The H Subunit (Vma13p) of the Yeast V-ATPase Inhibits the ATPase Activity of Cytosolic V$_1$ Complexes

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V-ATPases are composed of a peripheral complex containing the ATP-binding sites, the V$_1$ sector, attached to a membrane complex containing the proton pore, the V$_o$ sector. In vivo, free, inactive V$_1$ and V$_o$ sectors exist in dynamic equilibrium with fully assembled, active V$_1$V$_o$ complexes, and this equilibrium can be perturbed by changes in carbon source. Free V$_1$ complexes were isolated from the cytosol of wild-type yeast cells and mutant strains lacking V$_o$ subunit c (Vma3p) or V$_1$ subunit H (Vma13p). V$_1$ complexes from wild-type or vma3Δ mutant cells were very similar, and contained all previously identified yeast V$_1$ subunits except subunit C (Vma5p). These V$_1$ complexes hydrolyzed CaATP but not MgATP, and CaATP hydrolysis rapidly decelerated with time. V$_1$ complexes from vma3Δ cells contained all V$_1$ subunits except C and H, and had markedly different catalytic properties. The initial rate of CaATP hydrolysis was maintained for much longer. The complexes also hydrolyzed MgATP, but showed a rapid deceleration in hydrolysis. These results indicate that the H subunit plays an important role in silencing unproductive ATP hydrolysis by cytosolic V$_1$ complexes, but suggest that other mechanisms, such as product inhibition, may also play a role in silencing in vivo.

V-ATPases$^*$ are highly conserved proton pumps distributed throughout the vacuolar network in all eukaryotic cells. V-ATPases maintain organelle acidification and affect cytosolic pH and ion balance, and their activity has been linked to a diverse array of cellular processes ranging from zymogen activation to protein sorting to viral membrane fusion events (1, 2). V-ATPases are comprised of two structural domains, the V$_1$ domain, which consists of a complex of peripheral subunits containing the nucleotide-binding sites attached to the cytoplasmic face of membrane, and the V$_o$ domain, which is comprised of several integral membrane and tightly associated peripheral proteins that contain the proton pore (1, 2). The yeast V-ATPase has at least eight V$_1$ subunits (designated A, B, C, D, E, F, G, and H) and five V$_o$ subunits (designated a, c, c', d, and d) (1, 3, 4). Genetic and biochemical approaches have converged to show that all of the subunits are required for function of V$_1$V$_o$ complexes (3, 4). Other eukaryotic V-ATPases have very similar subunit compositions.

Functional interdependence of V$_1$ and V$_o$ has been clearly established. Only fully assembled V$_1$V$_o$ complexes can couple ATP hydrolysis to H$^+$ translocation, and in vitro experiments indicate that upon V$_1$ dissociation, the V$_o$ domain does not conduct protons and the V$_1$ domain does not perform MgATP hydrolysis (5–8). Nevertheless, many different cells have been shown to contain free V$_1$ and free V$_o$ sectors in addition to fully assembled V$_1$V$_o$ complexes (9–12). Independent experiments in yeast and Manduca sexta have indicated that the disassembled V$_1$ and V$_o$ sectors exist in a dynamic equilibrium with fully assembled complexes and that this equilibrium can be shifted in response to changes in extracellular conditions (10, 13, 14). Starvation appears to stimulate disassembly of V$_1$ from V$_o$; but this disassembly is fully reversible upon refeeding (13–15). This reversible association between V$_1$ and V$_o$ is believed to regulate V-ATPase function in vivo: disassembly of V-ATPase complexes may conserve ATP when energy reserves are low and reassembly of the enzyme may provide the renewed proton pumping capacity necessary to prevent cytosolic acidification when active metabolism resumes (16, 17).

A constitutively active free V$_1$ in the cytosol could quickly become lethal to the cell by hydrolyzing cytosolic reserves of ATP. Graf et al. (14) have isolated cytosolic V$_1$ complexes from M. sexta and shown that these complexes exhibit Ca$_{2+}$-dependent ATP hydrolysis at nonphysiological Ca$_{2+}$ concentrations but hydrolyze MgATP only in the presence of melathon. The properties of V$_1$ complexes have also been examined by reconstitution of expressed subunits and biochemically isolated subcomplexes of the bovine clathrin-coated vesicle ATPase (18–21). These studies have also revealed a shift from Mg$_{2+}$-dependent to Ca$_{2+}$-dependent ATPase activity in V$_1$ complexes detached from the membrane subunits and have suggested that CaATPase activity is a partial reaction characteristic of dissociated V$_1$ sectors that is functionally related to the MgATPase activity of the fully assembled proton pump (18).

