Loose Binding of the DF Axis with the A₃B₃ Complex Stimulates the Initial Activity of Enterococcus hirae V₁-ATPase

Md. Jahangir Alam¹,², Satoshi Arai¹,³, Shinya Saijo¹,⁴,⁵, Kano Suzuki¹, Kenji Mizutani¹,³, Yoshiko Ishizuka-Katsura⁶, Noboru Ohsawa⁶, Takahiro Terada⁶, Mikako Shirozumi⁶, Shigeyuki Yokoyama⁶, So Iwata⁶,⁷, Yoshimi Kakinuma⁸, Ichiro Yamamoto¹, Takeshi Murata¹,³,⁶,⁹

1 Department of Biological Science and Technology, Tokyo University of Science, Chiba, Japan, 2 Department of Genetic Engineering and Biotechnology, School of Life Sciences, Shahjalal University of Science and Technology, Sylhet, Bangladesh, 3 Department of Chemistry, Graduate School of Science, Chiba University, Chiba, Japan, 4 RIKEN SPring-8 Center, Hyogo, Japan, 5 Structural Biology Research Center, Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK), Ibaraki, Japan, 6 RIKEN Systems and Structural Biology Center, Yokohama, Japan, 7 Department of Cell Biology, Faculty of Medicine, Kyoto University, Kyoto, Japan, 8 Laboratory of Molecular Physiology and Genetics, Faculty of Agriculture, Ehime University, Ehime, Japan, 9 JST, PRESTO, Chiba, Japan

Abstract

Vacuolar ATPases (V-ATPases) function as proton pumps in various cellular membrane systems. The hydrophilic V₁ portion of the V-ATPase is a rotary motor, in which a central-axis DF complex rotates inside a hexagonally arranged catalytic A₃B₃ complex by using ATP hydrolysis energy. We have previously reported crystal structures of Enterococcus hirae V-ATPase A₃B₃ and A₃B₃DF (V₁) complexes; the result suggested that the DF axis induces structural changes in the A₃B₃ complex through extensive protein-protein interactions. In this study, we mutated 10 residues at the interface between A₃B₃ and DF complexes and examined the ATPase activities of the mutated V₁ complexes as well as the binding affinities between the mutated A₃B₃ and DF complexes. Surprisingly, several V₁ mutants showed higher initial ATPase activities than wild-type V₁-ATPase, whereas these mutated A₃B₃ and DF complexes showed decreased binding affinities for each other. However, the high ATP hydrolysis activities of the mutants decreased faster over time than the activity of the wild-type V₁ complex, suggesting that the mutants were unstable in the reaction because the mutant A₃B₃ and DF complexes bound each other more weakly. These findings suggest that strong interaction between the DF complex and A₃B₃ complex lowers ATPase activity, but also that the tight binding is responsible for the stable ATPase activity of the complex.

Citation: Alam MJ, Arai S, Saijo S, Suzuki K, Mizutani K, et al. (2013) Loose Binding of the DF Axis with the A₃B₃ Complex Stimulates the Initial Activity of Enterococcus hirae V₁-ATPase. PLoS ONE 8(9): e74291. doi:10.1371/journal.pone.0074291

Editor: Paul Hoskisson, University of Strathclyde, United Kingdom

Received May 17, 2013; Accepted July 30, 2013; Published September 13, 2013

Copyright: © 2013 Alam et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Targeted Proteins Research Program, grants-in-aid (23370047, 23118705), and Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

E-mail: t.murata@faculty.chiba-u.jp

Introduction

Vacuolar ATPase (V-ATPase) functions as a proton pump in acidic organelles and plasma membranes of eukaryotic cells and bacteria [1,2]. The acidic environment is essential for processes such as receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, protein processing, and degradation [1]. V-ATPase contains a globular catalytic domain, V₁, that hydrolyses ATP, and this domain is attached by central and peripheral stalks to an integral membrane domain, Vₒ, that pumps ions across the membrane. ATP hydrolysis triggers the rotation of the central stalk and an attached membrane ring of hydrophobic subunits. Ions are pumped through a channel formed at the interface between the rotating ring and a static membrane component, which is linked to the outside of the Vₒ domain by the peripheral stalks [1].

