Proton-translocating ATPases are central to biological energy conversion. Although eukaryotes contain specialized F-ATPases for ATP synthesis and V-ATPases for proton pumping, eubacteria and archaea typically contain only one enzyme for both tasks. Although many eubacteria contain ATPases of the F-type, some eubacteria and all known archaea contain ATPases of the A-type. A-ATPases are closely related to V-ATPases but simpler in design. Although the nucleotide-binding and transmembrane rotor subunits share sequence homology between A-, V-, and F-ATPases, the peripheral stalk is strikingly different in sequence, composition, and stoichiometry. We have analyzed the peripheral stalk of Thermus thermophilus A-ATPase by using phage display-derived single-domain antibody fragments in combination with electron microscopy and tandem mass spectrometry. Our data provide the first direct evidence for the existence of two peripheral stalks in the A-ATPase, each one composed of heterodimers of subunits E and G arranged symmetrically around the soluble A₁ domain. To our knowledge, this is the first description of phage display-derived antibody selection against a multi-subunit membrane protein used for purification and single particle analysis by electron microscopy. It is also the first instance of the derivation of subunit stoichiometry by tandem mass spectrometry to an intact membrane protein complex. Both approaches could be applicable to the structural analysis of other membrane protein complexes.

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**1 To whom correspondence should be addressed: Victor Chang Cardiac Research Institute, 384 Victoria St., Darlinghurst NSW 2010, Australia. Tel.: 61-2-9295-8560; Fax: 61-2-9295-8501; E-mail: d.stock@victorchang.edu.au.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

§ Most current models of V- and A-ATPases assign both the E and G subunits to the peripheral stalk, but considerable uncertainty still exists regarding the stoichiometry of these subunits, their precise location, and whether they associate as homodimers or heterodimers (7, 11, 15, 17–19). It has been shown that the E and G subunits of the yeast V-ATPase interact tightly and specifically (20, 21) and are both present at more than one copy per V₁ complex (21). These data are consistent to F-ATPases (5). However, A-ATPases are functionally more similar to F-ATPases because both synthesize ATP using energy derived from proton translocation (5). V-ATPases work in reverse by actively pumping protons through membranes using ATP hydrolysis as the driving force (6).

Although eukaryotes contain both types of ATPases, each one highly specialized in its physiological function, archaea and eubacteria typically contain only one. Both eubacterial F-ATPases and eubacterial and archaeal V-ATPases are simpler than their eukaryotic counterparts but are functionally more versatile in that they can operate in both directions. Archaeal and eubacterial V-ATPases are closely related and are often referred to as A-ATPases (4).

F-, V-, and A-ATPases share an overall conservation of structure that includes a water-soluble F₁/V₁/A₁ domain and a membrane-bound Fₒ/Vₒ/Aₒ domain (7–10). The Thermus thermophilus ATPase-active A₁ domain is composed of a head group that contains a heterotrimer of the nucleotide-binding proteins A and B and a central stalk composed of proteins C, D, and F (11). The proton-translocating Aₒ domain contains a ring of L proteolipids and a single copy of protein I that is located adjacent to the ring. The L ring, in association with the central stalk components (CDF), forms the rotor of the A-ATPase, whereas all other subunits (A₃B₃GEI) form the stator (11). Part of the stator is the peripheral stalk, which counteracts the rotation of the catalytic A₅B₃ head with respect to the spinning central rotor in all types of ATPases. It has been visualized by electron microscopy (EM) in various F-ATPases (9, 12–14), in V-ATPases (8, 10), and in other eubacterial/archaeal V/A-ATPases (7, 15, 16). These studies suggest that F-ATPases contain a single peripheral stalk, A-ATPases contain two, and eukaryotic V-ATPases might contain up to three. However, the observation of multiple peripheral stalks has often been attributed to artifacts introduced by the averaging procedures used in EM, and direct evidence for multiple peripheral stalks was missing.

Most current models of V- and A-ATPases assign both the E and G subunits to the peripheral stalk, but considerable uncertainty still exists regarding the stoichiometry of these subunits, their precise location, and whether they associate as homodimers or heterodimers (7, 11, 15, 17–19). It has been shown that the E and G subunits of the yeast V-ATPase interact tightly and specifically (20, 21) and are both present at more than one copy per V₁ complex (21). These data are consistent
with recent EM models showing at least two peripheral stalks connecting the V₁ and V₀ domains of the eukaryotic V-ATPase (18, 19, 22), and it has been hypothesized that both peripheral stalks contain EG complexes (21). Similarly, previous EM and image analysis of the T. thermophilus A-ATPase prompted us to assign two peripheral stalks to the A-ATPase (16) and not a single one, as generally proposed (11, 17), but direct evidence was missing.

In this study we locate and quantify the stator subunits E and G within the intact A-ATPase from T. thermophilus by using phage display-derived single-domain antibody fragments (dAbs) followed by immuno-EM. In addition we have performed tandem mass spectrometry (MS) on the intact A-ATPase complex. These studies have allowed us to derive a revised model of the intact A-ATPase.

