Dimer formation of subunit G of the yeast V-ATPase

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Abstract The G subunit of the vacuolar ATPase (V-ATPase) is a component of the stalk connecting the V_1 and V_O sectors of the enzyme and is essential for normal assembly and function. Subunit G (Vma10p) of the yeast V-ATPase was expressed in *Escherichia coli* as a soluble protein and was purified to homogeneity. The molecular mass of subunit G, determined by Native-polyacrylamide gel electrophoresis, gel filtration analysis and small-angle X-ray scattering, was approximately 28 ± 2 kDa, indicating that this protein is dimeric. With a radius of gyration (R_g) and a maximum size (D_{max}) of 2.7 ± 0.2 nm and 8.0 ± 0.3 nm, respectively, the G-dimer is rather elongated. To understand which region of subunit G is required to mediate dimerization, a $G_{38–144}$ form (the carboxyl-terminus) was expressed and purified. $G_{38–144}$ is homogeneous, with a molecular mass of approximately 12 ± 3 kDa, indicating a monomeric form in solution.

Key words: Vacuolar-type ATPase; V_1V_O ATPase; V_1 ATPase; Vma10p; Dimerization; Small-angle X-ray scattering

1. Introduction

Vacuolar ATPases (V-ATPases) are present in every eukaryotic cell where they are responsible for acidification of lysosomes, the Golgi apparatus, and endosomes; these enzymes are involved in protein sorting, pH and calcium homeostasis, and zymogen activation [1–3]. The V-ATPases are composed of a water-soluble V_1 ATPase and an integral membrane subcomplex, V_O. ATP is hydrolyzed on the V_1-headpiece consisting of an A_3:B_3 hexamer, and the energy released during that process is transmitted to the membrane-bound V_O domain, to drive ion translocation. This energy-coupling occurs via the so-called 'stalk' structure, an assembly of the V_1 and V_O subunits C–H and *a*, respectively, that forms the functional and structural interface [3–5]. The proposed subunit stoichiometry of V_1 is A_3:B_3:C_1:D_1:E_1:F_1:G_2:H_x [6]. The integral V_O domain contains five different subunits in a stoichiometry of a_1:d_1:$c_{4–5}$:c'_1:c''_1 [3]. V-ATPases have a structure similar to F-ATPases and several of their subunits probably evolved from common ancestors [5,7]. A comparison of independently identified V_1 structures [8–10] with the F_1 ATPase [11] indicates mushroom-shaped enzymes, composed of a headpiece, which is formed by the hexagonally arranged nucleotide-binding subunits A and B of V_1 and the corresponding α and β subunits of F_1, and one stalk, comprised of the V_1 subunits, C–H, and the F_1 subunits γ–ε (mitochondrial nomenclature [11]). Structural comparison of the V_1 and F_1 stalks indicates different shapes and lengths of these domains [5,12].

Understanding the structural and functional roles of the V-ATPase stalk subunits is essential, because they may impart to V-ATPases the characteristics that distinguish them from the F-ATPases, including their activity as dedicated ion pumps rather than ion-driven ATP synthases, and their susceptibility to multiple forms of regulation in vivo [3,13,14]. We have turned our attention to examination of the structure of stalk subunit G, which has been proposed to be essential for the assembly and activity of the V_1V_O ATPase [15–19]. Here we describe the expression, purification and characterization of subunit G (Vma10p) from yeast. Independent approaches demonstrate that this subunit forms a dimer in solution. The overall dimension of subunit G in solution was determined using small-angle X-ray scattering (SAXS). In addition, an N-terminally truncated form of G was analyzed to clarify the role of the conserved N-terminal region of this subunit in dimerization.

2. Materials and methods

2.1. Biochemicals

ProofStart™ DNA Polymerase and Ni^{2+}-nitrilotriacetic acid (NTA) chromatography resin were obtained from Qiagen (Hilden, Germany); restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany). The expression vector pET9d-His_6 was provided by G. Stier, EMBL (Heidelberg, Germany). Chemicals for gel electrophoresis and trypsin used for in-gel digestion were purchased from Serva (Heidelberg, Germany) and Promega (Madison, WI, USA), respectively. All other chemicals were at least of analytical grade and obtained from BIOMOL (Hamburg, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), or Serva (Heidelberg, Germany).

