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Sodium or Lithium Ion-Binding-Induced Structural Changes in the K-Ring of V-ATPase from *Enterococcus hirae* Revealed by ATR-FTIR Spectroscopy

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Supporting Information

ABSTRACT: V-ATPase from *Enterococcus hirae* forms a large supramolecular protein complex (total molecular weight ~700 000) and physiologically transports Na^+ and Li^+ across a hydrophobic lipid bilayer. Stabilization of these cations in the binding site has been discussed on the basis of X-ray crystal structures of a membrane-embedded domain, the K-ring (Na^+- and Li^+-bound forms). Here, sodium or lithium ion-binding-induced difference IR spectra of the intact V-ATPase have for the first time been measured at physiological temperature under a sufficient amount of hydration. The results suggest that sodium or lithium ion binding induces the deprotonation of Glu139, a hydrogen-bonding change in the tyrosine residue, and a small conformational change in the K-ring. These structural changes, especially the deprotonation of Glu139, are considered to be important for reducing energetic barriers to the transport of cations through the membrane.

V-ATPases couple ion movement with ATP hydrolysis, and their structure and mechanism resemble those of F-ATPases.[1] V-ATPases have a globular catalytic domain, V_1 (equivalent to F_1), where ATP is hydrolyzed. This domain is attached by central and peripheral stalks to the intrinsic membrane domain, V_o (equivalent to F_o), which pumps ions across the membrane. V-ATPase from *Enterococcus hirae* physiologically transports Na^+ and Li^+.[2] This enzyme is encoded by nine *ntp* genes (*ntp*FIKECGABD) organized in the *ntp* operon.[3] The amino acid sequences of NtpF, -I, -K, -E, -C, -G, -A, -B, and -D were homologous with those of subunits G, a, c, E, d, F, A, B, and D of eukaryotic V-ATPases, respectively.[4] The V_1 domain responsible for ATP-driven rotation consists of the NtpA, -B, -C, -D, -E, and -G subunits (Figure 1A). In V_1, the three A subunits and the three B subunits are arranged alternately around a central D subunit. The V_o domain, which utilizes the rotation of V_1 for the transport of Na^+ (or Li^+), is composed of oligomers of 16 kDa NtpK, which form a membrane rotor ring (the K-ring), and a single copy of the NtpI subunit. The $NtpA_3B_3D$ complex and the V_o domain are connected by central subunits NtpG and -C of V_1 and peripheral stalks composed of subunits NtpF and -E.[5] In 2005, the crystal structure of the Na^+-bound K-ring was reported as the first high-resolution ring structure[6] (Figure 1B,C). Recently, the crystal structure of the Li^+-bound K-ring (Figure 1D) was also reported.[7] The overall structure is almost identical to that of the Na^+-bound form except

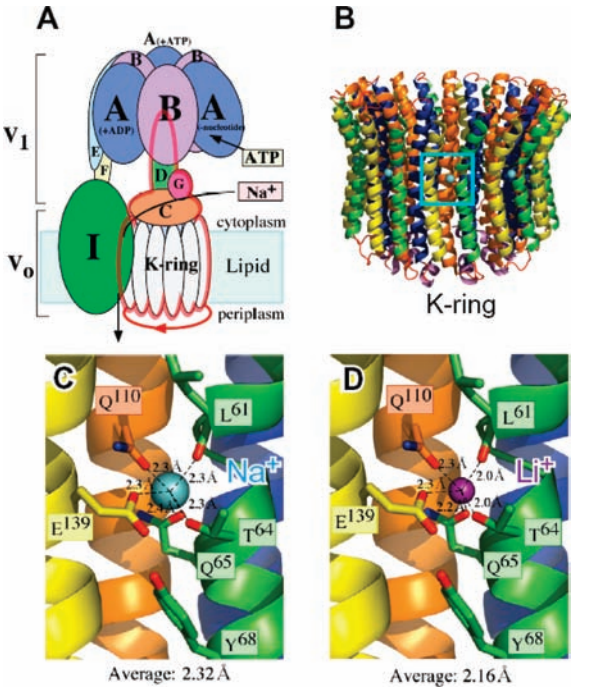

Figure 1. (A) Schematic structure of the entire V-ATPase complex. (B) X-ray crystal structure of the K-ring, which consists of 10 NtpK rotor proteins. (C, D) Close-up views of the ion-binding site of the K-ring: (C) Na^+-bound form; (D) Li^+-bound form.