**EXPERIMENTAL PROCEDURES**

**Materials**—pETDuet-1, pET-12a, and pET-25b vectors were purchased from Novagen. Taq DNA polymerase was purchased from Promega. Endonuclease restriction enzymes and DNA ligase were from New England Biolabs. Recombinant protein A, alkaline phosphatase-conjugated protein A, and tablets for Triton X-100, were obtained from Amersham Biosciences. Streptavidin-bead, and Superdex 200 and Superose 6 gel filtration columns were obtained from GE Healthcare Life Sciences. Protein A-Sepharose (Streamline ProteinA), peroxidase-conjugated anti-M13 monoclonal antibody single-domain fragment library was provided by Schwer Research Inc. (New York, New York). The human antibody single-domain antibody fragments (dAbs) were obtained from Biogen Idec (Cambridge, MA). The DNA encoding the cassette dAb-HSV-His₆ was digested from pET-25b with Sall and Blpl and cloned into the same restriction sites of pET-12a giving the plasmid pET12a-dAb.

**Construction of dAb Expression Vectors**—The selected dAb antibodies were cloned into the expression plasmid pET-25b, where a HSV-His₆ tag was introduced at their C-terminal end. The DNA encoding the cassette dAb-HSV-His₆ was digested from pET-25b with Sall and Blpl and cloned into the same restriction sites of pET-12a giving the plasmid pET12a-dAb. For construction of dAbs covalently attached to a C-terminal His₁₀ tag, a double-stranded DNA adapter encoding the His₁₀ tag was generated by annealing of single-stranded phosphorylated oligonucleotides Not-rev (5'-GGCG CGC ACG GTG CAA TGG TGA TGA TGG TGA TGG TGA TGG TGT GC-3') and Not-for (5'-GGGC CGC ACA CCA TCA CCA CCA TCA CCA TCA CCA CCA CCA TCA CCA TCA CCA CCA TCA CCA CCA CCA TGG CAG CGT GC-3'), which produced NotI-compatible overhangs (underlined in the above sequences) and cloned into NotI-digested pET12a-dAb.

**Expression and Purification of dAbs**—The vectors containing the dAb inserts were transformed into Escherichia coli C41 cells (23). dAb expression in 0.5-liter cultures was induced with 1 mM isopropyl thiogalactopyranoside at 30 °C for 48 h. After centrifugation, the supernatants were dialyzed against ATPase buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 0.05% DDM, 0.02% NaN₃). Protein purity was evaluated by SDS-PAGE on 12% acrylamide Tris-glycine gels. Protein concentration was determined by BCA assay.

**Purification of A-ATPase**—Purification of A-ATPase from T. thermophilus was performed as previously described (16). When A-ATPase was purified by Ni-NTA chromatography via His-tagged dAbs, membranes from 10 g of T. thermophilus cells were resuspended in ATPase buffer (supplemented with protease inhibitor tablet) and an additional 1% DDM) and sonicated for 1 min. After centrifugation at 30,000 rpm for 30 min, the supernatant was incubated with purified dAbs at room temperature for 1 h. 1 ml of Ni-NTA was applied to the mixture through the entire encoding sequence to verify the absence of undesired mutations introduced by PCR. To coexpress Hisᵣ₆-G and E subunits, Rosetta 2 (DE3) cells were transformed with the bicistronic vector. Liquid cultures were shaken in 2× TY medium containing ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml) at 37 °C until A₆₀₀ of 0.6–0.7 was reached. To induce the cells, 0.2 mM isopropyl thiogalactopyranoside was added, and expression was carried out at 20 °C for 16 h. After expression the cells were harvested at 4000 rpm for 15 min before being resuspended in 20 mM Tris, pH 7.4, 100 mM NaCl with an EDTA free protease inhibitor tablet. The cells were freeze-thawed twice in liquid nitrogen before sonication in an ice bath for 3 min with 1-s bursts. The lysed cells were centrifuged at 13,000 rpm, and the supernatant passed over a 5-ml Ni²⁺-NTA column to capture Hisᵣ₆-G and E. The eluting fractions containing the complex were pooled, concentrated, and loaded on a preparative Superdex 200 gel filtration column. The fractions were analyzed by SDS-PAGE on 4–12% Bis-Tris acrylamide gels (NuPage, Invitrogen), pooled, and concentrated with a Vivaspin concentrator. Protein concentration was determined by BCA assay.