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Abbreviations: BSA, bovine serum albumin; IPTG, isopropyl (thio)-β-D-galactoside; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, Tris-(hydroxymethyl)aminomethane

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2.2. Expression and purification of subunit G (Vma10p)

To amplify the *VMA10* coding region, oligonucleotide primers 5′-CAT GCC ATG GCG TCC CAA AAA AAC-3′ (forward primer) and 5′-CGG GAT CCT TAC AAG GCA TTG ATA T-3′ (reverse primer), incorporating *Nco*I and *Bam*HI restriction sites, respectively (underlined), were designed. *Saccharomyces cerevisiae* genomic DNA was used as template for the polymerase chain reaction (PCR). Following digestion with *Nco*I and *Bam*HI, the PCR product was ligated into the pET9d-His_6 vector. The pET9d-His_6 vector containing the *VMA10* insert was then transformed into *Escherichia coli* cells (strain BL21) and grown on 100 μg/ml kanamycin-containing Luria-Bertani (LB) agar plates. To express His_6-Vma10p, liquid cultures were shaken in LB medium containing kanamycin (30 μg/ml) for about 20 h at 21°C until an optical density at 600 nm (OD_{600}) of 0.6–0.7 was reached. To induce expression of His_6-Vma10p, the cultures were supplemented with isopropyl (thio)-β-D-galactoside (IPTG) to a final concentration of 1 mM. Following incubation for another 4 h at 21°C, the cells were harvested at 6000×*g* for 20 min, 4°C. Subsequently, they were lysed on ice by sonication for 3×1 min in buffer A (50 mM Tris-(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.0, 100 mM NaCl, 8 mM Pefabloc SC (BIOMOL)). The lysate was cleared by centrifugation at 10 000×*g* for 30 min. The supernatant was filtered (0.45 μm; Millipore) and passed over a 5 ml Ni^{2+}-NTA resin column to isolate the His_6-Vma10p, according to a modified version of the manufacturer's protocol [20]. The His-tagged protein was allowed to bind to the matrix for 2 h at 4°C and eluted with an imidazole gradient (0.02–0.2 M) in buffer A. Fractions containing His_6-Vma10p/subunit G were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [21], pooled and concentrated as required using Centricon YM-10 (10 kDa molecular mass cut off) spin concentrators (Millipore). Imidazole was removed by dialysis against buffer A using membrane tubes with a molecular mass cut off of 3.5 kDa.

In order to obtain the truncated G subunit, including the residues Ala_{38}–Ile_{144}, the primers 5′-CAT GCC ATG GCA GCC AAG GAA ATC-3′ (forward primer) and 5′-CGG GAT CCT TAC AAG GCA TTG ATA T-3′ (reverse primer), incorporating *Nco*I and *Bam*HI restriction sites, respectively (underlined), were used. Following digestion with *Nco*I and *Bam*HI, the pair PCR product was ligated into the pET9d-His_6 as described for subunit G. Liquid cultures were shaken at 30°C for about 20 h. The G_{38-144} form was isolated using a Ni^{2+}-NTA resin column as described above, followed by a size exclusion column (Superdex 75 HR 10/30, Amersham Biosciences) using a buffer of 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl. The protein was concentrated using Centricon YM-3 spin concentrators (Millipore).

2.3. Determination of native molecular mass

Gel filtration chromatography was performed on a Superdex 75 HR 10/30 column (Amersham Biosciences) using a buffer of 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl. To construct a calibration curve, a set of standard proteins (Amersham Biosciences and Sigma) was analyzed. The K_{av} parameter was determined ($K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e represents the elution volume, V_0 the void volume, and V_t the total bed volume). The K_{av} values for standard proteins were plotted as a function of the logarithm of molecular mass, and the resulting calibration curve was used to derive a molecular mass for subunit G and G_{38-144}.

