Crystal and NMR Structures Give Insights into the Role and Dynamics of Subunit F of the Eukaryotic V-ATPase from Saccharomyces cerevisiae*

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**Background:** Subunit F is a stalk subunit in V-ATPases.

**Results:** This work is the first crystallographic and NMR structure of eukaryotic V-ATPase subunit F from *Saccharomyces cerevisiae*.

**Conclusion:** Subunit F plays a central role during reversible disassembly and ATP hydrolysis by transmitting movements of subunit d and H.

**Significance:** Insights into the structure and dynamics of subunit F are essential for the understanding of V1VO disassembly and ATP hydrolysis regulation.

Subunit F of V-ATPases is proposed to undergo structural alterations during catalysis and reversible dissociation from the V1VO complex. Recently, we determined the low resolution structure of F from *Saccharomyces cerevisiae* V-ATPase, showing an N-terminal egg shape, connected to a C-terminal hook-like segment via a linker region. To understand the mechanistic role of subunit F of *S. cerevisiae* V-ATPase, composed of 118 amino acids, the crystal structure of the major part of F, F(1–94), was solved at 2.3 Å resolution. The structural features were confirmed by solution NMR spectroscopy using the entire F subunit. The eukaryotic F subunit consists of the N-terminal F(1–94) domain with four-parallel β-strands, which are intermittently surrounded by four α-helices, and the C terminus, including the α5-helix encompassing residues 103 to 113.

Two loops 26GQITPETQEK35 and 60ERDDI64 are described to be essential in mechanistic processes of the V-ATPase enzyme. The 26GQITPETQEK35 loop becomes exposed when fitted into the recently determined EM structure of the yeast V1VO-ATPase. A mechanism is proposed in which the 26GQITPETQEK35 loop of subunit F and the flexible C-terminal domain of subunit H move in proximity, leading to an inhibitory effect of ATPase activity in V1.

Subunits D and F are demonstrated to interact with subunit d. Together with NMR dynamics, the role of subunit F has been discussed in the light of its interactions in the processes of reversible disassembly and ATP hydrolysis of V-ATPases by transmitting movements of subunit d and H of the VO and V1 sector, respectively.

Eukaryotic V-ATPases (V1VO-ATPases) are ATP-dependent ion pumps, which are situated in intracellular organelles and involved in various important cellular processes. It is one of the major proteins that maintain pH homeostasis at the cellular and the whole organism level (1, 2). The multifunctional enzyme complex also plays an important role in the endosomal pH-sensing machinery (3). Most recently, the V-ATPase has been described as a signaling receptor that modulates activity of cytohesin-2 and Arf GTP-binding proteins (4).

V1VO-ATPases are multisubunit enzymes, consisting of a cytosolic V1 and a membrane-embedded VO sector (4, 5). The subunit stoichiometry of the V1 and VO part is proposed as A2B2C2D2E3F1G3H1 (5, 6) and a1b1c1d2e4f4g4h4 (7), respectively. The cleavage of ATP into ADP + Pi in the A3:B3 head-piece of the V1 headpiece of V-ATPases drives the proton translocation at the interface of subunits a and c. The coupling of both events occurs via the stalk structure, which is an assembly of the V1 subunits C-H and the VO subunits a and d, which form a structural and functional interface.

So far, of all of the *Saccharomyces cerevisiae* V1VO-ATPase stalk subunits, the high-resolution structure of subunit C (8), E (9, 10), G (10–12), and H (13) are known. Subunit C is formed by three distinct domains, an upper globular head domain, an elongated neck domain, and a lower globular foot domain (8). Subunit E consists of an N-terminal extended α-helix with a globular domain, composed of α-helical and β-sheet content (9, 10). Subunit G consists mainly of an elongated α-helical structure (10–12), whereas stalk subunit H is characterized by a superhelical structure, forming a shallow groove extending from one end of the amino-terminal domain to the other. The N- and C-terminal domains of H, which are assumed to be flexible, are connected by a four-residue loop (13).

The eukaryotic V1VO ATPases are able to disassemble into the V1 and VO part, a process that is reversible (14) and discussed to be sensed by ATP/ADP ratio and/or phosphorylation of subunit C (15, 16), depending on physiological conditions. Such disassembly leads to a loss in H+-pumping and a reduc-
tion in ATPase activity of the V₁ part (2, 17). Subunit H has been described to undergo a rearrangement during disassembly and moves in proximity to subunit F. This proximity is discussed in context with the role it plays to prevent F from rotation with subunit D and the subsequent drop in ATPase activity of the V₁ sector (18).