In an attempt to gain more insight into the cellular mechanisms of V$_1$-ATPase silencing, we have purified and characterized native yeast cytosolic V$_1$ complexes. Cytosolic V$_1$ were isolated from wild-type cells and from two vma mutant strains. We found that V$_1$ subunit C was not present in any of the isolated complexes. All of the isolated V$_1$ complexes hydrolyzed ATP in the presence Ca$^{2+}$, but only V$_1$ complexes lacking subunit H had MgATPase activity. The current study also indicates that product inhibition of ATPase activity may occur in cytosolic V$_1$ complexes but cannot fully account for the inactivation of these complexes. In addition, structural changes within the V$_1$ complex itself, such as loss of an activator subunit (C subunit) and presence of at least one inhibitory subunit (H subunit)

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$^*$ The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; V$_p$, peripheral sector of V-ATPase; V$_m$, membrane sector of V-ATPase; F-ATPase, F$_0$F$_1$-ATP synthase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
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subunit) may be critical for silencing the MgATPase activity in vivo.

### EXPERIMENTAL PROCEDURES

#### Materials and Strains—Zymolase 100T was purchased from ICN. Concana
cymycin A was obtained from Wako Biochemicals. Prestained molecular mass markers (high range) were obtained from Life Technolo

gies. For Western blotting and anti-Myc monoclonal antibody 9E10 were purchased from Roche Molecular Biochemicals. All other reagents were purchased from Sigma.

The wild-type yeast strain used in these experiments was SF838-1D (MATa ade6 leu2-3,112 ura3-52 pep4-3 gal2-22). The vma3Δ strain was congenic with the wild-type strain except for the vma3Δ:URA3 mutant allele, which was constructed by PCR using the wild-type allele and a HindIII fragment containing the vma3Δ::LEU2 allele from the deletion plasmid described below, and integrating into the VMA13 locus by a one-step gene disruption (24). Replacement of the wild-type allele by the deletion allele was confirmed by PCR of chromosomal DNA

- **PCR products** by the deletion allele was confirmed by PCR of chromosomal DNA
- **Replacement of the wild-type allele** by the deletion allele was confirmed by PCR of chromosomal DNA
- **Myc epitope including the ACC generating a 1912-base product which contained 27 bases of the 9-CAAAGCTTATTTCTGAAA-9 base sequence. Two separate PCR reactions were performed using low
- **vma3Δ** strain was constructed by excising a BamHI-SacI fragment containing the vma3Δ::LEU2 allele from the deletion plasmid described below, and integrating into the VMA13 locus by a one-step gene disruption (24). Replacement of the wild-type allele by the deletion allele was confirmed by PCR of chromosomal DNA

- **Preparation of Cytosolic V₁ Complexes—Cytosolic V₁ complexes from wild-type yeast cells and vma3Δ mutant cells, treated both with and without a brief glucose deprivation, were purified by a variation of the methods reported by Graf et al. (14). In wild-type cells, the population of V₁ complexes in the cytosol was increased by depriving the cells of glucose for 5 min. This treatment has been shown to trigger dissociation of approximately 75% of assembled V₁ complexes (13, 15). vma3Δ mutant cells lack the gene encoding the proteolipid subunit c of the V₁ sector (28) and provide an alternative source of V₁.

