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Na^+- and K^+-Dependent Oligomeric Interconversion among αβ-Protomers, Diprotomers and Higher Oligomers in Solubilized Na^+/K^+-ATPase

Takayuki Kobayashi[1], Yoshikazu Tahara[1], Hitoshi Takenaka[1], Kunihiro Mimura[2] and Yutaro Hayashi[1,*]

[1]*Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611; and*
[2]*Department of Environmental Security System, Faculty of Risk and Crisis Management, Chiba Institute of Science, Choshi, Chiba 288-0025, Japan*

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Protein fractions of a higher-oligomer (H), $(\alpha\beta)_2$-diprotomer (D) and αβ-protomer (P) were separated from dog kidney Na^+/K^+-ATPase solubilized in the presence of NaCl and KCl. Na^+/K^+-dependent interconversion of the oligomers was analysed using HPLC at 0°C. With increasing KCl concentrations, the content or amount of D increased from 27.6 to 54.3% of total protein, i.e. $\Delta C_{max} = 26.7\%$. ΔC_{max} for the sum of D and H was equivalent to the absolute value of ΔC_{max} for P, regardless of the anion present, indicating that K^+ induced the conversion of P into D and/or H, and Na^+ had the opposite effect. When enzymes that had been denatured to varying degrees by aging were solubilized, ΔC_{max} increased linearly with the remaining ATPase activity. The magnitude of the interconversion could be explained based on an equilibrium of D $\rightleftharpoons$ 2P, assuming 50-fold difference in the K_d between KCl and NaCl, and coexistence of unconvertible oligomers, which comprised as much as 39% of the eluted protein. Oligomeric interconversion, determined as a function of the KCl or NaCl concentration, showed $K_{0.5}$s of 64.8 μM and 6.50 mM for KCl and NaCl, respectively, implying that oligomeric interconversion was coupled with Na^+/K^+-binding to their active transport sites.

Key words: dog kidney, oligomeric interconversion, oligomeric structure, Na^+/K^+-ATPase, solubilized membrane protein.

Abbreviations: $C_{12}E_8$, octaethyleneglycol *n*-dodecylether; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid;CHES, 2-(cyclohexylamino)ethanesulfonic acid; D, $(\alpha\beta)_2$-diprotomer; ΔC_{max}, maximum induction in oligomeric protein by changing the K^+ or Na^+ concentration; E_1, enzyme conformation with a higher affinity for Na^+ which binds ATP with high affinity and accepts phosphate from ATP; E_2, enzyme conformation with a higher affinity for K^+, which can accept Pi; E_1-P, an ADP-sensitive phosphoenzyme; E_2-P, a K^+-sensitive phosphoenzyme; $E_1{\cdot}Na^+$, Na^+-bound enzyme; $E_2{\cdot}K^+$, K^+-bound enzyme; HPGC, high-performance gel chromatography; G, aggregates; H, higher-oligomer; $K_{0.5}$, concentration of K^+ or Na^+ to induce a half-maximum change in the amount of oligomer; K_d, dissociation constant for an association-dissociation equilibrium; LALLS, low-angle laser light-scattering photometer; MES, 2-morpholinoethanesulfonic acid, monohydrate; P, αβ-protomer; M_p, molecular weight of protein moiety for solubilized membrane protein; *p*NPPase, *p*-nitrophenyl phosphatase; *p*-NPP, *p*-nitrophenyl phosphate; PS, phosphatidylserine; TEA, triethanolamine.