During catalysis, subunit F is proposed to undergo structural alterations by interacting with subunits A, D, and E in a nucleotide-dependent manner (19–21). Recently, the solution shape of subunit F of V-ATPase from S. cerevisiae (118 amino acids) has been described as a long egg-shaped protein connected to a hook-like segment via a linker region (22). The comparison of the shape of the C-terminal truncated form of subunit F (F(1–94)) revealed that the hook-like region forms the very C terminus of subunit F (22). The NMR structure of the C-terminal peptide F(90–116) shows that the hook-like region consists of an α-helical segment (22).

Understanding the important functional features and domain structures of subunit F requires a high-resolution structure. Here, we present the crystallographic structure of F(1–94) of the S. cerevisiae V₁V₀-ATPase at 2.3 Å resolution. The structure revealed an alternating arrangement of four-stranded β-sheets and four α-helices and an extended polar loop (28GQITPETQEK35) between α1–β2, discussed to be important in coupling. NMR spectroscopy has been used to further characterize the structural traits and dynamics of the full length F subunit. The data form a platform for a mechanistic model of subunits D and F as well as D and H interaction via the novel 28GQITPETQEK35 loop of subunit F of eukaryotic V-ATPases and the C-terminal domain of subunit H in V₁, and its coupled effect in ATPase inhibition in the catalytic A₃B₃ headpiece. In addition, the key role of F in the subunit D, F, and d arrangement is described.

EXPERIMENTAL PROCEDURES

Biochemicals—Pfu DNA polymerase and N¹²⁺-nitrotri-acetic acid chromatography resins were obtained from Qiagen (Hilden, Germany); restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany). Chemicals for gel electrophoresis and trypsin used for in-gel digestion were purchased from Merc (Darmstadt, Germany), Sigma, or Serva (Heidelberg, Germany). 15N-labeled NH₄Cl and [13C]glucose were obtained from Bior (Hamburg, Germany), Sigma, or Serva (Heidelberg, Germany). 15N-labeled NH₄Cl and [13C]glucose were purchased from Cambridge Isotope Laboratories (Andover, MA).

Protein Production, Purification, and Crystallization of F(1–94) and Its Mutant Forms—Single F(1–94) mutants I8M, I11M, L20M, L67M, L68M, I69M, and one double mutant L20M/L67M gene were produced by overlap extension PCR method and cloned into pET9d1 vector (23) with an N-terminal His tag. The proteins were then expressed in the Escherichia coli BL21(DE3) strain. Recombinant mutant proteins were purified in two steps and crystallized as described previously (24). High-quality crystals were obtained for the SeMet F(1–94)/I69M mutant of S. cerevisiae V-ATPase by hanging drop method, and diffracted to 2.3 Å. The crystal belongs to an orthorhombic C222₁ space group (unit cell parameters were a = 47.21, b = 160.26, and c = 102.49 Å) with four molecules in the asymmetric unit. Phasing was carried out by three-wavelength multiple anomalous dispersion technique. Analysis with SHELXC (25) indicated a maximum resolution of 2.9 Å for substructure determination and initial phase calculation. All four selenium sites were found using SHELXD program (26) with a correlation coefficient of 43.62%. The correct hand for the substructure and initial phases were calculated after density modification using the program SHELXE (27). The phases were improved by density modification and phase extension to 2.4 Å resolution was done using the program RESOLVE (28). Automated model building was carried out by Buccaneer program (29), which gave the initial model of the selenomethionine F(1–94)/I69M mutant protein with 312 residues for four molecules. The phases were extended up to 2.3 Å, and iterative cycles of model building and refinement were carried out using the programs COOT (30) and REFMAC5 (31) of the CCP4 suite to build the final model. Refinement was done until convergence and the geometry of the final model was validated with PROCHECK (Table 1) (32). The final figures were drawn using the program PyMOL (33), and structural comparison analysis was carried out by using the SUPERPOSE program (34) as included in the CCP4 suite.