V-ATPases are found in bacteria such as Thermus thermophilus and Enterococcus hirae. T. thermophilus V-ATPase functions physiologically as an ATP synthase [3], whereas the E. hirae V-ATPase, which transports Na⁺ or Li⁺ instead of H⁺ [4-8], is not an ATP synthase and acts instead as a primary ion pump like eukaryotic V-ATPases. The amino acid sequences and subunit structures of E. hirae V-ATPase are more similar to those of eukaryotic V-ATPases than to those of...
ATP synthases of the F- and V-type ATPase families. The enzyme has 9 subunits whose amino acid sequences are homologous to sequences from corresponding subunits of eukaryotic V-ATPases [9-12]. The core of *E. hirae* V$_1$ domain is composed of a hexameric arrangement of alternating A and B subunits that are responsible for ATP binding and hydrolysis [13]. The V$_o$ domain, which uses rotational energy to drive Na$^+$ translocation, is composed of oligomers of the 16-kDa c subunits and an a subunit [7,8]. The V$_i$ and V$_o$ domains are connected by a central stalk, composed of the D, F, and d subunits, and 2 peripheral stalks comprising the E and G subunits of V$_i$ (Figure 1) [11,13]. ATP hydrolysis induces the rotation of the central axis (DFd complex) and the attached membrane c ring, which results in ions being pumped through a channel at the interface between the c ring and the a subunit [6]. Recently, we purified the A$_B_3$ and DF complexes and reconstituted the V$_i$-ATPase using the 2 complexes [14] and we determined the crystal structures of the DF, A$_B_3$, and A$_D_3$B$_D$DF complexes [15,16]. This structural information suggests that the DF complex binds the A$_B_3$ complex tightly through 19 polar interactions and 101 nonpolar (van der Waals) interactions; through these interactions, the DF complex induces conformational changes in the A$_B_3$ complex (Figure 2C-H), and ATP hydrolysis appears to be stimulated by the approach of a conserved arginine residue (arginine finger) [15].

The general architectures and mode of actions of V-ATPase and F-ATPase are similar [17]. The isolated F$_1$ domain, like the V$_i$ domain, is composed of a$_B_3$y(5x) and hydrolyses ATP [18], with the y-subunit serving as the rotation axis. The contact between y and a$_B_3$, especially at the DELSEED region, has been examined extensively by mutagenesis to elucidate the rotation mechanism [19-22].

To date, 120 polar and nonpolar (van der Waals) interactions have been identified between the DF and A$_B_3$ complexes (Figure 2C-H) [15]. In this study, we chose 10 residues for creating site-directed mutants of A, B, and D subunits (1 mutation in A, 2 in B, and 7 in D subunits (Figure 2, residues in red boxes)). These residues located at the interface between the D subunit and the C-terminal domain of A and/or B subunits [15] (and likely, correspond to the DELSEED region in F$_1$) were selected for mutation, taking into account the conservation, importance, and location of each amino acid. All 10 residues appeared to interact with each of the other 2 subunits in at least 1 of their 3 conformational states (A$_D_3$-B$_D$CR pair, "tight" form; A$_C_3$-B$_C_3$ pair, "bound" form; and A$_C'-B_3$ pair, "empty" form; Figure 2B). We reconstituted mutant V$_i$-domains, and measured the ATPase activities and binding affinities of the mutant A$_B_3$ for the DF axis (wild-type or mutant DF). It has been suggested that ATPases showing higher activities have lower subunit-subunit binding affinities and vice versa [23,24].

In this study, we determined the critical reciprocal relationship between ATPase activities and subunit-subunit binding affinities by using mutant V$_i$-ATPases, suggesting that loose binding of the DF axis with the rotary A$_B_3$ ring enhances the activity of V$_i$-ATPase. A similar reciprocal relationship has been observed recently in *Escherichia coli* F-ATPase [25]. Our results also suggest that tight binding of the DF complex with the A$_B_3$ complex lowers ATPase activity but that the strong interaction ensures stable ATPase activity in the A$_B_3$DF complex.

### Materials and Methods

**Expression and Purification of the A$_B_3$ Complex**

Synthesized DNA fragments corresponding to the A and B genes with optimal codon usage for an *Escherichia coli* expression system were cloned into the plasmid vector pET23d [14]. Mutant A and B subunits were constructed using the wild-type A and B genes, respectively, in the plasmids as template for PCR mutagenesis. A and B subunits were expressed separately in *E. coli* BL21 (DE3) grown in a modified-Davis Migioli-Casamino Acid (m-DM-CA) medium [26] at 30°C, and the 2 subunits were then purified and reconstituted as described previously [14]. Briefly, the purified A and B subunits (3.4 and 2.7 mg of A and B subunits, respectively, in a 1: 1 molar ratio) were mixed and incubated for 1 h on ice in buffer A (20 mM MES-Tris, pH 6.5; 50 mM KCl; 10% glycerol; 5 mM MgSO$_4$; 0.1 mM DTT) in the presence of 2 mM ATP and were then concentrated to 100 µL by ultrafiltration using Amicon filters.