2.4. Mass spectrometric analysis of the isolated Vma10p

The band was cut out from the SDS–polyacrylamide gel and automatically digested with trypsin in ProGest Investigator robot (Genomic Solutions Ltd.) according to a procedure modified from Hellmann et al. [22] and Roos et al. [23]. For matrix-assisted laser desorption ionization (MALDI) mass spectrometry, 0.5 μl aliquots of the digested solution were applied to the SCOUT 384 Maldi sample plate and allowed to dry. Subsequently, 0.5 μl of matrix solution (1% w/v α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were acquired automatically under fuzzy logic control using Bruker AutoXecute software on an AUTOFLEX MALDI-time-of-flight mass spectrometer. For protein identification, the intranet MASCOT software package (Matrix Science Ltd.) was used.

2.5. X-ray scattering experiments and data analysis

The Synchrotron radiation X-ray scattering data were collected following standard procedures on the X33 camera [24,25] of the EMBL on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) using multiwire proportional chambers with delay line readout [26]. Solutions with a protein concentration of 2.5 mg/ml were measured. At sample detector distances of 2.0 m and a wavelength $\lambda = 0.15$ nm the range of momentum transfer $0.22 < s < 2.6$ nm^{-1} was covered ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). The data were normalized to the intensity of the incident beam and corrected for the detector response; the scattering of the buffer was subtracted and the difference curves were scaled for concentration using the program SAPOKO (Svergun and Koch, unpublished). The maximum dimension, D_{max}, of subunit G, the distance distribution function $p(r)$ and the radii of gyration, R_g, were computed with the indirect Fourier transform program GNOM [27]. The molecular masses of the solutes were estimated by comparison of the forward scattering with that of reference solutions of lysozyme and bovine serum albumin (BSA).

2.6. Other methods

The purity and homogeneity of the protein sample were analyzed by Native-PAGE[2] (12% Tris–glycine gel; NOVEX [28]) and SDS–PAGE [21]. SDS gels were stained with Coomassie brilliant blue G250. Protein concentrations were determined by the bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA). Western blot analyses were performed as described [29]. Anti-Vma10p antibodies used in these experiments were a generous gift from Prof. T. Stevens, University of Oregon, Eugene, OR, USA.

3. Results

3.1. Expression and purification of subunit G (Vma10p)

To express and isolate recombinant Vma10p/subunit G, its DNA coding region was amplified by PCR and cloned into the bacterial vector pET9d-His_6. Bacteria (strain BL21) transformed with this vector were grown to OD_{600} 0.6–0.7 at 21°C. SDS–PAGE and Coomassie staining revealed a prominent band of the expected molecular mass in crude lysates following IPTG induction (Fig. 1A, lane 2) compared with lysates of

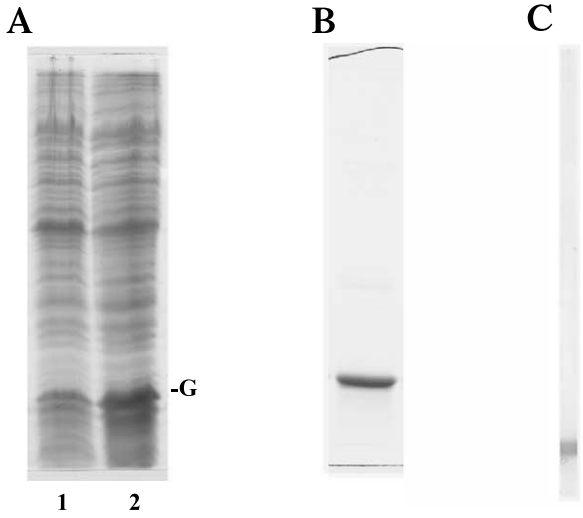

Fig. 1. Recombinant expression and purification of *S. cerevisiae* G subunit. A: *E. coli* lysates analyzed as described in Section 2. Lanes 1 and 2, *E. coli* BL21 containing pET9d-HIS_6-*VMA10* uninduced (lane 1) and induced (lane 2) by IPTG. B: SDS–PAGE of purified recombinant subunit G. Proteins were separated by SDS–PAGE (17.5% total acrylamide and 0.4% cross-linked acrylamide gel) and stained with Coomassie blue G250. C: The purified protein was applied to an SDS gel and transferred to nitrocellulose. The nitrocellulose was cut into a strip, and Western blotting was carried out using protein A-Sepharose-purified antibody (1:1000 dilution) prepared against subunit G.