Cloning, Protein Production, and Purification of a Subunit DF Complex—The genes encoding subunits F and D of S. cerevisiae V-ATPase were amplified by PCR using genomic DNA as a template. In these reactions, forward primer, 5’-AACATATG-CATCACCATCATCACCACATGTCCTGGAATAGA-3’ and reverse primer, 5’-CCCTCGAGTCAAGATAAACATTGCTTTCTTGTACGCAA-3’, incorporating NdeI and Xhol (underlined) restriction sites were used to amplify the gene encoding subunit D. For amplification of the subunit F-encoding gene, the following primers were used: 5’-AAGGATCCATGGCT-GAGAAACGTACTCTT-3’ (forward) incorporating a BamHI site, and 5’-GTCTTCTGAGCTTCTACTACCCGAAACTTTTC-3’ (reverse) incorporating a SacI site. Both genes were then inserted into the pETDuet-1 vector and transformed into E. coli DH5α cells, for plasmid propagation and purification. Finally, the verified plasmid was transformed into E. coli BL21(DE3) cells (Stratagene) for protein production. Cells were grown on 100 µg/ml carbenicillin-containing Luria-Bertani medium. The protein was induced by isopropyl β-D-thiogalactopyranoside to a final concentration of 0.8 mM at 37 °C. Cells were lysed on ice by sonication for 3 × 1 min in buffer A (50 mM Tris-HCl, pH 8.5, 200 mM NaCl, 2 mM PMSF, and 2 mM PefablocSC (4-(2-aminoethyl) benzzenesulfonfyl fluoride hydrochloride) (BIOMOL)). Cell debris was separated by centrifugation at 10,000 × g for 35 min. The supernatant was then filtered (0.45 µm; Millipore) and allowed to bind with Ni²⁺-nitrotri-acetic acid matrix, which was then eluted with an imidazole gradient (25–400 mM) in buffer A. Fractions containing the DF heterodimer were identified by SDS-PAGE (35), pooled together, and diluted with 50 mM Tris-HCl, pH 8.5 to reduce the salt concentration to 50 mM and applied onto an anion exchange column (Resource Q, 6 ml). The eluted protein was applied onto a gel filtration column (S200), which was equilibrated with 50 mM Tris-HCl, pH 8.5, 200 mM NaCl, and 5 mM EDTA. Purity and homogeneity of the protein samples were analyzed by SDS-PAGE (35). The gels were stained with Coomassie Brilliant Blue G250.
Protein concentrations were determined by the bicinchoninic acid assay (Pierce).

**Characterization of Subunit F by NMR**—All NMR experiments were performed at 293 K on a 700 MHz NMR spectrometer (Bruker Avance), equipped with 5-mm triple-resonance (1H/15N/13C) single axis gradient probes and a cryoprobe. 0.5 mM of recombinant subunit F subunit, we have collected TROSY-based experiments: two-dimensional 1H-15N TROSY-HSQC, three-dimensional TROSY-HNCACB with improved signals (−10–50% increase) unlike non-TROSY experiments, showing signal broadening for many residues. As the earlier small-angle x-ray scattering data at the same concentration confirmed the monomeric state of F subunit (22), the signal broadening is probably due to slow conformational exchange on the microsecond to millisecond time scale. To measure the heteronuclear steady-state NOEs, using NOE-TROSY in an interleaved mode (36), two spectra with and without proton saturation were measured. Proton presaturation was achieved by a train of 120° pulses spaced at 5 ms, for a 3-s period. In addition, a 3-s recycle delay was employed in both experiments. 15N-[1H]NOE values were obtained from the ratio of signal intensities in the presence ($I_{sat}$) and in the absence of proton saturation ($I_{unsat}$). Errors were estimated from the standard deviation of the NOE,

\[ \sigma_{NOE} = \left( \frac{\sigma_{sat}}{I_{sat}} \right)^2 + \left( \frac{\sigma_{unsat}}{I_{unsat}} \right)^2 \]  

where $\sigma_{sat}$ and $\sigma_{unsat}$ are their measured background noise levels (37). All NMR spectra were processed using NMRpipe (38) and analyzed with the SPARKY program (39).