Na^+/K^+-ATPase is an integral membrane protein that co-transports three Na^+ ions out of and $2K^+$ ions into the cell at the expense of hydrolysing one ATP molecule (*1–3*). According to the Post-Albers mechanism for Na^+/K^+-ATPase and other P-type ATPases, the enzyme is phosphorylated by ATP at the β-carboxyl group of an Asp in two conformational states, designated E_1 and E_2, to form E_1-P and E_2-P during ATP hydrolysis coupled with cation-transport (*4*, *5*). The enzyme forms an $E_1{\cdot}Na^+$ complex in K^+-free medium, and an $E_2{\cdot}K^+$ complex in the presence of K^+. The $E_2{\cdot}K^+$ complex is stable, even in the presence of equimolar amounts of Na^+, because the affinity of the enzyme for K^+ is much higher than for Na^+ (*6*). Toyoshima and colleagues (*7–11*) reported that Ca^{2+}-ATPase of the sarcoplasmic reticulum, belonging to the P-type family, exists in the two three-dimensional conformations—E_1 and E_2. The conformational change between E_1 and E_2 postulated for Ca^{2+}-ATPase probably also applies to Na^+/K^+-ATPase, as indicated by Rice *et al*. (*12*), and Sweadner and Donnet (*13*).

The two polypeptides comprising Na^+/K^+-ATPase, catalytic subunit α and glycoprotein β, are non-covalently linked in a minimal structural unit—an αβ-protomer (*14*). Recent studies have shown that a regulatory subunit also is present in the enzyme unit obtained from mammalian kidney (*15*, *16*). It is, however, controversial as to whether the Na^+/K^+-ATPase and Ca^{2+}-ATPase can function in the protomeric form (*17–24*) [monomeric for Ca^{2+}-ATPase (*25*)] or in the oligomeric form, which consists of several protomers (*26–36*) or monomers (*37–43*). Electron microscopy has verified that membrane-bound Na^+/K^+-ATPase

*To whom correspondence should be addressed. Tel: +81-422-76-7651, Fax: +81-422-76-7651, E-mail: yutahaya@kyorin-u.ac.jp

and Ca^{2+}-ATPase contain oligomeric, as well as protomeric (monomeric for Ca^{2+}-ATPase), proteins (*44–46*). To identify the oligomeric structure of the enzyme that functions in active transport, purified membrane-bound Na^+/K^+-ATPase from mammalian kidneys was solubilized with nonionic surfactants such as $C_{12}E_8$ (*28*, *47–50*) and Lubrol (*51*), as described previously. The solubilized enzyme was analysed using HPGC at 0°C, and the M_p of the eluted protein determined using HPGC-LALLS (*28*, *52*). The enzyme typically separated into two major protein components, which comprised 85% of all eluted protein: αβ-protomers (P, $M_p = 1.56 \times 10^5$) and $(\alpha\beta)_2$-diprotomer (D, 3.02×10^5). Two minor protein components, which accounted for the remaining 15% of the eluted protein also were identified: one component with a molecular weight greater than D ($M_p \geq 7 \times 10^5$) (H) and an aggregate (G) that eluted with the void volume of column (*28*, *49*). The simultaneous measurement of M_p and ATPase activity of the solubilized enzyme indicated that: (i) P and D had essentially the same specific ATPase activity in the gel column using an elution buffer containing exogenous PS; (ii) P and D in equilibrium, $D \rightleftharpoons 2P$ at 20°C and pH 7, which was shifted forward or backward by addition of NaCl or KCl, respectively (*28*). Thus, the results of this study indicate that Na^+ and K^+ cause enzyme to alternate between the P and D oligomeric states and that the respective oligomers hydrolysed ATP. Although the function of switching between oligomeric forms remains unclear, the characterizing the relationship between the oligomeric structure, i.e. D or P, and the conformational states, i.e. E_1 or E_2, is essential for understanding the molecular mechanism of Na^+/K^+-ATPase. Therefore, the relationship between oligomeric switching and enzyme conformation and activity were examined in the present study.