**Figure 1. Schematic model of *E. hirae* V-ATPase (adapted from [15] and [16]).** The V$_i$ domain of V-ATPase is composed of a hexameric arrangement of alternating A and B subunits responsible for ATP binding and hydrolysis; it also contains the DF subunits (shown by a dotted red line), the focus of this study. The V$_o$ domain of V-ATPase comprises an a subunit and an attached membrane c ring. The V$_i$ and V$_o$ domains are connected by a central stalk, which is composed of D, F, and d subunits, and 2 peripheral stalks assembled from the E and G subunits of V$_i$. ATP hydrolysis induces the rotation of the central axis (DFd complex) together with the c ring, which causes Na$^+$ to be pumped through the channel at the interface between the c ring and the a subunit. doi: 10.1371/journal.pone.0074291.g001
Figure 2. Structure of nucleotide-free A₃B₃DF and protein-protein interactions between the A₃B₃ and DF complexes in *E. hirae* V₁-ATPase. A, Side view of the nucleotide-free A₃B₃DF (V₁) complex of *E. hirae*. D and F subunits are represented in green and red, respectively. B, Top view of the C-terminal domain of *E. hirae* V₁-ATPase. Empty (O and O'), bound (C), and tight (CR) conformations of *E. hirae* A and B subunits are shown in light, dark, and darker colors, respectively. Red arrows indicate nucleotide-binding sites. The side-view ribbon representations show the residues of B₃C(C), A₀(D), B₀(E), A₀(F), B₀R(G), and A₀R(H) that interact with the residues of the DF complex in *E. hirae* V₁-ATPase. The sticks represent the residues with a buried surface area > 10 Å², as calculated by PDBePISA (http://pdbe.org/pisa/). The residues in the red boxes were mutated in this study.

doi: 10.1371/journal.pone.0074291.g002
Ultra-4 30K filters (Millipore Corporation, USA). Next, 4 mL of buffer A containing ATP was added to dilute the protein solution, and the solution was concentrated again to 100 µL. This dilution/concentration process was then repeated thrice without adding ATP, and the A₃B₃ heterohexamer was purified finally using a Superose 6 pg column (500 × 16 mm ID) (GE Healthcare). The formation of the complex was confirmed by using basic native polyacrylamide gel electrophoresis (PAGE) as described [14].

Expression and Purification of the DF Complex

An E. coli cell-free protein expression system [27] was used to synthesize the DF complex by using plasmids carrying D and F subunit genes. More than 0.5 mg of the complex was synthesized with this system in 1 mL of the reaction solution in the presence of 3 µg of plasmid [27] and the expressed proteins were purified as described previously [11]. The D subunit was mutated using the QuikChange site-directed mutagenesis kit (Agilent Technologies).

Reconstitution of the V₁ (A₃B₃DF) Complex

The catalytic V₁ (A₃B₃DF) complex was reconstituted from purified A₃B₃ and DF complexes as described in earlier studies [14,15]: Briefly, purified A₃B₃ and DF complexes were mixed in a 1: 5 molar ratio and incubated on ice for 1 h, and complex formation was confirmed by using basic native-PAGE [14].

Measurement of ATPase Activity of Mutant A₃B₃DF Complexes

ATPase activities of the reconstituted A₃B₃DF complexes were measured using an ATP regenerating system [28]. The reaction mixture contained various concentrations of ATP, 2.5 mM phosphoenolpyruvate, 50 µg/mL pyruvate kinase, 50 µg/mL lactate dehydrogenase, and 0.2 mM β-NADH (dipotassium salt) in 1 mL of buffer B (25 mM MES-Tris, pH 6.5; 4 mM MgSO₄; and 10% glycerol). Reactions were initiated by adding 1–2 µg of proteins, and ATP hydrolysis rates (at 25°C) were determined in terms of the rate of NADH oxidation, which was measured as a decrease in absorbance at 340 nm. Specific activities were calculated as units/mg proteins, with 1 unit of ATPase activity being defined as hydrolysis of 1 µmol ATP/min. Initial ATPase activity was calculated by measuring the specific activity during the first minute (starting from the 16th second) after adding the protein. Because the measurement curve was concave even for wild-type V₁, we concluded that the activity was not stable in the reaction mixture with ATP. Thus, the stability of reconstituted A₃B₃DF mutants was estimated using time-course experiments: ATPase activity was measured at 2-min intervals for 20 min. The measurements were repeated thrice and averaged, and the standard deviations were calculated. Kᵣ and Vₑₐₘₐₓ were then calculated by fitting the average values as straight lines in Lineweaver-Burk plots.

