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Nucleotide-Binding Sites in V-Type Na^+-ATPase from *Enterococcus hirae*[1]

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***Enterococcus hirae* V-ATPase, in contrast to most V-type ATPases, is resistant to *N*-ethylmaleimide (NEM). Alignment of the amino acid sequences of NtpA suggests that the NEM-sensitive Cys of V-type ATPases is replaced by Ala in *E. hirae* V-ATPase. Consistent with this prediction, the V-ATPase became sensitive upon substitution of the Ala with Cys. The three-dimensional structure of the NtpB subunit of V-ATPase was modeled based on the structure of the corresponding subunit (α subunit) of bovine F_1-ATPase by homology modeling. Overall, the 3D structure of the subunit resembled that of α subunit of bovine F_1-ATPase. The NtpB subunit, which lacks the P-loop consensus sequence for nucleotide binding, was predicted to bind a nucleotide at the modeled nucleotide-binding site. Experimental data supported the prediction that the *E. hirae* V-ATPase had about six nucleotide-binding sites.**

Key words: *Enterococcus hirae*, homology modeling, Na^+-translocating ATPase, nucleotide binding, V-ATPase.

Ion-motive ATPases that do not form phosphorylated intermediates are divided into two types: F_1F_0-ATPase (F-ATPase) and V_1V_0-ATPase (V-ATPase). F-ATPase functions as an ATP synthase in mitochondria, chloroplasts and oxidative bacteria (*1*). V-ATPase functions as a proton pump in acidic organelles, in plasma membranes of eukaryotic cells (*2*) and in some bacteria (*3*). Both ATPases are similar multisubunit enzymes consisting of hydrophilic catalytic portions (F_1 and V_1) and membrane-embedded portions (F_0 and V_0, respectively) that constitute the proton pathway (*4, 5*). Each hydrophilic portion contains three copies of catalytic subunits (F-ATPase β subunit or V-ATPase A subunit), three copies of noncatalytic subunits (F-ATPase α subunit or V-ATPase B subunit), one copy of a central subunit, and several minor subunits (*4, 5*). Studies of F_1-ATPase have been progressing much better than those of V_1-ATPase. Crystal structures of F_1-ATPase from bovine mitochondria have been obtained at atomic resolution (*6*). Further, the "rotation catalysis" mechanism, proposed by P.D. Boyer (*7*) has now been verified as the mechanism of F_1-ATPase. As F_1-ATPase and V_1-ATPase resemble each other both structurally and functionally, it is generally accepted that the reaction mechanism of V_1-ATPase is similar to that of F_1-ATPase (*2, 5, 8*).

On the other hand, there are several notable differences between the F_1-ATPase and V_1-ATPase molecules. First, a sequence stretch (about 90 amino acid residues) not found in the F_1-ATPase β subunit is conserved in the N-terminal region of V_1-ATPase A subunit (*9*). V_1-ATPase is sensitive to sulfhydryl reagents such as *N*-ethylmaleimide (NEM) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), but F_1-ATPase is not (*10*). The B subunit of V_1-ATPase lacks the P-loop consensus sequence (G*XX*G*X*GKTV) that is conserved at the nucleotide-binding sites of β and α subunits of F_1-ATPase and the A subunit of V_1-ATPase (*11*). To understand the differences between these enzymes, it is necessary to determine the structure of V_1-ATPase.

We found a variant of V-ATPase that physiologically transports sodium ions in a fermentative bacterium, *Enterococcus hirae*. The enzyme, consisting of nine subunits encoded by a Na^+-responsive operon (designated *ntp*) (*9, 12*), has been purified on a large scale using the cloned genes (*13, 14*) and characterized biochemically (*15–17*). In the V_1 domain, the NtpA and NtpB subunits both appear to show about 40% sequence similarities (about 25% sequence identities) with β and α subunits of bovine F_1-ATPase (*9, 12*). Homology modeling based on such high similarity is known to give fairly good results. In this study, homology modeling of the NtpB subunit of *E. hirae* V_1-ATPase was done based on the three-dimensional structure of the corresponding subunit (α subunit) of bovine F_1-ATPase. The obtained structure implies new information that was consistent with experimental data.

