ATP-dependent Affinity Change of Na\(^+\)-binding Sites of V-ATPase*

V-type Na\(^+\)-ATPase of Enterococcus hirae binds about six (6 \pm 1) Na\(^+\) ions/enzyme molecule with a high affinity (Murata, T., Igarashi, K., Kakinuma, Y., and Yamato, I. (2000) J. Biol. Chem. 275, 13415–13419). After the addition of 5 mM ATP, the binding capacity dropped to about 2 (1.8 \pm 0.3) Na\(^+\) ions/enzyme molecule, returning to the initial value concomitant with the decrease of ATP hydrolysis rate. These findings suggest that the affinity of four of six Na\(^+\)-binding sites of the enzyme changes (lowers) in enzyme reaction. The ATP analogs (adenosine 5’-O-(3-thiotriphosphate) or 5’-adenylylimido-diphosphate), ADP, or aluminum fluoride that is postulated to trap ATPases at their transition state did not inhibit the Na\(^+\) binding capacity significantly. Therefore, the affinity decrease of Na\(^+\)-binding sites was unlikely to be due to ATP binding alone or at the transition state of ATP hydrolysis. In the presence of 5 mM ATP, the ATPase showed strong negative cooperativity (\(n_H = 0.16 \pm 0.03\)) for Na\(^+\) stimulation of ATPase activity. The Hill coefficient (\(n_H\)) increased to 1 in parallel to the decrease of ATP concentration in the reaction mixture. Thus, the ATP-dependent affinity change cooperatively occurs in continuous enzyme reaction.

Ion-transporting ATPases are divided into three types: P-ATPase, F-ATPase, and V-ATPase. P-ATPase is found in plasma membranes and is sequentially phosphorylated and dephosphorylated during the pumping cycle (1, 2). F-ATPase functions as an ATP synthase in mitochondria, chloroplasts, and oxidative bacteria (3). V-ATPase is a proton pump in acidic organelles, plasma membranes of eukaryotic cells (4), and bacteria (5). Because V-type and F-type ATPases resemble each other both structurally and functionally, it is accepted that the reaction mechanism and energy coupling mechanism of the two ATPases are similar (4, 6, 7).

The reaction mechanism of P-type ATPase has been studied and explained by affinity change of the ATPase; ions are bound tightly on the low concentration side of the membrane and a conformational change caused by high energy intermediate of ATP hydrolysis results in their exposure to the high concentration side. Because the ion binding affinity is lowered by the conformational change, they dissociate easily (1, 2, 8). The “rotation catalysis” mechanism of F-ATPase, proposed by P. Boyer (9), has now been verified; the energy of ATP hydrolysis is converted into the physical force in the form of rotation of the y subunit, with three ATP molecules being hydrolyzed/rotation (10–13). However, the mechanism of ion translocation by F-ATPase and V-ATPase is still not clear.

A V-type ATPase transports Na\(^+\) or Li\(^+\) in the eubacterium Enterococcus hirae (14). The enzyme, consisting of nine subunits encoded by a Na\(^+\)-responsive operon (designated ntp) (15–17), has been purified on a large scale using the cloned genes (18) and characterized biochemically (18, 19). The kinetics of Na\(^+\) binding to purified V-ATPase suggested that 6 \pm 1 Na\(^+\) ions bind per enzyme molecule with a single high affinity (\(K_d = 15 \pm 5 \mu M\)) (20). However, the apparent affinity (\(K_m\)) for Na\(^+\) in the enzyme reaction was not single but bimorphic; the \(K_m\) values for Na\(^+\) were estimated to be about 20 \mu M and 4 mM (18, 19). Taking advantage of Na\(^+\) binding by the V-ATPase, we further characterized the enzyme properties aiming to understand the above inconsistency and the ion-translocating mechanism of F- and V-type ATPases.

In this study, we report ATP-dependent affinity change of Na\(^+\)-binding sites of the V-ATPase in the catalytic reaction, suggesting that the reaction mechanism of the V-ATPase is explained by an affinity change of cation-binding sites similar to those of P-type ATPases. We discuss the reaction mechanism of the V-ATPase on the basis of our observation.