Measurement of Binding Affinity Using Surface Plasmon Resonance (SPR)

The binding affinity of the DF complex for the reconstituted A₃B₃ complex was measured using SPR analysis on a Biacore T100 instrument (GE Healthcare Bio-sciences, AB, Sweden) as described previously [15,16]. The Biacore Ni-NTA sensor chip (GE Healthcare Bio-sciences) was activated using 0.5 µM NiCl₂ as per the manufacturer’s instructions. For analyses, we used His-tagged A₃B₃ as the ligand and DF as the analyte. His-tagged A₃B₃ was reconstituted using A subunits treated with TEV protease (tobacco etch virus protease) and His-tagged B subunits; the complex was reconstituted following protocols described above (in “Expression and purification of the A₃B₃ complex”). The reconstituted His-tagged A₃B₃ complex was immobilized on the sensor chip at a concentration of 35 µg/mL in running buffer (20 mM MES-Tris, pH 6.5; 150 mM NaCl; 50 µM EDTA-Na; 0.005% polyoxyethylene [20] sorbitol monolaurate) by passing the protein solution through the Biacore flow cell at a rate of 10 µL/min. A flow cell containing no immobilized protein served as the negative control. Several concentrations of the DF complex were prepared in running buffer and used as the analyte. The sensograms obtained were examined using the Biacore T100 evaluation software, and the equilibrium constant for dissociation (Kᵣ) was calculated using the Langmuir binding model (1:1 binding).

Other Experimental Procedures

Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories) with bovine serum albumin serving as the standard. Protein purification steps were evaluated using sodium dodecyl sulfate-PAGE (SDS-PAGE) [29] and samples were stained with Coomassie Brilliant Blue R-250. Restriction enzymes were purchased from Nippon Gene Japan, New England BioLabs Japan, and Wako Pure Chemical Industries Ltd. All other chemicals were of analytical grade and were obtained from Sigma-Aldrich Japan KK or Wako Pure Chemical Industries Ltd.

Results and Discussion

Properties of Central Axis D Mutants

From the crystal structures of A₃B₃ and A₃B₃DF [15], we identified 120 polar and nonpolar (van der Waals) interactions between the DF complex and the A₃B₃ complex in the A₃B₃DF complex (Figure 2C-H). In this study, we selected 6 amino acids in the D subunit for mutation (L₁⁶₉, L₁₇₀, R₁₆₅, R₁₆₆, A₁₆₉, and L₁₇₀); these residues are located at the binding interface between the A, B, and D subunits and reside close to the C-terminal domain of the A and/or B subunits of the E. hirae V₁-ATPase [15]. We constructed D(L₁⁶₉F), D(L₁₇₀F), D(R₁₆₅A), D(R₁₆₆A), D(A₁₆₉S), and D(L₁₇₀N) F mutants and reconstituted the corresponding mutant catalytic V₁ domains using the wild-type A₃B₃ heterohexamer. As shown in Figure 3, all DF mutants reconstituted V₁ domains to a similar extent as wild-type. Among the mutants, D(L₁⁶₉F) and D(L₁₇₀N) F showed almost wild-type levels of initial ATPase activities and binding affinities, whereas D(A₁₆₉S) F showed...
slightly lower initial ATPase activity and slightly higher binding affinity than the wild-type DF (Table 1).

In contrast to other mutants, D(R165A) F and D(R166A) F showed higher initial ATPase activities and lower binding affinities than wild-type DF (Figure 4A and Table 1): D(R165A) F and D(R166A) F had 1.2- and 1.8-times higher initial ATPase activities than the wild-type and lower binding affinities (K\textsubscript{D} values) for the A\textsubscript{3}B\textsubscript{3} heterohexamer, that is, 6.5 and 7.3 nM, respectively, than the wild-type (K\textsubscript{D}, 1.6 nM) (Table 1).

Considering the high initial specific activities of the mutant V\textsubscript{1}-ATPases and their lower binding affinities of these 2 single mutants, we constructed the double mutant D(RR165-6AA) F and

Table 1. Summary of ATPase activities of V\textsubscript{1} complexes containing wild-type A\textsubscript{3}B\textsubscript{3} and mutant DF and their binding affinities measured using SPR assays.

| Protein                  | (% of initial specific activity)\* of initial specific activity \^ to the assay\*   | K\textsubscript{D} (nM) (using wild-type A\textsubscript{3}B\textsubscript{3} as ligand and mutant DF as analyte) |
|--------------------------|---------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
| Wild-type A\textsubscript{3}B\textsubscript{3}DF                               | 100                                                                            | 1.6 ± 0.1                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(L\textsuperscript{28}N)F                | 91                                                                             | 2.7 ± 0.2                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(L\textsuperscript{29}N)F                | 31                                                                             | 5.0 ± 0.9                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(R\textsuperscript{165}A)F              | 121                                                                            | 6.5 ± 1.7                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(R\textsuperscript{166}A)F              | 31                                                                             | 7.3 ± 0.6                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(165-6AA)F                             | 5                                                                              | 17.0 ± 0.1                                                                                                        |
| A\textsubscript{3}B\textsubscript{3}D(A\textsuperscript{169}S)F              | 82                                                                             | 0.9 ± 0.1                                                                                                         |
| A\textsubscript{3}B\textsubscript{3}D(L\textsuperscript{170}N)F              | 31                                                                             | 5.3 ± 0.5                                                                                                         |

