Structural Basis for a Unique ATP Synthase Core Complex from Nanoarchaeum equitans*

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Background: Structural asymmetry upon nucleotide binding is crucial for catalytic activity of ATP synthases.

Results: The structures of apo and bound A3B3 hexamer of N. equitans ATP synthase show conformational inflexibility.

Conclusion: The N. equitans A3B3 hexamer is an inactive form.

Significance: The structure of inactive form of N. equitans ATP synthase A3B3 is shown for the first time.

ATP synthesis is a critical and universal life process carried out by ATP synthases. Whereas eukaryotic and prokaryotic ATP synthases are well characterized, archaeal ATP synthases are relatively poorly understood. The hyperthermophilic archaeal parasite, Nanoarchaeum equitans, lacks several subunits of the ATP synthase and is suspected to be energetically dependent on its host, Ignicoccus hospitalis. This suggests that this ATP synthase might be a rudimentary machine. Here, we report the crystal structures and biophysical studies of the regulatory subunit, NeqB, the apo-NeqAB, and NeqAB in complex with nucleotides, ADP, and adenylyl-imidodiphosphate (non-hydrolysable analog of ATP). NeqB is ~20 amino acids shorter at its C terminus than its homologs, but this does not impede its binding with NeqA to form the complex. The heterodimeric NeqAB complex assumes a closed, rigid conformation irrespective of nucleotide binding; this differs from its homologs, which require conformational changes for catalytic activity. Thus, although N. equitans possesses an ATP synthase core A3B3 hexameric complex, it might not function as a bona fide ATP synthase.

Cellular respiration and bioenergetics are vital functions of life, and these processes are dominated by the multimeric molecular machine, the ATP synthase. This ubiquitous enzyme complex includes a specific arrangement of several subunits, with a membrane-embedded ion pump and a soluble, hexameric “mushroom”-like catalytic head, connected by central and peripheral stalks (1). In multicellular eukaryotic organisms, ATP synthases are predominantly found in mitochondria and chloroplasts, whereas in unicellular organisms, such as bacteria and archaea, they localize to the plasma membrane. Depending on subcellular location or environmental demands, these machines either generate ATP by utilizing the ion gradient across the membrane or hydrolyze it by acting as an ion pump (2, 3).

There are three major categories of ATP synthases as follows: F-type, V-type, and A-type. F1F0-ATP synthases are the major respiratory enzymes in eukaryotes and prokaryotes and employ the membrane proton gradient to synthesize ATP through chemiosmosis coupling (4–7). V1V0-ATPases are found in certain eukaryotic vesicles, such as vacuoles and lysosomes, and in bacteria they are responsible for acidification by proton influx upon ATP hydrolysis (8, 9). Finally, A1A0-ATP synthases are found in most archaeal organisms and some hyperthermophilic bacteria, such as Thermus thermophilus, and are acquired through horizontal gene transfer (10). The A-type ATP synthases are structurally similar to the V-type ATPases but, instead, function as ATP generators, which is reminiscent of the function of the F-type; thus, A-type ATP synthases are considered as chimeras of the F-/V-types (2, 11). The significant structural resemblance between V- and A-types and the fact that V-type ATPases are known to have originated in archaea are the reasons why these two ATPases are often compared (12). However, among all of the ATP synthase structures to date, evidence points to similarity in the overall morphology of the complexes, with differences mostly in the stoichiometry of the subunits, particularly those of the membrane rotors (13, 14).

Archaea are a highly diverse group of organisms, often found to reside at extreme temperatures, pH, and salinity, and they have been shown to employ unique modes of respiration and bioenergetics to derive their energy (15, 16). Irrespective of their energy source, ATP synthesis is carried out in these organisms by archaeal ATP synthases (17, 18). However, an understanding of the evolution of ATP synthases and their mechanisms of cellular energetics under extreme conditions has been challenging to ascertain because of the inherent difficulties in

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is isolating and culturing these organisms, concomitant with the lack of operons in most of these organisms. The most well-known model of archaeal ATP synthases arose from cryo-electron microscopy studies of the ATP synthase from *Methanococcus jannaschii* and *Methanosarcina mazei*, where the presence of complete ATP synthase operon provided an opportunity to characterize this nine-subunit complex (19, 20). Subunits A and B form the core of the enzyme or the catalytic hexamer (A$_3$B$_3$); subunits E and H form the peripheral stalk that stabilizes rotation; subunits D, C, and F comprise the central stalk required for rotation; and subunits K and I form the ion translocating ring complex (20).

*Nanoarchaeum equitans* is a recently discovered hyperthermophilic archaeal species and an obligate parasite on *Ignicoccus hospitalis* (16, 21). Genomic analysis of *N. equitans* revealed its primitive and unusual characteristics, such as a highly reduced genome, a large number of split genes and tRNAs, and lack of enzymes involved in various important metabolic pathways (21). Further experimental studies indicated the dynamic exchange of enzymes and metabolites between *N. equitans* and *I. hospitalis* (22). The study on this system will provide potential insight into this unique organism and its intriguing host-parasite relationship.

The ATP synthases/ATPase family of proteins is one of the most primitive enzyme systems. Because of its highly conserved nature and complex organization, the study of its evolution has sparked recent interest. Intriguingly, genomic interrogation of *N. equitans* indicates that it has only five representative subunits of the ATP synthase (16). The simplest ATP synthase to date is the F$_1$F$_0$-ATP synthase of *E. coli*, which has eight subunits (23). Thus, of all the known ATP synthases, *N. equitans* harbors the smallest number of representative subunits. A comparison of the *N. equitans* ATP synthase subunits with its archaeal homolog (*M. mazei* ATP synthase) revealed the presence of representative elements of the ATP synthase, such as the A and B subunits (which form the catalytic core hexamer, A$_3$B$_3$), the central stalk subunit D, the rotor subunit I, and the proteolipid subunit C, which forms the membrane-embedded proton channel (Fig. 1). However, some of the other central stalk subunits, such as F and C, and the peripheral stalk subunits E and H appear to be completely absent in *N. equitans*. Therefore, it has been widely speculated that this organism either possesses a compact, minimalistic ATP synthase or a rudimentary ATP synthase, as the parasite relies on its host, *I. hospitalis* for its energy source (24–26).