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MATERIALS AND METHODS

Alignments of Amino Acid Sequences of NtpA and NtpB Subunits—Alignments of the amino acid sequences were created by using DAYHOFF (*18*) and modified by hand based on conserved residues found by using PROSITE (*19*) and Clustal W (*20*) (Fig. 1). The amino acid sequences of the NtpA and NtpB subunits had approximately 25 and 22% sequence identities, respectively, with those of β and α subunits of bovine F_1-ATPase. The NtpA subunit has 90 non-matching amino acids as shown by the blue box in Fig. 1A. About 30 amino acids are lacking in the NtpB C-terminal sequence corresponding to that of the α subunit of bovine mitochondrial F_1-ATPase (Fig. 1B).

Site-Directed Mutagenesis of NtpA Subunit—Site-directed mutagenesis for replacement of Ala236 by Cys was done using the PCR-amplified product obtained with a mutated oligonucleotide primer. The oligonucleotide used was as follows, with the substitution site underlined: A236C, 5′-TTCCAGGGCCCTTTGGTTGTGGGAAGAC-3′.

The PCR product was inserted into the corresponding site in the *ntpA* gene on pHEexA as described previously (*21*). The base replacement was confirmed by DNA se-

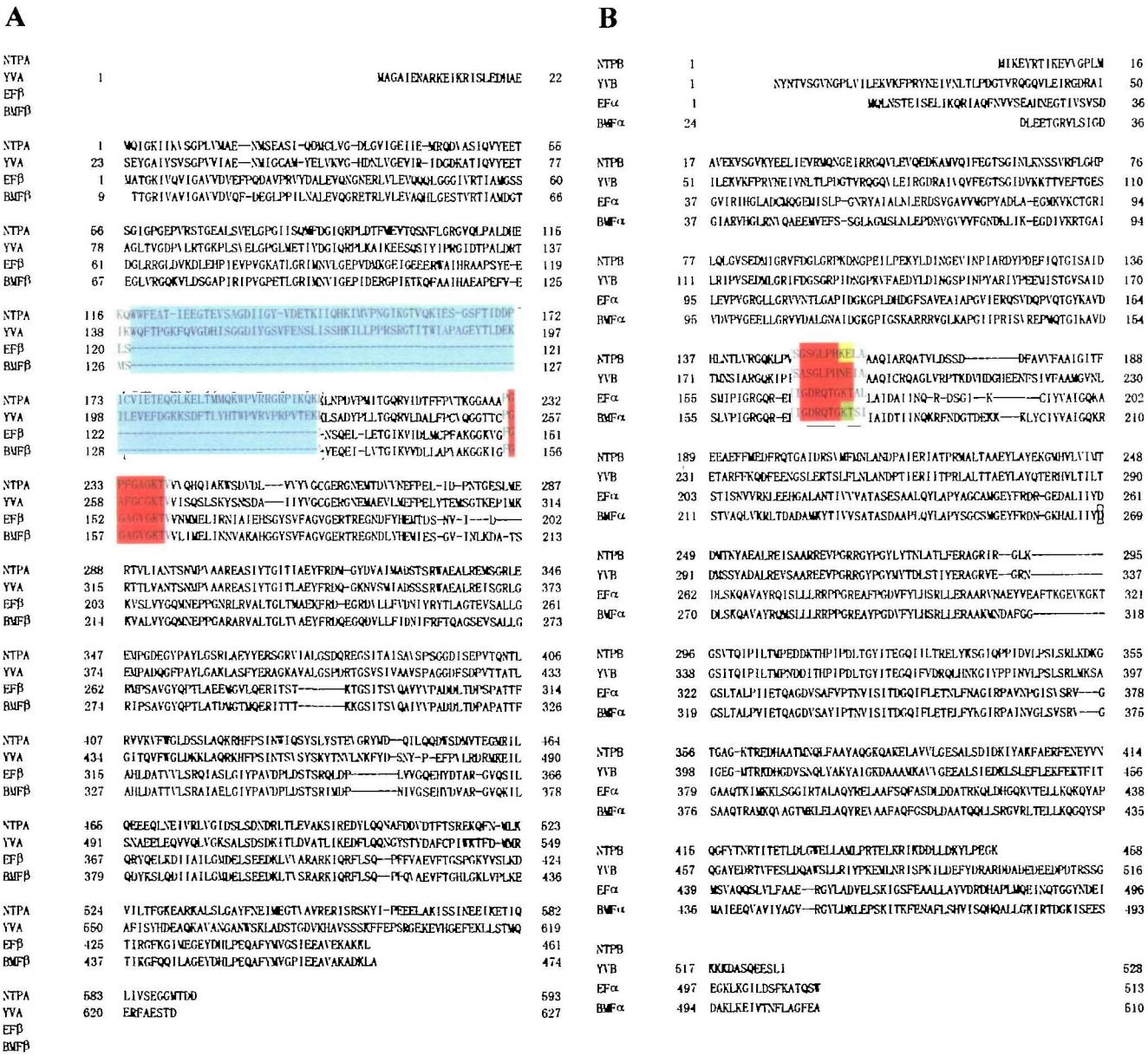

Fig. 1. **Alignments of amino acid sequences of *E. hirae* V-ATPase subunits with those of other ATPases.** Numbers at both ends of each line are those of amino acid residues of different ATPase subunits starting from the amino termini. Alignment of the amino acid sequence of the NtpA or NtpB subunit of *E. hirae* V-ATPase (NTPA or NTPB) with that of the A or B subunit of *Saccharomyces cerevisiae* (YVA or YVB) V-ATPase, β or α subunit of *E. coli* (EFβ or EFα) and bovine (BMFβ or BMFα) F-ATPase, respectively, is shown. Red boxes indicate P-loop sequence regions. The blue box indicates the inserted 90 amino acid sequence region. Green boxes indicate important residues for nucleotide binding in bovine F-ATPase α subunit, and yellow boxes indicate predicted corresponding important residues for nucleotide binding in NtpB. A, Alignment of NtpA. B, Alignment of NtpB.

quencing using the dideoxy method (*22*). The constructed plasmid (pHEexAA236C) or pHEexA (having wild-type *ntpA*) was introduced into *E. hirae* mutant Nak1 (nonsense mutation of *ntpA* gene) (*23*). Membranes were obtained from these strains and the ATPase activities of the membranes were measured according to the reported method (*21*). NEM was added to the reaction mixture 10 min before the ATPase activity assay.