ATPase activities of reconstituted A\textsubscript{3}B\textsubscript{3}DF mutants were analyzed as described in Materials and Methods, with assays being started by adding 1–2 µg protein, depending on the mutants. For SPR assays, DF heterodimers at various concentrations (analyte) were injected onto the sensor chip Ni-NTA surface with immobilized wild-type A\textsubscript{3}B\textsubscript{3} heterohexamers, as described previously [15,16]. Reconstituted wild-type A\textsubscript{3}B\textsubscript{3} heterohexamers and mutant DF heterodimer were diluted in running buffer; the experimental procedures used are described in Materials and Methods.

\*"Initial specific activity" was calculated by measuring the specific activity during the first minute of the assay (starting from the 16\textsuperscript{th} second) after adding proteins. We calculated the percentage of the initial specific activity of the mutant V\textsubscript{1} domains, considering the specific activity of the wild-type A\textsubscript{3}B\textsubscript{3}DF as 100%.

\"Activity after 15 min" was the specific ATPase activity measured at the 16\textsuperscript{th} min (from 15: 01 to 16.00 min) during the extended assay. These values were calculated considering the initial ATPase activity of each mutant as 100%.

doi: 10.1371/journal.pone.0074291.t001

Figure 3. Basic native-PAGE patterns for the reconstitution of wild-type/mutant catalytic domains (V\textsubscript{1} domains). Purified wild-type or mutant A\textsubscript{3}B\textsubscript{3} and DF complexes were mixed in a 1: 5 molar ratio and incubated on ice for 1 h to reconstitute the catalytic domain A\textsubscript{3}B\textsubscript{3}DF, as described in Materials and Methods. Lanes 1, 10, 12, and 14: purified wild-type and mutant A\textsubscript{3}B\textsubscript{3} complexes; lane 2: wild-type A\textsubscript{3}B\textsubscript{3}DF; lanes 3, 9, 11, 13, 15, 17: reconstituted mutant catalytic domains; and lanes 18 and 19: B and A monomers, respectively. Three micrograms of proteins were loaded in lanes 1, 9, 16, and 17, and 2 µg in lanes 10, 15, 18, and 19.
doi: 10.1371/journal.pone.0074291.g003
reconstituted the corresponding catalytic domain with wild-type A3B3 heterohexamer (Figure 3). Surprisingly, D(RR165-6AA) F had almost 2-times higher initial ATPase activity (191%, Figure 3A and Table 1) than wild-type DF and also had considerably lower binding affinity (K_D = 17 nM, Table 1) for the A3B3 heterohexamer than wild-type DF. The ATPase activities of the 3 mutants, D(R165A) F, D(R166A) F, and D(RR165-6AA) F, had similar K_m values (0.33, 0.27, and 0.26 mM, respectively) and higher V_max values (22.2, 27.8, and 33.3 s^{-1}, respectively) than those of the wild-type form (K_m = 0.35 mM, V_max = 18.2 s^{-1}) (Figure 4B). Therefore, we focused on these 3 mutants while selecting residues to mutate in the A and B subunits.

Our results suggested that when nonpolar leucine was substituted with polar asparagine (D(L28N), D(L29N), and
D(L176N)), the ATPase activities of the mutants were almost the same as the activity of the wild-type complex (Table 1). Although these 2 amino acids with similar sizes differ in their hydrophobicity and polarity, they do not affect the activity and stability of V1-ATPase. The mutant in which the nonpolar/weakly hydrophobic alanine residue mutated to polar/weakly hydrophilic serine (D(A166S)) showed slightly lower ATPase activity and higher binding affinity for binding A3B than the wild-type (Table 1). However, basic and neutral/neutral, helix-forming alanine increased the ATPase activities of the mutants and lowered the binding affinities for A3B; especially in the double RR165-6AA mutant (Table 1). We interpreted this to mean that the association/dissociation of the axis determines the ATPase activity level and possibly the rotational activity.

