Interaction and Stoichiometry of the Peripheral Stalk Subunits NtpE and NtpF and the N-terminal Hydrophilic Domain of NtpI of Enterococcus hirae V-ATPase

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The vacuolar ATPase (V-ATPase) is composed of a soluble catalytic domain and an integral membrane domain connected by a central stalk and a few peripheral stalks. The number and arrangement of the peripheral stalk subunits remain controversial. The peripheral stalk of Na"+ -translocating V-ATPase from Enterococcus hirae is likely to be composed of NtpE and NtpF (corresponding to subunit G of eukaryotic V-ATPase) subunits together with the N-terminal hydrophilic domain of NtpI (corresponding to subunit a of eukaryotic V-ATPase). Here we purified NtpE, NtpF, and the N-terminal hydrophilic domain of NtpI (NtpINterm) as separate recombinant His-tagged proteins and examined interactions between these three subunits by pulldown assay using one tagged subunit, CD spectroscopy, surface plasmon resonance, and analytical ultracentrifugation. NtpINterm directly bound NtpF, but not NtpE. NtpE bound NtpF tightly. NtpINterm bound the NtpE-F complex stronger than NtpF only, suggesting that NtpE increases the binding affinity between NtpINterm and NtpF. Purified NtpE-F-NtpINterm complex appeared to be monodisperse, and the molecular masses estimated from analytical ultracentrifugation and small-angle x-ray scattering (SAXS) indicated that the ternary complex is formed with a 1:1:1 stoichiometry. A low resolution structure model of the complex produced from the SAXS data showed an elongated “L” shape.

The vacuolar ATPases (V-ATPases) function as ATP-dependent proton pumps in the membranes of acidic organelles and in plasma membranes of eukaryotic cells. This acidification is involved in concentration of neurotransmitters, processing of secretory proteins, endocytosis, and other important cellular processes (1). The V-ATPase contains a globular catalytic domain, V1, which hydrolyzes ATP, attached by central and peripheral stalks to an integral membrane domain, V0, which pumps ions across the membrane. ATP hydrolysis generates rotation of the central stalk and an attached membrane ring of hydrophobic subunits. Ions are pumped through a pathway at the interface between the rotating ring and a static membrane component, which is linked to the outside of the V1 domain by the peripheral stalk (1).

In yeast, the V1 domain contains subunits A–H, while the membrane-bound V0 is made of subunit a, c, c', c'', d, and e. The core of the V1 domain is composed of a hexameric arrangement of alternating A and B subunits responsible for ATP binding and hydrolysis. The V0 domain consists of a ring of proteolipid subunits (c, c', and c'') adjacent to subunits a and e. The V1 and V0 domains are connected by a central stalk, composed of subunits D and F of V1 and subunit d of V0, and a few peripheral stalks, composed of subunits C, E, G, and H together with N-terminal domain of subunit a (1). Because V-ATPases function in various physiological processes, regulation of their activity is very important. The peripheral stalk subunits have been shown to play an important role in the regulation of the enzyme via a reversible dissociation and re-association mechanism (2, 3). To understand the structural mechanism of the regulation, information on the stoichiometry of the subunits comprising...
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the peripheral stalk and the nature of their interaction is essential. Interactions within the V₁ domain, between subunits E, G, and C (4), H and a (5), a and C (6), a and G (7), and E and G (8) have been demonstrated by cross-linking studies and/or in vitro pulldown techniques. Electron microscopic studies have indicated that subunits E and G formed a rod-shaped heterodimer (8, 9) and are both present in two or three copies per complex (10–12). These findings suggest that the peripheral stalk of V-ATPase is composed of two or three EG heterodimers together with subunits H and C, and the N-terminal domain of subunit a. However, the precise subunit arrangement in the stalk regions remains controversial.

V-ATPases are also found in bacteria (13–15). We have identified a variant of V-ATPase in a fermentative bacterium Enterococcus hirae, which physiologically transports Na⁺ rather than H⁺ (16–18). The enzyme is encoded by nine ntp subunit genes (ntpFIKECGABD) organized in the ntp operon (19, 20). Amino acid sequences of NtpF, -I, -K, -E, -C, -G, -A, -B, and -D are homologous to those of subunits G, a, c, e, d, f, A, B, and D of eukaryotic V-ATPases, respectively (21). Bacterial V-ATPases have no counterpart for subunit C and H of eukaryotic V-ATPases that are responsible for modulating the interaction between V₁ and V₉ (22). Therefore, E. hirae Na⁺-ATPase is a homolog of eukaryotic V-ATPase, with a simpler subunit composition. The V₁ domain responsible for ATP-driven rotation is composed of the Ntp-A, -B, -C(d), -D, -E, -F(G), and -G(F) subunits. The V₉ domain responsible for utilization of the rotation energy of V₁ for Na⁺ translocation is composed of oligomers of the 16-kDa NtpK(c) forming a membrane rotor ring and a single copy of the NtpI(a) subunit (Fig. 1). The peripheral stalk of this enzyme is likely to be composed of NtpF(G) and NtpE with the N-terminal domain of NtpI(a) (Fig. 1, shown in gray), although the subunit arrangement and stoichiometry of these subunits are unclear.