Homology Modeling of NtpB Subunit—The 3D structure of the NtpB was constructed based on the X-ray crystal structure of the α subunit of bovine mitochondrial F_1-ATPase [PDB code: 1BMF] using Insight II/Discover Homology software. The structures of the inserted portions predicted by the alignment process were randomly generated and refined on the structure of F_1-ATPase. Finally, the 3D structure was optimized by energy minimization using Insight II/Discover. The 3D–1D compatibility of the obtained structure was examined by use of its 3D–1D profile (*24*). Electrostatic potential of the surface of nucleotide-binding sites was calculated using Insight II/Delphi (*25*).

Measurement of Nucleotide Binding to Purified V-ATPase—V-ATPase was purified by anion-exchange and gel filtration chromatographies as described previously (*14*). The equilibrium-dialysis method was applied for the measurement of nucleotide binding of the purified sample using a microequilibrium-dialysis apparatus as described previously (*16*). The purified enzyme (final concentration, 3 μM ATPase) in 50 μl of buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, 100 mM NaCl, and 0.05% dodecyl maltoside; pH 7.5) was placed in a chamber, and various concentrations of [α-^{32}P]ATP in 50 μl of buffer A was placed in another chamber. The solutions in the two chambers, separated by a membrane filter (Spectra/Pro, MWCO: 3,500), were equilibrated for 3 h at room temperature. The total amount of nucleotide binding to the purified sample was calculated from the difference in radioactivities (Cerenkov ray) in the two chambers measured with a liquid-scintillation counter (LS5000TD, Beckman). The amount of specific binding of nucleotide to the enzyme was estimated by subtracting the amount of nonspecific binding, which was measured by diluting the radioactive substrate with 5 mM nonradioactive ATP. The measurement was repeated three times, averaged, and the standard deviation (SD) was calculated.

Others—Protein was determined according to the method of Lowry *et al.* (*26*) with bovine serum albumin used as standard. [α-^{32}P]ATP (111 TBq/mmol) was purchased from Amersham Corp.