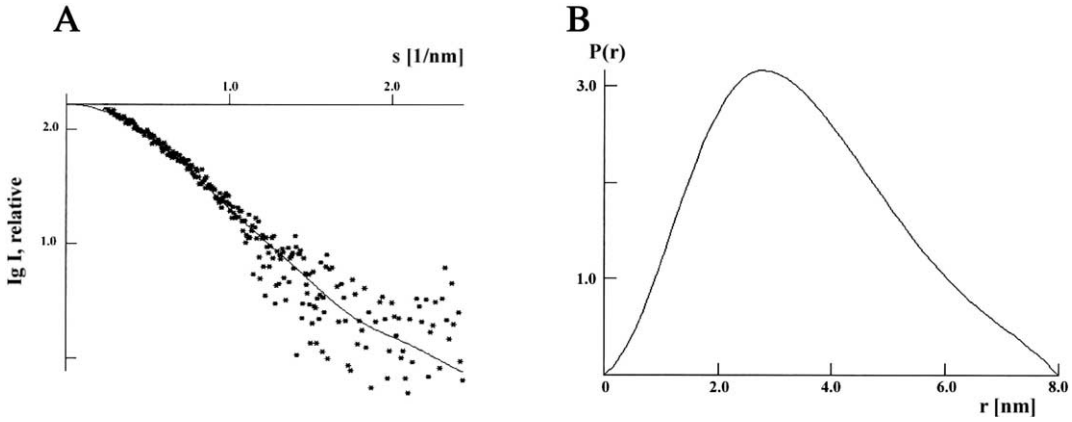

Fig. 2. (A) Experimental scattering curve and (B) distance distribution function of subunit G (2.5 mg/ml) of the V-ATPase from yeast.