In this study, NtpE, NtpF, and the N-terminal hydrophilic domain of NtpI (NtpI(Nterm)) were individually purified, and the interactions between the three subunits were investigated. The purified NtpI(Nterm) was shown to bind directly to NtpF but not NtpE, and the three subunits make up the ternary complex with a 1:1:1 stoichiometry. A low resolution structure model of the complex obtained by SAXS reveals an elongated “L”-shape.

**EXPERIMENTAL PROCEDURES**

**Initial Expression Trials of NtpE, NtpF, and NtpI(Nterm).**—Full-length ntpE and ntpF genes, and nine DNA constructs encoding the N-terminal soluble domain of NtpI with different C-terminal boundaries were amplified by PCR. The amplified DNA fragments were incorporated by overlap PCR to the T7 promoter sequence, the ribosome-binding site, the His tag, the cleavage site for tobacco etch virus protease, a linker sequence, and the T7 terminator sequence, which are coding following protein sequence from N terminus: His tag (His-7)/tobacco etch virus cleavage site (EHLYFQGQ)/linker (SSGSSG)/protein sequence (23). The His-tagged proteins were synthesized by the dialysis mode of cell-free protein expression using these PCR DNA templates, as described elsewhere (24). The total reaction mixtures were centrifuged to remove insoluble proteins, and the total and soluble fractions were subjected to SDS-PAGE to assess the quantity and the solubility of the expressed proteins.

**Expression and Purification of the Subunits.**—The DNA fragments expressed in the cell-free system were cloned into the plasmid vector, pET23d, and expressed in Escherichia coli BL21(DE3) by isopropyl (thio)-β-d-galactoside induction. The E. coli lysate was loaded onto a HiTrap HP column (GE Healthcare, Ireland) equilibrated with A buffer (50 mM Tris-HCl, 750 mM NaCl, 5 mM 2-mercaptoethanol, and 10 mM imidazole, pH 8.0), and bound proteins were eluted with B buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM 2-mercaptoethanol, and 500 mM imidazole, pH 8.0). The sample buffer was exchanged to A buffer using a HiPrep 26/10 desalting column. To obtain non-tagged protein, the proteins were treated with tobacco etch virus protease at 4 °C for 12 h. The reaction solution was loaded onto a HisTrap HP column again, and the flow-through fractions containing the non-tagged proteins were pooled. This step was not included in protocol for the purification of His-tagged proteins. The protein samples were then dialyzed against C buffer (50 mM Tris-HCl, 10 mM NaCl, 5 mM 2-mercaptoethanol, pH 8.5), loaded onto a HiTrap Q HP (GE Healthcare, England) column equilibrated with C buffer and eluted with a linear gradient of 10 mM-1.0 mM NaCl. Finally, the samples were loaded onto a HiLoad 16/60 Superdex 200pg (GE Healthcare) column equilibrated with D buffer (50 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, pH 7.5) and eluted using D buffer. The purified sample was concentrated by ultrafiltration (YM 10 filter, Amicon).

**Pulldown Assay Using Only One Tagged Subunit and Co-purification of the Complex.**—His-tagged sample (NtpE or NtpI(Nterm)) and an excess of non-tagged sample(s) (NtpE, NtpF, and/or NtpI(Nterm)) were mixed and incubated for 90 min on ice. The mixture was loaded on a HiTrap HP column equilibrated...
with A buffer, the column was washed with 5 column volumes of A buffer, and bound proteins were eluted with B buffer. All eluted proteins were analyzed by SDS-PAGE. To obtain non-tagged complex (NtpE-F or NtpE-F-I\textsuperscript{Nterm}), the histidine-affinity tag was removed by tobacco etch virus protease treatment at 4 °C for 12 h. The reaction solution was loaded onto a HisTrap HP column again, and the flow-through fraction was pooled. Finally, concentrated pooled samples were loaded onto a HiLoad 16/60 Superdex 200pg (GE Healthcare) column equilibrated with D buffer and eluted with D buffer. Purified complex was concentrated by ultrafiltration (YM10 filter).

**Measurements of CD Spectroscopy**—The CD spectroscopy was performed with a Jasco J-725 spectropolarimeter (Jasco, Japan). The purified subunits and complexes were dialyzed in phosphate-buffered saline (pH 7.4), and diluted to 0.25 mg of protein/ml which were determined using the BCA Protein Assay Kit (Pierce) using bovine serum albumin as the standard. Far UV scans were collected between 200 nm and 240 nm in 0.2 nm steps at 20 °C. All CD measurements were corrected for background buffer contribution. The raw data were converted to molar residual ellipticity (θ) using standard analysis.