To measure the stability of the mutant catalytic domains, we used ATPase time-course assays. We allowed reactions to proceed for approximately 20 min and estimated the specific activity at 2-min intervals. The ATPase activities of all mutant catalytic domains after 15 min of the assay are listed in Table 1 footnotes. We observed that the specific activity of the mutants decreased continuously during the entire assay, with the A3B, D(D(RR165-6AA) F mutant showing the most rapid reduction in activity during the assay: Only 5% of the mutant’s original activity remained after 15 min (Figure 4C and Table 1); the activities of the A3B, D(D(RR165-6AA) F and A3B, D(RR165-6AA) F mutants decreased more slowly than that of the A3B, D(D(RR165-6AA) F mutant, but slightly faster than that observed with the wild-type protein.

Properties of the Mutated A3B Complexes

When V1-ATPase hydrolyzes ATP, the D subunit rotates inside the hexagonally arranged A3B complex and comes into contact with conserved residues of A and/or B subunits, which likely correspond to the conserved DELSEED loop of the β subunit of F-ATPase [19-21]. From structural and sequence analyses of E. hirae V1-ATPase, we considered residues460DELSLSDND466 of the A subunit to correspond to the DELSEED loop of F-ATPase. Furthermore, from the crystal structures of A3B and A3B with or without the nucleotide AMP-PNP [15], we determined that residues R475, L478, and V477 of the A subunit and V389 and L389 of the B subunit were located near R165 and R166 of the D subunit. R475 of the A subunit appears to reside close to, but not in direct contact with R165 and R166 of the D subunit. Therefore, we mutated the residues near the sequence of the A subunit. We changed all the residues to alanine and found that we could still reconstitute the catalytic domains, A(R475A)ββDF, A3B(V389A)ββDF, and A3B(L389A)ββDF, in the same manner as the wild-type (Figure 3) [14] with similar biochemical properties and stability in the presence of nucleotides [14]. In other cases, we could not reconstitute the catalytic domain as with the wild-type. The 3 mutant catalytic domains, A(R475A)ββDF, A3B(V389A)ββDF, and A3B(L389A)ββDF, showed initial ATPase activities that were 1.9-, 1.4-, and 2.1-times higher, respectively, than the wild-type complex (Table 2).

Table 2. Summary of ATPase activities and binding affinities of V1 complexes containing mutant A3B and wild-type or mutant DF.

| Protein | (% of initial specific activity) | (% of ATPase activity after 15 minutes of the assay) | $K_D$ (nM) (using mutant A3B as ligand and wild-type or mutant DF as analyte) |
|---------|---------------------------------|-----------------------------------------------|-----------------------------------------------|
| Wild-type A3BDF | 100 | 44 | 1.6 ± 0.1 |
| A(R475A)ββDF | 186 | 29 | 1.0 ± 0.1 |
| A3B(V389A)ββDF | 138 | 29 | 5.2 ± 1.1 |
| A3B(L389A)ββDF | 214 | 32 | 10.9 ± 0.9 |
| A(R475A)ββββDF | 193 | 12 | 14.8 ± 3.2 |
| A3B(V389A)ββββDF | 191 | 15 | 10.8 ± 0.8 |

*See the Table 1 footnotes.

**Details presented in Table 1 footnotes.

ATPase activities of the reconstituted A3BDF mutants were measured, and SPR assays were performed as described in Materials and Methods.

doi: 10.1371/journal.pone.0074291.t002
substitution of these amino acids with the small amino acid alanine is expected to increase the ATPase activities by lowering binding affinities, which was the measured result with all mutants except the R^{475}A mutant noted above (Figure 4D, Table 2). Recently, for yeast V-ATPase, “loosening” the V-ATPase complex was suggested to increase catalytic activity [32], and mutational studies on the ε-subunit of E. coli F-ATPase [25] showed that lowering binding affinity increased ATPase activity.

We once again used time-course experiments to determine the stability of the ATPase activities of these mutants (Figure 4D), and the results are summarized in Table 2. Specific activities decreased continuously during the assay for the mutants as with the wild-type and the D subunit mutants, and, after 15 min, the specific activities dropped to 29%, 29%, and 32% of initial activities for A(R^{475}A) B, A(B(V^{388}A)) DF, and A(B(L^{389}A)) DF, respectively. All 3 mutants showed slightly higher reduction in activity than the wild-type complex, likely due to their lower binding affinities.

Properties of the Reconstituted Double-Mutant A,B,DF Complexes

After constructing A, B, and D subunit mutants, we reconstituted double-mutant Vᵢ complexes with mutations in the D subunit and A or B subunit: A(R^{475}A) B D(R(R^{165-6}AA) F and A(B(V^{388}A)) D(R(R^{165-6}AA) F (Figure 3). We selected these combinations based on interactions of the mutants suggested by the crystal structures of E. hirae Vᵢ-ATPase [15,16]. Both double mutants showed higher initial ATPase activities (1.9 times for both A(R^{475}A) B D(R(R^{165-6}AA) F and A(B(V^{388}A)) D(R(R^{165-6}AA) F) and lower binding affinities than the wild-type (Table 2).