**Construction, Expression, and Purification of Soluble EG Complexes**—DNA encoding subunits E and G from T. thermophilus was cloned by PCR into the bicistronic pETDuet-1 vector with subunit G carrying an N-terminal His₉ tag. The E and G subunits were amplified from T. thermophilus genomic DNA as a template with the oligonucleotide pairs forG100 (5'-GGCGATCCGGATTATGTATATTCTACACCAGGAGATCTTTATTTTCAGGCCACCGCCGGGTGGTCTTGGTGAGGCCGCC-3') and revG101 (5'-GGCGATCCGGATTATGTATATTCTACACCAGGAGATCTTTATTTTCAGGCCACCGCCGGGTGGTCTTGGTGAGGCCGCC-3'), respectively. The primer forG100 was modified at its 5' end to introduce a unique BamHI restriction site followed by a linker sequence and a TEV cleavage site. The PCR products encoding subunits E and G were cloned into pETDuet-1 expression vector using the BamHI and HindIII (subunit G) and Ndel and EcoRV (subunit E) restriction sites, respectively. The plasmid was sequenced by the University of Georgia fermentation facility (Athens, Georgia). The human antibody single-domain fragment library was provided by Domantis (Cambridge, UK).

**Construction, Expression, and Purification of dAbs**—The selected dAb antibodies were cloned into the expression plasmid pET-25b, where a HSV-His₆ tag was introduced at their C-terminal end. The DNA encoding the cassette dAb-HSV-His₆ was digested from pET-25b with Sall and Blpl and cloned into the same restriction sites of pET-12a giving the plasmid pET12a-dAb. For construction of dAbs covalently attached to a C-terminal His₁₀ tag, a double-stranded DNA adapter encoding the His₁₀ tag was generated by annealing of single-stranded phosphorylated oligonucleotides Not-rev (5'-GGCG CGC ACG GTG CAA TGG TGA TGA TGG TGA TGG TGA TGG TGT GC-3') and Not-for (5'-GGGC CGC ACA CCA TCA CCA CCA TCA CCA CCA TCA CCA CCA CCA TCA CCA CCA CCA TCA CCA CCA CCA TGG CAG CGT GC-3'), which produced NotI-compatible overhangs (underlined in the above sequences) and cloned into NotI-digested pET12a-dAb.
and incubated at room temperature for 1 h. The resin was packed into a column and washed with ATPase buffer with 20 mM imidazole, and bound dAb-ATPase complexes were eluted in ATPase buffer with 200 mM imidazole (pH 8.0). The protein sample was concentrated using 15-mL Vivaspin columns (molecular mass cut-off, 100 kDa) and applied to a Superose 6 column equilibrated in ATPase buffer.

**Biotinylation of A-ATPase and Subunits E and G**—Purified A-ATPase and recombinant EG complex were modified with a 5-fold molar excess of sulfo-NHS-LC-biotin at 25 °C for 1 h in 20 mM Na-HEPES, pH 8.0, and 100 mM NaCl (plus 100 mM sucrose, 10% glycerol and 0.05% DDM when A-ATPase was biotinylated). Unreacted biotin was removed by dialysis, and biotin incorporation was determined by matrix-assisted laser desorption ionization time-of-flight MS and Western blotting with streptavidin-alkaline phosphatase.

**Phage Display Selections**—A phage library of human immunoglobulin heavy chain variable domain dAbs (~10^9 phages) was provided by Domantis Ltd. The selection protocol consisted of three rounds of panning; the first one was performed with biotinylated recombinant EG heterodimers as antigen followed by two consecutive rounds with biotinylated A-ATPase at decreasing concentration (supplemental Fig. S1). Phage selections were performed in ATPase buffer with 2% Marvel milk powder. The phage library was incubated at room temperature for 1 h with 100 nM of recombinant EG complex (round 1) or 10 nM and 1 nM A-ATPase (rounds 2 and 3, respectively). The complexes were captured with streptavidin magnetic particles and washed 10 times with ATPase buffer and once with phosphate-buffered saline. Bound phages were eluted with 1 mg/ml trypsin solution. *E. coli* TG1 cells at an A_{600} of 0.5 were infected with the eluted phages and then plated on large LB agar plates containing 15 μg/ml tetracycline. The plated cells were incubated overnight at 37 °C, and the cells were scraped off the next day to inoculate 100 ml of 2× TY containing 15 μg/ml tetracycline. The culture was incubated at 220 rpm, 37 °C overnight for phage production. The phages were precipitated from the culture supernatant with 25 ml of polyethylene glycol solution (20% polyethylene glycol 6000, 2.5 M NaCl) and redissolved in 1 ml of phosphate-buffered saline. After the third round, 60 clones were randomly picked and analyzed by ELISA, and the positive clones were sequenced.

**ELISAs**—For phage ELISA screening, MaxiSorp plates were coated with 5 μg/ml neutravidin in carbonate buffer overnight and blocked for 1 h at room temperature with 3% Marvel dried milk in phosphate-buffered saline. All of the subsequent incubations and washes were performed in ATPase buffer at room temperature. Biotinylated A-ATPase, EG heterodimers, or negative controls were immobilized by incubation for 1 h. Monoclonal phages displaying selected dAbs diluted in 2% Marvel milk powder in ATPase buffer were added into the MaxiSorp wells and incubated for 2 h. Bound phages were detected with anti-M13 antibody horseradish peroxidase conjugate followed by development with p-nitrophenyl phosphate or tetramethylbenzidine substrate, respectively. Absorbance at 405 or 650 nm was measured with a 700 Plus plate reader.