Our objective was to explore the structural and functional aspects of the *N. equitans* A$_3$B$_3$ ATP synthase core hexamer. We report the four crystal structures, such as the nucleotide-free *N. equitans* ATP synthase regulatory subunit B (NeqB), as well as that of the core complex (composed of subunits A and B) in its nucleotide-free or apo-form, and the ADP-bound and the AMP-PNP (4) (a non-hydrolysable analog of ATP)-bound forms along with biophysical studies. A comparison of these structures with the homologous V/A/F-ATPase structures revealed key features as well as unique aspects of this ATP synthase, with solution studies showing that the core complex (NeqAB) forms a hexamer. This hexameric ring (A$_3$B$_3$) is constructed using the dimeric AB complex of the asymmetric unit along with the neighboring symmetry-related AB complexes. We observed conformational inflexibility in the NeqAB dimers, irrespective of nucleotide binding, which is contradictory to the binding change model, which is the mechanism of action for typical ATP hydrolysis (27, 28). This leads us to speculate that the core complex is not functional, possibly due to the loss of function in association with its parasitic lifestyle (29, 30).

**Experimental Procedures**

**Sequence Alignment and Phylogenetic Analysis**—Representative V/A/F-type subunit homologs were selected from a BLAST search with NeqA and NeqB and were converted into FASTA format. Sequence alignment was done using the T-coffee software (31), and the figures for sequence alignment were generated using Boxshade (32). The same sequences were used for phylogenetic analysis and tree building. This was performed using the Phylogeny.fr software platform using the “advanced” mode (33). In this module, the sequence alignment was done using MUSCLE (34), curation using G-blocks (35), phylogeny using PhyML (36), and final tree building using TreeDyn (37). The bootstrapping value in the phylogeny mode was set to 100 iterations.
Structure of the NeqAB ATP Synthase Core Complex

Cloning, Expression, and Purification of N. equitans ATP Synthase Subunits—Genes coding for NeqA (NEQ103) and NeqB (NEQ263) (consisting of 570 and 416 amino acids, respectively) were created through gene synthesis and were subcloned into the pMK-RQ vector with a constitutively active glucose isomerase promoter and a C-terminal hexahistidine tag (without any additional residues between the tag and the gene). Both NeqA and NeqB were cloned into pMK-RQ using NheI and XhoI restriction sites. For studies with the complex, the genes for the A and B subunits were subcloned into the pETDuet vector; the gene for subunit A was cloned into the MCS1 using BamHI and HindIII restriction sites, whereas the gene for subunit B was cloned into the MCS2 using NdeI and XhoI restriction sites and was expressed under the control of the inducible T7 promoter.

NeqA and NeqB were transformed into the Escherichia coli strain, BL21-De3, and were cultured in TB media containing 50 μg/ml kanamycin at 37 °C for 24 h. The AB_pET-Duet constructs were grown in TB media with 100 μg/ml ampicillin at 37 °C until an A600 of 0.6, and then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 16 °C for 18 h. The cell pellets were resuspended in Pierce BPER lysis buffer (Pierce) containing lysozyme and DNase and were lysed enzymatically for 1 h. The lysates were heated at 75 °C and spun down at 13,000 × g. The supernatants were applied onto the HiTrap columns (GE Healthcare, Buckinghamshire, UK) for affinity purification. The elution fractions were desalted and exchanged into 50 mM Tris, pH 8.0, using PD10 desalting columns (GE Healthcare). The gel filtration elution fractions were concentrated using Vivaspin concentrators (GE Healthcare) for matically for 1 h. The lysates were heated at 75 °C and spun down at 13,000 × g. The supernatants were applied onto the HiTrap columns (GE Healthcare, Buckinghamshire, UK) for affinity purification. The elution fractions were desalted and exchanged into 50 mM Tris, pH 8.0, and crystallization screens were set up using the sitting-drop method. Crystals of the nucleotide-free NeqAB complex were obtained from optimized conditions with 0.1 M MES, pH 6.5, 1.6 M ammonium sulfate, and 10% dioxane as an additive. A 3.0 Å resolution diffraction data set were collected at 100 K using the in-house Rigaku MicroMax-007 HF equipped with Saturn 944 + CCD detector.

To obtain NeqAB crystals with nucleotides, the purified NeqAB protein was separately incubated with 10 mM Mg-ADP and AMP-PNP (a non-hydrolysable analog of ATP). Drops were set up with conditions selected from the initial NeqAB native crystal screening trials, and the best quality crystals were obtained from the same conditions as the native crystals. Data were collected at 100 K for the ADP-bound complex at 2.0 Å and the AMP-PNP-bound complex at 2.63 Å, respectively, at the National Synchrotron Radiation Research Center beamline BL13B1 (ADSC quantum 315R detector).

All data sets were processed using HKL2000 software, and the structure was solved by molecular replacement method using Phenix PHASER (38). The coordinates of V-ATPase from Thermus thermophilus (PDB code 3GQB) (39) was used as the search model. The required manual model building was done in Coot, and refinement was performed using Phenix refine (40). There was no non-crystallographic symmetry constraints used in the refinement of NeqB or NeqAB structures. To rule out any ambiguity regarding pseudosymmetry, the space group was verified using the Zanuda program (41). The structure-related figures presented in this paper were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC).

Blue Native-PAGE—Blue native-PAGE experiments were carried out using native-PAGE Novex BisTris 4–16% gradient gels from Life Technologies, Inc. Gels were washed and stained with Simply Blue safe stain (Life Technologies, Inc.), and the protein bands were compared with the Native Mark protein ladder (Life Technologies, Inc.).

