

Structure of the rotor ring modified with *N,N'*-dicyclohexylcarbodiimide of the Na⁺-transporting vacuolar ATPase

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The prokaryotic V-ATPase of *Enterococcus hirae*, closely related to the eukaryotic enzymes, provides a unique opportunity to study the ion-translocation mechanism because it transports Na⁺, which can be detected by radioisotope (²²Na⁺) experiments and X-ray crystallography. In this study, we demonstrated that the binding affinity of the rotor ring (K ring) for ²²Na⁺ decreased approximately 30-fold by reaction with *N,N'*-dicyclohexylcarbodiimide (DCCD), and determined the crystal structures of Na⁺-bound and Na⁺-unbound K rings modified with DCCD at 2.4- and 3.1-Å resolutions, respectively. Overall these structures were similar, indicating that there is no global conformational change associated with release of Na⁺ from the DCCD-K ring. A conserved glutamate residue (E139) within all 10 ion-binding pockets of the K ring was neutralized by modification with DCCD, and formed an "open" conformation by losing hydrogen bonds with the Y68 and T64 side chains, resulting in low affinity for Na⁺. This open conformation is likely to be comparable to that of neutralized E139 forming a salt bridge with the conserved arginine of the stator during the ion-translocation process. Based on these findings, we proposed the ion-translocation model that the binding affinity for Na⁺ decreases due to the neutralization of E139, thus releasing bound Na⁺, and that the structures of Na⁺-bound and Na⁺-unbound DCCD-K rings are corresponding to intermediate states before and after release of Na⁺ during rotational catalysis of V-ATPase, respectively.

V₁/V_o-ATPase | inhibitor | sodium binding | rotary motor

Vacuolar ATPase (V-ATPase), which resembles ATP synthase (F-ATPase), functions as a proton pump in the acidic organelles and plasma membranes of eukaryotic cells and bacteria (1). Like F-ATPases, V-ATPases have a globular catalytic domain, V₁ (equivalent to F₁) which hydrolyzes ATP. This V₁ domain is attached by central and peripheral stalks to an intrinsic membrane domain, V_o (equivalent to F_o) that pumps H⁺ (or Na⁺) across the membrane. In both F- and V-ATPases, ATP hydrolysis drives rotation of the central stalk and the membrane ring attached to it, which is composed of hydrophobic c subunits. H⁺ (or Na⁺) is pumped at the interface between the rotating ring and a static membrane component (the "a" subunit) (1). It is widely believed that electrostatic interaction at the glutamate (aspartate) residues within the H⁺ (or Na⁺) binding sites of the rotor ring and the conserved arginine residue of the a subunit is crucial for the ion-transport mechanism (2–4). However, the precise mechanism of ion translocation by F- and V-ATPases is still not clear because of the lack of the atomic structure of the a subunit, although high-resolution crystal structures of Na⁺- and H⁺-coupled rotor rings have been obtained (5–7).

N,N'-dicyclohexylcarbodiimide (DCCD) specifically inhibits ATPase activity and proton translocation by reacting with the conserved Glu (or Asp) residues of the rotor ring of both F- and V-ATPases (8–10). The exact mechanism by which DCCD reacts with the carboxyl groups of the acidic residues is unclear, but it is widely assumed that the reaction occurs with the protonated form of the carboxyl group. The reaction takes place in two steps as follows (Fig. 1): (i) The carboxylic acid forms a hydrogen bond to one of the nitrogen atoms in DCCD. The electron rearrangement in DCCD leads to formation of an unstable dicyclohexyl-O-acylurea intermediate. (ii) Further rearrangement of the molecule leads to formation of the stable dicyclohexyl-N-acylurea (DCNU) (11). Hence the ion-binding site accommodating the DCNU structure should have no bound H⁺ (or Na⁺) available for transport because of neutralization of the conserved carboxylic groups. DCCD has been proposed to influence the ring structure in a way that mimics the arginine residue in the a subunit (7, 9). Recently, Pogoryelov et al. reported a crystal structure of the c ring modified with DCCD in the F-type H⁺-ATPase from *Spirulina platensis*, even though the structure was obtained from native c-ring crystals after soaking with 5 mM DCCD and the occupancy of DCCD was low because of partial reaction (modified 27% of the c subunits) (12). The structure of the c ring revealed a small conformational change caused by side-chain movement of E62 modified with DCCD breaking off its hydrogen-bond interactions with Q29 and F60. They proposed that the open conformation of the DCCD-modified E62 corresponds to the neutralized conformation of E62 by forming the salt bridge with the conserved arginine of the a subunit (12).