**RESULTS**

### Purification of Cytosolic V₁ Complexes—In order to better understand how cytosolic V₁ sectors are inactivated and possibly to gain insights into how V₁ dissociation is triggered, we isolated and characterized cytosolic V₁ sectors from yeast. Cytosolic V₁ complexes from wild-type yeast cells and vma3Δ mutant cells, respectively. Glucose deprivation improved the yield of V₁ sectors from wild-type cells, but did not significantly affect the yield of V₁ sectors from the vma3Δ cells. Briefly, the purification consisted of isolation of a soluble fraction by high speed centrifugation, followed by protein precipitation with 50% ammonium sulfate and two
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Fig. 1. Purification of cytosolic V$_1$ sectors. A and B, ion exchange chromatography of yeast cytosol. A, supernatant protein obtained after high speed centrifugation of a yeast cell lysate was precipitated with 50% ammonium sulfate, desalted, and applied to a Mono-Q ion exchange column as described under “Experimental Procedures.” After an initial wash, proteins were eluted from the column with three sequential linear gradients. Protein concentration was monitored by measuring absorbance at 280 nm (A$_{280}$). A profile of the stepwise salt gradient used is superimposed on the protein elution profile and is described in more detail under “Experimental Procedures.” B, the indicated fractions collected from the chromatogram shown in A were precipitated with 10% trichloroacetic acid, solubilized, and separated by SDS-PAGE, and blotted to nitrocellulose. The blot was probed with mouse monoclonal antibodies 7A2, which recognizes the C subunit, 13D11, which recognizes the A subunit, and 8B1, which recognizes the B subunit, followed by alkaline-phosphatase-conjugated goat anti-mouse antibodies (11). The fractions containing assembled V$_1$ complexes were identified by nondenaturing immunoprecipitation with monoclonal antibody SB1 as described under “Experimental Procedures.” C and D, isolation of cytosolic V$_1$ complexes by gel filtration. C, fractions 43–54 from the ion exchange chromatography column shown in A were pooled, concentrated, and loaded on a Bio-Rad Sec400 gel filtration column. Protein concentration in fractions eluted from the column was monitored by measuring the A$_{280}$ of D$_{2}V$_1 subunits eluted from the gel filtration column in a single peak, centered at fraction 20. The indicated fractions were subjected to SDS-PAGE and immunoblotting. The A and B subunits were recognized with monoclonal antibodies SB1 and 13D11 as described above, and the E subunit was recognized by polyclonal antisera raised against the yeast E subunit (generously provided by Dr. Tom Stevens).

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Fig. 2. Subunit composition of cytosolic V$_1$ complexes isolated from vma3A cells. The combined fractions containing assembled V$_1$ complexes from the ion exchange column (Q2 Pool) and fraction 20 from the gel filtration column (Sec 400 Pool) were precipitated, solubilized, and subjected to SDS-PAGE. The gels were then silver stained (23). The positions of known V$_1$ subunits are indicated; the identities of the A, B, H, and E subunits were confirmed by immunoblotting.

Sequential chromatographic columns: ion exchange on a Mono-Q column and gel filtration on a Biosilect Sec400 column. Fig. 1A shows the protein elution profile from the ion exchange column, and Fig. 1B is a Western blot analysis of the elution pattern of A, B, and C V$_1$ subunits. The C subunit failed to bind to the column even at the lowest salt concentration and fractionated away from the rest of the cytosolic V$_1$ subunits. Because the A and B subunits were detected throughout the gradient, we assessed whether they were assembled with other subunits by nondenaturing immunoprecipitation of selected pooled fractions (not shown). Assembled V$_1$ complexes were present only in fractions eluted with 0.2 M salts (0.1 M NaCl, 0.1 M KCl); A and B subunits that eluted elsewhere from the column were either partially or fully dissociated from the remaining V$_1$ subunits. The assembled V$_1$ complexes eluted from the Mono-Q column (Fig. 1B, fractions 43–54) were concentrated and subjected to gel filtration chromatography. The elution profile for the gel filtration column is shown in Fig. 1C. The V$_1$ complexes eluted in a single peak (Fig. 1D) with an estimated molecular mass of 445 kDa.

Purified V$_1$ complexes from wild-type and vma3A cells, either with or without glucose deprivation, showed a similar subunit composition. The presence of the 27-kDa E subunit in the V$_1$ complexes was confirmed by Western blotting (Fig. 1D). Silver staining of the peak eluted from gel filtration column (Fig. 2) showed additional bands of 32, 16, and 14 kDa that correspond in molecular mass to the previously identified D, G, and F subunits, respectively (4). These data indicate that the V$_1$ complexes obtained from both strains contained the A, B, D, E, F, and G V$_1$ subunits (Figs. 1D and 2). Both the E and G subunit had a somewhat smeared appearance in the V$_1$ preparations. In addition to the previously characterized V$_1$ subunits, bands of approximately 25 and 80 kDa and several high molecular mass bands were consistently present in the fractions containing the V$_1$ complexes. We have not yet determined whether these proteins are associated with cytosolic V$_1$ complexes.