**Real-time Binding Assay by Surface Plasmon Resonance**—The BIAcore 3000 system and reagents, including sensor chips and the amine coupling kit, were obtained from GE Healthcare. Purified NtpE (50 μg/ml) or NtpI\textsuperscript{Nterm} (30 μg/ml) in 10 mm sodium acetate (pH 4.5) was immobilized on a CM5 sensor chip using an amine coupling kit according to the manufacturer’s instructions. Purified sample was passed over the surface of the sensor chip with a typical flow rate of 30 μl/min, and the interactions were monitored for 3 min at 20 °C. The sensor surface was then washed with D buffer containing 2% glycerol for 3 min. Chip regeneration conditions varied according to the interactions studied: for NtpE (ligand)-NtpF (analyte) interaction, the chip was washed with 10 mM glycine (pH 1.5) for 30 s; for NtpI\textsuperscript{Nterm} (ligand)-NtpF (analyte) interaction and NtpI\textsuperscript{Nterm} (ligand)-NtpEF complex (analyte) interaction, the chip was washed with D buffer containing 2% glycerol for 5 min, which is enough to dissociate the bound analyte. For high affinity interactions of the NtpE-NtpF and NtpI\textsuperscript{Nterm}-NtpEF complexes, the association and dissociation rate constants (k\textsubscript{on} and k\textsubscript{off}) were calculated using BIAevaluation software (Version 4.1, GE Healthcare) with the program, 1:1 (Langmuir) binding model. The dissociation constant (K\textsubscript{D}) was determined from the k\textsubscript{off}/k\textsubscript{on} values. In the case of the NtpI\textsuperscript{Nterm}-NtpF interaction, it was not possible to evaluate the rate constants from the k\textsubscript{off}/k\textsubscript{on} values because of low affinity. We obtained an approximate equilibrium dissociation constant (K\textsubscript{D}) by measuring the equilibrium resonance units (R\textsubscript{eq}) at several analyte concentrations at equilibrium. The fitted curve (R\textsubscript{eq} versus concentrations of analyte) and the K\textsubscript{D} values were obtained using the BIAevaluation software with a single 1:1 interaction binding isotherm.

**Analytical Ultracentrifugation**—Sedimentation velocity (AUC/SV) and equilibrium (AUC/SE) experiments were carried out using an Optimal XL-1 analytical ultracentrifuge (Beckman Coulter). For AUC/SV experiments, cells with a standard Epon two-channel centerpiece and sapphire windows were used. Samples in D buffer and reference buffer (D buffer) were loaded into cells. The rotor temperature was equilibrated at 20 °C in the vacuum chamber for 1–2 h prior to start-up. Absorbance (A\textsubscript{280}) scans were collected at 10-min intervals during sedimentation at 50 × 10\textsuperscript{3} rpm. The sample concentrations were 0.8, 0.4, and 0.2 mg of protein/ml protein for purified subunits (NtpE, NtpF, and NtpI\textsuperscript{Nterm}) or 0.8 mg of protein/ml proteins for mixed samples and the purified complex (NtpE-F-I\textsuperscript{Nterm}). Partial specific volume of the protein, solvent density, and solvent viscosity were calculated from standard tables using the program SEDNTERP, version 1.09 (25). The resulting scans were analyzed using the continuous distribution (c(s)) analysis module in the program Sedfit version 11.0 (26). In this analysis a differential sedimentation coefficient distribution, c(s), that deconvolutes diffusion effects, based on the direct boundary modeling with distributions of Lamm equation solutions, is determined (27). Sedimentation coefficient increments of 200 were used in the appropriate range for each sample, and the weight average frictional ratio (ff\textsubscript{w}) was allowed to float during fitting. The weight average sedimentation coefficient was obtained by integrating the range of sedimentation coefficients in which peaks were present. The s\textsubscript{20,w} value, sedimentation coefficient corrected to 20 °C in pure water, was calculated from the observed sedimentation coefficient value using the program SEDNTERP.

**AUC/SE experiments** were also carried out in cells with a six-channel centerpiece and quartz windows. The samples were diluted 0.4, 0.2, and 0.15 mg of protein/ml with D buffer. The absorbance wavelength was set at 280 nm, and data were acquired at 20 °C. Data were obtained at 12, 15, and 18 × 10\textsuperscript{3} rpm for NtpE and NtpF, at 9, 12, and 15 × 10\textsuperscript{3} rpm for NtpI\textsuperscript{Nterm}, at 7, 10, and 13 × 10\textsuperscript{3} rpm for mixed samples, and at 6, 9, and 12 × 10\textsuperscript{3} rpm for purified NtpE-F-I\textsuperscript{Nterm} complex. A total equilibrium time of 16 h was used for each speed, with a scan taken at 12 h and 14 h to ensure equilibrium had been reached. The optical baseline was determined by accelerating at 40 × 10\textsuperscript{3} rpm at the end of data collection. Data analysis was performed by global analysis of data sets obtained at different loading concentrations and rotor speeds using UltraSpin software (MRC Center for Protein Engineering, Cambridge, UK, www.mrc-cpe.cam.ac.uk/ultraspin).