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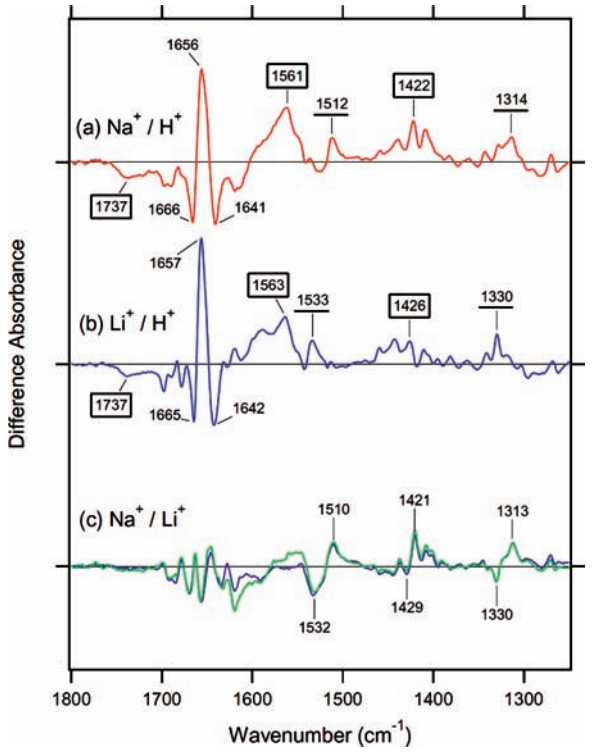

Figure 2. Ion-binding-induced difference IR spectra of the entire V-ATPase complex. (a, b) Difference spectra were recorded in the presence and absence of (a) 1 mM NaCl (red) or (b) 1 mM LiCl (blue). (c) Comparison of the calculated spectrum obtained as (a) − (b) (green) and the experimentally obtained difference spectrum recorded with 1 mM NaCl and LiCl (purple). One division of the *y* axis corresponds to 0.0015 absorbance unit.

around the ion binding site. The ion transport mechanism has been discussed on the basis of these structures; however, X-ray crystal structures lack information on hydrogen atoms. Therefore, precise structural differences such as the protonation states of carboxylate residues and the hydrogen-bonding structures of amino acid side chains remain unclear.

Perfusion-induced difference FTIR spectroscopy is a powerful tool for investigating structural changes in proteins upon ion or ligand binding.[8,9] The samples were physically adsorbed on an attenuated total reflection (ATR) crystal and soaked in a buffer solution containing ions at a concentration suitable for the investigation of the ion binding site (depending on the K_D values) (Figure S1 in the Supporting Information). Difference spectra calculated from the spectra recorded in the presence and absence of the ion showed spectral changes in the protein upon ion binding. Recently, we reported the ion-binding-induced difference spectra of *pharaonis* sensory rhodopsin II (*p*SRII, also called *pharaonis* phoborhodopsin, *p*pR),[10] which has a chloride ion binding site, and a subunit of flagellar motor protein, the PomA−PomB complex,[11] which has a sodium ion binding site. These results showed that the protonation states of the carboxylate residues in the binding site (Asp193 in *p*SRII and Asp24 in the PomB subunit) depend on the presence of the ions. In the present work, we applied perfusion-induced difference ATR-FTIR spectroscopy to a much more complicated protein complex, V-ATPase (total molecular weight ~700 000), the largest protein complex to which this technique has been applied to date.

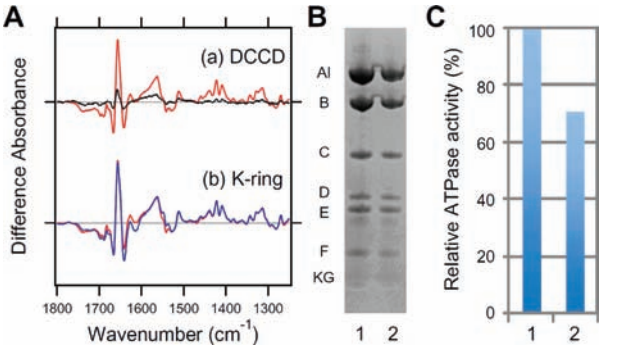

Figure 3. (A) Ion-binding-induced difference IR spectra of the entire V-ATPase complex (a) after DCCD treatment (black) and (b) of the K-ring alone (blue). The red-colored spectra were reproduced from Figure 2a. (B) SDS-PAGE before and after the ATR-FTIR experiment. (C) Na^+-dependent ATPase activity before and after the ATR-FTIR experiment.