We have identified a variant of V-ATPase in the fermentative bacterium *Enterococcus hirae*, which physiologically transports Na⁺ and Li⁺ (13). The enzyme is encoded by nine *ntp* genes (*ntpFIKECGABD*) organized within the *ntp* operon (14). Amino acid sequences of NtpF, -I, -K, -E, -C, -G, -A, -B, and -D are homologous to those of subunits G, a, c, E, d, F, A, B, and D of eukaryotic V-ATPases, respectively (15). The *E. hirae*

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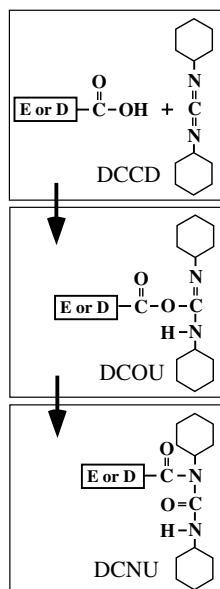


Fig. 1. Proposed model of the DCCD-reaction mechanism at the ion-binding sites of the rotor ring. DCCD reacts with protonated forms of the conserved Glu or Asp residues at the ion-binding site in the rotor-ring subunit forming a dicyclohexyl-O-acylurea (DCOU) intermediate with subsequent acyl migration to form the stable DCNU.

V-ATPase possesses a high-binding affinity for the substrate Na^+ . This property enabled the measurement of Na^+ binding to this ATPase using radioisotope ($^{22}\text{Na}^+$) (16). We previously proposed an ion-transport mechanism of V-ATPase based on the Na^+ binding properties of the rotor ring (K ring) and the crystal structure of the Na^+ -bound K ring (5, 17). To elucidate the precise ion-transport mechanism of V-ATPase, it is essential to understand the structure of Na^+ -unbound K ring after releasing bound substrate Na^+ . Here, we examined the inhibition mechanism of K ring by DCCD and found that the binding affinity for Na^+ decreased by the DCCD reaction. Subsequently, we succeeded in obtaining the structures of Na^+ -bound and Na^+ -unbound rotor rings modified with DCCD. On the basis of these findings, we present an ion-transport mechanism involving the K ring during rotational catalysis of V-ATPase.

Results

Inhibition of $^{22}\text{Na}^+$ Binding to the Purified K Ring by DCCD. DCCD is believed to inhibit $^{22}\text{Na}^+$ binding and ATPase activities of *E. hirae* V_1V_0 -ATPase, by attacking the conserved glutamic acid residue (E139) at the Na^+ -binding pocket of the NtpK proteolipid (16) (Fig. S1). In the present study, we examined the inhibitory effects of DCCD on $^{22}\text{Na}^+$ binding to the purified K ring and measured the kinetics of $^{22}\text{Na}^+$ -binding inhibition by 0.2 mM DCCD at pH 6.0 in the absence of Na^+ in the reaction buffer (Fig. 2A). Following 1 h of incubation with DCCD at room temperature, approximately 90% reduction in the $^{22}\text{Na}^+$ -binding ability was observed. The inhibition curve followed pseudo-first-order kinetics, suggesting that each NtpK monomer in the 10-mer K ring independently reacts with DCCD. The slope of the linear part of the data plotted as $\log ^{22}\text{Na}^+$ -binding versus incubation time gave an inhibition rate constant ($k_{\text{DCCD}} = 0.8 \times 10^{-3} \pm 1 \times 10^{-4} [\text{S}^{-1}]$) (see Fig. 2A, *Inset*) similar to that for V_1V_0 -ATPase inhibition (Fig. S1).