**Isothermal Titration Calorimetry to Study the Interaction of F and DF with Subunit d**—Isothermal titration calorimetry experiments were carried out using a MicroCal iTC200 microcalorimeter (MicroCal, Northampton, UK), to study the binding of DF complex and subunit F with subunit d of the S. cerevisiae V-ATPase. Purification of subunit d was done as described previously (40). All of the proteins were prepared in common buffer consisting of 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, and 5% D$_2$O. To assign the backbone of the F subunit, we have collected TROSY-based experiments: two-dimensional 1H-15N TROSY-HSQC, three-dimensional TROSY-HNCACB with improved signals (−10–50% increase) unlike non-TROSY experiments, showing signal broadening for many residues. As the earlier small-angle x-ray scattering data at the same concentration confirmed the monomeric state of F subunit (22), the signal broadening is probably due to slow conformational exchange on the microsecond to millisecond time scale. To measure the heteronuclear steady-state NOEs, using NOE-TROSY in an interleaved mode (36), two spectra with and without proton saturation were measured. Proton presaturation was achieved by a train of 120° pulses spaced at 5 ms, for a 3-s period. In addition, a 3-s recycle delay was employed in both experiments. 15N-[1H]NOE values were obtained from the ratio of signal intensities in the presence ($I_{sat}$) and in the absence of proton saturation ($I_{unsat}$). Errors were estimated from the standard deviation of the NOE,

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where $\sigma_{sat}$ and $\sigma_{unsat}$ are their measured background noise levels (37). All NMR spectra were processed using NMRpipe (38) and analyzed with the SPARKY program (39).

**Localization of Subunits inside of the S. cerevisiae V-ATPase**—The high resolution crystal structures of individual subunits were fitted in the EM map of the S. cerevisiae V$_1$V$_0$-ATPase, which was determined at 11 Å (6). The A-ATP synthase subunit A (Protein Data Bank code 3I4L (41)) and E (42) from *Pyrococcus horikoshii* OT3, subunit B from *Methanococcus mazei* G01
Structure and Dynamics of Eukaryotic V-ATPase Subunit F

(Protein Data Bank code 2C61 (43)), the c-ring (Protein Data Bank code 2BL2 (44)), subunit D (Protein Data Bank code 3AON) and F (Protein Data Bank code 3AON) from Enterococcus hirae (45), subunit G (46) and the homologue subunit d from Thermus thermophilus (Protein Data Bank code 1R5Z (47)), the N-terminal region of subunit a from Meiothermus ruber (Protein Data Bank code 3RRK (48)), and the subunits C (Protein Data Bank code 1U7L (8)) and H (Protein Data Bank code 1HOB (13)) as well as the structure of F(1–94) along with the α5-F(103–113) peptide of the eukaryotic S. cerevisiae V-ATPase (22) were fitted into the EM map using the Situs program (49). Because subunit H was not fitting properly into the EM map, normal mode analysis was carried out using ElNemo (50) to find an optimum conformation. Of the 55 conformations generated, the conformation that fit well linearly into the EM map, normal mode analysis was carried out. Situs program (49). Because subunit H was not fitting properly into the EM map, normal mode analysis was carried out using ElNemo (50) to find an optimum conformation. Of the 55 conformations generated, the conformation that fit well into the EM density was chosen. The overall linear correlation coefficient of the fitted structures to the EM map was 0.8357.

RESULTS

Overall Structure of F(1–94)—The native F(1–94) yielded crystals, which diffracted to a resolution of 2.64 Å (24). Attempts at phasing by molecular replacement using subunit F structures from the related A-ATP synthase from M. mazei G01 (M. mazei F (51)) and E. hirae (E. hirae F (45)) were unsuccessful. Selenomethionine-substituted native F(1–94) crystals yielded a weak signal for selenium; therefore, different single mutants of F(1–94) with the following substitutions were produced: I8M, I11M, L20M, L67M, L68M, I69M, and one double mutant L20M/L67M. The selenomethionine substitution did not yield crystals for all the mutants, except for the mutants F(1–94)L68M and F(1–94)I69M. Small crystals of the F(1–94)L68M mutant could be grown but were heavily twinned and only diffracted to 7 Å. In comparison, the F(1–94)I69M mutant produced good quality crystals, diffracting to 2.3 Å resolution. The structure of F(1–94)I69M was solved by a multiple anomalous dispersion technique using the selenomethionyl protein. There are four molecules in the asymmetric unit. The structure was solved at 2.3 Å (Rwork = 0.15 and Rfree = 0.21), and the final model fits to the electron densities very well along the polypeptide chains, including loops. The N-terminal His tag residues were disordered, and no clear electron density was visible in that region. The four strands of the β-sheet (β1–β4) of the N-terminal part are oriented parallel to each other and are linked by four α-helices (α1–α4) (Fig. 1A).