**SAXS**—SAXS experiments were carried out at the RIKEN Structural Biology Beamline I BL45XU at SPring-8, Japan (28). The x-ray wavelength used, λ, was 0.09 nm, and the beam size at the sample position was 0.25 × 0.5 mm\textsuperscript{2}. The sample-to-detector distance was determined to be 3343 mm by the silver benenate (d = 5.380 nm, The Gem Dagout) as a standard sample in the SAXS experiment. This setup covers the momentum transfer ranges 0.057 nm\textsuperscript{−1} < q < 2.133 nm\textsuperscript{−1} (where q = 4π sin θ/λ, 2θ is the scattering angle), however, the data points were discarded in q < 0.1 nm\textsuperscript{−1} to avoid the influence of parasitic scattering into the result. The sample temperature was controlled to 293.00 ± 0.01 K with a high precision thermoelectric controller. The sample solutions were brought into a sample cell, which has 30 μl in volume, a path length of 3 mm, and a pair of 0.02-mm thickness synthetic quartz windows. The SAXS patterns of protein solutions and corresponding buffer were measured by an on-line imaging plate detector, Rigaku R-AXIS IV+++. The sample cell was continuously moved during x-ray exposure to reduce the radiation damage. The SAXS
profiles were collected as three subsequent exposures of 100 s with the attenuated beam (∼1/100 against the intrinsic intensity). The radiation damage was tested by comparing subsequent exposures of the same sample, and only the data without the signs of radiation damage were analyzed. Exposures from sample and buffer were alternated to minimize the possible effects of drift in any experimental parameters. The SAXS patterns were circularly averaged and reduced to one-dimensional profiles using the in-house software (28). Those scattering profiles were then normalized by the x-ray intensity measured by the ionization chamber placed upstream of the sample, the exposure time, and the protein concentration c (mg/ml) of the solution. Data processing was performed using the program PRIMUS in the software package ATSAS 2.2 (Svergun). These data treatments resulted in scattering profiles, I(q). To eliminate inter-particle interference, I(q), recorded at three different protein concentrations (1.7, 2.4, and 3.0 mg of protein/ml) as described above, were scaled using a protein concentration c, 

\[ I(q, c) = \frac{I(q)}{c} \]  

(Eq. 1)

where I(q, c) denotes the SAXS profile normalized by weight percent concentration. The radius of gyration was determined by fitting the innermost portion of I(q, c) to,

\[ I(q, c) = I(0, c) \exp(-R_g(c)^2q^2/3) \]  

(Eq. 2)

where I(0, c) and R_g(c) are the concentration-normalized intensity at an angle of zero (q = 0) and the apparent radius of gyration at the protein concentration c, respectively. The value of R_g(c) was determined using Guinier approximation with q_{max}R_g(c) < 1.3. Data points for q < 0.143 nm⁻¹, which were affected by intermolecular interactions, were excluded from the data fit. The reported SAXS data were treated as monodisperse systems unless otherwise mentioned. The molecular mass of protein, M_m, was estimated by comparing the extrapolated forward scattering intensity, I(0, c), c→0, of NtpE-F-I^{Nterm} complex with that of bovine serum albumin (Sigma-Aldrich) as the standard protein in SAXS measurements. Bovine serum albumin protein is a 66.4-kDa monomeric protein, which is one of the most common standards for estimating the molecular mass by using SAXS. The pair distribution function, P(r), was calculated by the program GROMOS (29) based on an indirect Fourier transform algorithm in the q range from 0.1072 to 2.133 nm⁻¹ for NtpE-F-I^{Nterm} complex. The maximum molecular length, D_{max}, was estimated as a distance, r, where P(r) = 0 for r ≥ D_{max}.

Low Resolution Shape Reconstruction—After processing the scattering data with GNOM, low resolution shape reconstruction of the NtpE-F-I^{Nterm} complex was conducted with an ab initio bead-modeling program, DAMMIN (30). DAMMIN were applied to SAXS profiles in q < 2.133 nm⁻¹ and to those weighted by q⁻⁴ to ensure Porod’s law. Reproducibility of the structural solution was confirmed by repeating ten reconstructions without any shape and symmetrical constraints. To evaluate the credibility and accuracy of the reconstructed model, we employed the program, DAMAVER (31), which aligns all reconstructed models and removes outliers below a given cut-off volume, for setting up the criterion of the pairwise normalized spatial discrepancies (NSD). The ab initio model exceeding (NSD) (mean value of all pairs NSD) + 2σ (NSD) (variation of NSD) were discarded. The most probable model of NtpE-F-I^{Nterm} complex after DAMAVER processing is chosen by the criterion of NSD. The mean value of NSD and variation of NSD in the set of ten ab initio models was 0.406 and 0.040, respectively.

Others—Protein concentration was determined using the BCA Protein Assay Kit (Pierce) using bovine serum albumin as the standard. SDS-PAGE was carried out according to Laemmli (32) and stained with Imperial Protein Stain (Pierce). Densitometric analysis of SDS-PAGE gels was performed using ImageJ software (National Institutes of Health). All other chemicals were obtained from Sigma or Wako (Japan). Secondary structure prediction was carried out using the PSIPRED server (available on the World Wide Web).