Post and Suzuki (*53*, *54*) investigated the effect of Hofmeister anions on the equilibrium between E_1-P and E_2-P in the presence of Na^+. Chaotropic anions, such as iodide, thiocyanate and nitrate shifted the equilibrium toward E_1-P; while kosmotropic anions, such as citrate, sulphate and acetate shifted the equilibrium toward E_2-P. When chloride was used as the anion, $E_1 \cdot Na^+$ favoured P, whereas $E_2 \cdot K^+$ favoured D (*28*); however, the effect of K^+ or Na^+ on oligomeric form in the presence of anions other than chloride remains unknown. Based on our previous studies (*28*, *29*) and those of Post and Suzuki (*53*, *54*), stronger kosmotropic anions should favour D over P. Furthermore, oligomeric interconversions induced by K^+ or Na^+ should be independent of which anion is used. Thus, the effect of varying the anion and concentrations of K^+ and Na^+ on oligomeric interconversion of the enzyme solubilized with $C_{12}E_8$ was investigated in this study. Interconversion among the oligomers also was measured as a function of Na^+ or K^+ concentration to test whether interconversion was coupled to Na^+/K^+-ATPase activity. K^+-dependent association of the enzyme and its reversal by Na^+ was observed regardless of the type of anion present. The affinities of K^+ or Na^+ for the oligomers were comparable to the respective binding affinities for the active sites of the membrane-bound enzyme. Parts of this work have been described in preliminary reports (*55–57*).

MATERIALS AND METHODS

Chemicals—Special care was taken to minimize contamination by Na^+ and K^+. NaCl, KCl, glycerol (of 'Servo vaccine' grade), and imidazole (of 'Buffer Solution' purity) were special grade reagents purchased from Wako Pure Chemical Industries, Ltd and Tokyo Kasei Kogyo Co. Ltd, respectively. HEPES and free acids of EDTA and CDTA were products of Dojindo Laboratories. $C_{12}E_8$ (BL-8SY) and choline chloride (3× crystallized, C7017) were obtained from Nikko Chemical Co. and Sigma Chemical Co., respectively. All reagents described above, except EDTA and CDTA which were recrystallized once from boiling water and then neutralized with imidazole, were used without further purification.

Membrane-Bound Na^+/K^+-ATPase—The enzyme was purified from microsomes of the outer medulla of frozen dog kidneys according to the method of Jorgensen (*58*) with minor modifications (*28*). The microsomes were treated with SDS at final microsomal protein and SDS concentration of 1.40 mg/ml and 0.60 mg/ml, respectively. The purified enzyme was washed to remove Na^+ and K^+ contamination of the enzyme, described elsewhere (*49*), suspended in 20%(w/v) glycerol, 12 mM imidazole, 28 mM HEPES, pH 7.1, frozen and stored at −80°C until used, unless otherwise stated. The specific ATPase activity of the enzyme ranged from 37 to 50 μmol Pi/min/mg protein (U/mg) at 37°C under optimal conditions, as described elsewhere (*28*).

Isolation of Solubilized Na^+/K^+-ATPase and Separation of Oligomeric Protein Components—The membrane-bound enzyme was incubated in the presence of various concentrations of KCl and/or NaCl, or salts consisting of the mono-valent cations and Hofmeister series anions, with $C_{12}E_8$ at pH 7.0 and 0°C for 5 min, unless otherwise stated (*28*). The final composition was 2 mg/ml protein, 6 mg/ml $C_{12}E_8$, 1 mM EDTA, 10% (w/v) glycerol, 10–14 mM imidazole, and 13–23 mM HEPES. The solution was centrifuged at 436 000*g* for 5 min at 2°C (Beckman TL100). The supernatant, heretofore referred to as the solubilized Na^+/K^+-ATPase or the solubilized enzyme, was collected, stored at 0°C, and used within 12 h. The protein concentration of the solubilized enzyme was determined from the absorbance at 280 nm, corrected for light-scattering, as described by Reddi (*59*), using an absorption coefficient of 1.22 mg^{-1} ml cm^{-1}, as described in detail elsewhere (*49*).