RESULTS AND DISCUSSION

NEM Sensitivity of the ATPase—Eukaryotic V-ATPases, but not F-ATPases, are in general highly sensitive to sulfhydryl reagents such as NEM and NBD-Cl (*10*). Several reports showed that modification of the A subunit cysteine residue (Cys261 in the P-loop of the yeast enzyme) led to resistance of the V-ATPase activity to these reagents (*27–29*). In contrast, *E. hirae* V-ATPase was less sensitive to these sulfhydryl reagents (*30*). In this enzyme, the cysteine residue was not conserved at the corresponding position of the nucleotide-binding site (P-loop) in the NtpA subunit (Fig. 1A, red box), being replaced by alanine (Ala-236). The P-loop consensus sequence is thought to be critical for catalytic activity of ATP hydrolysis. To verify the role of Cys in the sensitivity to NEM, we constructed a subunit A mutant (A236→C) of *E. hirae* V-ATPase. Neither assembly nor the Na^+-ATPase activity was altered in the mutant enzyme (data not shown). Figure 2 shows the sensitivity to NEM of subunit A mutant (A236→C). The Na^+-ATPase activities of 9790 (wild type) and Nak1/pHEexA (Nak1 harboring the plasmid with native *ntpA* gene) were inhibited by NEM with K_i values of about 0.1–0.2 mM (Fig. 2; open and closed circles). In subunit A mutant (Nak1/pHEexAA236C; Nak1 harboring the plasmid with mutated *ntpA* gene: A236→C), the NEM sensitivity of the Na^+-ATPase activity was drastically altered; the K_i value was about 0.01 mM (Fig. 2, triangles). These results indicate that the cysteine residue at the nucleotide-binding site of the V-ATPase is the target of sulfhydryl reagent attack, and that the P-loop sequence is important for the catalytic activity.

Homology Modeling of NtpB Subunit—Figure 3, A and B, shows overall 3D structures of the α subunit of bovine F_1-ATPase and the NtpB subunit which was modeled on the basis of sequence alignment of these subunits, the coordinates of crystal structure of the α subunit, and energy minimization as described in "MATERIALS AND METHODS." The overall structure of the NtpB subunit mostly matched the structure of the α subunit. NtpB lacks the corresponding C-terminal region (A478–A510) of the α subunit (shown by green ribbon in Fig. 3A). The 3D–1D scores of the modeled structure of NtpB were satisfactory except for the C-terminal 30 amino acids (data not shown). The low scores of this part may suggest inappropriate structure modeling due to the absence of the region corresponding to the C-terminal region of the α subunit in F_1-ATPase (Figs. 1B and 3A).

The B subunits of V-ATPases including NtpB lack the P-loop sequence but possess another conserved sequence among V-ATPases from various species, which contains amino acids frequently found in random coil conformations or in turns (Fig. 1B) (*11*). Nucleotide binding to the isolated B subunit of prokaryotic V-ATPase from *Thermus thermophilus* was not observed (*31*). On the other hand, the B subunit of eukaryotic V-ATPase from *Saccharomyces cerevisiae*

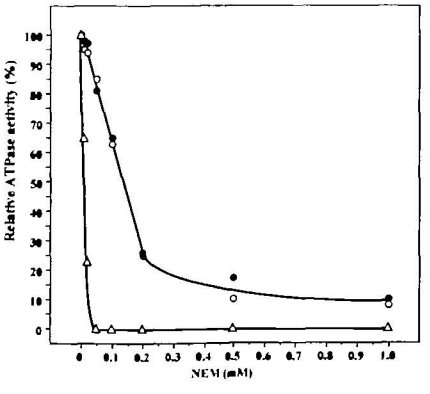

Fig. 2. **Sensitivity to NEM of the Na^+-ATPase activities of membrane vesicles from Ala-236 Cys mutant.** Membranes were prepared from each strain grown in NaTY medium containing 0.5 M NaCl. Assays were performed at pH 8.5 according to the method described previously (*21*). NEM was added 10 min prior to initiating the reaction. Symbols: ○, ATCC9790; ●, Nak1/pHEexA; △, Nak1/pHEexAA236C.