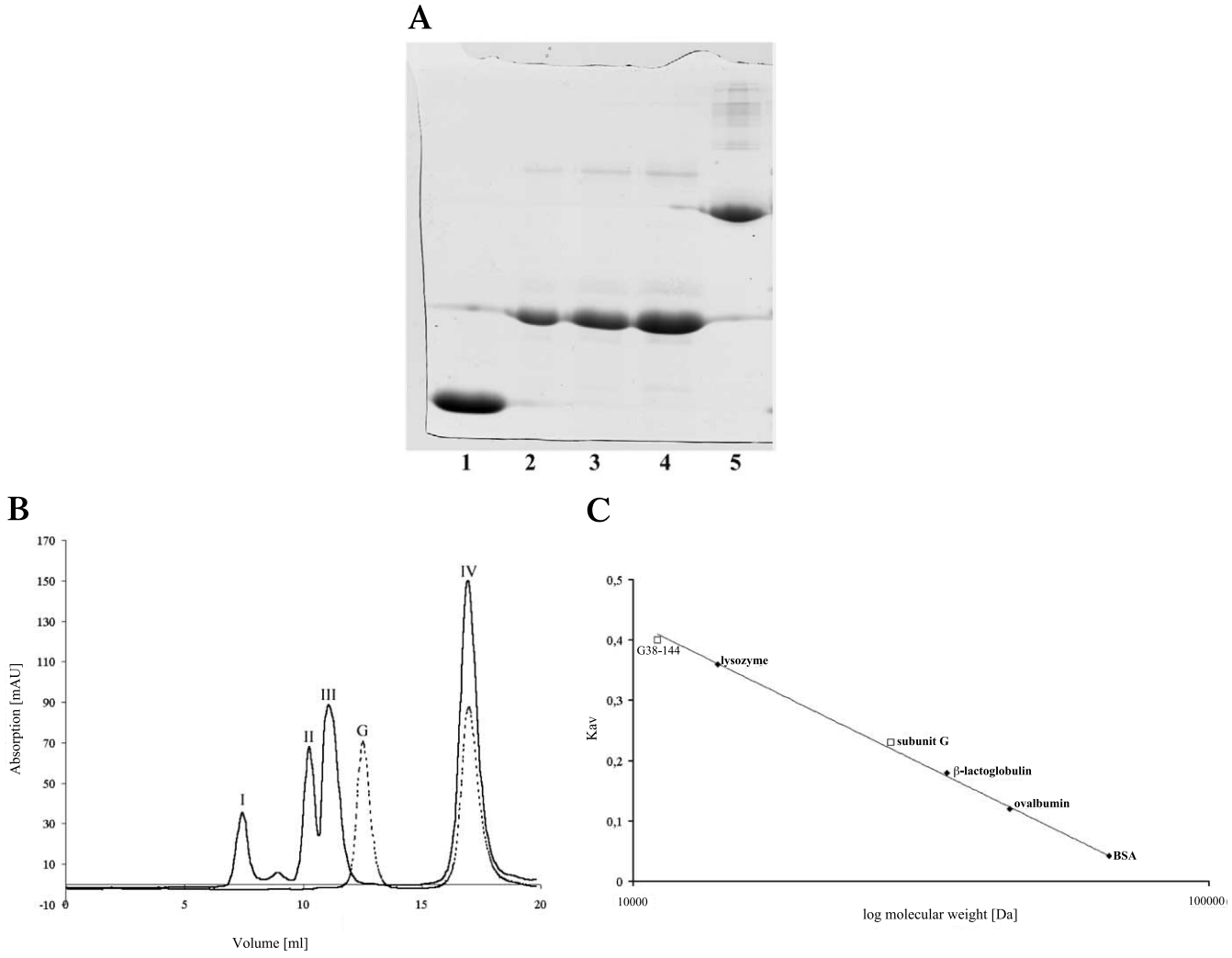

Fig. 3. Native-PAGE of the purified subunit G and determination of native molecular mass of this subunit. A: Comparison of subunit G (lanes 2–4) with marker (Amersham Biotech and Sigma) consisting of lysozyme (14.4 kDa, lane 1) and BSA (67 kDa; lane 5). Different protein concentrations of subunits G (1 mg/ml (lane 2), 2 mg/ml (lane 3), and 4 mg/ml (lane 4)) were applied to a native polyacrylamide gel (12% Tris–glycine gel; NOVEX). B: Superdex 75 gel filtration analysis of subunit G was performed as described under Section 2. (—) Elution diagram of molecular size standards composed of BSA (I; 67 kDa), ovalbumin (II; 45 kDa), β-lactoglobulin (III; 35 kDa) and lysozyme (IV; 14 kDa). The second elution diagram (— —) shows a mixture consisting of subunit G and lysozyme (IV), the latter of which has been added to demonstrate the identical conditions used for the molecular size standards (—). C: For each protein, a K_{av} parameter was derived as described under Section 2. The K_{av} for subunits G and G_{38-144} (□) and the molecular size standards (♦) are indicated.

uninduced cells (Fig. 1A, lane 1). His_6-Vma10p/subunit G was purified by passing the lysate over a Ni^{2+}-NTA resin column. An imidazole gradient (0.02–0.2 M) in buffer consisting of 50 mM Tris–HCl, pH 7.0 and 100 mM NaCl allowed its separation from the contaminating proteins. Subunit G eluted at 125 mM imidazole was collected and used for further analysis (Fig. 1B). MALDI mass spectrometry was used to confirm the identity and purity of subunit G. Five peptides consisting of the amino acid sequences N_5–K_{15}, K_{71}–K_{85}, A_{72}–K_{84}, A_{72}–K_{85} and I_{97}–L_{114} could be distinguished (Table 1). The identity of subunit G was also established by use of antiserum against this polypeptide (Fig. 1C).