**Native PAGE, SDS-PAGE, Nondenaturing, and Denaturing Western Blot Analysis**—Native gel electrophoresis was performed with the Novex gel system according to Betts et al. (24). Nondenaturing Western blotting was performed as described by Speed et al. (25) using EG-specific dAbs. For denaturing SDS-PAGE analysis and Western blots, the EG complexes were mixed with NuPAGE loading buffer, boiled for 5 min, separated on 12% NuPage Bis-Tris gels, and electrotransferred onto polyvinylidene difluoride membranes. After blocking the membranes with 3% bovine serum albumin, the membranes were incubated with dAbs for 2 h at room temperature. Alkaline phosphatase-conjugated protein A was used as secondary reagent. The membranes were developed with the chromogenic substrates bromo-4-chloro-3-indoly-phosphate and nitroblue tetrazolium.

**Immuno-EM**—For immuno-EM an excess of protein A or protein A-coupled colloidal gold was mixed with purified dAb-A-ATPase, incubated for 1 h, and subjected to gel filtration on a Superose 6 HR 10/30 column. Elution was monitored by absorbance at 280 nm (for protein) and 420 nm (for colloidal gold), and 0.5-ml fractions were collected. The fractions were analyzed by SDS-PAGE.

The eluted complexes were analyzed by EM on negatively stained grids. No averaging of particles was done so as to avoid the possibility of misalignment of particles, which could result in the appearance of multiple labels where there should only be one. Samples of purified complexes were directly absorbed onto glow-discharged 400 mesh grids containing a 100–200-Å-thick layer of evaporated carbon. Immediately following absorption, the grid was blotted with a piece of filter paper, and 4 μl of 2% methyleneamine tungstate prepared in 1 mM Tris, pH 8, was added. The negative stain was repeated a second time, and the grid was allowed to air dry. The data were collected on a FEI Tecnai 12 microscope operating at 120 kV, with magnification of 42,000 and a defocus of 1-μm underfocus. The images were recorded on Kodak SO-163 film and developed in full strength D19 developer for 12 min followed by 6 min in fixer. The images were scanned on the MRC-KZA scanner at a 6-μm step size that was later binned to 12 μm.

**Mass Spectrometry of A-ATPase**—Immediately prior to MS analysis under native conditions, aliquots of the solution containing the A-ATPase complex were buffer-exchanged using Bio-Rad micro biospin columns (molecular mass cut-off, 6 kDa; Bio-Rad) into 100 mM ammonium acetate solution, pH 7.0, and stored on ice. For analysis under denaturing conditions, the sample was then diluted twice in 50% acetonitrile, 0.1% formic acid. Electrospray....
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Ionization-MS and tandem MS (MS/MS) experiments were conducted on a high mass quadrupole time-of-flight (Q-TOF) type instrument (26) adapted for a QSTAR XL platform (27). An aliquot (2 μl) of the solution at ~0.5–1 mg/ml was introduced via gold-coated borosilicate capillaries prepared in-house as described (28). The following experimental parameters were used: ion spray voltage, 1.0–1.5 kV; curtain gas, 20 liters/h; declustering potential 1 and 2, 100–250 and 15 V, respectively; and focusing potential, 250 V. For MS/MS experiments, ions of a mass to charge ratio (m/z) were selected in the quadrupole and subjected to acceleration in the collision cell (collision energy, 150–200 V).

The spectra were analyzed using Masslynx to give molecular masses for the various charge states of the subunits and complexes. Possible subunit compositions were explored using SUMMIT3 using the experimentally determined masses of the denatured subunits.

**MS of Coexpressed EG Complexes**—Electrospray ionization-MS spectra of coexpressed subunits E and G were recorded as for intact A-ATPase on a modified Q2 mass spectrometer. The protein concentration was ~40 μM in 100 mM ammonium acetate, pH 7.0.

**RESULTS**

**Expression and Purification of Recombinant EG Complex**—Both subunits E and G from yeast V-ATPase and subunits E and H (equivalent to *T. thermophilus* and yeast subunit G) from *Pyrococcus horikoshii* have been shown to interact (17, 20, 21). However, no data have been reported for the *T. thermophilus* subunits E and G. Overexpression of subunit E or G on their own resulted in unstable products that were not suitable for antibody selection. Therefore, subunits E and G from *T. thermophilus* were coexpressed from a bicistronic vector with only subunit E carrying an N-terminal His6 tag. Both subunits accumulated at high soluble levels in *E. coli*, and specific complexes could be isolated by copurification via Ni-NTA affinity chromatography followed by gel filtration (Fig. 1). Gel filtration, native-PAGE (Figs. 1B and 3B), MS under native conditions (supplemental Fig. S3B), and analytical ultracentrifugation (supplemental Fig. S4) suggested the presence of a homogeneous population of single EG heterodimers, and no higher aggregates were found.