We were particularly interested in determining whether the H subunit, encoded by the VMA13 gene in yeast (29), was associated with the cytosolic V$_1$ complexes. This protein has a molecular mass of 54 kDa and is often masked by the 60-kDa subunit, so the VMA13 gene was tagged with a Myc epitope to allow it to be clearly identified. The tagged protein was expressed in a vma13A yeast strain and shown to fully complement the growth defects of the strain. Co-purification of subunit H with cytosolic V$_1$ complexes was confirmed using anti-Myc antibodies against V$_1$ complexes purified from vma13A cells expressing the Myc-tagged VMA13 gene (Fig. 3). Therefore, the cytosolic V$_1$ sectors appear to contain all the previously characterized V$_1$ subunits except subunit C.

Enzymatic Activities of Cytosolic V$_1$ Complexes—The isolated V$_1$ domain of the M. sexta V-ATPase is not active as a MgATPase except in the presence of organic solvents (14). Similarly, the isolated yeast V$_1$ complexes did not hydrolyze ATP if the divalent cation supplied was Mg$^{2+}$. In an attempt to activate the yeast V$_1$ ATPase activity, the purified V$_1$ was treated with 25% methanol, 30 mM octylglucoside, 5–50 mM sodium sulfite, 0.5% N,N-dimethyldecycleamine-N-oxide, and 5–10 mM dithiothreitol, treatments which had effectively activated the
MgATPase activity of the *Manduca* V$_1$ (14) or F$_1$-ATPases from various sources (30–33). None of these treatments elicited any MgATPase activity in the yeast V$_1$ complexes.

Cytosolic V$_1$ complexes from both wild-type and *uma3Δ* cells did hydrolyze ATP in a Ca$^{2+}$-dependent manner at nonphysiological (mM) Ca$^{2+}$ concentrations, however. CaATPase activity has been described in purified V$_1$ complexes from *M. sexta* (14), isolated chloroplast and *Bacillus firmus* F$_1$ complexes (30, 31), and reconstituted mixtures of bovine V$_1$ subunits (18–21). The enzymatic properties of complexes purified from glucose-deprived wild-type cells or *uma3Δ* cells with or without glucose deprivation were very similar. Because *uma3Δ* cells provided a more abundant source of cytosolic V$_1$ than wild-type cells, the kinetic analysis described below was performed on cytosolic V$_1$ complexes isolated from *uma3Δ* mutant cells.

CaATP hydrolysis was first examined as a function of the time at a constant CaATP concentration (1.4 mM). When the incubation time was varied from 0.5 to 20 min, the plot of micromole of Pi formed/mg. At a lower CaATP concentration (0.3 mM), it took longer for the activity to decay, but the activity was gone by 20 min. An apparent $K_m$ of 0.183 mM for CaATP, which is similar to the $K_m$ of yeast V$_1$ (0.210 mM; Ref. 34) was estimated from 1-min reactions performed at a larger range of concentrations. Based on this information, 1.4 mM CaATP should nearly saturate the enzyme, and the loss of activity over time seen in Fig. 4A cannot be attributed to substrate depletion.

The substrate specificity of the yeast V$_1$ complexes was examined. Ca$^{2+}$-dependent hydrolysis of GTP was observed (Fig. 4B). Interestingly, CaGTP hydrolysis was linear for at least 20 min under conditions where ATP hydrolysis had ceased after 3 min. The V$_1$ complexes exhibited a specific activity for GTP hydrolysis of 0.47 $\mu$mol/min/mg of protein. Once again the hydrolysis was Ca$^{2+}$-dependent; Mg$^{2+}$ did not support any GTPase activity in the cytosolic V$_1$ complexes.

