Mechanism Responsible for Oligomycin-induced Occlusion of Na\(^+\) within Na/K-ATPase*

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The mechanism whereby oligomycin occludes Na\(^+\) within Na/K-ATPase was investigated to study Na\(^+\) and K\(^+\) transport mechanisms. Oligomycin stimulated Na\(^+\) binding to Na/K-ATPase but inhibited Na-K and Na-Na exchange. The oligomycin concentration required to stimulate Na\(^+\) binding to half-maximal was 4.5 \(\mu\)M, which was close to the concentration that reduced Na-Na and Na-K exchange and ATPase activity to half-maximal, suggesting that Na/K-ATPase possesses an oligomycin binding site responsible for stimulating Na\(^+\) binding and reducing ion exchange and ATPase activity. In contrast, neither K\(^+\) binding nor K\(^+\) transport was affected by oligomycin. Limited tryptic digestion of Na/K-ATPase showed that, unlike Na\(^+\), K\(^+\), and ouabain, oligomycin treatment did not result in a specific digestion pattern. Oligomycin appeared to inhibit ouabain binding in a noncompetitive manner, whereas it did not affect ATP binding. Na/K-ATPase isoforms with low and high sensitivities to ouabain were equally sensitive to oligomycin. These results suggest that the oligomycin binding site is located on the extracellular side of Na/K-ATPase, at a different position from the ouabain binding site, and this antibiotic did not induce a conformational change of Na/K-ATPase. We propose that oligomycin interacts with the Na\(^+\) occlusion site from the extracellular side of Na/K-ATPase, which delays Na\(^+\) release to the extracellular side without inducing a conformational change, suggesting that the pathways responsible for Na\(^+\) and K\(^+\) transport differ.

Na/K-ATPase is an integral membrane protein responsible for the active transport of Na\(^+\) and K\(^+\) across the cell membrane using ATP as a driving force (1–3). This ATPase is composed of \(\alpha\)- and \(\beta\)-subunits in an equimolar ratio. The \(\alpha\)-subunit contains the phosphorylation site and binding sites for ATP, cardiac glycosides, and probably cations, which suggests that it plays a central role in ion transport and the ATP hydrolysis reaction, and the \(\beta\)-subunit is involved in stabilization of the \(\alpha\)-subunit in the plasma membrane (4, 5). According to the recently proposed Na/K-ATPase reaction scheme (6, 7), the enzyme is phosphorylated by Na\(^+\) and ATP, and the resulting phosphorylated intermediate with a high energy phosphate bond, Na\(^+\)E\(_2\)-P, occludes Na\(^+\) within its molecule. The transition of Na\(^+\)E\(_2\)-P to E\(_2\)-P by oligomycin binding to E\(_2\)-P (6). In our previous report (21, 22), we demonstrated that oligomycin increased the affinity of nonphosphorylated ATPase for Na\(^+\). Esmann and Skou (23) showed that oligomycin occluded Na\(^+\) within nonphosphorylated ATPase. These results showed clearly that oligomycin participated in the interactions of Na\(^+\) with both nonphosphorylated and phosphorylated ATPase. The mechanism whereby oligomycin promotes Na\(^+\) occlusion within Na/K-ATPase, however, is unclear, but if it is elucidated, this antibiotic will almost certainly be a very useful tool for the study of Na\(^+\) and K\(^+\) transport mechanisms.

This study was designed to investigate how oligomycin occludes Na\(^+\) within Na/K-ATPase, and we obtained the following results. Na/K-ATPase possesses a single oligomycin binding site, which is responsible for enhancing Na\(^+\) binding to the enzyme and inhibiting Na\(^+\) transport, ouabain binding, and ATPase activity but is not responsible for K\(^+\) binding and K\(^+\) transport. A tryptic digestion study showed that oligomycin did not result in Na/K-ATPase adopting a specific conformation. Oligomycin appeared to inhibit ouabain binding in a noncompetitive manner, and Na/K-ATPase isoforms with high and low sensitivities to ouabain were equally sensitive to oligomycin. These results suggest that oligomycin binds to a site different from the extracellular ouabain binding site but does not induce a conformational change of Na/K-ATPase. Therefore, we concluded that oligomycin occludes Na\(^+\) within Na/K-ATPase by interacting only with the Na\(^+\) occlusion site on the extracellular side without affecting the conformation of the enzyme.