Isothermal Titration Calorimetry (ITC)—Isothermal titration calorimetry was used to study the interaction of NeqA and NeqB subunits with and without nucleotides. All ITC experiments were performed using a VP-ITC calorimeter (Microcal, LLC, Northampton, MA) at 25 °C. For nucleotide binding studies, 10 μM protein (NeqA and NeqB) was used in the cell for titration against 300 μM to 1 mM of Mg-ATP in the injection syringe. For binding studies of NeqA and NeqB, 100 μM NeqA or NeqB in the syringe was titrated against 10 μM NeqB or NeqA in the cell. All samples were dialyzed into the same buffer, degassed, and centrifuged to remove any precipitates. Volumes of 10 μl per injection were used for all experiments, and consecutive injections were separated by 4 min to allow the calorimetric signal (thermal power) to return to baseline. For experiments with NeqB, the stirring speed was reduced to 220 rpm, and the spacing between injections was increased to 5 min as the protein is prone to precipitation. ITC data were analyzed with a single ligand-binding site model using Origin 7.0 software (OriginLab Corp., Northampton, MA).

Analytical Ultracentrifugation (AUC)—The NeqAB complex was subjected to sedimentation velocity experiments using analytical ultracentrifugation to verify complex formation. The AB complex was purified and dialyzed into PBS buffer. A range of concentrations up to 2 mg/ml was used for conducting trials.
Sedimentation velocity profiles were collected by monitoring the absorbance at 280 nm. The samples were sedimented at 40,000 rpm at 24 °C for 5 h in a Beckman Optima XL-I centrifuge (Beckman Coulter Inc., Brea, CA) fitted with a four-hole AN-60 rotor and double-sector aluminum center pieces, and equipped with absorbance optics. A total of 95 scans were collected and analyzed using Sedfit. The data were fitted to a $c(s)$ model with a reasonable residual distribution map. The r.m.s.d. value for the fit of the model with the data is 0.006 OD, in a working range of 6.09 to 7.30 cm. The confidence interval used in the SEDFIT data analysis was 0.95. Finally, the $c(s)$ profile was transformed to a $c(M)$ distribution to depict the size and amount of individual components in solutions.

**Results**

**Sequence Homology of N. equitans ATP Synthase Subunits**—A PSI-BLAST was performed for the N. equitans catalytic subunit NeqA (gi:40068623) and regulatory subunit NeqB

![Figure 2](image-url)
Structure of the NeqAB ATP Synthase Core Complex

TABLE 1
Crystallographic statistics and refinement details

|                | NeqB   | NeqABapo | NeqABADP | NeqABANP |
|----------------|--------|----------|----------|----------|
| Data collection|        |          |          |          |
| Space group    | P2,2,2 | R3       | R3       | R3       |
| Molecules in asymmetric unit | Four monomers | One heterodimer | One heterodimer | One heterodimer |
| Cell dimensions a, b, c (Å) | a = 77.23 b = 155.23 c = 177.45 | a = b = 192.61 c = 108.94 | a = b = 192.46 c = 110.24 | a = b = 193.48 c = 108.85 |
| Wavelength (Å) | 0.97913 | 1.5418   | 0.97913  | 1.5418   |
| Resolution (Å) | 0.50–2.8 | 33.0–3.0 | 50–2.0 | 50–2.63 |
| R0.6[^a]      | 0.10 (0.56) | 0.23 (0.39) | 0.132 (0.54) | 0.09 (0.51) |
| I/|         | 23 (2.0) | 5.6 (2.6) | 37.12 (2.87) | 22 (2.8) |
| Completeness (%) | 98.3 (91.8) | 96.9 (87) | 99.8 (98.5) | 99.8 (97.3) |
| Redundancy    | 13.0 (8.9) | 4 (2.5) | 5.5 (4.5) | 5.7 (4.3) |

[a] The high resolution bin details are in parentheses.
[^b] R0.6 = Σ[I−|I|]/[Σ[I]], where I is the intensity of the i-th measurement, and (I) is the mean intensity for that reflection.
[^c] Reflections with I > σ were used in the refinement.
[^d] Rwork = Σ[|Fobs|−|Fcalc|]/Σ[Fobs], where Fobs and Fcalc are the observed and calculated structure factor amplitudes, respectively.
[^e] Rfree = as for Rwork but for 5–7% of the total reflections chosen at random and omitted from refinement.

The effect of parasitism, a trait seen in many obligate bacte-
ria, such as *T. thermophilus*, and archaeal species, such as *Pyrococcus horikoshii*. The sequence alignment of NeqA with other subunit A homologs showed ~50% sequence identity with A-type homologs from *T. thermophilus, P. horikoshii,* and *M. mazei*, and V-type from *Enterococcus hirae,* and ~30% sequence identity with the F-type ATP synthase from *Saccharomyces cerevisiae* and *E. coli* (Fig. 2a). Similarly, NeqB exhibited 45% sequence identity with the V/A-type subunit B from hyperthermophilic archaean *Methanothermobacter* *thermoautotrophicus*, *Pyrococcus*, and *Thermococcus* species, but only 23 and 25% sequence identity, respectively, with the F-type *E. coli* and the yeast α-subunit (Fig. 2a).

The search for conserved motifs indicated that the 23[^a]GPFSGK[^240] and 23[^a]GER[^253] region of NeqA is the Walker A motif/P loop and Walker B motif, respectively. These motifs are highly conserved in proteins that bind to and hydrolyze ATP to ADP (6, 42). The search for conserved motifs indicated that the 13[^a]SPGPLPM[^141] and 16[^a]GVP[^165] motifs on NeqB aligned with the SXSGLPHN and GIT motifs on the V/A-type ATPases. The SXSGLPHN motif is known to be the Walker A homologous region in the regulatory subunit B of the V/A-type ATP synthase family (Fig. 2a) (43). The Arg-326 residue of NeqB was also found to be highly conserved across the homologs; this residue has been reported to be crucial for nucleotide hydrolysis (29, 39). Overall, our analysis suggests that the A and B subunits of the *N. equitans* ATP synthase possess most of the conserved features observed in V/A/F-ATP synthase complexes.