### RESULTS

**Expression and Purification of NtpE, NtpF, and NtpI^{Nterm}**—Both NtpE and NtpF expressed as well as soluble His-tagged proteins in the E. coli cell-free protein expression system and in vivo in E. coli BL21 (DE3) cells (Table 1). NtpE and His-tagged NtpE and NtpF were individually purified using column chromatography (see “Experimental Procedures” for details) yielding proteins of apparent molecular masses of 24, 28, and 15 kDa, respectively, as assessed by SDS-PAGE (Fig. 2A, lanes 1–3).

Subunit NtpI constitutes the membrane-embedded V_o domain together with an NtpK ring, and probably has two half channels for Na⁺ translocation across the membrane (33). The 76-kDa NtpI is composed of two regions; a hydrophilic N-terminal half domain, which is likely to form contacts with the peripheral stalk(s), and a hydrophobic C-terminal half domain with several transmembrane helices (predicted as seven by hydropathy analyses) responsible for Na⁺ translocation (34). We constructed nine DNA templates, which encode the N-terminal half domain together with an NtpK ring, and probably has two half channels for Na⁺ translocation across the membrane (33). The 76-kDa NtpI is composed of two regions; a hydrophilic N-terminal half domain, which is likely to form contacts with the peripheral stalk(s), and a hydrophobic C-terminal half domain with several transmembrane helices (predicted as seven by hydropathy analyses) responsible for Na⁺ translocation (34).
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NtpI\text{Nterm} fragments (1–365 and 1–355 residues), both of which do not contain the putative trans-membrane region, were expressed as soluble and also as insoluble proteins (Table 1). It was not possible to isolate these proteins, because they aggregated heavily during purification. Other shorter NtpI\text{Nterm} fragments (1–341, 1–334, and 1–326 residues) were expressed as all soluble proteins. In this study, we used the NtpI\text{Nterm} fragment (1–316, 1–306, and 1–300 residues) were detected with the molar ratio of 1:1.2:1.5 (Fig. 2B, lane 6). Although His-tagged NtpE did not bind NtpI\text{Nterm} directly (Fig. 2B, lane 3), it bound NtpI\text{Nterm} complexed with NtpF; densitometric analysis showed that the molar ratio of NtpE:NtpF:NtpI\text{Nterm} is 1:1.3:0.8. These findings suggested that NtpE binds NtpF, and NtpF binds NtpI\text{Nterm}, directly, and these three subunits form a ternary complex.

The NtpE-F and NtpE-F-I\text{Nterm} complexes were purified as described under “Experimental Procedures.” SDS-PAGE analysis of the purified NtpE-F complex reveals two bands: NtpE (24 kDa) and NtpF (15 kDa) with a molar ratio of 1:1.3 as estimated by densitometric analysis (Fig. 2B, lane 5). The purified NtpE-F-I\text{Nterm} complex has three bands for NtpI\text{Nterm} (45 kDa), NtpE (24 kDa), and NtpF (15 kDa) in SDS-PAGE gel, which were detected with the molar ratio of 1:1.2:1.5 (Fig. 2B, lane 6).

CD Spectroscopy of Purified Subunits and Complexes—The secondary structure of the purified samples were characterized by far UV CD spectroscopy. Fig. 3A shows the wavelength dependences of the ellipticities of NtpE (open diamond), NtpF (open square), NtpI (open triangle), NtpE-F complex (closed diamond), and NtpE-F-I\text{Nterm} complex (closed circular) are shown. B, the secondary structure composition based on secondary structure prediction using PSIPRED server is listed.

FIGURE 2. Purification of the subunits and subunit-subunit interaction by pulldown experiment. A, SDS-PAGE analysis of purified subunits. Lane 1, NtpE; lane 2, His-tagged NtpE; lane 3, NtpF; lane 4, NtpI\text{Nterm}; and lane 5, His-tagged NtpI\text{Nterm}. B, SDS-PAGE analysis of eluted fraction from HisTrap HP column after applying the following mixtures (lane 1, His-tagged NtpI\text{Nterm} and NtpF; lane 2, His-tagged NtpE and NtpF; lane 3, His-tagged NtpE and NtpI\text{Nterm}; and lane 4, His-tagged NtpE, NtpF, and NtpI\text{Nterm}), and of purified NtpE-F (lane 5) and NtpE-F-I\text{Nterm} complexes (lane 6).

FIGURE 3. CD spectroscopy of purified subunits and complexes. A, the wavelength dependences of the ellipticities of NtpE (open diamond), NtpF (open square), NtpI (open triangle), NtpE-F complex (closed diamond), and NtpE-F-I\text{Nterm} complex (closed circular) are shown. B, the secondary structure composition based on secondary structure prediction using PSIPRED server is listed.
of the molar ellipticities at 222 and 208 nm (θ_{222}/θ_{208}) of NtpE-F complex increased to 1.08 compared with those of NtpE (0.98) and NtpF (0.92). This characteristic increase of the ratio is often observed when two helical elements interact to form higher order structures such as coiled coils (35). Interaction of the corresponding subunits from yeast V-ATPase and Thermoplasma acidophilum A-ATPase (resembling V-ATPase structurally) to form a higher order coiled-coil has been previously described (8, 36). NtpINterm was predicted to contain a β-sheet of 17% and a random coil of 26% (Fig. 3B). Therefore, the magnitude of the molar ellipticity of the NtpE-F-1INterm complex at the two minima is likely to be smaller than that of NtpE-F complex.