Loss of activity over time could be an indication of product inhibition of the ATPase activity. Product inhibition has been studied in considerable detail in F$_1$-ATPases, and appears to be specific to ADP in many cases (35–37). Thus, although GTP is a substrate for F$_1$, GDP is much less efficient in product inhibition (36). To further explore the possibility that the ATPase activity of the cytosolic V$_1$ complexes was inhibited by ADP, V$_1$ complexes were preincubated in the presence of CaADP before measurement of the CaATPase activity. Isolated V$_1$ complexes were preincubated with 1.1 mM CaADP for 1 min, then the V$_1$-CaADP mixture was diluted 30-fold into assay medium and the CaATPase activity measured in the presence of 1.4 mM CaATP (Fig. 4A). The CaATPase activity of the V$_1$ complexes was not fully inhibited by CaADP preincubation. The initial ATPase activity was 51% that in the absence of ADP, and a decay in ATPase activity similar to that seen in the absence of ADP, preincubation was observed over the next 3 min. We also determined whether the cytosolic V$_1$ preparation contained tightly bound nucleotides after isolation that might be involved in inhibition of either Mg$^{2+}$-dependent or Ca$^{2+}$-dependent ATPase activity. Only substoichiometric amounts of ADP (0.02 mol/mol V$_1$) and ATP (0.005 mol/mol V$_1$) were detected in the isolated cytosolic V$_1$ complexes. Pyrophosphate was shown to enhance MgATPase activity of F$_1$-ATPases by removing tightly bound nucleotides (36). However, addition of 4.8–9.4 mM PPI did not activate the ATPase activity of cytosolic yeast V$_1$. The sensitivity of the yeast cytosolic V$_1$ to a variety of inhibitors was examined. The CaATPase activity of the cytosolic V$_1$ was not affected by addition of the specific P- and F-type ATPases inhibitors sodium orthovanadate (1 mM) and sodium azide (10 mM), respectively. Concanamycin A, a specific V-type ATPase inhibitor believed to interact with the $V_o$ domain at the membrane (38) had no effect on the ATPase activity of purified V$_1$. V-ATPases contain a set of three conserved cysteine residues that are essential for activity and render the enzyme sensitive to low concentrations of N-ethylmaleimide (39). Measuring N-ethylmaleimide sensitivity of the cytosolic V$_1$ sectors was difficult because the presence of reducing agent (β-mercaptoethanol) appeared to be essential for purification of an active V$_1$ and maintenance of its activity. However, addition of N-ethylmaleimide in excess to the concentration of β-mercaptoethanol in the isolated V$_1$ preparation allowed us to estimate
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FIG. 5. Subunit composition of cytosolic V₁ complexes isolated from vma13Δ cells. Cytosolic V₁ complexes were isolated from wild-type and vma13Δ mutant cells as described in the legend to Fig. 1B and under “Experimental Procedures.” The V₁ peak fraction after gel filtration chromatography was subjected to SDS-PAGE and stained with Coomassie Blue. The positions of known V₁ subunits are indicated; the identities of the A, B, and E subunits were confirmed by immunoblotting.

Discussion

Subunit Composition and Enzymatic Activities of Cytosolic V₁ Complexes—The yeast cytosolic V₁ complexes contain established V₁ subunits A, B, D, E, F, G, and H. Subunit C was the only V₁ subunit not associated with yeast cytosolic V₁ complexes; this subunit was present in a high speed supernatant, but fractionated away from the other V₁ subunits in ion exchange chromatography. Earlier immunoprecipitation experiments had indicated that the C subunit dissociated from both the V₁ and Vₒ sectors during V-ATPase disassembly (13). The subunit composition of the yeast cytosolic V₁ complexes closely resembles that of the cytosolic V₁-ATPase complexes from M. sexta (14, 40). The M. sexta complexes appear to contain subunits A, B, D, E, F, and G, along with substoichiometric amounts of the C subunit (40). Subunit H has only recently been identified in M. sexta (17), so it is unclear whether this subunit is really not present in the insect cytosolic V₁ complexes, or is hidden by the B subunit, which generally runs very close to the H subunit on SDS-PAGE.

Both the insect and yeast cytosolic V₁ complexes are active as CaATPases, indicating that this activity does not require the presence of the C subunit. There were other striking similarities in the activities of the two enzyme preparations. Both showed a loss of CaATPase activity over time and a lower level of CaATPase activity that did not decay over time. As noted by Graf et al. (14), these features are also shared by isolated F₁ sectors from chloroplasts and B. firmus (30, 31). One difference between the insect and yeast V₁ preparations is the activation of MgATPase activity in the insect enzyme in the presence of 25% methanol; no MgATPase activity was observed for the wild-type yeast V₁ preparation, even in the presence of a wide variety of potential activating agents. A shift from Mg²⁺-dependent to Ca²⁺-dependent ATP hydrolysis has been observed in V₁ subunit reconstitution experiments of the bovine clathrin-coated vesicle V-ATPase, as well. These subunit reconstitution experiments had indicated an essential role for the C subunit in CaATP hydrolysis (19), however, and this appears to conflict with results from the native cytosolic V₁ preparations.