EXPERIMENTAL PROCEDURES
Preparation of Na/K-ATPase

Microsomes were prepared from canine kidney outer medulla and treated with sodium deoxycholate, as described by Hayashi et al. (24). The ouabain-sensitive ATPase activity of the purified ATPase obtained was usually within the range of 10–15 \(\mu\)mol of P/min/mg protein. Na/K-ATPase from rat whole kidney, which was prepared using the same method, exhibited a specific activity of 2 \(\mu\)mol of P/min/mg. Canine Na/K-ATPase was used for the experiments in this study unless stated otherwise. The protein contents of the ATPase preparations were determined by the method of Lowry et al. (25) using bovine serum albumin as a standard.

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Ion Transport

Na-K and Na-Na Exchange—These experiments were carried out using the method of Garrahan and Glynn (10). Fresh citrated human blood samples were divided into two 1.5-ml portions; 50 μl of NaCl solution containing 185 kBq was added to one portion, which was stored at 4 °C for 1 or 2 days, after which, the erythrocytes were washed five times with choline buffer (151 mM choline chloride, 1 mM MgCl2, and 2.5 mM phosphate-Tris, pH 7.4), then mixed with this buffer to produce a 30–50% (v/v) suspension. Erythrocyte suspensions prepared in the same manner without radiolabeled NaCl were used to measure the NaCl concentration in erythrocytes with an atomic absorption spectrophotometer (Hitachi 208). Fifty μl of erythrocyte suspension with 22NaCl was mixed with 450 μl of Na solution (147 mM NaCl, 1 mM MgCl2, 11 mM glucose, and 2.5 mM phosphate-Tris, pH 7.4) or 450 μl of Na-K solution (150 mM NaCl, 6 mM KCl, 1 mM MgCl2, 11 mM glucose, and 2.5 mM phosphate-Tris, pH 7.4) containing 5 μl of 42KCl with 0–6.2 nmol of oligomycin and 5 μl of H2O with or without 50 nmol of ouabain for the Na-Na and Na-K exchange assays, respectively. Each reaction mixture (500 μl) was incubated at 37 °C for 60 min and centrifuged at 1600 × g for 4 min in a microcentrifuge (Tomy MC-150); then 450 μl of supernatant were transferred to a counting vial and mixed with 6 ml of Scintisol 500, and the radioactivity was measured using a liquid scintillation spectrophotometer (Aloka LSC-3050). The amount of each ion transport was specifically calculated from the difference between the radioactivities in the presence and absence of unlabelled excess ouabain. The amount of ouabain bound in the absence of oligomycin was taken as 100%, and the oligomycin concentration required to reduce this to half (I50) was determined for each ouabain concentration used.

Proteolytic Cleavage of Na/K-ATPase

Cleavage of Na/K-ATPase by trypsin was carried out using the method of Jørgensen and Farley (30). Thirty μg of Na/K-ATPase were incubated in a 24-μl reaction mixture composed of 5 nmol of EDTA, 750 nmol of imidazole-HCl, pH 7.5, and 0.6 μl of ET0H with or without 3.73 nmol of oligomycin for 5–10 min at room temperature. Various concentrations of NaCl, KCl, or ouabain were added, followed by 0.3 μg of N-tosyl-l-phenylalanyl chloromethyl ketone-trypsin (20 μl), and then incubated at 37 °C for 15 min. The reactions were stopped by adding 0.6 μg of soybean trypsin inhibitor; the reaction products were subjected to SDS-polyacrylamide gel electrophoresis with a 10.5% (w/v) separating gel using the method of Laemmli (31), and the gel was stained with Coomassie Brilliant Blue.