**Phage Display Selection and Antibody Expression**—To isolate EG-specific antibodies that recognize native A-ATPase from *T. thermophilus*, we used a two-stage selection scheme that involved a first round of selection with recombinant EG complex followed by two consecutive rounds with detergent-solubilized intact A-ATPase (supplemental Fig. S1). After these rounds of selection, specific phage clones were isolated and screened for binding by native ELISA. The dAb genes from the most reactive phage clones were subcloned into an expression vector and expressed with a C-terminal HSV-His6 tag in *E. coli*. The dAbs were further purified by affinity chromatography on protein A-Sepharose, and their integrity and expected molecular sizes were confirmed by SDS-PAGE (Fig. 2A). Bacterial supernatants were screened by ELISA for the presence of secreted dAbs against EG and native A-ATPase (supplemental Fig. S1). Specific binding of dAbs was observed to EG (Fig. 2B, light green) and native A-ATPase (Fig. 2B, dark blue) but not to subunit F of A-ATPase (Fig. 2B, yellow) or other unrelated antigens such as bovine serum albumin (Fig. 2B, cyan). To determine the nature of the epitope recognized by the dAbs, their reactivity against EG complexes was analyzed by ELISA and in native A-ATPase and non-denaturing Western blots. Whereas most selected dAbs including E4 only recognized intact EG heterodimers (Fig. 2C and supplemental Fig. 2), dAb D6 recognized isolated subunit G and to a small extent isolated subunit E (Fig. 2C) in ELISA. Similar results were obtained in denaturing and non-denaturing Western blot analyses (Fig. 3); dAb E4 showed no reaction against single subunits E or G in denaturing Western blots (Fig. 3A, left panel) but reacted against EG complexes in native Western blots (Fig. 3B, left panel), demonstrating that dAb E4 recognizes a conformational epitope. dAb D6 on the other hand formed an exception in that it showed binding to both native EG (Fig. 3B, right panel) but also to denatured subunit G and to a lesser extent to denatured subunit E (Fig. 3A, right panel). This suggests that the D6 epitope is formed by amino acids at the subunit interface, which are still recognized under denaturing conditions. Based on these binding properties and on expression levels, we selected dAbs D6 and E4 for further structural analyses.

**Purification of A-ATPase from Membrane Extract and EM Analysis**—Binding of dAbs to the intact A-ATPase was examined by gel filtration followed by SDS-PAGE analysis, which confirmed stoichiometric binding of the two components (Fig. 4). The tight interaction between the dAbs and A-ATPase led us to purify the A-ATPase complex directly from crude extract by Ni-NTA affinity chromatography using a His10 tag covalently attached to the dAbs. Extracts of solubilized membranes from *T. thermophilus* were incubated with purified His10-tagged dAbs and subjected to Ni-NTA chromatography and gel filtration. The protein composition was examined by SDS-PAGE (Fig. 5) and was the same as in samples purified by

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3 Taverner, T., Hermandez, H., Sharon, M., Ruotolo, B. T., Matak-Vinkovic, D., Devos, D., Russell, R. B., and Robinson, C. V. submitted for publication.
the conventional protocol but with an additional band of apparent molecular size of 14 kDa corresponding to the dAb. Overall the A-ATPase preparation using dAbs was much faster and cleaner than that from the conventional procedure.

Because averaging of particles from EM could potentially lead to multiple peripheral stalks caused by a possible misalignment of particles, we searched for the location of dAbs on the A-ATPase particles in raw, unaveraged EM images. We used several different approaches. First, we attempted to label dAbs using Ni-NTA nanogold conjugates, which should be specific to the His10 tag. This approach, however, proved to be ineffective because the Ni-NTA nanogold resin had very low affinity for the His10 tag and produced either no labeling with a harsh wash or too much background with a mild wash. In a second approach, we used protein A covalently bound to 5- or 10-nm colloidal gold particles. In both cases we observed an abundance of pairs of gold particles in close proximity to A-ATPase (23% of the total particle number analyzed), suggesting that each A-ATPase complex has two dAb binding sites. Closer inspection showed that the binding sites of both dAbs are at two symmetrical locations close to the top of the A3B3 headpiece, although the large size of the colloidal gold particles prevented accurate location of the binding sites (Fig. 6, A and B). In an attempt to improve the resolution, we used unlabeled recombinant protein A, a 42-kDa protein that can bind up to four dAb molecules. His10-tagged dAb-ATPase complexes eluted from a Ni-NTA column were incubated with a molar excess of protein A and subjected to gel filtration. Protein A-dAb-ATPase complexes eluted in a broad peak near the exclusion volume of the column and well separated from excess protein A (data not shown). Samples from individual fractions of the peak were loaded onto EM grids, negatively stained, and examined by EM. Although most of the electron micrographs showed strong aggregation, some regions on the grid of the dAb E4-bound sample showed A-ATPases cross-linked via protein A (Fig. 6C). In all cases one A-ATPase complex clearly shows two binding sites to two protein A molecules that must be mediated by the dAbs. In one case, two A-ATPase molecules are cross-linked, A1 headpiece to headpiece, in a 2-fold symmetrical arrangement mediated by two protein A molecules and two dAbs per A-ATPase (top panels of Fig. 6C). In another case, four A-ATPase molecules formed a complex with three protein A molecules and again two dAbs per A-ATPase (Fig. 6C, bottom panels). At higher A-ATPase concentrations, the combination of four dAb-binding sites of protein A and two dAb-binding sites of the A-ATPase leads to aggregation. The images indicate that the binding site for dAb E4 is at the very
Stoichiometry of A-ATPase Peripheral Stalk