Surface electrostatic potential shows that one side of the protein is hydrophobic and the opposite side is composed of both positive and negative charge (Fig. 1B). The hydrophobic site is comprised of the residues Ile9, Ala10, Val11, Ile12, located on β1, Ile64, Ala65, Ile66, Leu67, and Leu68 on β3, Ala69, Ile71, Leu72, and Ile74 on β4, Gly19, Leu20, Leu21, and Leu22 on α1. The electrostatic potential for the β-sheet contains highly hydrophobic residues (β1, 7LIAVIAD13; β2, 37FFVY40; β3, 66IAILM49; β4, 99AILE93), whereas the helices are mainly dominated by hydrophilic residues. The longer α2 helix contains mostly hydrophilic residues (15KKEITDKENHFTP89), whereas the shorter helices, α3 (17QHIAEN75) and α4 (78RARVD92) contain alternating hydrophilic and hydrophobic residues. In contrast, the first helix, α1, starts with a hydrophilic patch (14EDTTT18) of five residues followed by a hydrophobic patch containing four residues (19GLLL22). Sequence analysis of subunit F with other eukaryotic V-ATPases and the related A-ATP synthases revealed two important loops in the F(1–94) structure (see Fig. 3). One unique large polar loop between α1-β2 (26GQITPTEQK52) and a highly con-
served loop with a RDDI motif between α2-β3 have been observed, which are present only in F subunits of eukaryotic V-ATPases.

Comparison of the F(1–94) crystal structure with the low resolution solution structure of the entire S. cerevisiae F subunit derived from solution x-ray scattering (22) showed that F(1–94) occupies the egg-shaped domain with an root mean square deviation of 1.2 Å. The C-terminal peptide structure of F(90–116), which was found to occupy the hook-like region (22), is positioned orthogonally to the protein body and connected by the loop between β4 and α5 (Fig. 1C).

NMR Characterization of Full-length F Subunit Backbone—To compare the F(1–94) x-ray crystal structure and full length F subunit in solution, we performed NMR studies on the entire S. cerevisiae F subunit. Sequential backbone assignment for 118-residue F subunit was first carried out, and 84 of ~90 observable peaks (excluding proline and sidechains) were assigned (Fig. 2A). Most of the missing peaks fall within the unique loop, Gly26–Lys35 (Fig. 3), and can be attributed to increased exchange rate of the amide protons with the solvent at pH 7.5 or line broadening, due to chemical exchange, either of which is consistent with the flexibility of the loop. The secondary structure prediction based on assigned Cα and Cβ chemical shifts, subtracted from their random coil values (ΔCα/ΔCβ), indicates that there is an alternating arrangement of four β-strands and five α-helices (Fig. 2B). The four β-strands are β1 (Thr6–Asp13), β2 (Asn36–Gln41), β3 (Arg61–Leu94) and β4 (Phe88–Ile94), whereas the five α-helices are α1 (Glu14–Leu22), α2 (Lys47–Thr58), α3 (Asn76–Asn79), α4 (Arg78–Phe84), and α5 (Lys110–Phe119). These are consistent with the x-ray crystal structure of F(1–94) and the recently determined solution structure of α5-peptide solved in the presence of trifluoroethanol (F(90–116)) (Fig. 2B) (22). In addition, the helical propensity based on the aforementioned backbone ΔCα/ΔCβ values and 13C and 1H chemical shifts using TALOS+ (52) is consistent, which suggests a C-terminal helix α5 in solution (Fig. 2, B and C).

Dynamic Study of Subunit F by NMR—The ATPase/ATP synthase family members, A- and F-ATP synthases and V-ATPase have structural similarities, composed of a catalytic portion (A1/F1/V1) and a membrane-embedded portion (A2/ F2/V2). Presently, it is unclear how the individual subunits may independently affect the rotary mechanisms and interplay in ion-pumping and/or energy conversion. Subunit F of A-ATP synthases and V-ATPase have been proposed to be functionally similar in their coupling function like subunit ε of F-ATP synthases (7). During coupling, the C-terminal α-helical segment of ε moves up in proximity to the C termini of the αsβ3 hexamer and down to its N-terminal globular domain (53). Here, we have used the recombinant form of the proposed rotary F subunit of S. cerevisiae V-ATPase and studied its dynamics using the 15N/[1H] heteronuclear NOE experiment. This experiment provides residue-specific information about the conformational dynamics on the pico- to nanosecond time scale. In general, high NOE values reflect rigidity in the tertiary structure. Based on the mean NOE value (error, σ) of 0.8 (0.12), strands β1 to β4 and helices α2 to α4 are less flexible (NOE values > 0.8) than both the N- and C-terminal helices α1 and α5 with smaller