Fluorescence Measurement

Twenty μg of Na/K-ATPase were suspended in 2 ml of 10 mM imidazole-HCl, pH 8.0, which was stirred and kept at 25 °C during the measurement. The fluorescence was measured by excitation at 295 nm and emission at 325 nm (32), slitwidth 10 nm both for excitation and emission, and response time of 0.1 s on a fluorescence spectrophotometer (Hitachi F-2000). When the effects of various ligands were examined, 4 μl of 5 mM NaCl, 8 μl of 3 mM KCl, 4 μl of 0.5 μM strophedalin in a solution of N,N-dimethylformamide, and 4 μl of 2.5 mg/ml oligomycin in ET0H were added to give final concentrations of 10, 12, and 15 μM, respectively.

Measurement of Na/K-ATPase Activity

Two μg of canine Na/K-ATPase or 5 μg of rat Na/K-ATPase were preincubated in a 450-μl reaction mixture composed of 50 μmol of NaCl, 5 μmol of KCl, 2 μmol of MgCl2, 0.5 μmol of EDTA, and 25 μmol of imidazole-HCl, pH 7.4, with 5 μl of ET0H containing 0–18.6 nmol oligomycin at 37 °C for 5–10 min. The ATP hydrolysis reaction was started by adding 50 μl of 20 mM ATP, followed by incubation at 37 °C for 15 min, and the amount of Pi liberated was measured using the method of Fiske and Subbarow (33). Ouabain-insensitive ATPase activity was measured using the same reaction mixture, except NaCl and KCl were omitted.

Materials

Oligomycin B and soybean trypsin inhibitor were purchased from Sigma; N-tosyl-l-phenylalanyl chloromethyl ketone-trypsin was from Merck; 22NaCl, 62RbCl, and [3H]ATP were from Amersham Corp.; [3H]ouabain was from DuPont NEN; and 42K was obtained from the 42Ar-42K generator developed by Dr. Morinaga, as described previously (21). The protein molecular mass markers were purchased from TEFICO Co. Ltd. (Tokyo, Japan).

RESULTS

The effects of oligomycin on the transport and binding of Na+ and K+ and Na/K-ATPase activity were estimated and compared.

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1 The abbreviations used are: TEA, triethanolamine; AMP-PNP, adenylylimido diphosphate; I50, the oligomycin concentration required to reduce ligand binding and transport by one-half; P0.5, the oligomycin concentration that stimulated Na+ binding to half-maximal; H, transmembrane segment.
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Effects of Oligomycin on Na-K, Na-Na, and K-K Exchange—The Na-K, Na-Na, and K-K exchange activities of erythrocytes and their ghosts were measured in the presence of various concentrations of oligomycin (Fig. 1). The Na-Na and Na-K exchange activities declined as the oligomycin concentration increased. The \( I_{50} \) value of oligomycin for Na-Na exchange was 1.4 \( \mu M \), which was close to the \( I_{50} \) values of 1.2 and 2.2 \( \mu M \) for Na-K exchange and ATPase activity (Fig. 8), respectively. However, the K-K exchange activity was affected little by oligomycin, even when its concentration was increased up to its maximal solubility was reached. These results show that Na/K-ATPase possesses a single oligomycin binding site responsible for inhibiting Na\(^+\) transport and ATP hydrolytic activity and that oligomycin only inhibited Na-Na exchange, which consequently reduced Na-K exchange.

Effects of Oligomycin on Na\(^+\) and K\(^+\) Binding to Na/K-ATPase—The effects of oligomycin on Na\(^+\) and K\(^+\) binding to purified and nonphosphorylated Na/K-ATPase were examined in the presence of 0.1 mM Na\(^+\) or 1 \( \mu M \) K\(^+\), respectively (Fig. 2). These concentrations were lower than the apparent dissociation constants \( (K_{d}) \) for Na\(^+\) and K\(^+\) binding, which were determined as described in our previous report (21) and in Fig. 3 in this report, respectively. These low ion concentrations were selected to enable the effects of oligomycin on Na\(^+\) and K\(^+\) binding to be detected easily. As the concentration of oligomycin increased, the amount of Na\(^+\) bound to Na/K-ATPase increased up to a maximum of 6-fold. The oligomycin concentration that stimulated Na\(^+\) binding to half-maximal \( (K_{50}) \) was 4.5 \( \mu M \), which was close to the \( I_{50} \) values for Na-Na and Na-K exchange and ATPase activity, confirming that oligomycin binding to its specific site on Na/K-ATPase enhanced Na\(^+\) binding as well as the inhibiting Na\(^+\) transport and ATPase activity.