Inhibition of $^{22}\text{Na}^+$ -binding and ATPase activities of the whole complex by DCCD is specifically prevented by the presence of Na^+ (16, 18). The protective effects of Na^+ against DCCD inhibition have also been observed in bacterial F-type Na^+ -ATPase (19). In order to examine the protective properties of Na^+ against DCCD inhibition, we assessed the ability of

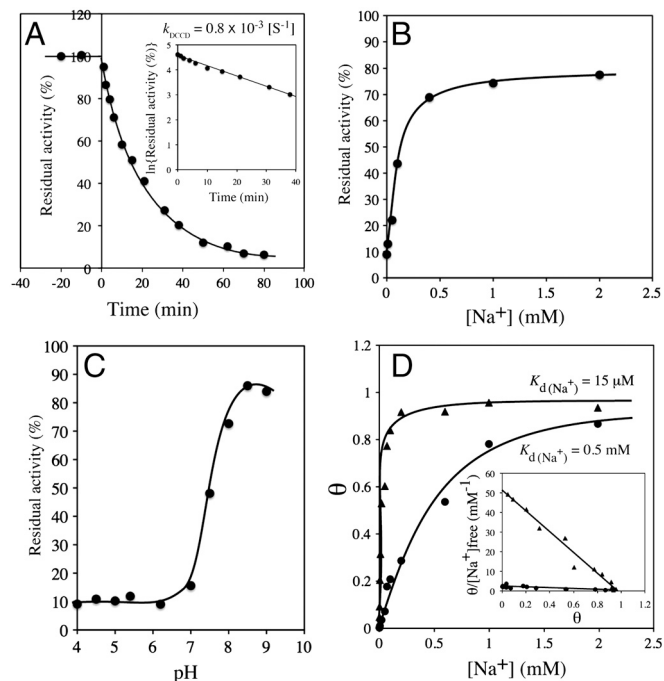


Fig. 2. DCCD inhibition of $^{22}\text{Na}^+$ -binding to the purified K ring. (A) Kinetics of $^{22}\text{Na}^+$ -binding inhibition by DCCD. The time course was initiated by addition of 0.2 mM DCCD. The inhibition rate constant (k_{DCCD}) was estimated from a semilogarithmic plot (*Inset*). (B) Protective effect of Na^+ on the DCCD inhibition of $^{22}\text{Na}^+$ binding. (C) pH dependence of DCCD inhibition. (D) Na^+ -concentration dependence of $^{22}\text{Na}^+$ binding to the K ring (\blacktriangle) and DCCD-modified K ring (\bullet). θ is defined as the number of moles of bound Na^+ per mole of K subunit but not K ring. (*Inset*) Scatchard plot of the specific binding of Na^+ .

DCCD to block $^{22}\text{Na}^+$ binding to the K ring in the presence of a range of Na^+ concentrations (Fig. 2B). Absence of Na^+ led to approximately 90% inhibition of Na^+ binding by 0.2 mM DCCD. The protective effect of Na^+ against DCCD inhibition increased depending on the increment of Na^+ concentrations, and the residual activity was retained up to 75% in the presence of more than 1 mM NaCl. Thus, Na^+ bound to the K ring probably prevents DCCD binding and inhibition. We previously reported that the K ring binds Na^+ and H^+ competitively (17). DCCD binding and inhibition were prevented by alkaline pH (>7.5) (Fig. 2C) in the absence of Na^+ where the E139 residue ($\text{pK}_a = 5.5$) were deprotonated (17). Thus, DCCD reacts with the protonated E139 in the K ring but not with the deprotonated E139 that binds Na^+ , consistent with the reaction model described in Fig. 1 whereby DCCD reacts with protonated forms of acidic residues.