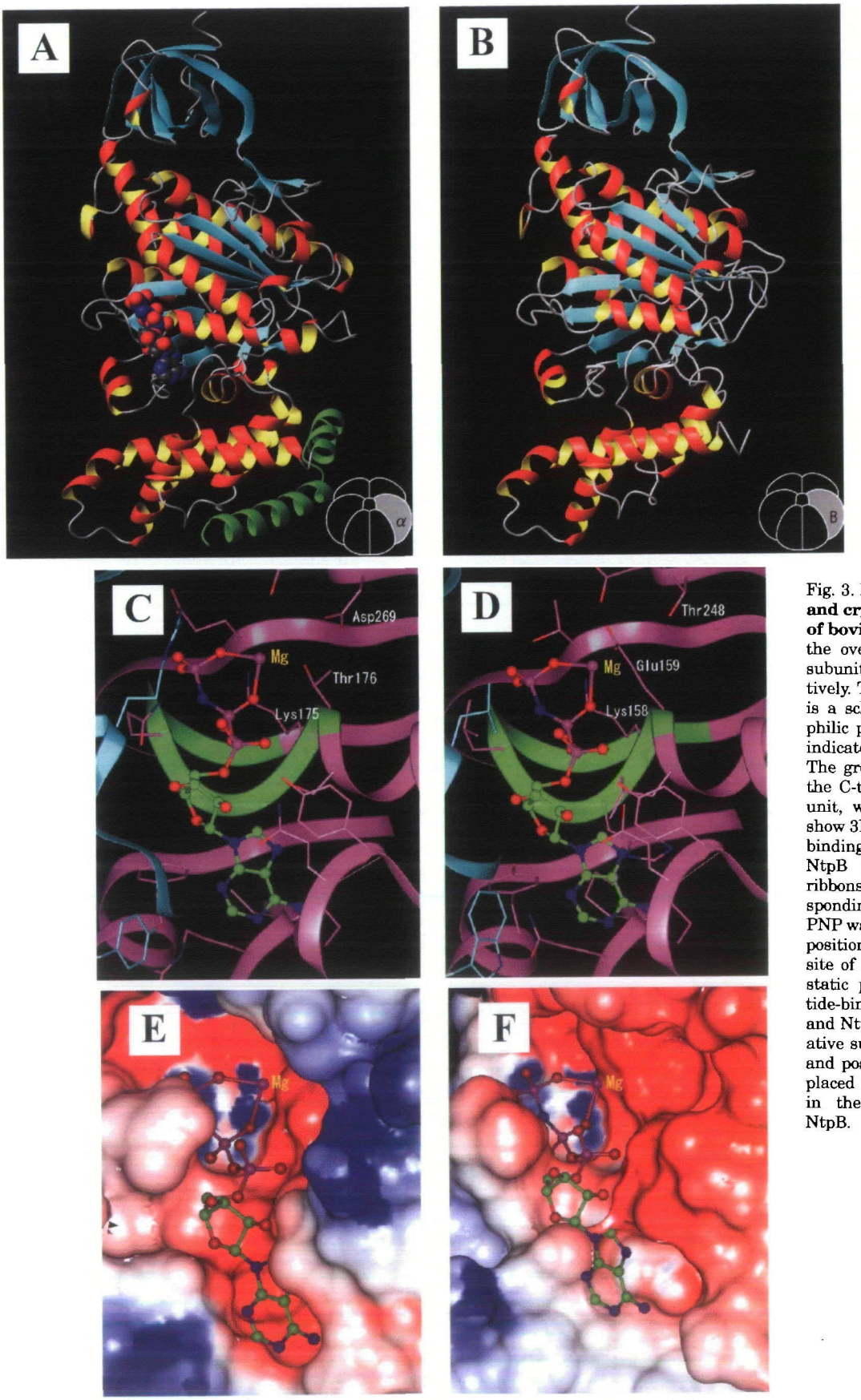

Fig. 3. **Modeled structure of NtpB and crystal structure of α subunit of bovine F_1-ATPase.** A and B show the overall 3D structures of the α subunit and NtpB subunit, respectively. The gray part of the diagram is a schematic model of the hydrophilic portion of these ATPases and indicates which subunit is depicted. The green ribbon in panel A shows the C-terminal region of the α subunit, which NtpB lacks. C and D show 3D structures of the nucleotide-binding sites of the α subunit and NtpB subunit, respectively. Green ribbons show the structures corresponding to the P-loop regions. AMP-PNP was placed at the corresponding position in the nucleotide-binding site of NtpB. E and F show electrostatic potential surfaces of nucleotide-binding sites of the α subunit and NtpB subunit, respectively. Negative surface charge is shown in red and positive in blue. AMP-PNP was placed at the corresponding position in the nucleotide-binding site of NtpB.