**Binding Affinity of the Subunit Interactions Analyzed Using the Biacore System**—We performed surface plasmon resonance analysis to estimate the binding affinities for the subunit interactions detected by pulldown assay as described above; interactions of NtpE-NtpF, NtpINterm-NtpF, and NtpINterm-NtpEF complex. When various concentrations of NtpF were overlaid on NtpE immobilized on a sensor chip, a specific binding response between these subunits was observed (Fig. 4A). An association rate constant, \( k_{\text{on}} = 2.3 \times 10^8 \text{M}^{-1}\text{s}^{-1} \), and a dissociation rate constant, \( k_{\text{off}} = 4.8 \times 10^{-5} \text{s}^{-1} \), were obtained. The consequent dissociation constant of \( K_D = k_{\text{off}}/k_{\text{on}} \) was \( 2.1 \times 10^{-8} \text{M} \). As shown in Fig. 4B and C, the immobilized NtpINterm bound both NtpF and NtpEF complex. NtpF binding to NtpINterm was extremely fast, reaching equilibrium immediately after NtpF injection, and the complex dissociated as soon as the flow solution was switched to a running buffer (Fig. 4B). Therefore, it was difficult to calculate the kinetic parameters \( (k_{\text{on}} \text{ and } k_{\text{off}}) \) directly. The affinity of the interaction was determined from the level of binding at equilibrium as a function of the sample concentrations; equilibrium dissociation constant \( (K_D) = 1.9 \times 10^{-5} \text{M} \). When various concentrations of purified NtpE-F complex were added to immobilized NtpINterm, \( k_{\text{on}} = 2.2 \times 10^7 \text{M}^{-1}\text{s}^{-1} \) and \( k_{\text{off}} = 3.3 \times 10^{-2} \text{s}^{-1} \) were obtained. The dissociation constant of \( K_D = k_{\text{off}}/k_{\text{on}} \) was \( 1.5 \times 10^{-7} \text{M} \). The equilibrium dissociation constant \( (K_D = 1.9 \times 10^{-7} \text{M}) \) was also obtained (Fig. 4C), consistent with the \( K_D \) value calculated from \( k_{\text{off}}/k_{\text{on}} \). Thus, the interaction affinity of NtpINterm-NtpEF complex was 100-fold higher than that of NtpINterm-NtpF.

**Monodispersity and Stoichiometry of Purified Samples and Subunit-Subunit Interactions Demonstrated by AUC**—Sedimentation velocity (AUC/SV) provides information about the hydrodynamic property of a sample and establishes the size distribution of proteins due to their different rates of migration in the centrifugal field depending on their masses and shapes (37). Sedimentation equilibrium (AUC/SE) is suitable for determination of the weight-average molecular mass and for the study of protein self-association (38). Fig. 5 shows the continuous distribution c(s) versus observed sedimentation coefficient of the samples by AUC/SE, the apparent molecular masses determined by AUC/SE using a simple monomeric analysis model, and \( s_{20,w} \) values calculated from the weight average sedimentation coefficient of the c(s) peak. The \( s_{20,w} \) values were much larger than the observed s values owing to the high viscosity of the D buffer containing 10% glycerol. The c(s) distribution of purified NtpE showed a single broad peak (Fig. 5A). By increasing the protein concentrations (0.2, 0.4, and 0.8 mg of protein/ml), the weight averages of the peak increased (1.40, 1.41, and 1.48 S, respectively; data for 0.2 and 0.4 mg of protein/ml were not shown). The apparent molecular mass determined by AUC/SE was 34.6 ± 0.2 kDa, which was greater than the theoretical value (23 kDa) based on the amino acid sequence. These findings suggested that the NtpE exists in a relatively rapid equilibrium between the monomer-dimer (or oligomer) species. We applied several models for fitting the AUC/SE data. The monomer-dimer equilibrium model using
the theoretical molecular mass (23 kDa) gave a good global fit and an equilibrium constant of $2.4 \pm 0.1 \times 10^{-5} \text{ M}$ was estimated. The $c(s)$ distribution of purified NtpF showed a single prominent peak (Fig. 5B). By increasing the protein concentrations (0.2, 0.4, and 0.8 mg/ml), the weight averages of the peak were constant (1.37, 1.37, and 1.36 S, respectively; data for 0.2 and 0.4 mg/ml not shown). The apparent molecular mass was 31.6 ± 0.2 kDa, which agreed well with that of the dimer (theoretical value for NtpF: 15 kDa). These findings suggested that the NtpF exists as a dimer in the protein concentration range (0.2–0.8 mg/ml). The $c(s)$ distribution of purified NtpINterm showed a single prominent peak (Fig. 5C). The weight averages of the peak remained constant (1.70, 1.68, and 1.69 S) at different protein concentrations (0.2, 0.4, and 0.8 mg/ml respectively; data for 0.2 and 0.4 mg/ml not shown). The apparent molecular mass was 41.0 ± 0.1 kDa, which agreed well with the theoretical value (40 kDa). These findings suggested that the NtpINterm exists as a monomer in the protein concentration range (0.2–0.8 mg/ml).