Binding of $^{22}\text{Na}^+$ to the Purified K Ring Modified with DCCD. Functional analysis has shown that the purified K ring binds one Na^+ per K monomer with high affinity ($K_d(\text{Na}^+) = 15 \pm 4 \mu\text{M}$) (Fig. 2D, filled triangles) as described previously (17), consistent with the crystal structure of the Na^+ -bound K ring (5). The Na^+ -binding capacity of the K ring, measured in the presence of 15 μM $^{22}\text{Na}^+$ ($K_d(\text{Na}^+)$ of the purified K ring) decreased after preincubation with DCCD as described in the previous section. Fig. 2D (filled circles) shows that $^{22}\text{Na}^+$ binding of the DCCD-K ring was also dependent on NaCl concentration, although higher concentrations of NaCl were necessary to stimulate binding. The Scatchard plot (Fig. 2D, *Inset*; filled circles) of this data gave an intercept on the abscissa of approximately one, indicating that one DCCD-K subunit binds one Na^+ (SD 0.91 ± 0.12). The slope of the Scatchard plot indicates that the dissociation constant ($K_d(\text{Na}^+)$) is 0.5 mM (SD $0.5 \pm 0.04 \text{ mM}$), suggesting that the DCCD-K ring can bind Na^+ with low affinity.

pocket was much weaker (Fig. 3J). Based on biochemical analysis indicating low affinity of the DCCD-K ring for Na^+ (Fig. 2D) and the crystallographic data obtained, we concluded that the structure obtained in the absence of Na^+ corresponds to the Na^+ -free form of the K ring (Fig. 3J and K). Similarity between Na^+ -bound and Na^+ -unbound structures indicates that there is no global conformational change associated with release of Na^+ from the binding pocket modified with DCCD.

Discussion

Mechanism of DCCD Inhibition. V-ATPases have been implicated in a number of diseases and are known as important drug targets in diseases such as osteoporosis and cancer (1). Therefore, inhibitors of V-ATPase have potential as drugs for these diseases, and it is important to understand the precise inhibition mechanism of V-ATPase in order to develop drugs targeting the V-ATPases. In this study, we examined the inhibition mechanism of DCCD in the ion-binding pocket of the K ring by using a $^{22}\text{Na}^+$ -binding inhibition assay and X-ray crystallography. DCCD inhibited $^{22}\text{Na}^+$ binding at acidic pH in the absence of Na^+ (Fig. 2A and B), suggesting that DCCD reacted with the protonated form of the carboxyl group (E139) located in the binding pocket. These findings correspond with the studies of F-type Na^+ -ATPase and H^+ -ATPases (8, 9). The electron density corresponding to DCCD fits well to the DCNU structure, consistent with the proposed model in Fig. 1. The DCCD-K ring still retained Na^+ -binding ability but the affinity for Na^+ was much lower than that of the wild-type K ring (Fig. 2D). Previous results suggested that any Na^+ bound to the DCCD K ring is not transported in the whole complex, because ATPase activity and $^{22}\text{Na}^+$ -transport activity were completely inhibited by DCCD (18). We demonstrated that the rate of inhibition by DCCD for ATPase activity of the whole complex was approximately 6.5 times faster than that of $^{22}\text{Na}^+$ -binding (Fig. S1). Under these inhibition conditions, DCCD did not inhibit the V_1 -ATPase. These findings suggest that modification of one or two NtpK subunits in the 10-mer K ring of the whole complex is sufficient for complete inhibition of the enzymatic activity, which is also reported in previous studies of F- and V-ATPases (9, 10). The additional bulk modification with DCCD can possibly cause steric hindrance at the interface between the K ring and NtpI (corresponding to the a subunit of other V-ATPases).