![FIGURE 2. NMR spectroscopic characterization of S. cerevisiae subunit F. A, 15N-[1H] HSQC spectrum of the entire subunit F of V-ATPase from S. cerevisiae. B, backbone characterization of full-length subunit F. The secondary structure elements (β1 to β4), which correspond to the F(1–94) crystal structure and the NMR solution structure of α5-F(103–113), are shown above. Based on NMR assignment, contiguous positive and negative chemical shift index values correspond to α-helix and β-strand, respectively. Missing amide stretches ≥ 3 residues are marked with an asterisk. C, helix propensity values, predicted using TALOS+ (53), show the existence of a short α5 at the C terminus. D, 15N-[1H] heteronuclear NOE with high values > 0.8 (rigid) and low values < 0.8 (flexible). NOE values with error > 15% (white box) and < 15% (black box) are also shown.](image-url)
NOE values, 0.78 (0.14) and 0.76 (0.14), respectively (Fig. 2D). Mapping onto the F(1–94) tertiary structure shows that the buried core of the protein is more rigid than its outermost surface, with increasing flexibility around helices α1 and α5, probably conferred by the long loops adjacent to them (Fig. 2D). Taken together, the high mean NOE value suggests a
rigid F subunit with less rigid helices α1 and α5 in the central stalk rotor.

Isothermal Titration Calorimetry, to Study the Interaction of F and DF with Subunit d—In the related E. hirae A-ATP synthase, it has been shown using surface plasmon resonance spectroscopy, that both central stalk subunits D and F are necessary to bind subunit d, which links the rotating DF assembly with the rotating c-ring (45). Here, we used isothermal titration calorimetry to investigate a possible interaction of the eukaryotic S. cerevisiae V-ATPase subunit F and DF with subunit d. As shown in Fig. 4A, no interaction of subunit F with subunit d could be observed in the injection profile of subunit F after base-line correction (top panel) with the profile of heat release/mole of injected subunits (bottom panel). In contrast, after titration of the DF heterodimer with subunit d, an overall negative heat enthalpy can be detected, indicating an exothermic reaction (Fig. 4B). By using a single-site model equation, the binding isotherm could be fitted nicely and reflects an equimolar binding of the DF heterodimer and subunit d. The dissociation constant ($K_d$) 52.9 μm indicates a weak (or somewhat weak) binding of subunit d by the DF complex, which might be stronger in the entire enzyme complex.