**MATERIALS AND METHODS**

**Strains and Culture**—The E. hirae strain 25Dp/Comp18, a mutant defective in the production of F$_{\mathrm{F}}$$_{\mathrm{F}}$-ATPase harboring a plasmid containing the whole Na\(^+\)-ATPase (ntr) operon (19) was used in this study. The cells were cultured at 37 °C in KTY medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% glucose, and 1% K$_2$HPO$_4$) containing 0.5 mM NaCl supplemented with 10 mg/ml erythromycin.

**Purification of Na\(^+\)-ATPase**—Purification of Na\(^+\)-ATPase was performed using anion exchange and gel filtration chromatographies as described previously (18).

**Measurement of Na\(^+\) Binding Capacity of Purified Enzyme**—The incubation mixture contained 0.6 \mu M purified enzyme and 20 \mu M 22NaCl (35,000 cpm/nmol) in 50 \mu l of buffer A (100 mM Tris-HCl, 5 mM MgCl$_2$, 20% glycerol, 0.02% n-dodecyl-b-d-maltoside, pH 8.5). The concentration of contaminating Na\(^+\) in the mixture before the addition of 22NaCl was measured by flame photometry. The contaminating Na\(^+\) was taken into account to estimate the total concentration of Na\(^+\) in the mixture. The mixture was incubated for 60 min at room temperature, which was sufficient to saturate the Na\(^+\)-binding sites of the purified enzyme. The free 22Na\(^+\) was quickly separated by the Dowex-50 method as described previously (20).

To examine the effect of ATP on Na\(^+\) binding of the enzyme, the reaction mixture contained 0.6 \mu M purified enzyme and 20 \mu M 22NaCl (35,000 cpm/nmol) in 0.5 ml of buffer A was incubated for 60 min at room temperature. The ATP hydrolysis reaction was started by the addition of ATP (final concentration, 5 mM) into the reaction mixture. The Na\(^+\) binding capacity was measured at various time intervals by separating the free 22Na\(^+\) as described above. At every time point,
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RESULTS

Effect of ATP on Na\(^+\) Binding—Na\(^+\) binding to the purified enzyme in the presence of 20 \(\mu\)M \(^{22}\)Na\(^+\) was 2.8 \pm 0.3 mol/mol enzyme. This specific binding of Na\(^+\) lowered to 0.9 \pm 0.2 mol/mol enzyme (about 32% of the initial value) 1 min after the addition of 5 mM ATP (Fig. 1). Then the ion binding increased depending on the decrease of ATP hydrolysis rate and increased to 2.5 \pm 0.3 mol/mol enzyme (about 90% of the initial value) after 15 min when the rate of ATP hydrolysis had decreased to less than 5% of the initial rate. This decrease of the rate was due to product inhibition by ADP, because ATPase activity in the presence of 1 mM ATP is strongly inhibited by 4 mM ADP (data not shown). These findings suggest that the affinity of Na\(^+\)-binding sites of the enzyme is lowered in the enzyme reaction. No decrease in Na\(^+\) binding was observed when 5 mM ATP analogs (AMP-PNP\(^3\) or ATP\(\gamma\)S) or 5 mM ADP replaced 5 mM ATP, although these compounds inhibited the ATPase activity (Table I). Therefore, the affinity change appeared not to be caused by ATP binding to the enzyme.

Na\(^+\) concentration dependence of the \(^{22}\)Na\(^+\) binding in the presence of ATP was examined (Fig. 2). The dissociation constant \((K_d)\) for Na\(^+\) and the number of moles of bound Na\(^+\)/mol of the enzyme were 20 \pm 4 and 1.8 \pm 0.3 \(\mu\)M, respectively (Fig. 2, inset). Purified Na\(^+\)-ATPase bound about six Na\(^+\) ions/enzyme molecule with a single high affinity (\(K_d = 15 \pm 5 \mu\)M) (20). This suggests that the affinity of about two-thirds of the binding sites for Na\(^+\) (about four sites) decreases in the enzyme reaction and that the affinity of the remaining one-third of the binding sites for Na\(^+\) (about two sites) does not change.

It is possible that the enzyme releases bound \(^{22}\)Na\(^+\) in the presence of ATP during the separation step in the column of Dowex-50 by centrifugation. However, the \(^{22}\)Na\(^+\) release also occurred in the column of Dowex-50 that was equilibrated with the buffer containing 20 \(\mu\)M \(^{22}\)Na\(^+\) and 5 mM ATP (data not shown), suggesting that the affinity actually changes in the presence of ATP.