**Phylogenetic Analysis**—A phylogenetic analysis was performed for NeqA and NeqB to understand the evolutionary path of the *N. equitans* ATP synthase subunits. It is well known that the ATP synthase family arose from a common ancestor and subsequently diverged into the F- and V/A-types (2, 44). Our phylogenetic analysis shows that *N. equitans* appears after the divergence of the F- and V/A-types and belongs to the V/A family of ATP synthases (Fig. 2, b and c). *N. equitans* subunits arose early during evolution of the ATP synthases and share a common ancestor with the rest of its archaean and prokaryotic homologs. It can be deduced from the branch lengths of NeqA and NeqB in their respective phylogenetic trees that these sequences have undergone significant evolutionary remodeling (Fig. 2, b and c). This can be an effect of the early association of *N. equitans* and *I. hospitalis* and the subsequent evolution of *N. equitans* as an energetically dependent parasite, leading to loss of catalytic function of its ATP synthase (26, 45). The absence of several subunits of the *N. equitans* ATP synthase can also be attributed to genomic loss due to parasitism, a trait seen in many obligatory bacterial endoparasites (21, 46).

Structure of the Regulatory Subunit NeqB—The nucleotide-free NeqB structure was determined at 2.8 Å resolution (Table 1). There are four molecules in the asymmetric unit. We did not observe electron density for the first five N-terminal residues, Glu-50–Ile-54 and Ile-98–Tyr-103, and hence these stretches of amino acids are not included in the model. The model was well refined with good stereo-chemical parameters (Table 1). The structure of NeqB consists of an N-terminal β-barrel (Pro-6–Ile-115) region, a central αβ-region (Ser-116–Leu-327), and a C-terminal α-helical region (Ala-328–Leu-410) comprising three helices (Fig. 3, a and b). All four molecules in the asymmetric unit are similar (r.m.s.d. is <0.5 Å). A structural homology search with DALI revealed that NeqB bears significant structural similarity to subunit B from the V1 complex structure of *E. hirae* (PDB code 3VFR) and *T. thermophilus* (PDB code
3GQB) as well as the independent A-type subunit B from *M. mazei* (PDB code 2C61) (Table 2). Moreover, despite its low sequence identity (24%), NeqB was found to be equally similar to the /H9251-subunit of mitochondrial F-type ATPase (PDB code 1BMF), suggesting that the three-dimensional structure of the NeqB subunit is well conserved (Table 2). The sequence of NeqB was found to be shorter at the C terminus by /H1101120 residues as compared with its close homologs from *T. thermophilus* and *E. hirae*. However, this did not affect the overall tertiary structural features of NeqB.

The /H150SASGLPHN/157 and /H184GIT/186 motifs of subunit B of the A-type *M. mazei* ATP synthase are homologous to the P-loop/Walker A and Walker B regions of the *N. equitans* subunit A. The P-loop homologous regions of the B subunits of the V/A-type ATPases appear to have lost the conserved residues found on the Walker motif that are important for nucleotide binding (Fig. 2a). Nucleotide binding to subunit B of the V/A-type ATPases has been shown previously in the crystal structure of *M. mazei* A-type subunit B (PDB code 3B2Q), in which Ser-150 and Ala-151 make contact with the phosphate group of 

![FIGURE 3. Structure of *N. equitans* subunits. a, bar diagram representing conserved motifs on the NeqA and NeqB subunits. b, crystal structure of NeqB (in corresponding colors). The Walker homologous and GVP motif are labeled.](image)

### TABLE 2
Comparison of independent NeqB structure with its homologs

| PDB/protein name | PDB chain ID | Subunit | Type | Organism | Ligand | r.m.s.d. | Ca atoms | DALI Z score |
|------------------|--------------|---------|------|----------|--------|----------|----------|-------------|
| 2C61 A           | B            | A1      | V/A  | *M. mazei* | None   | 0.764    | 272      | 48.5        |
| 3W3A L           | B            | V1      | V/A  | *T. thermophilus* | None   | 1.298    | 314      | 41          |
| 3W3A M           | B            | V1      | V/A  | *T. thermophilus* | ADP   | 2.010    | 323      | 38.3        |
| 3VR3 D           | B            | V1      | V/A  | *E. hirae* | None   | 1.132    | 304      | 47.4        |
| 3VR3 E           | B            | V1      | V/A  | *E. hirae* | ANP    | 0.836    | 293      | 47.7        |
| 3GQB B           | B            | V1      | V/A  | *T. thermophilus* | None   | 0.758    | 290      | 47.7        |
| 1BMF A           | α            | F1      | *Bos taurus* | AMPPNP | 2.140  | 273      | 41       |
| NeqB_apo B       | V/A          | N. equitans | None   | 1.432  | 323    |
| NeqB_ADP B       | V/A          | N. equitans | ADP    | 1.466  | 326    |
| NeqB_ANP B       | V/A          | N. equitans | ANP    | 1.365  | 329    |
the bound nucleotide (43). However, the nature of this binding is different from the interactions of the nucleotide with the P-loop of the A subunit, suggesting that it is a transitional binding site (43). The $K_d$ value for the binding between the wild-type $M. mazei$ subunit B and Mg-ATP was shown to be $22 \mu M$ (43).

From our sequence alignment, we observed that the equivalent regions in NeqB are $^{134}$SPPGLPM$^{141}$ and $^{163}$GVP$^{165}$, and we found these regions to be structurally similar to that of $M. mazei$ subunit B. Using ITC (Fig. 4b), we observed a $K_d$ of 62 $\mu M$ for NeqB in its interaction with Mg-ATP, which corresponds to a binding affinity 3-fold lower than that of $M. mazei$ subunit B. The binding between NeqB and ATP is weak, resulting in a $c$ value lower than 1 ($c$ is the product of the association constant and the initial concentration of protein in the calorimetric cell, $c = K_a \times [M]$) and a non-sigmoidal binding isotherm (48). Higher $c$ values could not be used as obtaining the corresponding concentrations of the ligand and the proteins was not practically possible in this case, keeping in mind the protein to ligand ratio. However, the $K_d$ values for low $c$ values are reliable (49). In addition, the concentration of Mg-ATP was increased to 1 mM to reach significant saturation of NeqB in the calorimetric cell (50). It is important to point out that in cases of low $c$ values, the intercept with the $y$ axis in the binding isotherm does not resemble the binding enthalpy but a small fraction of that binding parameter equal to $c/(c + 1)$ (51). The $K_d$ value of NeqA with Mg-ATP under similar conditions was 5.3 $\mu M$ (Fig. 4a), which is slightly over 2-fold lower than the $K_d$ value of the $M. mazei$ subunit A (2.38 $\mu M$) (52). However, our attempts to co-crystallize NeqB with nucleotide did not yield any complex crystals.