A(R^{475}A) B D(R(R^{165-6}AA) F and A(B(V^{388}A)) D(R(R^{165-6}AA) F showed remarkably rapid reduction in specific activities compared to the wild-type complex, but as with A,B,D(R(R^{165-6}AA) F, the original D subunit mutant, these 2 mutant complexes retained approximately 12% and 15% activities after 15 min (Table 2). To ensure that the low ATPase activity measured was not due to the substrate being depleted during the ATPase assay, we added excess NADH twice during the assay and found no noticeable change in activity (data not shown). We therefore speculate the activity decreased because of continuous dissociation of some amount of mutant DF from the A,B, stator barrel during high-speed rotation.

ATPase activities and binding affinities exhibited reciprocal relationships in all mutants except A(R^{475}A) B,D. These findings (Tables 1 and 2) indicate that the high ATPase activity, which likely depends on the rotation speed, results from the loose binding of the DF axis to the rotary ring A,B₃.

Wild-type A,B,DF (Vᵢ) Complex is an Optimized Rotary Motor

If close contact between 2 amino acids determines a protein’s function, a combined mutation of the amino acids may either produce a larger effect than a single mutation or compensate for the effect produced by a single mutation. The D(R(R^{165-6}AA) F mutant with A,B(V^{388}A)₁ showed higher ATPase activity with lower binding affinity than A,B(V^{388}A) DF, but nearly similar ATPase activity as A,B,D(R(R^{165-6}AA) F (Tables 1 and 2), which indicates that RR^{165} of the D subunit interacts with V^{388} of the B subunit in the “tight” form of the complex (A₁CR– B₁₂pair, Figure 2B and G), as suggested by our crystal structures [15,16]. We consider this to be a compensation effect. Unexpectedly, the D(R(R^{165-6}AA) mutant with A(R^{475}A) B₁ showed no change in ATPase activity or binding affinity (Tables 1 and 2) compared to D(R(R^{165-6}AA) with wild-type A,B₃, indicating that R^{475} of the A subunit locating near RR^{165} of the D subunit does not contact directly but interacts functionally with RR^{165}, again producing a compensation effect.

Our ATPase assay showed that the Vᵢ domains were less stable when containing D(R(R^{165-6}AA) F, D(R(R^{165-6}AA) F, or D(R(R^{165-6}AA) F mutants (Figure 4C), which may be due to their lower binding affinities (Table 1). After 15 min, we found only 5% of the original ATPase activity for A,B,D(R(R^{165-6}AA) F (Figure 4C and Table 1) and 12% and 15% for A(R^{475}A) B D(R(R^{165-6}AA) F and A,B(V^{388}A) D(R(R^{165-6}AA) F, respectively (Table 2). In contrast, wild-type and other DF mutants retained higher stability, possibly because of their higher binding affinities (Table 1). Moreover, incubation with ATP (especially under alkaline high-salt conditions), but not with ADP or AMP-PNP, stimulated the disassembly of the wild-type Vᵢ complex (unpublished observation, part of a Ph. D. thesis of Arai S [2009]: Study on the resolution and assembly of Vᵢ-ATPase from Enterococcus hirae. Tokyo University of Science, Japan). Therefore, we propose that tight binding of the DF axis is critical for stable ATPase activity. Considering its stability and rotation speed (activity), the wild-type A,B,DF (Vᵢ) complex appears to be a well-optimized rotary motor.

Acknowledgements

We thank Dr. Suhaila Rahman for suggestions during the experiments.

Author Contributions

Conceived and designed the experiments: IY TM. Performed the experiments: MJA KS KM YIK NO TT MS. Analyzed the data: MJA SA SS IY TM. Contributed reagents/materials/analysis tools: SY SI YK IY TM. Wrote the manuscript: MJA IY TM. Synthesized D subunit mutants: KS KM YIK NO TT MS. Conceived and designed the experiments: IY TM. Performed the experiments: MJA. Contributed reagents/materials/analysis tools: SY SI YK IY TM. Contributed reagents/materials/analysis tools: MJA SA SS IY TM. Wrote the manuscript: MJA IY TM. Synthesized D subunit mutants: KS KM YIK NO TT MS. Constructed A and B subunit mutants: MJA.
Rotation mechanism of Enterococcus hirae V-ATPase based on asymmetric crystal structures. Nature 493: 703–707.

16. Saio S, Arai S, Hossain KMM, Yamato I, Suzuki K et al. (2011) Crystal structure of the central axis DF complex of the prokaryotic V-ATPase.

Prochazkaova A, Brezina M, Pollock B, Lenard T, Hool D et al. (2004) The structure of the V1 domain of V-ATPase from Aspergillus niger at 3.5 Å resolution. Structure 12: 367–377.