We had anticipated that cytosolic V₁ sectors isolated from wild-type yeast cells and vma3Δ mutant cells before and after glucose deprivation might show differences in subunit composition that reflected their different histories and provided indications as to how glucose deprivation signals V₁ dissociation. This did not prove to be the case, at least at the level of analysis reported here. Both the subunit composition and basic enzymatic properties of the cytosolic V₁ sectors from different sources were very similar. It may still be that there are subtle differences in post-translational modifications of the subunits that we have not yet identified, and we plan to look at the different preparations in more detail in the future. It is also possible, however, that cytosolic V₁ sectors from wild-type cells before and after glucose deprivation have the same structure, and that different amounts of V₁ are present because the equilibrium of an ongoing dissociation and reassociation is shifted when glucose becomes limiting. Along the same lines, the cytosolic V₁ sectors that are formed in vma3Δ cells but never attach to the membrane may be stable because they resemble the cytosolic wild-type V₁ sectors that are normally cycling on and off the membrane.

How Is Mg²⁺-dependent ATP Hydrolysis by Cytosolic V₁ Sectors Silenced?—Reversible disassembly of V-ATPases has been proposed to be a mechanism of down-regulating V-ATPase activity when growth conditions are unfavorable (16, 17). Under-
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Characterization of V₁ ATPase activity from a vma13Δ mutant suggests that the H subunit may play an important role in inhibiting both Mg²⁺- and Ca²⁺-dependent ATP hydrolysis by cytosolic V₁ sectors. An inhibitory role for the H subunit was not expected from previous data. The yeast vma13Δ mutant, which lacks the H subunit, assembles V₁V₀ complexes in the membrane (29), but the complexes are unstable and inactive. Addition of sub-57-kDa dimer, which consists of two isoforms of the H subunit, to a V₁ complex reconstituted from bovine clathrin-coated vesicle subunits enhances CaATPase activity of the complexes, and sub-57-kDa dimer or either of the individual H subunit isoforms appears to be essential for MgATPase activity and proton pumping by the fully assembled bovine clathrin-coated vesicle pump (21, 41). Taken together, these data have suggested that the H subunit may act as an activator, not an inhibitor, of V-ATPase activity, but these experiments have focused predominantly on the intact V₁V₀ complex, not isolated V₁ sectors. The experiments presented here suggest that the H subunit may play a role similar to that of the ε subunit of the E. coli F₁-ATPase, which inhibits the F₀-ATPase when it is detached from the membrane (33), but may be critical for proper structural and function coupling of F₁ and F₀ (42, 43).

Comparison of CaATP hydrolysis by cytosolic V₁ sectors with and without the H subunit (Fig. 6A) indicates that the H subunit may be particularly critical for the decay in ATP hydrolysis rate after the first few minutes of turnover. Cytosolic V₁ complexes from vma13Δ cells showed only a slightly higher initial rate than those from vma3Δ cells, but they were able to maintain this rate for at least 20 min, under conditions where complexes from vma3Δ cells were almost completely inactive after less than 5 min. The higher activity of the complexes from vma13Δ cells could not be attributed to loss of another V₁ sector. However, it is notable that the M. sexta enzyme appeared to lose Ca²⁺-dependent activity under conditions where it gained Mg²⁺-dependent activity, but both Ca²⁺ and Mg²⁺-dependent activities appear to be activated in the cytosolic V₁ complexes from vma13Δ cells. This result suggests that the methanol does not

TABLE I

| Addition                  | μmol Pi/min/mg |
|---------------------------|---------------|
| None                      | 0.265         |
| Octylglucoside (30 mM)    | 0.113         |
| Methanol (25%, v/v)       | 0.475         |
| Sodium sulfite (60 mM)    | 0.217         |

Effect of activators on the MgATPase activity of cytosolic V₁ complexes isolated from vma13Δ cells

Cytosolic V₁ complexes were isolated from vma13Δ cells, and 1.5 μg of the isolated complexes were incubated in the presence of 1.4 mM MgATP for 15 min in 500-μl of assay buffer either without additions or with the indicated concentrations of potential activators present throughout the incubation. Pi release was determined colorimetrically after 15 min and used to calculate the apparent specific activity.