FIGURE 5. dAb mediated purification of A-ATPase from membrane extracts of T. thermophilus. A, SDS-PAGE of dAb-A-ATPase complex eluted from Ni-NTA columns. Lane 1, molecular mass standards; lane 2, dAb; lanes 3–5, eluate fractions. B, gel filtration chromatography of the Ni-NTA purified dAb-A-ATPase complexes. The peak fractions were pooled and analyzed on a Coomassie-stained SDS-PAGE (inset).

FIGURE 6. Electron micrographs of protein A-dAb-ATPase complexes. A, 5-nm colloidal gold-Protein A-E4-ATPase complex. B, 10-nm colloidal gold-protein A-D6-ATPase complex. Approximately 23% (166 of 739) of the gold particles were within 30 nm of each other and assumed to form a pair induced by dAb-A-ATPase binding. One 10-nm colloidal gold particle associates with 4–5 protein A molecules according to the manufacturer. C, cross-linked protein A-E4-ATPase complex raw images with interpretation on the right. Scale bar, 10 nm. D, single particles of protein A-D6-ATPase complexes with interpretation underneath. E, legend for interpretations.

TABLE 1
Molecular weights for T. thermophilus A-ATPase subunits

| Subunit | Full length predicted mass from sequence | Predicted molecular mass | Observed denatured molecular mass |
|---------|----------------------------------------|-------------------------|---------------------------------|
| atpA    | 64,523.4                                | 64,523.4                | 63,830 ± 0.6                    |
| atpB    | 53,232.9                                | 53,232.9                | 53,062 ± 1.3                    |
| atpC    | 35,918.6                                | 35,787.4                | 35,797 ± 2.3                    |
| atpD    | 24,523.1                                | 24,391.9                | 24,385 ± 9.5                    |
| atpE    | 20,659.6                                | 20,528.4                | 20,485.0 ± 0.3                  |
| atpF    | 11,283.0                                | 11,570.4                | 11,573 ± 0.5                    |
| atpG    | 13,142.3                                | 13,541.3                | 13,542 ± 0.1                    |
| atpI    | 71,733.2                                | 71,733.2                | 72,143 ± 2.3                    |
| atpL    | 9,833.8                                 | 8,784.2                 | 8,241 ± 4.6                     |

* Predicted by MassLynx.

** Taking N-terminal sequencing results into account (16).

The peak fractions were pooled and analyzed on a Coomassie-stained SDS-PAGE (inset).}

lanes 3–5 contrast led either to aggregation or, at very low A-ATPase concentration, to formation of single D6-A-ATPase complexes bound to protein A (Fig. 6D). This could be related to the different location of the D6 epitope at the interface of subunit E and G along the sides of the A-ATPase complex. At high magnification, extra density corresponding to protein A could be seen in this area (Fig. 6D).

MS of Denatured and Intact A-ATPase—MS of conventionally purified A-ATPase under denaturing conditions allowed the determination of the masses of the individual subunits as shown in Table 1. The masses broadly agree with previous findings, which used N-terminal sequencing to derive the molecular masses of processed subunits (16).

In the MS of intact A-ATPase, broad peaks arise because of a distribution of DDM still present in the sample after buffer exchange (supplemental Fig. 1A). DDM forms micelles and adheres to the A-ATPase complex. It is, however, possible to extract information by applying MS/MS. In this approach an m/z value is selected in the quadrupole of the MS and subjected to acceleration in the collision cell. We isolated a wide range of values covering the features of the mass spectrum. Similar spectra were recorded for all isolations between ~11,500 and 12,000 m/z (supplemental Fig. 3). The tandem MS spectrum shown in Fig. 7 is from ions isolated at 11,800 m/z. A low intensity charge state series is observed close to the region of the spectrum that was isolated. This series corresponds to intact ions that have survived the acceleration process. The majority of the isolated ions undergo partial dissociation upon acceleration through the collision cell, with relatively highly charged, unfolded proteins being ejected from the complex. The signals for these highly charged subunits G and E are readily identified by their masses at low m/z. The loss of these subunits leaves “stripped” complexes with relatively lower charge, which are therefore found at higher m/z than the isolated ions.