In contrast to its effects on Na\(^+\) binding, oligomycin did not affect K\(^+\) binding. Binding curves for Rb\(^+\), a K\(^+\) congener, in the absence and presence of oligomycin are shown in Fig. 3. The apparent \( K_{d} \) values for Rb\(^+\) binding were 5.5 and 4.0 \( \mu M \) in the presence and absence of oligomycin, respectively. This result contrasted considerably with the Na\(^+\) binding results in which...
the apparent $K_d$ value declined to one-sixth of the control value after adding oligomycin, as shown previously (21). The effect of oligomycin on $K^+$ binding was examined in the presence of Mg$^{2+}$ in detail (Fig. 4). The addition of Na$^+$, ATP, adenyl-limido diphosphate (AMP-PNP, an unhydrolyzed ATP analogue) or Na$^+$ + AMP-PNP, under which conditions formation of various nonphosphorylated intermediates occurs (Fig. 4, lanes 3–6 and 9–11), and the addition of Na$^+$ + ATP or P$_i$, under which conditions formation of phosphorylated intermediate (E$_2$P) occurs (34), affected $K^+$ binding to some extent. In these conditions, oligomycin significantly reduced $K^+$ binding only when Na$^+$ was present. As Na$^+$ binding is stimulated by oligomycin and competes with K$^+$ binding (21, 22), the present data indicate that oligomycin amplified the inhibitory effect of Na$^+$ on K$^+$ binding. In other words, oligomycin did not affect K$^+$ binding to various intermediates without Na$^+$. 

**Proteolytic Cleavage of Na/K-ATPase in the Presence of Oligomycin—**If oligomycin affects ion transport, ion binding, and ATPase activity by inducing a conformational change of the enzyme, treatment with this antibiotic would be expected to result in Na/K-ATPase adopting a specific conformational state. To explore this possibility, purified Na/K-ATPase was partially cleaved by trypsin in the presence of Na$^+$, K$^+$, ouabain, or oligomycin, as described by Jørgensen and Farley (30). In the presence of 0.12 mM oligomycin, the cleavage pattern was the same as that with buffer alone (Fig. 5, lanes 1, 2, 16, and 17) and in the presence of 0.1 mM NaCl (Fig. 5, lanes 3 and 4). As the concentration of NaCl increased, a 40-kDa peptide appeared (Fig. 5, lanes 3, 5, and 7), and when oligomycin and Na$^+$ were added to the reaction mixture, the 40-kDa peptide band was much clearer than in the absence of oligomycin (Fig. 5, lanes 3–8), supporting the result that oligomycin increases the affinity of Na/K-ATPase for Na$^+$. In the presence of K$^+$, at least five peptides were observed in the 35- to 50-kDa range (Fig. 5, lanes 9, 11, and 13). When oligomycin was added with K$^+$, the cleavage patterns did not change (Fig. 5, lanes 9–14), the patterns in the presence of ouabain were similar to those with K$^+$ (Fig. 5, lanes 9, 11, 13, 18, 20, and 22), and the addition of oligomycin did not affect the cleavage patterns in the presence of ouabain (Fig. 5, lanes 18–23). These results showed that oligomycin did not induce the conformation of Na/K-ATPase distinguishable from ones induced by Na$^+$, K$^+$, and ouabain and did not disturb the conformations induced by Na$^+$, K$^+$, and ouabain. Therefore, it appeared that this antibiotic influenced neither ion transport, ion binding, nor ATPase activity by inducing a conformational change of Na/K-ATPase.