3.2. Determination of overall dimensions and molecular mass of subunit G

X-ray solution scattering patterns of solutions of subunit G were recorded (Fig. 2A). The radius of gyration (R_g) of subunit G is found to be 2.7 ± 0.2 nm and its maximum dimension (D_{max}) is 8.0 ± 0.3 nm as deduced from a Guinier plot and from the distance distribution function $p(r)$ (Fig. 2B), calculated with the GNOM program [27]. Comparison of the forward scattering with the values obtained for a reference solution of lysozyme (14 ± 1 kDa) and BSA (66 ± 2 kDa) yields a molecular mass of 28 ± 2 kDa which is about twice the value calculated from the amino acid sequence [15], indicating that subunit G is dimeric at the concentration used. This is in agreement with qualitative estimates from a Native-PAGE, in which subunit G was compared with standard proteins (Fig. 3A). Independently of the concentration used (1, 2, and 4 mg/ml), the monodisperse G subunit appeared as a single band in the native electrophoresis gel, yielding an apparent molecular mass of 26 ± 2 kDa (Fig. 3A). In a third complementary approach to determine the native molecular mass of subunit G, we calibrated a Superdex 75 gel filtration column by determining the K_{av} values for a set of standard proteins of known molecular mass (Fig. 3A). A calibration curve based on these K_{av} values is shown in Fig. 3C. Comparison of the K_{av} for subunit G versus the standards suggests a native molecular mass of approximately 28 ± 3 kDa.

3.3. Expression and purification of the $G_{38\text{-}144}$ form

G subunits show higher conservation of the amino-terminal halves than the carboxyl-terminal regions [15,16,18,29]. In order to find out whether the N-terminus of subunit G is involved in dimer formation, a $G_{38–144}$ form of the yeast protein was constructed, where the subscript indicates that this fragment includes residues Ala_{38} through Ile_{144} of the native subunit sequence. The expressed polypeptide was purified by metal chelate affinity chromatography and gel filtration (Superdex 75 column (Amersham Biosciences)) (Fig. 4A). The identity of subunit G was resolved by use of antiserum against this polypeptide (Fig. 4B).

Fig. 4. SDS–PAGE of *S. cerevisiae* $G_{38–144}$ expressed and purified from *E. coli*. A: Recombinant $G_{38–144}$ was applied onto an SDS–PAGE (17.5% total acrylamide and 0.4% cross-linked acrylamide gel) after Ni^{2+}-NTA resin column (lane 1) and size exclusion column (Superdex 75 HR 10/30; lane 2). B: After gel chromatography the protein was transferred to nitrocellulose. Western blotting was carried out using an antibody (1:1000 dilution) against subunit G.

3.4. Analysis of the native molecular mass of the $G_{38\text{-}144}$ form

The mobility of $G_{38–144}$, subunit G and lysozyme were compared by non-denaturing gel electrophoresis (Fig. 5). The monodisperse $G_{38–144}$ form appeared as a single band below lysozyme with an apparent molecular mass of 14.4 kDa. As another measure of molecular mass and stoichiometry of $G_{38–144}$ gel filtration chromatography was performed using a Superdex 75 column as described above. Comparison of the K_{av} for $G_{38–144}$ form versus the standards suggests a native molecular mass of approximately 12 ± 3 kDa, in agreement with the value calculated from the amino acid sequence (Fig. 3C).

4. Discussion

The goal of this work was to express efficiently the stalk subunit G (Vma10p) of the V-ATPase in a prokaryotic system to yield pure and monodisperse protein retaining structural characteristics of the subunit which is proposed to be present in two copies per enzyme [6]. Physical characterization of this subunit by gel filtration, native gel electrophoresis and SAXS indicates that subunit G (Vma10p) forms a dimer in solution independently of protein concentration. This finding suggests that the two copies of G in V-ATPases [6] are present as a dimeric structure with an apparent mass of 28 ± 2 kDa. X-ray scattering yields a maximum dimension of 8.0 ± 0.3 nm and a

Table 1
MALDI mass spectrometry analysis of peptides from subunit G (Vma10p)

Start residue	End residue	Observed mass	Expected mass	Calculated mass	Delta mass (ppm)	Sequence
5	15	1157.73	1156.72	1156.65	0.07	NGIATLLQAEK
71	85	1571.04	1570.03	1569.87	0.16	KAEAGVQGELAEIKK
72	84	1314.87	131386	131368	0.18	AEAGVQGELAEIK
72	85	1442.93	1441.93	1441.78	0.15	AEAGVQGELAEIKK
97	114	1960.35	1959.34	1959.14	0.20	ILIETVIKPSAEVHINAL

Five peptides cover 38% of the sequence.