DISCUSSION

Unique Structural Features of Subunit F of the Eukaryotic V-ATPase—Subunit F has an N-terminal elliptical shape with a size of 30 × 16 × 38 Å. It embodies four parallel β-strands, which are intermittently surrounded by four α-helices forming an egg-shaped structure. The overall structure of F(1–94) belongs to an α-β class of proteins with Rossmann fold topology, which is ubiquitously found in kinases and dehydrogenases (54). The presented solution NMR data of the entire subunit F of the S. cerevisiae V-ATPase confirm the structural features of the crystallographic F(1–94) structure as well as the recently determined C-terminal peptide structure of F(90–116) (22). These first high resolution data of subunit F of an eukaryotic V-ATPase enable comparison with the NMR solution and crystallographic features of the previously solved structures of subunit F of the related A-ATP synthase, which share a sequence similarity <23%. As shown in Fig. 5A, the superimposition of the yeast F(1–94) crystal structure, including the C-terminal NMR structure of F(90–116) (22) with subunit F of the A$_1$A$_3$-ATP synthase from M. mazei G61 (51) and E. hirae (45), respectively, reveals an overall similar structure with an overall root mean square deviation of 2.51 and 2.16 Å, respectively. By comparison, when the yeast F(1–94) structure with the C-terminal F(90–116) was superimposed with subunit F of the T. thermophilus A-ATP synthase (Fig. 5B) (55), a root mean square deviation of 4.38 Å was calculated, reflecting the differences in the arrangement of α3 and β3 in the N-terminal globular domain as well as the major difference of the C-terminal β4-sheet-helix-loop-helix domain. This C-terminal portion of the T. thermophilus subunit F has been described as the extended conformation, which is proposed to make contacts with subunit B of the A-ATP synthase of T. thermophilus (56) and M. mazei G61 (57, 58). The most important features of this superimposition are the two loops between α1-β2 (26GQITPETQEK$^{35}$) and α2-β3 (66ERDDI$^{34}$) (Fig. 5, A and B). As confirmed by the amino acid sequence alignment (Fig. 3) the 26GQITPETQEK$^{35}$ containing loop is only present in eukaryotic F subunits. The second loop contains a conserved RDDI sequence, a motif also found only among eukaryotic F subunits. When the crystallographic structure of F(1–94) is fitted into the assembled A$_1$B$_1$DF complex (Fig. 6A), the 26GQITPETQEK$^{35}$ loop becomes exposed (Fig. 3). The relevance of this exposed loop becomes obvious when we fitted the high-resolution crystal structures of individual subunits into the recently determined EM structure of the S. cerevisiae V$_1$V$_{10}$ ATPase (6) (see details under “Experimental Procedures”). First, this approach revealed that the 26GQITPETQEK$^{35}$ loop of S. cerevisiae subunit F occupies well the density of the EM map that was previously unoccupied when the T. thermophilus F subunit (55) was fitted (Fig. 6B). Second, as shown in Fig. 6B, the 26GQITPETQEK$^{35}$ loop of S. cerevisiae subunit F is facing subunit H with a short distance of 23 Å. Previously, the serine residue 381 of subunit H, which is facing the central stalk subunit F was revealed to be involved in cross-linking subunit F of the disassembled V$_1$ sector (18). In this experiment, Ser$^{381}$ has been substituted by a cysteine residue to make use of the 10 Å long photo-activated cross-linking reagent 4-(N-maleimido)benzophenone. These experiments also demonstrated that an F-H
Structure and Dynamics of Eukaryotic V-ATPase Subunit F

The complex was modeled by fitting the P. horikoshii subunit A structures (orange; Protein Data Bank code 3I4L (41)), the M. mazei G01 subunit B (green; Protein Data Bank code 2C61 (43)), the DF complex from E. hirae (subunit D; yellow, subunit F; magenta (45)) on to the related A-, B-, D-, and F subunit of the low resolution (4.8 Å) A3B3DF complex of E. hirae V-ATPase (Protein Data Bank code 3AON), subunit C (Protein Data Bank code 1R5Z (44)), N-terminal residue Asp63 of the60ERDDI64 loop of the S. cerevisiae F(1–94), which was modeled, might conduct the disturbance along the flexible 26GQITPETQEK35 loop (pink) was used for fitting. The sulfhydryl cross-linker 4-(N-maleimido)benzophenone (stick; green), which links Ser93 of subunit H (pale green) with subunit F (magenta) in the free S. cerevisiae V1 domain of S381C mutant (18) is also shown. The interaction of subunit H (Ser93) through the cross-linker 4-(N-maleimido)benzophenone (ball and stick; green) to the S. cerevisiae subunit F, F(1–94), (Glu31), which was modeled, might conduct the disturbance along the flexible 26GQITPETQEK35 loop (gray; NOE values at or less than average; white, NOE values not assigned). Because of this, the interaction between Glu77, Gly78, and Asp85 of F(1–94) with Val95 (substituted by Arg91 in yeast), Glu86 (Thr89 in yeast), and Ser89 (Lys93 in yeast) of subunit D, respectively, are at a distance of 32 Å, therefore too far to generate a cross-link via the 10 Å long sulfhydryl linker 4-(N-maleimido)benzophenone. The C-terminal domain of subunit H is linked to the larger N-terminal part via a linker, giving the C terminus of H conformational flexibility (13) as recently confirmed by mutagenesis studies (6). Therefore, we propose that in the process of V1 and VO dissociation the flexible C-terminal domain moves slightly closer to its nearest neighbor, the exposed 26GQITPETQEK35 loop of subunit F, where it causes conformational changes, leading to an inhibitory effect on ATPase activity of the V1-ATPase (see Fig. 6C and below). This model is consistent with the fact that an F-H cross-link product can only be detected in the V1 dissociated complex (Fig. 6C) (18).