Figure 2 shows (a) sodium and (b) lithium ion-binding-induced difference IR spectra measured in a buffer [20 mM Bis-Tris (pH 6), 2.5 mM $MgSO_4$, 8% glycerol] containing 1 mM NaCl or LiCl against the buffer without these salts as reference. The K_D values reported for Na^+ [$K_{D(Na^+)}$] are 15 μM for the entire complex[12] and 12 μM for the K-ring.[7] Therefore, the spectral changes are considered to be caused by the binding of ions to the binding site. In fact, a similar Na^+-binding spectrum was obtained at 100 μM NaCl (Figure S2). The Li^+ affinity for the K-ring was examined by an inhibition assay, and its K_i value was estimated to be 48 μM.[7] The concentration of LiCl (1 mM) was also sufficiently high. The difference spectra between the sodium and lithium ion-binding forms were also measured and compared with the spectrum calculated by subtracting the lithium ion spectrum (Figure 2b) from the sodium ion spectrum (Figure 2a). The spectra were very similar to each other, which indicates that the negative sides in Figure 2a,b were correctly normalized and completely canceled out by the subtraction.

The negative bands at 1737 cm^{-1} in Figure 2a,b correspond to the characteristic frequency of the C═O stretching vibration of a protonated carboxyl group of Asp or Glu. On the other hand, the positive bands at 1561 and 1422 cm^{-1} (Figure 2a) and at 1563 and 1426 cm^{-1} (Figure 2b) correspond to the characteristic frequency of antisymmetric and symmetric COO^- stretching vibrations of carboxylate groups. The difference spectrum was reduced after the reaction with *N*,*N*′-dicyclohexylcarbodiimide (DCCD) [Figure 3A(a), black], which binds to Glu139 in the K-ring and significantly decreases the value of $K_{D(Na^+)}$.[12] Moreover, the K-ring alone produced an almost identical difference spectrum [Figure 3A(b), blue]. In the K-ring, there are two aspartate and four glutamate residues (Asp3, Glu39, Glu50, Asp83, Glu126, and Glu139). Among them, only Glu139 constitutes the sodium ion binding site. The other binding sites in the aqueous phase are on the cytoplasmic surface (Glu50 and Glu126), on the extracellular surface (Asp3 and Asp83), and in the inner ring (Glu39). These residues are easily accessible to bulk water at pH 6 and are always deprotonated independently of the presence of sodium or lithium ions. Therefore, these bands could reasonably be considered to originate from Glu139 residues in the K-ring.

The frequency difference between the antisymmetric and symmetric stretches of carboxylate depends on the interaction with the cation.[13] A difference larger than 200 cm^{-1} indicates a

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monodentate structure, where only one C−O group interacts with the cation and the other is free. The differences in the Na^+- and Li^+-bound forms, 139 and 137 cm^{-1}, respectively, are less than the criterion. According to the literature,[13] the carboxylate group interacts with cations in the pseudobridging structure, where one C−O group interacts with a cation and the other interacts with water or another OH group. This is consistent with the X-ray crystal structures (Figure 1C,D).

The bands at 1512 and 1314 cm^{-1} (Figure 2a) and at 1563 and 1330 cm^{-1} (Figure 2b) exhibit frequency changes that depend upon the binding of sodium and lithium ions. These modes are characteristic of phenol ring stretching and phenol C−O stretching modes of tyrosine residues.[14] The frequency shift is more pronounced in the difference spectrum shown in Figure 2c (1510/1532 and 1313/1330 cm^{-1}). The most probable candidate is Tyr68 in the K-ring. From the X-ray crystal structures, the structural difference between the Na^+- and Li^+-bound forms that can attributed to Tyr68 is not clear, but the IR spectra showed a significant effect due to this residue. The cations do not directly interact with Tyr68, but it may be important for the regulation of the electronic structure of Glu139 thorough the hydrogen bond. When the cations are removed from the binding site, Glu139 is protonated, and the C═O stretching mode of carboxylic acid appears at 1737 cm^{-1}. The frequency suggests that Glu139 forms one moderate hydrogen bond with a polar side-chain group,[15] which implies that Glu139 keeps a hydrogen bond with Tyr68 regardless of its protonation state.