Fig. 6. CaATPase activity of cytosolic V₁ complexes from vma13Δ cells. A, cytosolic V₁ complexes were isolated from vma13Δ cells, and 10.3 μg of the isolated complexes were incubated with 1.4 mM CaADP in a 500-μl total volume for the indicated times (closed circles). The activity from 11.2 μg of V₁ complexes isolated from vma3Δ cells and assayed under identical conditions is shown for comparison (open circles). Pi release was monitored by colorimetric assay (25). B, CaATPase activity was also measured after a 1-min preincubation of the complexes with 1.1 mM CaADP followed by a 30-fold dilution into assay buffer (500-μl final volume) containing 1.4 mM CaATP (closed circles), and compared with the activity in the absence of CaADP preincubation (open circles). For this set of experiments, 1.5 μg of protein was used.

Fig. 7. MgATPase activity of cytosolic V₁ complexes from vma13Δ cells. Cytosolic V₁ complexes were isolated from vma13Δ cells, and 1.5 μg of the isolated complexes were incubated with 1.4 mM MgATP in a 500-μl total volume for the indicated times. Pi release was monitored by colorimetric assay (25).

lying this proposal is the assumption, consistent with in vitro data (6, 8), that the cytosolic V₁ sectors are inactive in ATP hydrolysis. As expected, native cytosolic V₁ complexes purified from wild-type yeast cells could not hydrolyze ATP when Mg²⁺ was provided as the divalent cation, indicating that under physiological conditions, the yeast V₁ complexes are catalytically inactive. The results reported here suggest several potential reasons cytosolic V₁ sectors are not active in vivo.
act on the M. sexta enzyme simply through release of the H subunit or a functional equivalent. The MgATPase activity of yeast vma13Δ complexes showed a loss of activity with time similar to that seen for the CaATPase activity of cytosolic V₁ complexes from wild-type cells, indicating that cytosolic V₁ complexes are still prevented from exhibiting high levels of unproductive ATP hydrolysis in vma13Δ cells in vivo. These data suggest that the H subunit is important in inactivating cytosolic V₁-ATPase activity, but there are probably other silencing mechanisms that act in combination, as described below.

One of these other mechanisms may be inhibition by ADP. The data presented here suggest that ADP could play a rather complex role in inhibiting the activity of cytosolic V₁ complexes. The loss of CaATPase activity in the wild-type complexes or MgATPase activity in the vma13Δ complexes over time could have at least two explanations. First, enzyme activity may destabilize the complex so that one or more subunits is lost, inactivating the enzyme. We cannot eliminate this possibility at present, but it should be possible to address it by careful determination of the subunit composition before and after catalysis. Alternatively, the loss of activity is suggestive of prod-
determination of the subunit composition before and after ca-
at present, but it should be possible to address it by careful
inactivating the enzyme. We cannot eliminate this possibility
have at least two explanations. First, enzyme activity may
been reported previously (45). Further experiments will be
necessary to characterize the mechanisms of ADP inhibition of the yeast cytosolic V₁ complexes and fully assess their physio-
logical significance.

The data presented here suggest that the inhibitory H sub-
unit and inhibition by product ADP may play important roles is silencing unproductive hydrolysis by cytosolic V₁ complexes in yeast, but it is important to emphasize that they do not exclude other mechanisms of silencing. We have demonstrated release of the C subunit from cytosolic V₁ complexes, but have not yet determined whether this release plays a functional role. We have not yet assessed whether there are post-translational modifications of any of the V₁ subunits when they are released from the membrane, but with the purification protocol developed here, we are poised to determine both whether there are reversible modifications and whether these modifications affect activity. Silencing cytosolic V₁ complexes in vivo is likely to be a synergistic effect rather than a simple event. Considering that inhibition of cytosolic V₁ complexes is vital, it would not be surprising if cells had more than one mechanism to lock the catalytic conformation of the complex and prevent futile ATP hydrolysis.

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