The low intensity charge state series close to the isolated region is assigned to 37+ to 39+ and gives rise to a mass of 451,557.6 ± 48.7 Da. The only combination of T. thermophilus A-ATPase subunits that can satisfy this mass is A3B3DFG2E (450,816 Da). The charge state of the intact complex (38+) dissociates into 14+ for the highest intensity peak assigned to subunit E and 24+ for the stripped complex A3B3DFG2E (loss of one subunit E). Because both mass and charge apportion...
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**FIGURE 7. Tandem MS of intact A-ATPase.** Ions were isolated in the quadrupole MS at 11,800 m/z, isolating ions of A1. The dissociation of A1 into the observed species is shown schematically. The denatured, ejected subunits are observed at low m/z, and the ejected complexes were observed at high m/z.

During dissociation to give two complementary products, this increases our confidence in the assignment. Further stripped complexes in which additional subunits are dissociated have the following compositions: A1,B1,DFG1,E1, A1,B1,DFGE2, and A1,B1,DFGE3, confirming the A1,B1,DFG1,E1 stoichiometry of the isolated ion. The preferential loss of subunit E indicates its position on the periphery of the complex (29, 30).

**MS of dAb E4-bound A-ATPase**—We compared MS/MS spectra of *T. thermophilus* A-ATPase with and without dAb E4 bound. The spectrum of dAb E4-bound A-ATPase shows a decrease in the intensity of subunit E compared with free A-ATPase but no decrease in intensity for subunit G (supplemental Fig. S3C). The ratio of intensity for dissociated subunit E over G falls from ~10 to 3 upon antibody binding. This indicates that subunit E is protected from dissociation by E4 binding, indicating in turn that dAb E4 binds to a conformational epitope located in subunit E.

**DISCUSSION**

**Phage Display-derived Antibodies and Their Binding to the EG Complex**—Monoclonal antibodies specific to natively folded proteins are becoming increasingly important for structural studies of membrane proteins because they can stabilize conformations, help purification, allow labeling in EM studies, and extend the hydrophilic surface of the protein that is likely to form ordered crystal contacts. A promising alternative to traditional methods of obtaining monoclonal antibodies (31) is phage display antibody technology (32). However, examples of successful selection of antibodies against native membrane proteins via phage display are scarce (33, 34). This is mainly due to the difficulty of immobilizing the membrane proteins while keeping them in a native conformation in detergent solution during phage selections (35, 36) and the availability of suitable antibody libraries.

To characterize the peripheral stalks in the *T. thermophilus* A-ATPase, we developed a two-stage phage display strategy for isolation of dAbs to native epitopes of EG heterodimers, also present in the native detergent-solubilized *T. thermophilus* A-ATPase. The dAbs were expressed in a soluble form fused to a polyhistidine tag allowing the direct purification of the native A-ATPase from *T. thermophilus* membrane extracts by Ni-NTA affinity chromatography. The number of subunits in the affinity-purified dAb-A-ATPase complex was the same as that found in A-ATPase complexes isolated by the much more time-consuming conventional procedure using ion exchange chromatography (16) but with the addition of a stoichiometric portion of dAb.

An important factor for the successful application of antibody fragments in structural studies is the affinity and binding mode of the antibody to its epitope. The tight interaction between native A-ATPase and the antibodies E4 and D6 was demonstrated by gel filtration chromatography (Figs. 4 and 5). Furthermore, analysis of dAb binding in native and denaturing Western blots revealed that most selected dAbs such as E4 recognized a conformational epitope in the native EG complex, whereas denatured subunits were not recognized (Fig. 3 and data not shown). The only exception to this was D6, which recognized both native EG complex, but also denatured subunit G and to a lesser extent denatured subunit E, indicating that dAb D6 must bind at the interface of the two subunits. The sequences of both E and G subunits display regions predicted to have coiled coil structure, and the coiled coils are likely to form the interaction between the two subunits, which may be an important factor in the stabilization of the stator and of the entire A-ATPase complex. It should also be noted that the low ELISA reactivity of dAb D6 against subunit E could be related to a partial degradation of the D6 epitope in unassociated subunits E along the ELISA procedure.

**Immuno-EM and Model of A-ATPase Stator**—The two different dAb-A-ATPase complexes were examined by EM in two different approaches. In all cases the raw electron micrographs suggested the presence of two peripheral stalks per A-ATPase molecule, each containing one EG heterodimer. dAb E4, which binds to the native EG heterodimer, is located at the top of the complex, whereas D6, which recognizes preferentially subunit G, binds further down along the sides of the complex. Subunit E was previously located near the top of the yeast A3B3 complex by chemical cross-linking experiments, which suggested binding to subunit B (37). In addition, the limited sequence identity to the OSCP/delta subunit in eukaryotic/bacterial F-ATPases prompted an analogy in the locations of these subunits. Our experiments are consistent with a location of subunit E near the top of the A-ATPase, similar to the location of OSCP/delta in F-ATPases (38, 39), with the striking difference that there are two E subunits in the intact A-ATPase compared with only one OSCP/delta subunit in F-ATPases. A previous three-dimensional reconstruction of *T. thermophilus* A-ATPase to 23 Å showed that the two peripheral stalks are arranged 120° about A-ATPase.
Stoichiometry of A-ATPase Peripheral Stalk

the hexameric A$_3$B$_3$ headgroup of A$_1$ (16). Docking of the nucleotide binding subunits A and B into this density suggested that the peripheral stalks are in contact with two of the three B subunits (40), and it is therefore likely that the same interactions are used to affix both peripheral stalks to the A$_3$B$_3$ headgroup. A recent x-ray structure of subunit E from *P. horikoshii* (17) suggested a homodimeric arrangement of this subunit, but the authors speculate the homodimer to be located asymmetrically to the A-ATPase complex in only one peripheral stalk.

