucose signalling pathways are not essential for communicating changes in carbon source to the V-ATPase.

Many of the glucose signalling pathways in yeast seem to sense the very early stages of glucose metabolism, including transport of glucose into the cell and formation of glucose 6-phosphate. To test whether accumulation of glucose 6-phosphate was sufficient to maintain the V-ATPase in an assembled state or to reassemble the enzyme after glucose deprivation, we took two different approaches. We determined

the assembly state of the V-ATPase in the presence of 2-deoxyglucose, which can be imported and phosphorylated but cannot be further metabolized. Under these conditions, the V-ATPase disassembled rapidly (Parra and Kane, 1998). We also determined the effects of glucose in a yeast phosphoglucoisomerase (*pgi1*) mutant, which cannot interconvert glucose 6-phosphate and fructose 6-phosphate (Pascual et al., 1979). In a *pgi1* mutant, the V-ATPase disassembles in the presence of glucose, conditions that result in the accumulation of glucose 6-phosphate, but stays assembled (and can be reassembled) in the presence of fructose, which can be phosphorylated directly to fructose 6-phosphate and enter the glycolytic pathway beyond the point affected by the *pgi1* mutation (Parra and Kane, 1998). Taken together, these results indicate that metabolism of glucose beyond the formation of glucose 6-phosphate is necessary for communicating the presence of glucose to the V-ATPase complex and suggest, once again, that the major glucose signal-transduction pathways are not involved in transmitting glucose signals to the V-ATPase. Respiratory metabolism is not necessary for glucose to induce assembly of the V-ATPase. The mitochondrial complexes of the respiratory chain are present at very low levels in yeast cells grown in glucose, and *rho*$^{0}$ cells, which lack mitochondrial DNA and thus fail to form an intact respiratory chain, still support normal assembly, disassembly and reassembly of the V-ATPase (Parra and Kane, 1998).

Given the evidence that glucose metabolism is essential for maintaining maximal assembly of the V-ATPase and the fact that the V-ATPase has multiple sites for nucleotide binding, it is reasonable to speculate that the V-ATPase is directly sensing cytoplasmic nucleotide levels and translating changes in nucleotide binding to changes in $V_1/V_o$ sector interactions. This is still a possible mechanism, but the data indicate that it is not as simple as the V-ATPase directly sensing the concentration of its ATP substrate at the catalytic sites. We measured changes in cellular ATP levels upon glucose deprivation and determined that ATP levels did show an immediate drop on a time scale rapid enough to account for disassembly of the enzyme (Parra and Kane, 1998). There was, however, a partial recovery of cellular ATP levels over the next 20 min, presumably as a result of metabolism of cellular carbohydrate stores, even though there was no reassembly of the V-ATPase on this time scale. Furthermore, varying the ATP concentration in incubations with the V-ATPase in isolated vacuolar vesicles does not seem to change the assembly state of the enzyme (K. J. Parra and P. M. Kane, unpublished data).

The results described above suggest that changes in ATP concentration, or possibly the ATP/ADP ratio, could be one factor in generating disassembly and/or reassembly of the enzyme, but other cellular factors are probably necessary in combination with nucleotide binding to dictate the final assembly state of the V-ATPase. *In vitro* experiments have indicated that one of these factors may be cytoplasmic pH. The yeast V-ATPase can be disassembled *in vitro* by treatment of vacuolar vesicles with relatively low concentrations of chaotropic anions in the presence of MgATP (Kane et al., 1989). We were able to reassemble an active yeast V-ATPase from the $V_1$ subunits obtained by chaotrope stripping and either $V_o$ sectors in the stripped membranes or $V_o$ sectors in vacuolar vesicles isolated from a mutant that failed to assemble $V_1$ sectors (Parra and Kane, 1996). However, the reassembly process proved to be pH-dependent; reassembly and restoration of ATPase activity were optimal at pH 5.5, but at pH 7.5, the $V_1$ sectors neither bound to the $V_o$ sectors nor displayed any restoration of ATPase activity. This result is entirely consistent with the physiological context for V-ATPase reassembly described above and suggests that intracellular acidification resulting from increased rates of metabolism upon glucose readdition might be both a physiological justification and a direct stimulus for V-ATPase reassembly.