Na\(^+\) Binding of the Aluminofluoride-inhibited Enzyme—Fluorolunate complexes have been shown to mimic the transition state of several ATPases such as F\(\gamma\)-ATPase (23), myosin (24), nitrogenase (25), and nucleoside-diphosphate kinase (26). Although ATPase activity of the purified V-ATPase treated with Mg\(^2+\)-ADP and aluminum fluoride was strongly inhibited (93% inhibition), Na\(^+\) binding capacity did not change significantly (12% inhibition) (Table I). If the aluminum fluoride traps the enzyme at its transition state of ATP hydrolysis as in the case of other ATPases (23–26), the above result indicates that the significant affinity change of Na\(^+\)-binding sites that was observed in the presence of ATP (Fig. 1) does not occur at the transition state.

Negative Cooperativity of Na\(^+\)-binding Sites—The decrease

![Graph](image)

**Fig. 1.** Effect of ATP on the Na\(^+\) binding to purified Na\(^+\)-ATPase. 5 mM ATP was added at time 0 (shown by an arrow) to the mixture containing purified Na\(^+\)-ATPase that was incubated with 20 \(\mu\)M \(^{22}\)Na\(^+\) as described under “Materials and Methods.” Specific binding of Na\(^+\) (□) and residual ATP concentration (○) at various time intervals were determined.

**Table I**

| Reagent                           | Na\(^+\) binding capacity | ATPase activity |
|-----------------------------------|---------------------------|-----------------|
|                                   | mol/mol enzyme            | \(\mu\)mol Pi/min/mg protein | %            |
| No addition                       | 2.8 \pm 0.3               | 25 \pm 1         | 100          |
| 5 mM AMP-PNP                      | 2.7 \pm 0.2               | 3 \pm 0.2        | 95           |
| 5 mM ATP-S                        | 2.7 \pm 0.3               | 0.5 \pm 0.1      | 98           |
| Mg\(^2+\) ADP-aluminium fluoride  | 2.8 \pm 0.3               | 13 \pm 0.6       | 100          |

\(^{1}\)The abbreviations used are: AMP-PNP, 5'-adenylylimido-diphosphate; ATP\(\gamma\)S, adenosine 5'-O-(3-thiotriphosphate).
of the Na\(^+\) binding capacity in the presence of ATP occurs at either the cytoplasmic side (binding site) or outer side (release site). Therefore, the Na\(^+\) dependence of the whole ATPase reaction was examined, which should show the enzymatic properties at the cytoplasmic side (binding site). Fig. 3A shows the effect of NaCl concentration on the initial rates of ATPase activity by the purified V-ATPase in the presence of 5 mM ATP using an ATP-regenerating system. The rate of ATP hydrolysis increased with increasing concentrations of Na\(^+\) until saturation was reached at 100 mM NaCl. Double reciprocal plots of the data indicate the presence of two \(K_m\) values for Na\(^+\): 20 \(\mu\)M (high affinity) and 4 mM (low affinity) (Fig. 3A, inset). These \(K_m\) values are similar to the two \(K_m\) values for Na\(^+\) (20 \(\mu\)M and 3 mM) of the reconstituted V\(_{0}\)V\(_{1}\)-liposomes (18). Analysis by Hill plot (Fig. 3B) demonstrated strong negative cooperativity (Hill coefficient; \(n_H = 0.16 \pm 0.03\) in the range of 1 mM NaCl (ln [Na\(^+\)] = -2) to 10 mM NaCl (ln [Na\(^+\)] = 2).

Fig. 4A shows the dependence of ATPase activity on Na\(^+\) concentration at various ATP concentrations in the presence of an ATP-regenerating system. At low concentrations of ATP (less than 20 \(\mu\)M ATP), ATPase activity reached saturation point at around 1 mM NaCl, and high \(K_m\) values (4 mM) were not observed in double reciprocal plots (data not shown). Fig. 4B shows Hill coefficients (\(n_H\)) at respective ATP concentrations in the range of 1 mM NaCl (ln [Na\(^+\)] = -2) to 10 mM NaCl (ln [Na\(^+\)] = 2). The Hill coefficient (\(n_H\)) increased to 1 in parallel with decrease of ATP concentration. This finding suggests two possibilities: (i) In addition to the high affinity Na\(^+\) -binding sites (\(K_m = 20 \mu\)M), the Na\(^+\)-ATPase also has low affinity Na\(^+\)-binding site(s) (\(K_m = 4\) mM) that do not function at low ATP concentration. (ii) All of the Na\(^+\)-binding sites of the enzyme are high affinity sites for Na\(^+\) at inactive state, and these affinities cooperatively lower during the active state in the presence of ATP. This negative cooperativity decreases in parallel with the decrease in the concentration of ATP. This latter possibility agrees with the result of ATP-dependent decrease of Na\(^+\) binding capacity (Fig. 1).