15. Araki T, Takase K, Yamato I, Igarashi K, Kakinuma Y (2013) Rotation mechanism of Enterococcus hirae V-ATPase based on asymmetric crystal structures. Nature 493: 703–707.

14. Araki T, Takase K, Yamato I, Igarashi K, Kakinuma Y et al. (2009) Reconstitution of the catalytic portion (NtpAβ3-Bγ3-D-G complex) of Enterococcus hirae V-type Na+-ATPase. Biochem Biophys Res Commun 390: 698–702. doi:10.1016/j.bbrc.2009.10.032. PubMed: 19822027.

13. Murata T, Takase K, Yamato I, Igarashi K, Kakinuma Y (1999) Properties of the Vγ-Vδ Na+-ATPase from Enterococcus hirae and its Vε moiety. J Biochem 125: 414–421. doi:10.1093/oxfordjournals.jbch.a022332. PubMed: 9990142.

12. Zhou M, Morgen N, Barrera NP, Politis A, Isaacson SC et al. (2003) Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding. Science 301: 380–385. doi:10.1126/science.1210148. PubMed: 12201838.

11. Yamamoto M, Unzai S, Saijo S, Ito K, Mizutani K et al. (2008) Interaction and stoichiometry of the peripheral stalk subunits NtpE and NtpF and the N-terminal hydrophilic domain of NtpI of Enterococcus hirae V-ATPase. J Biol Chem 283: 19422–19431. doi:10.1074/jbc.M107.617220. PubMed: 18460472.

10. Murata T, Yamato I, Kakinuma Y (2005) Structure and mechanism of vacuolar Na+-ATPase. Annu Rev Cell Dev Biol 13: 779–807. doi:10.1146/annurev.cellbio.13.101501.140005. PubMed: 15802565.

9. Murata T, Yamato I, Kakinuma Y, Shirouzu M, Walker JE et al. (2008) Structure and mechanism of Na+-ATPase from Escherichia coli. Science 322: 787–791. doi:10.1126/science.11309608. PubMed: 18460472.

8. Mizutani K, Yamamoto M, Suzuki K, Yamato I, Kakinuma Y et al. (2001) Structure of the rotor ring modified with N,N' dicholcysteinylation of the NtpA-transporting vacuolar ATPase. Proc Natl Acad Sci U S A 98: 13474–13479. doi:10.1073/pnas.151080098. PubMed: 11303276.

7. Zhou M, Morgner N, Barrera NP, Politis A, Isaacson SC et al. (2003) Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding. Science 301: 380–385. doi:10.1126/science.1210148. PubMed: 12201838.

6. Murata T, Yamato I, Kakinuma Y, Shirouzu M, Walker JE et al. (2008) Structure and mechanism of vacuolar Na+-ATPase from Enterococcus hirae. Science 308: 654–659. doi:10.1126/science.1110064. PubMed: 18598985.

5. Murata T, Yamato I, Kakinuma Y, Shirouzu M, Walker JE et al. (2008) Interaction and stoichiometry of the peripheral stalk subunits NtpE and NtpF and the N-terminal hydrophilic domain of NtpI of Enterococcus hirae V-ATPase. J Biol Chem 283: 19422–19431. doi:10.1074/jbc.M107.617220. PubMed: 18460472.

4. Murata T, Igarashi K, Kakinuma Y, Yamato I (1997) Purification and reconstitution of Na+-translocating vacuolar ATPase from Enterococcus hirae. J Biol Chem 272: 24885–24890. doi:10.1074/jbc.272.40.24885. PubMed: 9312089.

3. Murata T, Araki T, Igarashi K, Kakinuma Y (2005) Structure and mechanism of vacuolar Na+-translocating ATPase from Enterococcus hirae. J Bioenerg Biomembr 37: 411–413. doi:10.1007/s10863-005-9481-0. PubMed: 16691474.

2. Yamamoto M, Unzai S, Saijo S, Ito K, Mizutani K et al. (2008) Interaction and stoichiometry of the peripheral stalk subunits NtpE and NtpF and the N-terminal hydrophilic domain of NtpI of Enterococcus hirae V-ATPase. J Biol Chem 283: 19422–19431. doi:10.1074/jbc.M107.617220. PubMed: 18460472.

1. Yamamoto M, Unzai S, Saijo S, Ito K, Mizutani K et al. (2008) Interaction and stoichiometry of the peripheral stalk subunits NtpE and NtpF and the N-terminal hydrophilic domain of NtpI of Enterococcus hirae V-ATPase. J Biol Chem 283: 19422–19431. doi:10.1074/jbc.M107.617220. PubMed: 18460472.