Subunit-subunit interactions were analyzed from the increase of the sedimentation coefficients of mixed samples compared with those of independent subunits. The $c(s)$ distribution peak (at 1.49 S) of the mixed sample at molar ratio 1:1 (NtpE dimer:NtpINterm monomer) did not change much compared with that of NtpE (at 1.48 S) or NtpINterm (at 1.69 S) (Fig. 5D). The apparent molecular mass (36 kDa) was close to that of NtpE (35 kDa) or NtpINterm (41 kDa). These findings suggest that NtpE does not interact with NtpINterm, consistent with the results of pulldown experiments as described above. Mixed samples at the molar ratio 1:1 (NtpF dimer:NtpINterm monomer, NtpE dimer:NtpF dimer, and NtpINterm monomer:NtpE dimer:NtpF dimer) were also examined by AUC (Fig. 5, E–G). The sedimentation coefficients (1.90, 1.79, and 1.93 S, respectively) and apparent molecular masses obtained (47, 60, and 65 kDa, respectively) were clearly larger than those of any one of the subunits. Thus, the suggested interactions between NtpF-NtpINterm, NtpE-NtpF, and NtpE-NtpF-NtpINterm were confirmed.

Monodispersity and the molecular mass of the purified NtpE-F-INterm complex were analyzed by AUC. The sedimentation velocity experiments for the purified NtpE-F-INterm complex were conducted at a concentration of 0.8 mg/ml. The resulting $c(s)$ distribution of the complex showed the presence of a well defined peak with a sedimentation coefficient of 2.09 S (95% of the total fraction) and a minor broad peak with 1.31 S (<3% of the total) (Fig. 5H). The weight average $f_{\text{av}}$ value was optimized by least-squares regression and converged to a best-fit value of 2.02. The molecular mass calculated from these results was 81.7 kDa, which corresponds to that of a 1:1:1 ternary complex (78 kDa). The apparent molecular mass of the complex by AUC/SE, 70.9 ± 0.4 kDa, is smaller than the predicted 78 kDa because of the slow dissociation of the complex at the protein concentrations tested (0.15, 0.2, and 0.4 mg/ml).
These findings suggest that the ternary complex is formed with 1:1:1 stoichiometry.

**SAXS Measurement of Purified NtpE-F-I^term Complex**—Fig. 6A shows the SAXS profiles of the NtpE-F-I^term complex and associated Guinier plots (shown in inset). The Guinier plots at three different concentrations (1.7, 2.4, and 3.0 mg/ml) presented single regression lines, suggesting monodisperse properties and no aggregation effect of the NtpE-F-I^term complex in the concentration range. The normalized forward scattering intensity I(0, c) and apparent radius of gyration R_g(c) are also constants in the concentration range (1.7–3.0 mg/ml) as shown in Fig. 6B. I(0, 0) and R_g(0), which were determined by extrapolation of the dependence to infinite diluted condition (c = 0) were 9.92 ± 1.18 and 7.05 ± 0.11 nm, respectively. The pair distribution function, P(r), calculated by applying the SAXS profiles without the data points in q < 0.1072 nm⁻¹, suggested an elongated molecular shape with a maximum molecular length (D_max) of 23.0 nm (Fig. 6C). Comparison of the forward scattering with the value obtained from a reference solution of bovine serum albumin indicated a molecular mass of 73.2 ± 8.7 kDa. The estimated value is similar to the theoretical molecular mass (78 kDa) of the complex calculated from the amino acid sequence for the 1:1:1 ternary complex. SDS-PAGE analysis revealed the NtpE-F-I^term complex contained three clear protein bands (Fig. 2B, lane 6). In addition the complex sample was monodisperse. Therefore, we concluded that the NtpE-F-I^term complex is formed with 1:1:1 stoichiometry.

The low resolution model of the NtpE-F-I^term complex was reconstructed by DAMMIN. The shape obtained for the NtpE-F-I^term complex is a good fit to the experimental data in the entire scattering range. All ten independent reconstructions showed a similar elongated “L” shape and have been averaged as shown in Fig. 7A. The complex appears as an “L”-shaped molecule of ~16 nm in length.