One final factor that appears to be critical for regulation of V-ATPase assembly state in response to glucose is the activity of the V-ATPase itself. Disassembly of the yeast V-ATPase in response to glucose deprivation is partially inhibited in the presence of 1 μmol l$^{-1}$ concanamycin A, a highly specific inhibitor of V-ATPases (Drose et al., 1993). A mutation in the vacuolar form of the 100 kDa subunit (*vph1*-E789Q), which completely inhibits ATPase activity and proton pumping (Leng et al., 1996), allows levels of assembly of the enzyme comparable with those in wild-type cells in the presence of glucose, but partially inhibits disassembly in the absence of glucose. Finally, a mutation in the c′ subunit (*vma11*-E145L), which changes the membrane-embedded glutamate predicted to be directly involved in proton transport (Hirata et al., 1997), completely inhibits disassembly of the V-ATPase in response to glucose deprivation and may also result in ‘hyperassembly’ of the enzyme in the presence of glucose (Hirata et al., 1997; Parra and Kane, 1998). Taken together, these results suggest that active catalysis by the V-ATPase may be necessary to generate a conformation of the enzyme susceptible to the cellular signal(s) for disassembly. These *in vivo* results are reminiscent of *in vitro* experiments indicating that disassembly of the V-ATPase in response to low concentrations of chaotrope requires both the presence of the catalytic substrate, MgATP (Kane et al., 1989), and a catalytically competent enzyme (Liu and Kane, 1996).

### Biochemical characteristics of $V_1$ sectors following disassembly

The experiments described in the previous section view disassembly and reassembly of the V-ATPase from a cellular perspective and focus on the cellular factors required for generating changes in V-ATPase structure. We have also begun to examine the structural changes in the enzyme itself upon disassembly. In intact cells, it would appear to be absolutely critical that disassembled $V_1$ sectors are catalytically incompetent, because release of an active and uncoupled ATPase into the cytosol would deplete cellular

ATP stores unproductively. Although many *in vitro* experiments indicate that free $V_1$ sectors do not catalyze MgATP hydrolysis, it is still unclear how this catalytic activity is 'silenced' by detachment of $V_1$ from $V_o$. Careful analysis of the subunit composition of free $V_1$ sectors after glucose deprivation could both indicate how catalytic activity is downregulated and provide clues about the source of the structural changes in V-ATPases triggered by the absence of glucose. To address these questions, we have purified cytosolic $V_1$ sectors and begun their biochemical characterization.

We have initially compared $V_1$ sectors isolated from the cytosolic fraction of two different cell types, wild-type yeast cells deprived of glucose for 5 min, and *vma3*Δ mutant cells that lack the major proteolipid (c) subunit and thus fail to assemble $V_o$ sectors. Previous experiments indicated that $V_1$ sectors assembled, formed a stable structure and remained in the cytosol in *vma3*Δ cells (Doherty and Kane, 1993). We hypothesized that $V_1$ sectors from glucose-deprived cells might differ from those of *vma3*Δ cells because they were released from the membrane in response to a change in carbon source. In fact, we have detected no difference between cytosolic $V_1$ sectors isolated from the two cell types. The isolated $V_1$ sectors contain the A, B, D, E, F, G and H subunits at a stoichiometry that appears to be similar to that of the intact enzyme, solely on the basis of comparison of Coomassie-stained SDS–polyacrylamide gels of the two preparations. As suggested from the initial immunoprecipitation studies (Kane, 1995), the $V_1$ sectors completely lack the C subunit, which is also released into the cytosol by glucose deprivation but appears to dissociate from the rest of the $V_1$ sector and to fractionate away from the rest of the complex during the purification. This overall subunit composition is very similar to that observed for the *M. sexta* $V_1$ sector released from the membrane by starvation (Graf et al., 1996; Svergun et al., 1998) except that the H subunit has not been conclusively identified in *M. sexta* and the *M. sexta* $V_1$ preparation appears to contain the C subunit at sub-stoichiometric levels. We examined the isolated, cytoplasmic $V_1$ sectors for catalytic activity and found no $Mg^{2+}$-dependent ATP hydrolysis. Unlike the *M. sexta* $V_1$ complex, which can be activated to perform $Mg^{2+}$-dependent ATP hydrolysis by the addition of methanol, there appeared to be no methanol activation of the yeast $V_1$ complexes. We did observe $Ca^{2+}$-dependent ATP hydrolysis in the isolated $V_1$ sectors from either glucose-deprived wild-type cells or *vma3*Δ mutant cells in the absence of methanol, however.