**Measurement of Fluorescence Intensity—**We examined the effect of oligomycin on the fluorescence intensity derived from the intrinsic tryptophan residues, a technique often used to monitor enzyme conformational changes (15, 23). The changes in fluorescence intensity by NaCl and KCl were canceled out by strophanthidin (data not shown). Although the addition of oligomycin reduced the fluorescence intensity, this change was not canceled out by other ligands, such as ouabain, an oligomycin competitor (data not shown). Therefore, we could not establish whether this oligomycin-induced change in the fluorescence intensity reflected a specific conformational change of Na/K-ATPase.

**Effects of Oligomycin on ATP and Ouabain Binding to Na/K-ATPase—**The high affinity ATP binding site on Na/K-ATPase was located on the cytoplasmic side (35), and ATP shifts the ATPase conformation to the E$_1$ form (2). The effect of oligomycin on ATP binding to purified Na/K-ATPase was meas-
A different amino acid sequence between H1 and H2. We determined whether oligomycin inhibited ouabain binding competitively or noncompetitively using intact erythrocyte Na/K-ATPase (Fig. 5). When the addition of oligomycin did not inhibit ATP binding to the cytoplasmic side of the α-subunit. Enhancement of Na\(^{+}\) binding by oligomycin was affected by adding ATP to the binding mixture prior to oligomycin (data not shown). Therefore, it is not essential that Na/K-ATPase is in the E1\(_{\alpha}\)-ATP form for oligomycin to enhance Na\(^{+}\) binding.

The ouabain binding site is known to be located on the extracellular surface of Na/K-ATPase (1); therefore, we investigated whether oligomycin inhibited ouabain binding competitively or noncompetitively using intact erythrocyte Na/K-ATPase (Fig. 7). When the I\(_{50}\) of oligomycin for ouabain was close to that for Na-K exchange (39), the inhibition of Na-K exchange was close to that for Na-K exchange and ATPase activity (18–20), K\(^{+}\)-dependent phosphatase activity (18–20), and ATPase activity and its stimulation curve for Na\(^{+}\) binding were monophasic (Figs. 1, 2, and 8). The I\(_{50}\) values for ion exchange and ATPase activity and the K\(_{0.5}\) value for Na\(^{+}\) binding lay within the narrow range of 1.2–4.5 μM, as did the assumption K\(_{0.5}\) of 2 μM for oligomycin binding derived from the curve shown in Fig. 7. These results suggest strongly that Na/K-ATPase possesses an oligomycin binding site responsible for enhancing Na\(^{+}\) binding and inhibiting Na\(^{+}\) transport and ATPase activity and that its affinity for oligomycin binding was essentially unaffected by the phosphorylation of the enzyme.

Oligomycin had little effect on K-K exchange (39)(Fig. 1), K\(^{+}\)-dependent phosphatase activity (18–20), K\(^{+}\) binding (Figs. 2, 3, and 4), the transition rate of K-E\(_{2}\) to Na-E\(_{1}\) (40), or the fluorescence intensity of the K-E\(_{2}\)-N-lp-[2-benzimidazolylphenyl] maleimide complex (15). Two possible explanations for these findings are that oligomycin does not bind to Na/K-ATPase in the presence of K\(^{+}\) or that it does not interact with K\(^{+}\) bound to the enzyme. Fig. 1 shows that the I\(_{50}\) value of oligomycin for Na-Na exchange was close to that for Na-K exchange and, therefore, that adding 6 mM KCl to the Na-K exchange assay reaction mixture (see "Experimental Procedures") did not disturb the oligomycin binding. The addition of oligomycin to the reaction mixture containing Na\(^{+}\), Mg\(^{2+}\), and ATP markedly reduced K\(^{+}\) binding to the ATPase (Fig. 4). As

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M. Taguchi, H. Homareda, and H. Matsui, unpublished data.