An aliquot of 100 μl of the solubilized enzyme was charged on a TSKgel $G3000SW_{XL}$ (7.8 mm × 30 cm, Tosoh Co.) column equipped with a guard column (TSK guard column SW_{XL}, 6 mm × 4 cm). The column had been equilibrated with an elution buffer of 0.30 mg/ml $C_{12}E_8$, 1 mM EDTA, 10 mM imidazole, 13 mM HEPES, NaCl and/or KCl; the total salt concentration of the elution buffer was the same as that used for the solubilization procedures. The enzyme was run through the column at pH 7.0 and 0°C, at a flow rate of 0.30 ml/min, and the

Table 1. **Contamination of the solubilizing solution by cations.**

Contaminant cations	6 mg/ml $C_{12}E_8$ (μM)	The buffer (13 mM Imidazole, 15 mM HEPES, 1 mM EDTA, pH7.0) (μM)	10%(w/v) Glycerol (μM)	0.1 M Choline chloride (μM)	Sum[a] (whole solution) (μM)
Li^+	0.56 ± 0.48	0 (−0.08)[b] ± 0.11	0 (−1.44)[b] ± 0.91	0 (−0.36)[b] ± 0.43	0.56 ± 1.12
Na^+	1.17 ± 0.98	0.09 ± 0.02	3.05 ± 0.51	0 (−1.45)[b] ± 0.39	4.31 ± 1.17
NH_4^+	0 (−0.59)[b] ± 1.08	0.35 ± 0.01	4.99 ± 1.19	0 (−0.83)[b] ± 0.50	5.34 ± 1.68
K^+	1.95 ± 1.38	0.35 ± 0.01	0 (−1.53)[b] ± 0.3	0 (−0.95)[b] ± 0.43	2.30 ± 1.48
Mg^{2+}	3.60 ± 2.31	2.21 ± 3.29	0 (−2.06)[b] ± 0.58	—[c] ± —	5.81 ± 4.06
Ca^{2+}	3.40 ± 1.91	0.95 ± 0.99	0.75 ± 0.66	11.22 ± 2.25	16.32 ± 3.18

Four aliquots of the respective constituents, described in the top line in this table, for a solubilizing solution were added to a standard solution of cation as an internal standard, and subjected to ion-chromatography to estimate the amount of cation contamination. The amounts of cation estimated for the respective aliquots were plotted against the contents of the standard cation added, and the amounts of cation included in the respective constituents were determined by extrapolation of the plot to zero concentration. The extrapolated values and their errors were obtained by linear regression, and are displayed in the table.
[a]The summation was given as the amounts of cations included in the whole solubilizing solution. [b]The amounts of cation obtained as negative numbers (numbers in parentheses) were considered to be 'not detected (zero)' in the summations, while the errors accompanying the negative number were reckoned into calculation of error propagation. [c]It was impossible to estimate areas under Mg^{2+}-peak owing to interference from choline$^+$-peak.

eluted with the same elution buffer. The effluent was monitored with a UV spectrophotometer equipped with a diode array detector, and the output with absorbance at 280 nm was displayed as an elution pattern. The relative amounts of protein were estimated by the vertical division method from the area under protein peaks using Shimadzu CLASS-LC10 software (Shimadzu Co.). The absorption coefficients of the oligomers were assumed to be identical. The amounts of the oligomers thus obtained were essentially equivalent to those estimated by computer simulation where the observed elution pattern was assumed to consist of a linear summation of Gaussian distribution curves for the respective oligomeric components.

To estimate the effect of anions other than Cl^- on the type and amount of solubilized oligomers of Na^+/K^+-ATPase, the membrane-bound enzyme was solubilized in standard solutions containing 0.1 N Hofmeister series anions: citrate^{3-}, sulfate^{2-}, tartrate$^-$, fluoride$^-$, acetate$^-$, chloride$^-$, bromide$^-$, nitrate$^-$, iodide$^-$ or thiocyanate$^-$. A 50 μl aliquot of the solubilized enzyme obtained in the presence of each anion present as salt of Na^+ or K^+ was subjected to HPGC, as described above. The elution buffer always contained 0.1 M choline chloride, regardless of which salt was included in the solubilizing solution. See Table 2 below as to the HPGC.