**DISCUSSION**

The number of bound Na\(^+\) ions, which was estimated as six Na\(^+\) ions/each molecule of V-ATPase, is consistent with the model that the V\(_{1}\) part of V-ATPase forms a hexameric rotor ring (27, 28). In this paper, we demonstrated that affinity of four of six Na\(^+\)-binding sites cooperatively decreased in the presence of high ATP concentrations, whereas the remaining two binding sites were not affected. The affinity decrease was not due to ATP binding alone nor at the transition state of ATP.
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hydrolysis because ATP analogs (AMP-PNP or ATPγS) or aluminum fluoride, which is postulated to trap ATPases at their transition state, did not inhibit the Na⁺ binding capacity significantly; 12% inhibition of Na⁺ binding by fluoroaluminate may correspond to a decreased affinity of one of six Na⁺-binding sites at the most, but the value is difficult to distinguish from the experimental error. Therefore, we consider that Na⁺-binding sites of the enzyme are all high affinity in the absence of ATP, but the affinity of four of six Na⁺-binding sites changes (lowers) cooperatively in the continuous enzyme reaction when ATP is being hydrolyzed and Na⁺ is transported, most likely at the cytoplasmic side (binding side).

How do we interpret this affinity change? Why does the affinity of Na⁺-binding sites decrease cooperatively in the continuous enzyme reaction? Although our observations do not indicate any properties of Na⁺-binding at the outer side (release side) of the enzyme, we believe that the operating principles of V-type and F-type ATPases should be similar to the “affinity change” model of P-type ATPases (see the second paragraph in the Introduction) (1,2,8). Here we propose an ion translocation mechanism in the V-ATPase based on the affinity change model and our structure model (29) (Fig. 5) as follows. NtpK protolipids form a rotor ring consisting of hexamers, each having one high affinity Na⁺-binding site (Kₚ = 15 ± 5 μM). The four Na⁺-binding sites of six are readily accessible from the cytoplasm, the other two sites are difficult to access because of the steric hindrance by NtpI (29). Our data are consistent with the one channel model that has been proposed for Propionigenium modestum F-type Na⁺-ATPase (7,30). Thus, NtpI is likely to have one channel, and Na⁺ ion is transported through the channel by rotating the proteolipid rotor (Fig. 5) (7,30). The NtpK rotor cannot rotate without Na⁺ even in the presence of ATP. Upon Na⁺ binding (Kₚ = 15 ± 5 μM) ATP is hydrolyzed, causing rotation of NtpK rotor. The energy of one ATP hydrolysis drives the rotor through a 120° rotation, and then the rotor stops according to the model of F-ATPase (7,30,31). We propose that a conformational change of NtpK caused by the rotation at the interface between NtpK and NtpI lowers the Na⁺ binding affinity to dissociate Na⁺ through the channel in NtpI. The conformation of the Na⁺-binding site returns to the original high affinity state by coming out from the interface after dissociation of Na⁺ to the outer side. Before this resumption of the native high affinity conformation, the empty binding sites remain at the low affinity state for Na⁺. During the continuous catalytic process (in the presence of high concentration of ATP and Na⁺), several Na⁺-binding sites in NtpK rotor remain at such low affinity state. We believe that this explains why the BSₘₐₓ of Na⁺ detected in the presence of high ATP concentration was about one-third of the BSₘₐₓ without ATP and why the affinity of Na⁺-binding sites cooperatively changed in the presence of high ATP concentration. At low ATP concentration, the rotation would be intermittent because binding of ATP to the ATP-binding site(s) should be the rate-limiting step. Because the low affinity state for Na⁺ can resume the high affinity state during such intermittent period, we have observed the resumption of the BSₘₐₓ and the cooperativity.

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