Furthermore, we studied the interactions between the NeqA and the NeqB subunits through pulldown assays, native-PAGE, AUC, and ITC. The results from each assay clearly indicated complex formation between NeqA and NeqB (Fig. 5). Moreover, the interaction between NeqA and NeqB is strengthened in complex with ADP (100-fold decrease in the complex dissociation constant), with a change in the $K_d$ from $11 \pm 2$ to $0.11 \pm 0.01 \mu M$ for NeqA/NeqB in the presence and absence of ADP, respectively (Fig. 6). Thus, Mg-ADP pre-bound to the subunit enhances the binding affinity between the subunits ($\Delta G = -3$ kcal/mol, approximately), and this effect is entropic in nature, because the binding enthalpy for NeqA in its interaction with NeqB is minimally affected by the presence of Mg-ADP.

Structure of Nucleotide-free NeqAB Complex—Next, we solved the structure of the core complex of the $N. equitans$ ATP synthase to gain a better understanding of its catalytic mechanism. The nucleotide-free form of the NeqAB complex was determined at $3.0 \AA$ (Table 1). The asymmetric unit consisted of a NeqAB heterodimer. The hexameric $A_3B_3$ core NeqAB complex has been generated using crystallographic symmetry-related neighboring molecules.

NeqA in the NeqAB complex comprised an N-terminal $\beta$-barrel region (Asn-2–Thr-204), followed by a central $\alpha\beta$
region (Arg-205–Val-379) housing the P-loop, and an α-helical C-terminal region (Ser-380–Ala-566) comprising six helices (Fig. 7a). A DALI structural homology search showed that NeqA most closely resembled the subunit A of the *T. thermophilus* (PDB code 3W3A) and *E. hirae* V₁-ATPases (PDB code 3VR3) (Table 3). Moreover, the NeqA β-barrel region (Asp-109–Tyr-179) appeared as a peripheral bulge, compared with the β-subunit of the bovine mitochondrial F₁-ATP synthase (PDB code 1BMF); this bulged region and part of the C-terminal α-helical region (Asp-492–Ala-566) of NeqA did not superimpose well with the F₁-ATPase β-subunit (Fig. 8). These features are signatures in V/A-types as compared with the structurally distinct F-types (39, 53). We observed a sulfate ion in the nucleotide-binding site of NeqA in the β-phosphate position that also interacts with Arg-326 of NeqB. A similar sulfate ion has been reported in the nucleotide-free *P. horikoshii* subunit A (PDB code 3I72), which interacts with the P-loop region in a similar manner (52). A comparison of the *P. horikoshii* subunit A structures with (PDB code 3I72) and without (PDB code 1VDZ) the sulfate ion showed no significant conformational changes (Table 3).

The overall structure of NeqA resembles NeqB, despite the low sequence identity of ~25% (r.m.s.d. is 1.945 Å for 382 Ca atoms); this is consistent with previous suggestions that the catalytic A and regulatory B subunits are from a common ancestor through a gene duplication event (2).
The nucleotide-free NeqAB heterodimer was superimposed with nucleotide-bound and nucleotide-free AB heterodimers from V-type E. hirae and T. thermophilus ATPases (Table 4). In the homologous nucleotide-free structures, hydrophobic interactions between subunits A and B were observed mostly at the N-terminal region, which left the C-terminal regions of both...
subunits wide apart in an “open” conformation; a “closed” conformation was seen to occur upon binding of a nucleotide in a pocket formed at the interface of A and B subunits, as seen in case of the V-type ATPases from E. hirae (PDB code 3VR6) and T. thermophilus (PDB code 3W3A) (29). Superimposition of the NeqAB structure, however, showed that the nucleotide-free NeqAB heterodimer was most similar to the nucleotide-bound closed form rather than nucleotide-free open forms of these homologous V-type complexes, i.e. NeqAB assumed a closed conformation even in the nucleotide-free state (Fig. 9 and Table 4). Furthermore, unlike its homologs, the NeqA and NeqB interface comprised 26 hydrogen bonding contacts and several hydrophobic interactions. The interface area between NeqA and NeqB was determined to be 2768 Å², which is similar to the nucleotide-free NeqAB complex (r.m.s.d. of 1.432 Å for 323 Ca atoms) as well as similar nucleotide binding positions. In the ADP-bound NeqAB, the interface area is 2729 Å², which is similar to the interface area calculated for the nucleotide-free NeqAB structure.

**Structure of the NeqAB ATP Synthase Core Complex**

Our structures showed that the nucleotide binding pocket is located at the interface of the NeqAB heterodimer, which is mainly composed of residues from NeqA, similar to that of other ATP synthase structures (Fig. 11, a and b). This pocket formed between the central αβ region of NeqA (encompassing the Walker A and Walker B (251GER253 loop) motifs) and a Ser-260–Gly-330 stretch, including the conserved Arg-326 from NeqB (Fig. 11, a and b). In the ADP complex, there were 16 hydrogen bonding contacts (<3.4 Å) between NeqA/NeqB and the ADP (Table 5). Eleven of these contacts were made with NeqA; the phosphate groups of ADP interacted with Gly-226, Gly-228, Lys-229, Thr-230, and Val-231 from the Walker A motif, and the adenine head groups made contact with Gln-491 and Ala-493 of NeqA (Fig. 11a) Arg-326 from NeqB made five hydrogen bonding contacts (<3.4 Å) with the β-phosphate of ADP. Superimposition of the ADP-bound NeqAB and V1-ATPase from T. thermophilus (PDB code 3W3A) showed similarity between them (r.m.s.d., 1.727 Å for 870 Ca atoms) as well as similar nucleotide binding positions. In the ADP-bound NeqAB, the interface area is 2729 Å², which is similar to the interface area calculated for the nucleotide-free NeqAB structure.