Ion-Binding/Releasing Mechanism of the K Ring. We have previously proposed an ion-binding/releasing mechanism of the K ring based on the crystal structures of the ion-bound K ring, and the ion-binding properties and selectivity of the K ring (5, 17) as follows: the bound ion is occluded by E139 and its carbonyl oxygen atom position is stabilized by hydrogen bonds with Y68 and T64 side chains (closed form) (Fig. 3G; shown as thick line). The bound ion can be accessed by changing the torsion angles of the E139 side chain by losing the hydrogen bonds (open form). Binding/releasing of the ion can then be easily achieved through the open space. In the DCCD-K-ring structures obtained, the hydrogen bonds with Y68 and T64 working for stabilization of the “closed” form are disrupted by modification with DCCD (Fig. 3D and J). Thus, the ion-binding site modified with DCCD probably corresponds to the open conformation, which is consistent with the low binding affinity for Na^+ of the DCCD-K ring (Fig. 2D). A high-resolution structure of the c ring from F-type H^+ -ATPase modified with DCCD has also been shown in a similar open conformation (Fig. S2), which was verified by the molecular dynamic simulations of the c ring (12).

Ion-Transport Mechanism of V-ATPase. We have previously proposed an ion-transport mechanism (5, 14, 17) as follows: clockwise rotation of the K ring driven by ATP-hydrolysis energy in V_1 -ATPase, as viewed from the cytoplasm, brings an occupied

Na^+ -binding site into the K ring–NtpI interface (Fig. 4A). The proximity of the Na^+ site to the essential R573 residue of NtpI produces an electrostatic interaction between R573 and E139, losing the hydrogen-bound network in the Na^+ -binding pocket of the K ring, resulting in the release of Na^+ into the periplasm

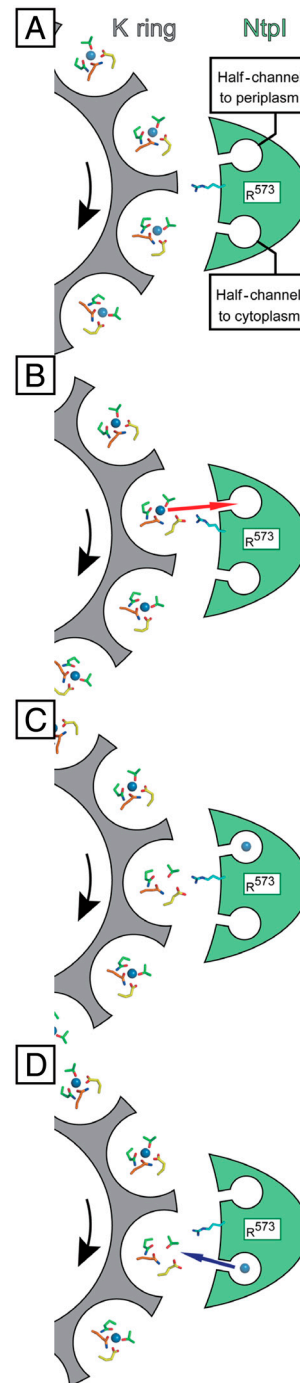


Fig. 4. A model for the ion-transport mechanism of Na^+ -transporting V-ATPase. The model is based on the crystal structures of the K ring with and without DCCD. The views are from the cytoplasm and show the K-ring–NtpI interface at the level of the Na^+ -binding sites. Residues involved in Na^+ binding are shown in stick representation. Colors correspond to Fig. 3. NtpI shown in green is in close proximity to the K ring with the essential R573 (blue stick representation), which has two half-channels connecting to the periplasm and cytoplasm, respectively. The Na^+ -transport mechanism of V-ATPase is represented by four intermediate states (A–D). See text and [Movie S1](#) for details.

via a half-channel in NtpI as indicated by the red arrow in Fig. 4B. Further rotation caused by ATP-hydrolysis energy in V_1 -ATPase, which may disrupt the Arg-Glu interaction, results in the binding of a cytoplasmic Na^+ as indicated by the blue arrow in Fig. 4D.