Subunit E and predominantly subunit G are predicted to have coiled coil regions, and therefore subunit G was speculated to form the side-parts of the peripheral stalk. Chemical cross-linking experiments located subunit G at the outer surface of subunit B (37), consistent with the location of dAb D6 (Fig. 6D). A recent small angle x-ray scattering study of the isolated subunit from *Methanocaldococcus jannaschii* confirmed an elongated structure consistent with this hypothesis (41), although the homodimeric nature of the rod shaped subunit G from *M. jannaschii* is not consistent with our findings for the *T. thermophilus* subunit.

As shown by gel filtration chromatography, native PAGE, MS, and analytical ultracentrifugation, subunit E and G from *T. thermophilus* form a heterodimer in solution, yet the native A-ATPase contains two (EG) heterodimers as shown by immuno-EM. We speculate that the two heterodimers are connected by the soluble part of subunit I, the only other component of the peripheral stalk in A-ATPases, which might stabilize the complex. According to our 23 Å electron density and a density obtained of bovine Vo (10), this structure must run parallel to the membrane and could thereby cross-link the two EG heterodimers. This hypothesis is corroborated by the recent finding that the homologs of subunits I and G in human V-ATPase interact (42). Intriguingly, subunit from F-ATPase, the homolog to subunit I of A-ATPase, has no soluble domain, and it is worthy to note that F-ATPases present only one peripheral stalk. The amphipathic F-type subunit b, which shows limited sequence homology to subunit G, has been shown to protrude straight out of the membrane, without making a 90° bend (13, 43).

**Mass Spectrometry**—In tandem MS we are isolating ions of the A$_1$ soluble portion, which must be dissociating from the full A-ATPase complex, known to be present in the sample. It is interesting to note, however, that the complex does not follow typical charging of soluble complexes in electrospray (44). For example the 800-kDa complex of GroEL gives rise to charge states at 12,000 m/z, close to the values observed here for the much smaller A$_1$ complex. It is likely that during the initial droplet stages of electrospray, the surface of the full A-ATPase complex is charged and that subsequent dissociation leads to the formation of the A$_1$ complex with lower charge states than anticipated. The absence of the A$_1$ complex in the MS is most likely due to its inability to ionize sufficiently.

Therefore, from the MS results of the entire A-ATPase complex, we can conclude that the complex dissociates with the soluble A$_1$ portion of the complex, giving rise to the most intense charge states during tandem MS. From this spectrum we are able to determine unequivocally the stoichiometry of A$_1$ and deduce that there are two copies of both subunits E and G, with E occupying a peripheral position. As a consequence these MS experiments provide unambiguous evidence for the existence of two stators in the A-ATPase. In addition, comparison of MS/MS spectra of A-ATPase with and without dAb E4 showed a decrease in dissociation of subunit E when dAb E4 was bound, thus indicating that the dAb E4 epitope is located in subunit E.

Taken together, the data described here provide direct evidence for a model of the *T. thermophilus* A-ATPase in which two peripheral stalks are associated to each A-ATPase particle. These findings together with previous biochemical and structural data allow us to propose a model of the intact A-ATPase shown in Fig. 8A, where two EG heterodimers form a rectangular scaffold around the soluble part of the A$\times$ATPase, presumably stabilized by the soluble part of subunit I (gray). The nucleotide binding subunits A and B are colored in light and dark blue, respectively. According to cross-linking data and docking of subunits A and B into the EM density, the stator forms symmetrical contacts with two B subunits (40). The central stalk subunits D, F, and C are colored in orange, magenta, and red, respectively, and the ring of subunit L is shown in yellow. B, 23 Å EM density of the intact *T. thermophilus* A-ATPase as described in Ref. 16.

**FIGURE 8. Model of T. thermophilus A-ATPase.** A, the peripheral stator of the A-ATPase consists of two heterodimers of subunits E (light green) and G (dark green) that form a rectangular scaffold around the soluble part of the A-ATPase, presumably stabilized by the soluble part of subunit I (gray). The nucleotide binding subunits A and B are colored in light and dark blue, respectively. According to cross-linking data and docking of subunits A and B into the EM density, the stator forms symmetrical contacts with two B subunits (40). The central stalk subunits D, F, and C are colored in orange, magenta, and red, respectively, and the ring of subunit L is shown in yellow. B, 23 Å EM density of the intact *T. thermophilus* A-ATPase as described in Ref. 16.

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