**DISCUSSION**

In this study, we purified NtpE, NtpF, and NtpI^term individually and showed that these three subunits make up a complex with a 1:1:1 stoichiometry. Does the resulting NtpE-F-I^term complex function as a structural element in the intact E. hirae V-ATPase? Previously, we have established a purification and reconstitution system for whole E. hirae V_1V_o-ATPase (20). Addition of EDTA to chelate the Mg²⁺ results in dissociation of the V_1 moiety from reconstituted V_1V_o-proteoliposomes. Recombination of the dissociated V_1 fraction with V_o-liposomes in the presence of Mg²⁺ resulted in a full recovery of the Na⁺/H⁺-stimulated ATPase activity and Na⁺-/H⁺-transporting activity, suggesting that dissociation did not cause inactivation of either the V_1 or V_o moieties of the V-ATPase (20). The dissociated V_1 fraction contained two subcomplexes (the NtpE-F complex and the NtpA3-B3-D-G complex retaining ATP hydrolytic activity) and NtpC, which were separated by anion-exchange and gel-filtration column chromatography. Based on the results of Thermus thermophilus V-ATPase studies (39), NtpC seems to be located between the NtpA3-B3-D-G complex and the NtpK ring in the V-ATPase (33). Therefore, the Ntp-EF complex is likely to comprise the peripheral stalk together with the N-terminal hydrophilic domain of NtpI (stator membrane subunit) (Fig. 1), consistent with the findings in this study.

AUC experiments showed that purified NtpI^term is stable as a monomer in solution (Fig. 5C), whereas purified NtpE exists...
Subunit Arrangement of the Peripheral Stalk of E. hirae V-ATPase

A

\[ \theta = 90^\circ \]

10 nm

B

\[ \theta = 90^\circ \]

FIGURE 7. Low resolution structure model of NtpE-F-I\textsuperscript{interm} complex determined from SAXS data. A, the molecular model of NtpE-F-I\textsuperscript{interm} complex was reconstructed by DAMMIN and DAMAVER. B, imaginary illustration of subunits arrangement fitted to the molecular model. NtpE, NtpF, and NtpI\textsuperscript{interm} are colored in blue, yellow, and red, respectively.

in a monomer-dimer (or oligomer) equilibrium (Fig. 5A) and purified NtpF forms a dimer in solution (Fig. 5B). It is not clear how dimers of NtpF and NtpE dissociate into monomers for formation of the 1:1:1 NtpE-F-I\textsuperscript{interm} complex. CD spectroscopy experiments showed that, upon NtpE-F complex formation, there was an increase in \( \alpha \)-helical secondary structure, most likely due to \( \alpha \)-helical coiled-coil formation between the two subunits as indicated by stronger minima at 208 and 222 nm and by the increased ratio of \( \theta_{222}/\theta_{208} \). The dissociation of the respective dimer of each subunit is likely to take place during formation of the interaction between NtpE and NtpF, as shown for studies on other V (or A)-ATPases (8, 36).

The observed \( K_d \) values estimated by surface plasmon resonance assay were calculated for all monomers in solution using evaluation software with a single 1:1 interaction binding isotherm. However, isolated NtpE and NtpF exist as oligomers in solution as described above. The observed \( K_d \) values must vary depending on the oligomeric states of the subunits. In contrast the dissociation rate constants \( (k_{\text{off}}) \) should not be dependent on the oligomeric states of the samples. The dissociation rate for the NtpI\textsuperscript{interm}-NtpF interaction was too rapid to be estimated (Fig. 4E). The dissociation rate for the NtpI\textsuperscript{interm}-NtpEF complex interaction was much slower \( (k_{\text{off}} = 3.3 \times 10^{-2} \text{s}^{-1}) \) than that of the NtpI\textsuperscript{interm}-NtpF complex. In addition, the purified NtpE-F-I\textsuperscript{interm} complex was monodisperse in solution as observed by AUC and SAXS. Thus, the interaction affinity of NtpI\textsuperscript{interm}-NtpEF complex is concluded to be much higher than that of NtpI\textsuperscript{interm}-NtpF. Although direct interaction between NtpE and NtpI\textsuperscript{interm} was not observed by pulldown assay (Fig. 2B, lane 3) and AUC (Fig. 5D), NtpE seems to help increase the binding affinity of NtpI\textsuperscript{interm} and NtpF.

The structure model of the NtpE-F-I\textsuperscript{interm} ternary complex obtained by SAXS revealed an elongated L-shape (Fig. 7A). Electron microscopic analysis of the EG complex of yeast V-ATPase with a 1:1 stoichiometry indicated a rod shape (8). Therefore, we can speculate that the NtpE-F complex corresponds to the blue and yellow parts in the L-shaped structure shown in Fig. 7B. Therefore, the remaining region shown in red is likely to correspond to the NtpI\textsuperscript{interm}. CD analysis implied that NtpE and NtpF interact through \( \alpha \)-helical coiled-coil formation (Fig. 3). The pulldown assay using one tagged subunit and AUC experiments show that NtpI\textsuperscript{interm} binds NtpF directly but not NtpE (Fig. 2B, lane 5). From these findings, we predict that NtpE and NtpF correspond to the blue and yellow regions in the L-shaped structure, respectively.

Most recently, analysis by mass spectrometry has indicated that there are three peripheral stalks (EG heterodimer) in yeast V-ATPase (11). In contrast, there are two EG heterodimers in the \( T. thermophilus \) enzyme (12), consistent with the electron microscopy images (40). In this study, we demonstrate that a single NtpE-F heterodimer of \( E. hirae \) V-ATPase binds to the N-terminal hydrophilic domain of NtpI (1–341 residues).

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