was shown to be modified by both 3′-*O*-(4-benzoyl)benzoyl-adenosine 5′-triphosphate (*32*) and 2-azido-ATP (*33*), and the purified recombinant B subunit could be labeled on irradiation in the presence of [α-^{32}P]ATP (*34*), suggesting that the B subunit of the eukaryotic V-ATPase has nucleotide-binding capability. The modeled structure of the sequence corresponding to the P-loop in NtpB was stable in energy minimization and closely matched that of the nucleotide-binding site of bovine F-ATPase α subunit. In studies of the α subunit of F_1-ATPase, it has been reported that three residues (BMFα: K175, T176, D269; shown by green boxes in Fig. 1B) are especially important for binding of a nucleotide and Mg^{2+} at the nucleotide-binding site (Figs. 1B and 3C) (*35*). K175 residue of the α subunit, the residue responsible for nucleotide binding, is conserved at the corresponding site (as K158; shown by yellow box in Fig. 1B) of the NtpB subunit. T176 and D269 residues, which are responsible for Mg^{2+} binding, are replaced by E159 and T248 at the corresponding sites (shown by yellow boxes in Fig. 1B and also see Fig. 3D). For protein function, the 3D structure rather than 1D sequence order is important. In Fig. 3, C and D, T176 and D269 of the α subunit structurally correspond to T248 and E159 of NtpB, respectively. Furthermore, the electrostatic potential surfaces of the nucleotide-binding sites of the α subunit and NtpB, especially the binding sites of Mg^{2+} and γ-phosphate of AMP-PNP, showed similar tendencies (Fig. 3, E and F). These characteristics suggest that a nucleotide binds to the corresponding nucleotide-binding site in NtpB as well as F-ATPase α subunit and yeast V-ATPase B subunit.

Nucleotide Binding to Purified V-ATPase—Figure 4 shows the ATP concentration dependence of the specific binding of nucleotide to purified ATPase. In this experiment, most of the added [α-^{32}P]ATP was hydrolyzed by the ATPase during incubation for 3 h at room temperature. Therefore, we think that the detected nucleotide-binding capacity was due to the binding of [α-^{32}P]ADP rather than [α-^{32}P]ATP. The Scatchard plot (Fig. 4, inset) of this data shows an intercept on the abscissa at 5.8, indicating that about 6 molecules (SD 5.7 ± 0.4) of nucleotide (ADP and/or ATP) bind per molecule of the enzyme. The slope of the Scatchard plot indicates that the dissociation constant ($K_{d(nucleotide)}$) is 17 μM (SD 18 ± 4 μM). This value is similar to the K_m value (20–40 μM) for ATP of the ATPase activity measured with ATP-regenerating system; in this system ADP seemed to competitively inhibit the ATPase activity with similarly high affinity, although we have not determined the precise K_i value for ADP (unpublished data). The amount of nucleotide binding per enzyme was similar to that of Na^+ binding to the enzyme (6 ± 1 Na^+ bound/enzyme molecule) (*16*).

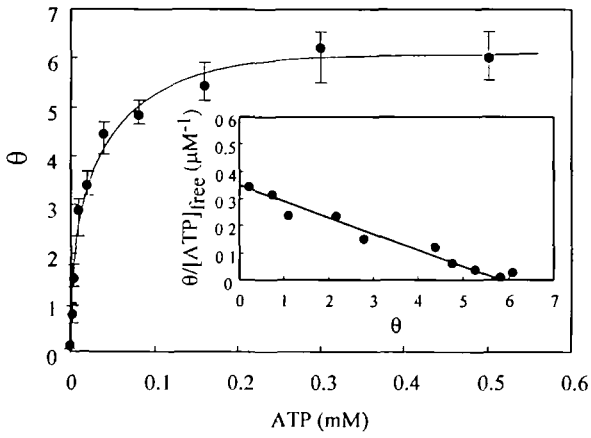

Fig. 4. **ATP concentration dependence of nucleotide binding to purified V-ATPase.** The inset shows the Scatchard plot of the specific binding of nucleotide ([α-^{32}P]ADP and/or [α-^{32}P]ATP) to the purified enzyme. θ is defined as the number of moles of bound nucleotide per mole of the enzyme. Each point is the averaged value of three experiments.

The ATPase has three catalytic and three non-catalytic subunits. Each catalytic subunit (NtpA) is thought to have one nucleotide-binding site. Therefore, the remaining three of the six bound nucleotides are likely to be bound to the three non-catalytic subunits (NtpB), which is consistent with the prediction from the modeled structure of NtpB. Thus, the models of these subunits were useful in providing an insight into the structure/function relationship of the protein. To elucidate the significance of this nucleotide binding, further experiments are necessary.

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