ATP hydrolysis in the A_3B_3 domain drives the rotation of the central stalk (D, G, and C subunits) and the connected K-ring, which drives the pumping of Na^+ ions through the interface between the K-ring and the I subunit. In the presence of a Na^+ ion, Glu139 is deprotonated, which generates an ion pair in the binding site. It would be interesting to know whether such a drastic change in the electrostatic field of the binding site could induce some conformational changes in a K-ring unit. The amide I mode mainly consists of a main-chain carbonyl C═O stretch whose frequency depends on the secondary structure. A positive peak at 1656 cm^{-1} and two negative peaks at 1666 and 1641 cm^{-1} were observed in the Na^+-binding-induced difference spectrum (Figure 2a). The amplitude was 0.00046 at 1656 cm^{-1}, which corresponds to about 0.4 and 1.7% of the entire amide I band of the entire complex (0.11 au) and the K-ring (0.026 au, estimated from the ratio of the molecular weights of the entire complex, 698 kDa, and the K-ring, 160 kDa), respectively. One subunit of the K-ring consists of 156 amino acid residues, and 1.7% corresponds to 2.7 residues. From the spectral decomposition analysis, the number of amino acid residues participating in conformational changes upon ion binding was 5−6 (Figure S3). Upon binding of Na^+ or Li^+ ions, 5−6 amide groups formed a rigid α-helix conformation, as indicated by a strong positive peak at 1656 cm^{-1}.

After the perfusion-induced difference ATR-FTIR experiment, the stability of the entire V-ATPase complex was examined by SDS-PAGE (Figure 3B) and Na^+-dependent ATPase activity (Figure 3C). The SDS-PAGE band patterns obtained (lane 1) before and (lane 2) after the experiment were almost identical. A and B subunits are the main components of the V_1 domain and are relatively easily detachable from the complex; however, they survived during the measurement. Not only the structure but also the function of V-ATPase was found to be retained, as indicated by the result that at least 70% of the Na^+-dependent ATPase activity remained. Desorption of the sample from the ATR cell surface was estimated to be ∼17% from the amide I intensity. Therefore, the substantial degeneration was limited to ∼13% after the experiment, which ensured that our ion-binding-induced difference spectrum was obtained from the intact V-ATPase sample.

Unexpectedly, the ion-binding-induced difference spectrum of the K-ring was very similar to that of the entire complex [Figure 3A(b)], because one or two subunits of the K-ring are considered to interact with the I subunit for Na^+ or Li^+ ion transport, where Arg573 was proposed to form an ion pair with Glu139 in the K-ring.[5,6] We calculated a double difference spectrum between the spectra obtained from the entire complex and the K-ring alone, but there was no significant difference in comparison with a difference spectrum calculated from two individual measurements of the entire complex (data not shown). This implies that protein interaction with the I subunit does not cause large structural changes in the K-ring. This may support the previously proposed Na^+ transport mechanism by V-ATPase, which states that a flip-flop movement of a carboxylate group of Glu139 without large conformational changes in the K-ring accelerates the replacement of a Na^+ ion in the binding site.[7]

In this work, using perfusion-induced difference ATR-FTIR spectroscopy, we have shown that binding of sodium and lithium ions to the K-ring induces deprotonation of the Glu139 residue and a small conformational change in the main chain around the binding site. These structural changes facilitate the transport of Na^+ and Li^+ ions across the hydrophobic lipid bilayer. Our measurement conditions did not impair the functionality of the V-ATPase complex; therefore, we will perform further experiments in the presence of ATP to elucidate the pumping mechanism coupled with ATP hydrolysis.

■ ASSOCIATED CONTENT

Supporting Information. Materials, methods, and supporting results. This material is available free of charge via the Internet at http://pubs.acs.org.

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