In this study, we found that the binding affinity of the K ring for Na^+ decreased approximately 30-fold by reaction with DCCD, and we obtained the structures of the DCCD-K ring with and without the substrate ion Na^+ . Binding of DCCD effectively neutralized the negative charge of the conserved E139 (Fig. 3 C–E and I–K), resulting in lower affinity for Na^+ in the binding pocket (Fig. 2D). Similarity of the Na^+ -bound and Na^+ -unbound DCCD-K ring structures to that of the wild-type K ring indicates that there is no global conformational change associated with neutralization of E139 and with release of Na^+ from the binding pocket modified with DCCD. We believe that E139 neutralized by DCCD adopts the open conformation corresponding to the similar open conformation through formation of an electrostatic interaction with R573 during the ion-transport process. A similar interpretation was proposed for the rotational model of F-type H^+ -ATPase (12). The structure of the Na^+ -bound K ring modified with DCCD (Fig. 3 C–E) seems to fit to the intermediate state prior to the release of Na^+ into the periplasm, as hypothesized in Fig. 4B. Neutralization of E139 in the binding site reduces the affinity for the bound Na^+ , which is readily released into the periplasm (Fig. 4B, indicated by the red arrow). The structure of the Na^+ -unbound DCCD-K ring (Fig. 3 I–K) is likely to correspond to another intermediate state after release of Na^+ into the periplasm, as shown in Fig. 4C. Thus, we propose an ion-transport mechanism of the V-ATPase whereby ion translocation can be accomplished through an affinity change of the Na^+ binding sites by neutralization of E139 due to formation of a salt bridge with R573 of the stator (see [Movie S1](#)).

Materials and Methods

Protein Preparation. V_1V_0 -ATPase was purified from *E. hirae* cells (18). The K ring was released from the isolated V_1V_0 -ATPase by treatment with 10% isopropanol and then purified by anion exchange and gel filtration chromatography (20).

DCCD Inhibition Kinetics of Na^+ Binding to the Purified K Ring. The K ring is comprised of ten 16 kDa K subunits. One milligram of purified K ring corresponds to 60 nmol K subunit (6 nmol K ring). The reaction mixture that contained 6 μM purified K subunit (0.6 μM K ring) and 15 μM $^{22}\text{NaCl}$ (3 cpm/nL) in Buffer A (20 mM MES-Tris, 20% glycerol, 0.05% n-dodecyl β -D-maltoside; pH 6.0) was incubated for 2 h at room temperature, which was sufficient to saturate Na^+ binding to the K ring. The time-course experiment of DCCD inhibition was initiated by addition of 0.2 mM DCCD to the incubation mixture at room temperature. Free $^{22}\text{Na}^+$ was rapidly separated using a Dowex-50 method at various time intervals (16). The inhibition curve followed pseudo-first-order kinetics. The rate constants (k_{DCCD}) for $^{22}\text{Na}^+$ -binding inhibition by DCCD were calculated by fitting the linear part of the data plotted as $\log^{22}\text{Na}^+$ binding versus incubation time. The slope of the line is the negative of the rate constant for inhibition. All $^{22}\text{Na}^+$ -binding experiments were repeated three times, the data were averaged, and the standard deviations were calculated.