Fig. 5. Non-denaturing gel electrophoresis (12% Tris–glycine gel; NOVEX) of the purified G_{38-144} form. Comparison of G_{38-144} (lane 2) with subunit G (lane 1) and lysozyme (14.4 kDa, lane 3).

radius of gyration of 2.7 ± 0.2 nm, indicating that subunit G is a rather elongated dimer. For comparison, lysozyme with a molecular mass of 14.4 kDa similar to that of a monomeric subunit G (13.5 kDa), has a maximum dimension of 4.6 nm and a radius of gyration of 1.4 nm. Secondary structure predictions of subunit G indicated that it forms a continuous, highly hydrophilic α-helix, followed by two further α-helices [16,30,31]. Such an α-helical arrangement would be consistent with the SAXS values.

The structure of the V-ATPase can be divided into three subregions, a headpiece, a stalk region and the membrane-embedded V_O part. As estimated from electron micrographs of negatively stained V_1V_O ATPase from bovine brain clathrin-coated vesicles [32,33] and V_1(-C) ATPase from *Manduca sexta* [10] the stalk is about 6 nm in height and 4 nm in diameter. A length of about 8 nm of the hydrated dimeric G subunit would exceed the distance of 6 nm. From recent studies using a bifunctional cross-link reagent it has been proposed that subunit G is located at the outer surface of subunit B of the yeast V-ATPase [34]. Also observed by cross-linking [6,35] and genetic approaches [17,18] was the close neighborhood relationship of subunit G and E, which interacts with the stalk subunit C [6,17,18], H [36], and F [35], the latter of which is proposed to be at the bottom of the stalk [3,35] and in close proximity to the V_O domain. Taken together, these data imply that the dimeric G subunit might span the length of the stalk and extend to the nucleotide-binding subunit B.

Subunit G is required for both ATPase activity as well as proton pumping catalyzed by the V_1V_O complex. A *vma10* null mutation resulted in loss of ATP-dependent proton uptake activity [15]. For the H^+-ATPase of clathrin-coated vesicles it has been demonstrated that the addition of recombinant subunit G to a recombinant A_3B_3CE complex significantly increased the ATPase activity [19]. Recent work on the relationship of nucleotide binding and interaction of subunit G with other stalk subunits showed a trapped CaATP conformation of the V_1 ATPase after $CuCl_2$ addition in which a zero-length cross-link E–G was formed. By comparison, no cross-link between these subunits can be obtained in the trapped CaADP form, implying a rearrangement of both subunits during coupling [35]. Therefore, the G subunit is likely to play a direct role in the transmission of energy derived from ATP hydrolysis to proton translocation across the membrane. Furthermore, previous genetic and biochemical data suggest that subunit G is essential in regulation and stabilization of V_1V_O interaction [17,18]. Point mutations in the N-terminal half of subunit G (E14A) resulted in destabilization of subunits E and C [18], which is believed to be involved in reversible dissociation of V_1 from V_O [37,38]. As demonstrated above, the absence of the N-terminal 37 residues in the truncated G_{38-144} form prevents dimer formation by the construct. It is possible that dimerization results in an extended form of subunit G which is required to bridge the stalk subunits like C and E and thereby a main part of the stalk region.

In summary, the data presented demonstrate that subunit G of the yeast V-ATPase exists in solution as an elongated dimer and that dimerization is mediated by interactions of the N-terminal domain. The dimeric formation is consistent with the suggestion of the presence of two copies of subunit G in V_1V_O ATPases [6]. These findings will allow a better understanding of the structural and functional role of this subunit inside the V_1V_O complex.

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