**Comparison of NeqA with its homologs**

The total number of Ca atoms for NeqA apo was 563 atoms. DALI Z scores have been calculated using chain A from NeqAB apo-model.

| PDB     | PDB chain ID | Subunit | Type | Organism | Ligand | r.m.s.d. | Ca atoms | DALI Z score |
|---------|--------------|---------|------|----------|--------|----------|----------|--------------|
| NeqA_ADP | A            | A       | V/A  | N. equitans | ADP    | 0.468    | 562      | 47.6         |
| NeqA_ADP | A            | A       | V/A  | N. equitans | ADP    | 0.468    | 562      | 47.6         |
| 3VR3    | B            | B       | V/1  | E. hirae  | ADP    | 0.966    | 473      | 46.9         |
| 3GQB    | A            | A       | V/1  | T. thermophilus | AMP-PNP | 1.761    | 452      | 44.7         |
| 3VR3    | A            | A       | V/1  | E. hirae  | None   | 1.787    | 457      | 44.7         |
| 3W3A    | A            | A       | V/1  | T. thermophilus | AMP-PNP | 1.711    | 456      | 42.3         |
| 3W3A    | I            | I       | V/1  | T. thermophilus | ADP    | 1.714    | 497      | 40.2         |
| 1VDZ    | A            | A       | A1   | P. horikoshii | AMP-PNP | 1.713    | 382      | 40.1         |
| 3W3A    | K            | K       | V/1  | T. thermophilus | ADP    | 1.669    | 504      | 40.1         |
| 3I72    | A            | A       | A1   | P. horikoshii | SO4    | 1.714    | 383      | 40.1         |
| 3I73    | A            | A       | A1   | P. horikoshii | AMP-PNP | 1.702    | 380      | 39.8         |
| 3I4L    | A            | A       | A1   | P. horikoshii | AMP-PNP | 1.676    | 381      | 39.6         |
| 1BMF    | D            | β       | F1   | B. taurus | ADP    | 2.080    | 313      | 32.1         |
| 1BMF    | F            | β       | F1   | B. taurus | AMP-PNP | 2.148    | 314      | 32.1         |
| 1BMF    | E            | β       | F1   | B. taurus | None   | 3.285    | 328      | 30.1         |
| %       | %             | %       | %    | %        | %      | %        | %        | %            |

a NeqA from the NeqAB apo-structure was compared with NeqA from the AB_ADP- and AB_ADP-PNP-bound structures.

**FIGURE 8. Cα trace representation of the bulge region on NeqA.** NeqA (magenta) overlaid with a, F-type β (yellow), and b, V-type A subunit (green) shows the Bulge region that is typical of the V/A-type ATPase A subunits.
In the AMP-PNP-bound complex, a total of 26 hydrogen bonding contacts (~3.4 Å) were observed between the AMP-PNP ligand and NeqA/NeqB, of which 21 contacts were with NeqA (Table 6). The phosphate groups of AMP-PNP were in contact with the P-loop residues (Gly-226–Val-231) of NeqA, with two additional contacts made between the -phosphates of the AMP-PNP moiety and the Arg-253 of the251GER253 loop and Glu-256 of NeqA with (Fig. 11b). This shows tight binding of NeqA with the nucleotide, and this was further confirmed by ITC analyses (Fig. 4a). The interface area between the A and B subunits of the AMP-PNP-bound NeqAB complex was 2629 Å², which is comparable with the interface areas observed for the apo and ADP complexes. Notably, a similar interface area was calculated for the nucleotide-bound and -unbound forms of the T. thermophilus V-type ATPase (PDB code 3W3A) and showed a difference of ~1000 Å². Likewise, an interface area difference of ~800 Å² was noted for the nucleotide-bound and -unbound forms of E. hirae (PDB code 3VR6). This comparison emphasizes the conformational rigidity of the NeqAB structures (Fig. 12).

In T. thermophilus, the ADP-bound form of the V₁-ATPase is in an inhibitory state to avoid any unnecessary hydrolysis of ATP upon the loss of proton transport (54, 55). The asymmetric state in the crystal structure of T. thermophilus (at 3.9 Å) demonstrated two distinct conformations of the AB complex in the presence of a nucleotide as follows: one “intermediate” and one “tight (closed)” (29). It has been suggested that the compactness of the nucleotide binding pocket in this “tight” form is a con-
A contributing factor to the molecular mechanism of ADP inhibition (54). We analyzed the interface area between the A and B subunits of *T. thermophilus* in these two states and found that the tight form has a higher interface area of $H_{11011} = 150 \text{ Å}^2$. After superimposing all of the C atoms of the ADP-bound NeqAB complex with these tight and intermediate forms, we found that the

**TABLE 5**

| Subunit | Residue | Ligand | Distance (Å) |
|---------|---------|--------|--------------|
| A       | Thr-230 (N) | ADP(O1B) | 3.1          |
| A       | Thr-230 (OG1) | ADP(O1B) | 3.0          |
| A       | Lys-229 (N) | ADP(O2B) | 2.9          |
| A       | Lys-229 (NZ) | ADP(O2B) | 2.6          |
| A       | Gly-226 (N) | ADP(O3B) | 2.8          |
| A       | Thr-230 (N) | ADP(O1A) | 3.1          |
| A       | Val-231 (N) | ADP(O1A) | 2.8          |
| A       | Gly-226 (N) | ADP(O3A) | 3.1          |
| A       | Thr-230 (N) | ADP(O1A) | 3.0          |
| A       | Gly-226 (N) | ADP(O3A) | 3.1          |
| A       | Gln-491 (O) | ADP(N6)  | 3.0          |
| A       | Ala-493 (N) | ADP(N6)  | 3.0          |
| B       | Arg-326 (NH1) | ADP(O3B) | 2.7          |
| B       | Arg-326 (NH2) | ADP(O3B) | 3.3          |
| B       | Arg-326 (NH3) | ADP(O2A) | 2.7          |
| B       | Arg-326 (NH4) | ADP(O3A) | 3.2          |
| B       | Arg-326 (O) | ADP(O3') | 3.3          |