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**Fig. 6. Effect of oligomycin on ATP binding to Na/K-ATPase.** The reaction mixture (100 μl) was composed of 20 μg of Na/K-ATPase, 1% (v/v) EtOH, 0–37.3 μM oligomycin, 0.5 μM [\(^{3}\)H]ATP, 1 mM EDTA, and 50 mM TEA-HCl, pH 7.4, with or without 0.5 mM ATP. The ATPase was incubated with or without unlabelled ATP in the presence of EDTA and TEA-HCl on ice for 5–10 min. Five to 10 min after adding EtOH with oligomycin, [\(^{3}\)H]ATP was added to the reaction mixture. The assay procedure is described in the legend to Fig. 2.

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**Fig. 7. Inhibition of ouabain binding by oligomycin.** The reaction mixture (100 μl) was composed of 10% (v/v) erythrocytes, 1% (v/v) EtOH, 0–12.4 μM oligomycin, 2–30 nM [\(^{3}\)H]ouabain, 150 mM NaCl, 1 mM MgCl\(_{2}\), 10 mM glucose, and 2.5 mM Na\(_{2}\)HPO\(_{4}\)-HCl, pH 7.4, in the presence and absence of 0.1 mM ouabain. The oligomycin concentrations required to reduce the binding to half-maximal were determined and plotted against the ouabain concentrations used; ●, means of four determinations (bars, S.D.).
oligomycin enhanced Na\(^+\) binding to Na/K-ATPase (Figs. 2–4), this result suggested that K\(^+\) bound to E\(_2\)-P formed in the presence of Na\(^+\), Mg\(^{2+}\), and ATP, and then, oligomycin shifted K\(_2\)-E\(_1\)-P to Na\(_2\)-E\(_1\)-P. Yoda and Yoda (41) demonstrated that oligomycin reduced the rate of K\(^+\)-dependent dephosphorylation of E\(_2\)-P formed in the presence of Na\(^+\), Mg\(^{2+}\), and ATP. Their observation also suggests that oligomycin binds to K\(_2\)-E\(_2\)-P and shifts the following equilibrium Na\(_2\)-E\(_2\)-P polymycin ⇔ K\(_2\)-polymycin ⇔ K\(_2\)-oligomycin + Pi to the left. In our previous report (22), we showed that the Na\(^+\) titration curve of K\(^+\) binding was shifted toward low Na\(^+\) concentrations by adding oligomycin. As the two titration were virtually superimposable, this result suggests that the affinity of Na/K-ATPase for oligomycin is affected little by Na\(^+\) and K\(^+\), whereas oligomycin enhances the affinity of the enzyme for Na\(^+\). Overall, our data strongly suggest the latter possibility. We have tried to measure specific oligomycin binding to Na/K-ATPase and permeation of oligomycin into erythrocytes by preparing \(\text{H}^\text{3}\)-labeled oligomycin, so far without success, because the specific binding and uptake of oligomycin cannot be distinguished from its high level of nonspecific binding due to its high hydrophobicity.

Oligomycin had little effect on ATP binding to the cytoplasmic side of the \(\alpha\)-subunit but inhibited ouabain binding to its extracellular side (Figs. 6 and 7). These results are consistent with the conclusion of Cornelius and Skou (11) and Halperin and Cornelius (42) that oligomycin exerts its inhibitory effect on Na\(^+\) transport from the extracellular side of Na/K-ATPase. However, oligomycin did not seem to inhibit ouabain binding in a simple competitive manner (Fig. 7), it inhibited two Na/K-ATPase isoforms with different sensitivities to ouabain to the same extent, showing identical I\(_{50}\) values (Fig. 8), and the structure of ouabain is markedly different from that of oligomycin (43). Hegyvary (13) showed that ouabain bound to the E\(_2\)-polymycin complex. Taken together, these results suggest that the oligomycin and ouabain binding sites are different. The domain between H1 and H2 of the \(\alpha\)-subunit is the ouabain binding site (37, 38), and Lemas et al. (44) demonstrated that the domain between H7 and H8 of the \(\alpha\)-subunit is essential for assembly with the \(\beta\)-subunit. Therefore, the oligomycin binding site may be located on the extracellular side of Na/K-ATPase in H3-H6 of its \(\alpha\)-subunit.