Protection Effect of Na^+ from $^{22}\text{Na}^+$ -Binding Inhibition by DCCD. The purified K ring in Buffer A was incubated with 0.2 mM DCCD in the presence of several concentrations of NaCl for 2 h at room temperature, which was sufficient to saturate the inhibition of Na^+ binding by DCCD. Free DCCD and NaCl in the

mixture were removed by quick spin column (Sephadex G-50) equilibrated with Buffer A. The eluate (6 μM purified K subunit) was incubated with 15 μM $^{22}\text{NaCl}$ (3 cpm/nL) for 2 h. The Na^+ -binding capacity was measured as described in the previous subsection.

pH Dependence of $^{22}\text{Na}^+$ Binding Inhibition by DCCD. The purified K ring was incubated with 0.2 mM DCCD at varying pH (pH 4–5.5, citrate-Tris; pH 5.5–7, Bistris; pH 7–9, Tris-HCl). Free DCCD and pH buffer were removed by a quick spin column (Sephadex G-50) equilibrated with Buffer A. The Na^+ -binding capacity was measured as described in the previous subsection.

Na^+ -Concentration Dependence of $^{22}\text{Na}^+$ Binding to the DCCD-Modified K Ring. DCCD-modified K ring was obtained by incubating with 0.2 mM DCCD for 5 h at 20 °C. The reaction mixture that contained 6 pmol DCCD-bound K ring (or DCCD-unbound K ring) and various concentrations of $^{22}\text{NaCl}$ (30,000 cpm) in 10 μL of Buffer A was incubated for 2 h at room temperature, which was sufficient to saturate Na^+ binding to the K ring. Na^+ -binding capacity was measured as described in the previous subsection.

Crystallization of DCCD-Modified K Ring in the Presence of Na^+ . The purified K ring (2.5 mg/mL) in buffer containing 10 mM MES-HCl (pH 6.0), 10% glycerol, 0.32 mM n-dodecyl β -D-maltoside, and 1.2 mM undecyl maltoside was incubated with 0.2 mM DCCD for 5 h at 20 °C. The protein sample (1 μL) was then mixed with reservoir solution (1 μL) consisting of 100 mM Tris-HCl (pH 7.5), 4% glycerol, 240 mM sodium citrate, and 32% PEG 400. Crystals grew at 20 °C in sitting drops by vapor diffusion. The crystals appeared after 5 d and grew to maximum dimensions (200 \times 200 \times 80 μm) in 1 mo, and were plunged into liquid nitrogen and stored at 100 K.

Crystallization of DCCD-Modified K Ring in the Absence of Na^+ . The purified K ring (2.5 mg/mL; 1 μL) modified with DCCD as described in the previous subsection was mixed with reservoir solution (1 μL) consisting of 100 mM Tris-HCl (pH 7.5), 4% glycerol, 240 mM potassium citrate, and 35% PEG 400. Crystals grew at 20 °C in sitting drops by vapor diffusion. The crystals appeared after 10 d and grew to maximum dimensions (150 \times 150 \times 40 μm) in 1 mo, and were plunged into liquid nitrogen and stored at 100 K.

Data Collection, Structure Determination, and Refinement. Diffraction data were collected from a single crystal at cryogenic temperature (100 K) on beamline BL26B1 and BL41XU at SPring-8. The data were indexed and integrated with MOSFLM (21) and processed further with the CCP4 programs (22). The structures were solved by molecular replacement with AMoRe (23) using the Na^+ -bound K-ring structure as a search model (PDB ID code 2BL2) in all subsequent refinement steps, 5% of the data was set aside for calculation of the free R factor. The atomic model was built using the program O (24) and refined using REFMAC5 (25). The coordinates for N-5-cyclohexyl-N-5-[(cyclohexylamino) carbonyl] glutamine were obtained from the PDB file (1E79) of bovine F_1 -ATPase inhibited with DCCD (26). Tight noncrystallographic symmetry (NCS) restraints (sigma 0.05 Å) were applied to the 10 K protomers (excluding regions in lattice contacts). Translation, libration, and screw-rotation refinement (TLS), with one TLS group per protomer, was carried out in the final stages without NCS restraints. The refined structures were validated with PROCHECK (27). Figures were generated with PyMOL (28).

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