**TABLE 6**

| Subunit | Residue (atom) | Ligand (atom) | Distance (Å) |
|---------|----------------|---------------|--------------|
| A       | Thr-230 (OG1) | ANP(PG)       | 3.0          |
| A       | Thr-230 (O1B) | ANP(O2G)      | 3.0          |
| A       | Asp-320 (OD1) | ANP(O2G)      | 3.3          |
| A       | Gln-226 (OE2) | ANP(O2B)      | 3.0          |
| A       | Gly-226 (N)  | ANP(O1B)      | 2.9          |
| A       | Lys-229 (OG) | ANP(O2B)      | 3.4          |
| A       | Ser-227 (N)  | ANP(O2B)      | 3.0          |
| A       | Thr-230 (N)  | ANP(O3B)      | 3.1          |
| A       | Val-231 (N)  | ANP(O3)       | 3.2          |
| A       | Gly-226 (N)  | ANP(N6)       | 3.0          |
| A       | Ala-493 (N)  | ANP(N6)       | 3.1          |
| B       | Arg-326 (NH1) | ANP(O1B)      | 2.9          |
| B       | Arg-326 (NH2) | ANP(O1B)      | 3.3          |
| B       | Arg-326 (NH3) | ANP(N3B)      | 3.4          |
| B       | Arg-326 (O)  | ANP(O3A)      | 3.2          |

**FIGURE 11. Interaction of nucleotides with NeqA and NeqB.** Stereo diagrams are as follows: a, side chains of NeqA (magenta) and NeqB (cyan) interacting with Mg-ADP (green); b, side chains of NeqA and NeqB interacting with Mg-AMP-PNP (blue). In both images, the P-loop motif (Walker A) residues of NeqA in its interaction with the ligand are shown in a cartoon loop representation, and the Arg-326 residue from NeqB is highlighted in red. The final electron density map ($2F_o - F_c$ map, contoured at 1.0σ) is shown for both ADP and AMP-PNP. c, nucleotide binding pocket of NeqA is shown in electrostatic surface representation, with AMP-PNP in stick representation (green). The inset shows the orientation of AMP-PNP on the surface of NeqA. For clarity, NeqB is not shown.
NeqAB-ADP complex was more similar to the tight (closed) form (r.m.s.d., 1.67 Å for 867 C\(^{\alpha}\)/H9251 atoms) than the intermediate form (r.m.s.d., 1.83 Å for 867 C\(^{\alpha}\)/H9251 atoms). We therefore speculate that the ADP-bound NeqAB complex possibly represents the inhibited form of the AB core complex.

Overall, the ADP- and AMP-PNP-bound forms were similar (r.m.s.d., 0.472 Å for 968 C\(^{\alpha}\)/H9251 atoms), and superimposition of the nucleotide-bound and nucleotide-free forms did not show any conformational changes (Fig. 12). In the recently reported _E. hirae_ A3B3 complex structure with AMP-PNP (PDB code 3VR3), three AB heterodimers were present in the asymmetric unit, two of which were in an AMP-PNP-bound (closed) state, but the third is in an open or nucleotide-unbound state (30). Similar open/closed differences have been shown for _T. thermophilus_ as described above (29). These closed and open forms have also been observed in the bovine mitochondrial F1-ATP synthase (6) as well as in the V-type ATPases from _T. thermophilus_ (PDB codes 3W3A, 3A5D, and 3A5C) (56). Furthermore, transitional states of ADP capture have been shown for the _M. mazei_ subunit B, indicating this C-terminal entry route for the nucleotide (43, 57, 58) and the nucleotide binding-induced conformational change required for its catalytic mechanism (29, 30). However, in our NeqAB complexes, we did not observe any significant conformational transitions as a result of nucleotide binding (Figs. 9 and 12). The transition from an open to a closed state has been shown to be the basis of the binding change mechanism of catalysis of the F/V-type ATP synthases, where the AB heterodimers have different binding affinities for the nucleotide (27).

Structure of Hexameric Ring of NeqAB Complexes (A3B3) and Comparison with Homologs—Our gel filtration, AUC, and native-PAGE data indicate that the NeqAB complex forms a hexamer (Fig. 5). The hexameric ring of the NeqAB complex (A3B3) was generated using crystallographic, symmetry-related neighboring molecules, and this was compared with the homologous structures of several nucleotide-free and nucleotide-bound catalytic hexamers (A3B3) as well as F1/V1 sub-complexes (Table 7). Most of the well characterized F1 and V1 complexes have an A3B3 hexamer in their structural, asymmetric states in one asymmetric unit of the crystal, which enables direct visualization of the entire A3B3 core complex and the central cavity. We also generated hexamers using crystallographic, symmetry-related neighboring AB dimers for the ATP synthase crystal structures of the _Bacillus PS3_ A3B3 complex.