The question arises as to how the interaction of the 26GQITPETQEK35 loop of subunit F with the C terminus of H may couple the inhibition of ATPase activity in the catalytic A3B3 headpiece of the S. cerevisiae V1-ATPase. When the presented structure of the eukaryotic F subunit is superimposed on the recently solved crystal structure of the subunit DF complex of the E. hirae A-ATP synthase (45), similar hydrophobic patches can be identified (data not shown). Furthermore, when the modeled subunit D and the crystallographic structure F of the S. cerevisiae V-ATPase were docked into a DF complex of the E. hirae A-ATP synthase (45), the peptide35XKMR95 in the β-hairpin region of the S. cerevisiae subunit D is in close proximity to the S. cerevisiae subunit F loops, including the peptides 24GIGQQXXPE31 and 60ERDDI94. It has been shown that this β-hairpin region of D subunit in the E. hirae A-ATP synthase stimulates ATPase activity two times in the enzyme complex (45). A sequence alignment of eukaryotic V-ATPases showed a conserved basic residue Lys93 in the β-hairpin of D subunit (data not shown), which is in close proximity to the acidic residue Asp53 of the 60ERDDI94 loop of the S. cerevisiae F subunit, a sequence motif conserved only among eukaryotic V-ATPases and not among F subunits of A-ATP synthases (Fig. 3). The close proximity of these two acidic-basic residues allows an interaction via a salt bridge to be formed, thereby driving the cross-talk between the two central stalks and rotating subunits D and F. In addition, the carbonyl side chain of residue Gin27 in the 26GQITPETQEK35 loop of the S. cerevisiae F subunit is
close to the guanidine side chain of residue Arg91 of subunit D, enabling the formation of a hydrogen bond between both residues. Taken together, the close proximity of 26GQITPETQEK35 loop and the 60ERDDI64 loop of the S. cerevisiae subunit F indicate that these loops may regulate the movement of the β-hairpin in subunit D, leading to further alteration in the N- and C-terminal helices of subunit D, which are in close neighborhood to the catalytic interfaces of the subunits A and B in V1.

The 15N-[1H] heteronuclear NOE studies on the entire subunit F of the S. cerevisiae V-ATPase revealed a rigid core formed by β-strands, β1 to β4, and α2 to α4 (Fig. 2D). In comparison, the N- and C-terminal helices α1 and α5 with their adjacent loops 26GQITPETQEK35 and 94IPSKDHPYD102 respectively, are more flexible in solution. This includes also the 60ERDDI64 loop, which together with the 26GQITPETQEK35 loop of subunit F is suggested to form hydrogen bonds with residues of the β-hairpin in subunit D (see above). Together with the subunit DF assembly and the F-H interaction discussed above (Fig. 6, B and C), the flexibility of the 26GQITPETQEK35 loop and 60ERDDI64 loop, determined by solution NMR, support a cross-talk of these regions with the polar residues of the β-hairpin in subunit D.

The process of reversible disassembly is a concerted action in which the V1 subunits C-H become dissociated from their V0 subunit partners, as with the central stalk subunits D and F and the V0 subunit d. The ITC data presented demonstrate that both subunits D and F are essential for the interaction with subunit d. Although the binding between the DF heterodimer and d may prove stronger in the V1V0 complex, the determined dissociation constant of 52.9 µM reflects a weaker binding between these two partners, which is essential for the dissociation of V1 and V0 to occur. By comparison, the Kd of the related subunits in the non-dissociating A1A0-ATP synthase from E. hirae is 82 nM (45). The N-terminal helix α1 of the S. cerevisiae F subunit and the bottom segment of subunit D form a neighborhood with subunit d, which is affected during the process of dissociation and reassembly of the V1 and V0 sectors. The higher flexibility of α1 in subunit F (Fig. 6C) would therefore not only allow transmission of alterations in subunit d during dissociation from the DF heterodimer but also the movement of subunit H closer to F via the neighboring 26GQITPETQEK35 loop.

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Structure and Dynamics of Eukaryotic V-ATPase Subunit F

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APRIL 26, 2013 • VOLUME 288 • NUMBER 17
JOURNAL OF BIOLOGICAL CHEMISTRY 11939