The Effect of Oligomycin on Conformational Change—We observed the effects of limited tryptic digestion of Na/K-ATPase in the presence of Na\(^+\), K\(^+\), and ouabain with and without oligomycin (Fig. 5). Oligomycin did not result in the enzyme adopting a conformation distinguishable from those in the presence of Na\(^+\), K\(^+\), or ouabain, nor did it disturb the specific conformations formed in their presence. Because the peptide bonds cleaved by limited tryptic digestion are located in the cytoplasmic domain of the \(\alpha\)-subunit (45), this result suggests that the conformation of the \(\alpha\)-subunit is changed little by oligomycin. We could not confirm that the change in fluorescence intensity induced by oligomycin reflected an oligomycin-dependent conformational state. Studies using extrinsic fluorescent probes (15, 23) appeared that oligomycin did not change the fluorescence intensity directly but did so by changing the affinity of ATPase for Na\(^+\). Therefore, the enhancement of Na\(^+\) binding and inhibition of ouabain binding, Na\(^+\) transport, and ATPase activity by oligomycin does appear to be attributable to oligomycin-induced conformational change.

A Possible Model of Na\(^+\) and K\(^+\) Transport—It is believed that Na\(^+\) binds to the internal high affinity Na\(^+\) site and then is released from the external low affinity Na\(^+\) site during an ATP hydrolysis cycle catalyzed by Na/K-ATPase (1–3, 6, 7). However, it is unclear whether the internal Na\(^+\) site is located at a position distinct from the external site or they share a site in the transmembrane and whether Na\(^+\) and K\(^+\) are antiported through common or separate pathways. Mutagenesis studies using Na/K-ATPase mutants have been expected to resolve this problem. Several amino acids with negatively charged or oxygen-containing side chains in transmembrane segments were picked up as candidates for cation selectivity and cation binding (46–49). These studies, however, often yielded results that were not easily explained. For example, a mutant in which Glu781 was substituted by Ala exhibited higher Na\(^+\)-ATPase activity than the wild-type enzyme, whereas mutants with Asp and Lys substituents exhibited no Na\(^+\)-ATPase activity, and all
the mutants increased the affinity of the enzyme for ATP, even though Glu781 was embedded in H5. It is generally unclear whether the candidate amino acids are implicated directly or indirectly in cation binding, because their locations on the three-dimensional structure of the enzyme are unknown. Mutagenesis, therefore, may be useful but is not a definitive technique.

An unique characteristic of oligomycin, stimulation only of Na\(^+\) occlusion within the Na/K-ATPase molecule, suggests that oligomycin only interacts with the Na\(^+\) occlusion site of this enzyme. Recently, Ishii et al. (50, 51) demonstrated that the Na\(^+\) and K\(^+\) sensors were located in the N- and C-terminal regions of the α-subunit, respectively. On the other hand, the inhibition of K\(^+\) binding by K congeners, e.g. Rb\(^+\) and Cs\(^+\), was due to simple competition, whereas its inhibition by Na\(^+\) was not (22). The substitution of some of the candidate amino acids by another amino acid affected the affinities of ATPase for Na\(^+\) and K\(^+\) to different extents (46–49). Therefore, the data available led us to conclude that the Na\(^+\) and K\(^+\) occlusion sites interact but do not share a common site. Examination of the three-dimensional structure of oligomycin B showed that it has a protruding structure attached to a circular structure (43) and the protruding portion, the length and width of which are around 7Å and 2Å, respectively, appears favorable for interacting with the Na\(^+\) occlusion site of Na/K-ATPase embedded in the transmembrane segments. Chandler et al. (52) proposed that Na\(^+\) was transported in the intrahelical space of transmembrane segments, the diameter of which is around 2Å, whereas K\(^+\) was transported in the interhelical space between the helices, which appears compatible with our conclusions. Consequently, we propose the following model. Oligomycin interacts only with the Na\(^+\) occlusion site in the transmembrane segments from the extracellular side of Na/K-ATPase, thereby delaying the release of Na\(^+\) to the extracellular side, as shown in Fig. 9.

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