### Table 7
Comparison of Neq AB3 (hexamer) with its homologs

| PDB code | Organism          | State    | Asymmetric unit | Bottom aperture of hexamer | Nucleotide binding | Ref.   |
|----------|-------------------|----------|-----------------|-----------------------------|--------------------|--------|
| 5BN5     | _N. equitans_     | Symmetric | AB              | Narrow                      | Empty              | Present study |
| 5BN3     | _N. equitans_     | Symmetric | AB              | Narrow                      | ADP                | Present study |
| 5BN4     | _N. equitans_     | Symmetric | (AB)\(_2\)     | Wide                        | Empty              | Present study |
| 3V92     | _E. hirae_        | Asymmetric | A\(_3\)B\(_3\)DG | Wide                        | AMP-PNP            | Present study |
| 3VR3     | _E. hirae_        | Asymmetric | A\(_3\)B\(_3\)DG | Wide                        | AMP-PNP            | Present study |
| 3VR4     | _E. hirae_        | Asymmetric | A\(_3\)B\(_3\)DG | Wide                        | AMP-PNP            | Present study |
| 3VB6     | _E. hirae_        | Asymmetric | A\(_3\)B\(_3\)DG | Wide                        | AMP-PNP            | Present study |
| 3V3A     | _T. thermophilus_ | Asymmetric | A\(_3\)B\(_3\)DG | Wide                        | ADP                | 29     |
| 1BMF     | Bovine            | Asymmetric | αββγ3/3         | Narrow                      | ADP                | 6      |
| 1MAB     | Rat               | Symmetric | αββγ3/3         | Narrow                      | ADP, ATP           | 59     |
| 2F43     | Rat               | Symmetric | αββγ3/3         | Narrow                      | ADP, ATP           | 60     |
| 1FX0     | Spinach           | Symmetric | αββγ3/3         | Narrow                      | Empty              | 71     |
| 1SKY     | _Bacillus PS3_    | Symmetric | αββγ3/3         | Wide                        | Empty              | 61     |
| 2QE7     | _Bacillus sp. TA2_ | Symmetric | αββγ3/3         | Wide                        | Empty              | 47     |
(PDB code 1SKY), the spinach chloroplast F$_1$-ATPase (PDB code 1FX0), and rat liver (PDB codes 1MAB and 2F43), which, as with our case, also do not have hexamers in their asymmetric units (59–61). We then compared the central cavity in all of these cases. We found that the central cavity of the nucleotide-bound NeqAB structure tapered toward the bottom of the hexameric ring (A$_3$B$_3$) and formed a much smaller aperture than that of its homologs (Fig. 13). Based on our analysis of the homologs, we have seen so far that the “tapered central cavity” conformation either depends on the presence of the central stalk or the nucleotide. However, the nucleotide-free NeqAB hexamer (A$_3$B$_3$) is highly similar to the nucleotide-bound form and therefore has the same tapered central cavity. It is noteworthy that this has not been observed in any nucleotide-free A$_3$B$_3$ F$_1$/V$_1$-ATP synthase to date (Table 7).

**Discussion**

The core catalytic ATP synthase subunits (subunits A and B) from eukaryotic and bacterial F-type and V-type are well characterized; yet limited structural details are available for the ATP synthases from archaea (30, 39). Among the archaeal ATP synthases reported, the *M. mazei* and *Pyrococcus* sp. ATP synthases are the most well studied. However, this information is limited to low resolution cryo-EM data and structures of indi-
Structure of the NeqAB ATP Synthase Core Complex

vidual subunits (19, 52, 57, 62, 63). To date, there is no crystal structure available for the core complex (A3B3) of ATP synthases from archaeal species. This study reports the crystal structure of the regulatory subunit B (NeqB) and core complex subunits AB (NeqAB) in its nucleotide-bound and -unbound forms of the N. equitans ATP synthase. It has been widely accepted that the A-type, F-type, and V-type arose from a common ancestor (44), with the A3B3 or αβ3 hexamer of the ATP synthase family being the first subcomplex to arise (64). As archaeal organisms, especially the hyperthermophiles, are thought to be one of the earliest living forms (65), the structural and functional studies of N. equitans ATP synthase may offer an evolutionary perspective.

There are several crystal and cryo-EM structures available for the F/V-type ATPase complexes, and these have already revealed the asymmetric states of the AB heterodimers in the A3B3 headpiece of the catalytic core (6, 19, 29, 56); these studies support the binding-change model of catalysis described previously (27). Although initially attributed to the interaction of the A3B3 headpiece with the central stalk (D and F subunits in V-type and γ, δ, and ε subunits in the F-type), recent studies have shown that the A3B3 headpiece, even in the absence of nucleotide, also exhibits asymmetric states (30). Furthermore, the αβ3 headpiece of the F-type ATP synthase, which is devoid of a central stalk, can rotate in the presence of nucleotides, which confirms the importance of the conformational transition from an open to a closed state for catalytic activity, consistent with the binding change mechanism (66, 67). Here, we have shown that, although the NeqAB core complex retains its nucleotide-binding traits, the lack of a conformational change (open to close transition) suggests that the NeqAB core complex is possibly functionally inactive and a rudimentary form of the ATP synthase complex (Fig. 14).

Several studies have shown that N. equitans relies on its host I. hospitalis for essential metabolites (22, 26). Although it is not yet clear whether N. equitans is a parasite or symbiont on I. hospitalis, it has been shown that N. equitans cannot survive for long periods when detached from its host (21). Previous studies have noted that lipids and amino acids are transported from I. hospitalis into N. equitans (26, 68), with transmission electron microscopy studies of I. hospitalis showing vesicles blebbing out from the inner plasma membrane into the periplasmic space (69). The probable energetic dependence of N. equitans on I. hospitalis was proposed by a recent study showing that the A3B3 complex is possibly functionally inactive and a rudimentary form of the ATP synthase complex (Fig. 14). Several studies have shown that N. equitans relies on its host I. hospitalis for essential metabolites (22, 26). Although it is not yet clear whether N. equitans is a parasite or symbiont on I. hospitalis, it has been shown that N. equitans cannot survive for long periods when detached from its host (21). Previous studies have noted that lipids and amino acids are transported from I. hospitalis into N. equitans (26, 68), with transmission electron microscopy studies of I. hospitalis showing vesicles blebbing out from the inner plasma membrane into the periplasmic space (69). The probable energetic dependence of N. equitans on I. hospitalis was proposed by a recent study showing that the A3B3 complex is possibly functionally inactive and a rudimentary form of the ATP synthase complex (Fig. 14).

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self-sufficiency in various other processes, such as DNA replication and repair, RNA transcription and recombination, and protein transportation, to name a few (21). An analysis of the proteome of N. equitans also shows the presence of the ATP synthase subunits from I. hospitalis (25). Therefore, it is possible that the N. equitans ATP synthase can assemble into a functional form by borrowing the missing subunits from I. hospitalis. Future projects will be aimed at deriving an understanding of the bioenergetics of this unique organism.

Author Contributions—C. W. V. H. and J. S. conceived this study; J. S. designed the study; S. M. performed the cloning, protein expression, purification, crystallization, and biophysical studies; S. M., C. J., J. S. and V. P. R. C. performed data collection and structure determination; A. V. C. performed the ITC data analysis; J. S. and B. C. L. provided tools and reagents; S. M. and J. S. wrote the paper.

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