The purified yeast $V_1$ fraction provides us with an ideal system in which to study the mechanisms of silencing the ATPase activity of disassembled $V_1$, and we plan to pursue these experiments. Graf et al. (1996) have initiated this type of experiment on the *M. sexta* cytoplasmic $V_1$ complex and have observed strong similarities between the catalytic properties of isolated $V_1$ and soluble $F_1$ complexes from *Bacillus firmus* (Hicks and Krulwich, 1986) and chloroplasts (McCarty and Racker, 1968), including a shift from $Mg^{2+}$-dependent to $Ca^{2+}$-dependent activity in the soluble enzyme and a requirement for methanol to achieve $Mg^{2+}$-dependent activity in the soluble enzyme. Because these conditions (high $[Ca^{2+}]$ and the presence of organic solvent) are unlikely to be mimicked *in vivo*, the soluble $V_1$ and $F_1$ sectors would be enzymatically silent under physiological conditions. Previous experiments on these $F_1$ systems suggest a number of mechanisms of silencing uncoupled ATPase activity *in vivo*, including the presence of inhibitory subunits, the formation of an inhibitory disulfide bond (reviewed by McCarty, 1992; Walker, 1994) and product inhibition at the catalytic site in the soluble enzyme (Zhou et al., 1988). Graf et al. (1996) have already observed product inhibition of the $Ca^{2+}$-dependent ATPase activity of the *M. sexta* $V_1$ complex; we will attempt to reproduce this result in yeast to determine whether it is a general feature of soluble $V_1$ sectors. The possibility that one of the subunits acts as an inhibitor of the soluble $V_1$ complex, similar to the action of the ε subunit of the *Escherichia coli* $F_1$-ATPase complex (for a review, see Senior, 1990), has not been explored, but can be addressed in yeast by isolating and characterizing $V_1$ complexes from strains lacking one of the $V_1$ subunits. Finally, another possibility is that loss of a critical subunit during dissociation might also silence the $Mg^{2+}$-dependent ATPase activity of the $V_1$ sectors. The C subunit, which is lost from the $V_1$ sector during glucose deprivation and does not appear to assemble with the rest of the $V_1$ in *vma3*Δ cells, is an excellent candidate for this type of 'activator subunit'. On the basis of the results of subunit reconstitution experiments, Peng et al. (1993) have proposed that the C subunit is essential for both $Ca^{2+}$- and $Mg^{2+}$-dependent activity of the bovine clathrin-coated-vesicle ATPase. This is not consistent with our results or with those of Graf et al. (1996), but it is still possible that dissociation of the C subunit is partially responsible for the shift from $Mg^{2+}$- to $Ca^{2+}$-dependent ATP hydrolysis in the yeast and *M. sexta* $V_1$ complexes. Given that V-ATPases show much more tendency to exist as separated $V_1$ and $V_o$ sectors than F-ATPases do, it would not be surprising if there were multiple mechanisms involved in activating ATP-driven proton transport and silencing uncoupled ATP hydrolysis by the free $V_1$ sector.

## Unanswered questions about V-ATPase assembly as a regulatory mechanism

Although it now appears to be clear that reversible disassembly of V-ATPases is a significant regulatory mechanism for V-type ATPases as a class, there are still many unanswered questions. The specific cellular signals that communicate extracellular nutrient changes to intracellular V-ATPase complexes are still unclear, although we now have some idea of the important cellular factors. The roles of the individual $V_1$ and $V_o$ subunits in disassembly and cytoplasmic silencing of $Mg^{2+}$-dependent ATPase activity are not known. Elucidating how individual subunits contribute to these processes could also provide important clues about their

roles in the intact and functional V-ATPase. Finally, characterization of the enzymatic activities present in the soluble $V_1$ complexes will not only provide important information about the regulation of V-ATPases, but may also yield specific insights into the mechanism of the membrane-bound V-ATPase.

Most of the experiments described in the previous sections are directed towards understanding what happens when the assembly state of the V-ATPase changes, but one of the most fundamental questions is how the cell establishes an 'assembly setpoint' for any given set of conditions. This question remains largely unaddressed. It is notable that we have not yet discovered any conditions in wild-type yeast cells in which the V-ATPase is 100 % assembled; instead, the cells seem to establish a distribution of disassembled and assembled V-ATPases that can be shifted on either a short or a long time scale (Kane, 1995; Parra and Kane, 1998). Recent data from Peng et al. (1999) have indicated that a second isoform of the a subunit (a2), present in certain bovine tissues, exists in a predominantly disassembled state even under conditions in which the first isoform of the a subunit (a1) would be predominantly assembled. This difference suggests the possibility that related but structurally distinct V-ATPases may respond differently to the same set of extracellular conditions or that the assembly state of these different sets of V-ATPases may be regulated by different extracellular conditions. On the basis of our results with the yeast V-ATPase, we believe that most of the observed changes in assembly state of the enzyme occur after its initial biosynthetic assembly, because the primary assembly pathway of the newly synthesized enzyme does not involve independent assembly of $V_1$ and $V_o$ sectors (Kane et al., 1999). On this assumption, we propose either that the newly synthesized V-ATPase assembles and then partially disassembles into $V_1$ and $V_o$ sectors in response to some cytosolic signal reflective of the growth conditions or that the $V_1$ sector of the assembled V-ATPase continuously cycles on and off the membrane, but that the rate of cycling is altered by cytosolic signals. Either of the proposed models is consistent with the observation that certain inactive V-ATPase complexes, which appear to be defective in disassembly, are actually hyperassembled at the vacuolar membrane (Hirata et al., 1997; Parra and Kane, 1998). Both models could potentially allow the cell to establish a rate of proton pumping appropriate for a given set of conditions, but at the same time to maintain a dynamic range of pumping capacity that is easily accessible by disassembly or reassembly of existing complexes. The available range of proton-pumping capacity could then be further altered, or modulated in specific intracellular compartments, by having structurally distinct V-ATPase complexes, such as the a1- and a2-containing complexes observed in bovine cells.

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