Contamination of the Solubilizing Solution by Na^+, K^+ and Other Cations—Contaminant cations were evaluated by ion chromatography on DX-500 in combination with a cation suppresser (CSRS ULTRA, 4 mm, Dionex Co.). A standard solution containing 72 μM Li^+, 87 μM Na^+, 111 μM NH_4^+, 128 μM K^+, 206 μM Mg^{2+} and 125 μM Ca^{2+} was added as an internal standard to the samples, in volume ratios (v/v) of 0.05, 0.1, 0.15 and 0.2 so that the samples included four different fractional concentrations of the standard cations. Total 10 or 25 μl of the resultant sample were applied onto an Ion Pac CS12A (4 mm × 25 cm) column equipped with an Ion Pac CG12A guard column (4 mm × 5 cm), which had been equilibrated with the eluent (20 mM methanesulphonic acid) at a flow rate of 1 ml/min, and eluted isocratically with the same eluent. The eluent was monitored with a conductivity detector (CD-20, Dionex, Co.). The sample cation concentrations were plotted against the concentrations of the standard cation in the sample, and the amounts of cation included in the constituent reagents were obtained by extrapolating the plots to zero standard. The results and errors are shown in Table 1.

ATPase and pNPPase Activities for the Enzyme Solubilized under the Various Conditions—Aliquots of 79 μl each of solubilized enzyme preparation were added to 1.50 ml of a buffer solution of 15 mM imidazole, 23 mM HEPES, 1 mM EDTA, 3.9 mM $MgCl_2$ at pH 7.0 and 0°C. The concentrations of NaCl and KCl in the buffer were the same as those used in the preparation of the solubilized enzyme, as well as 4 mM ATP or 10 mM *p*NPP for the ATPase or *p*NPPase assay, respectively. After incubating the resulting mixture for 2 min at 20°C, unless otherwise stated, a linear increase in Pi concentration was estimated in the ATPase and *p*NPPase assays using a Technicon Auto Analyzer II, as described elsewhere (*28*, *49*).

pH-Treatment of the Solubilized Enzyme—The membrane-bound enzyme was solubilized in the standard solubilizing solution containing 0.1 M NaCl or KCl, except that the buffer concentration was diluted 3-fold. A 50 μl aliquot of the solubilized enzyme was added to 10 μl of buffer: 170–70 mM imidazole/100–380 mM MES to make final pH 5.5 to 7.0; 500 mM imidazole/380–750 mM HEPES to give a final pH of 7.0 to 7.6; 500 mM Tris/300 mM HCl to give a final pH of 8.3; 500 mM TEA/50–330 mM CHES to make final pH 8.8 to 9.4. As a result, the solubilized enzyme was treated at pH of 5.5, 6.0, 6.5, 7.0, 7.6, 8.4, 8.9 and 9.4. After 5 min at 0°C, the solubilized enzymes treated at different pH were subjected to HPGC. For the solubilized enzymes treated at pH values from 5.5 to 7.0, a TSKgel $G3000SW_{XL}$ column was used; enzymes treated at pH 7.0 to 9.4 were run on a Superdex 200HR 10/30 (10 mm × 30 cm, Pharmacia-LKB Co.) column.

Table 2. **Comparison of two ways of elution for HPGC to analyze Na^+- and K^+-dependence of oligomer amount of the solubilized enzyme.**

X^+Cl^- for HPGC elution	Parameters[a]	Protomer (P)	Diprotomer (D)	Higher oligomer (H)
		Oligomer amount (%)		
Variable, but keeping [NaCl] + [KCl] = 0.1 M	C_0 (%)	66.8 ± 0.7	27.6 ± 0.7	5.29 ± 0.5
	ΔC_{max} (%)	−29.4 ± 0.8	26.7 ± 0.8	2.32 ± 0.49
	$K_{0.5}$ (mM)	12.0 ± 1.0	12.0 ± 1.0	9.4 ± 3.9
	n	2.00 ± 0.12	2.15 ± 0.15	1.44 ± 0.61
Constant at 0.1 M Choline chrolide	C_0 (%)	65.4 ± 1.8	26.6 ± 1.9	7.71 ± 0.88
	ΔC_{max} (%)	− 24.0 ± 2.0	17.9 ± 2.1	5.40 ± 0.98
	$K_{0.5}$ (mM)	7.3 ± 1.3	9.2 ± 2.2	3.0 ± 3.3
	n	2.13 ± 0.44	1.99 ± 0.53	6.55 ± 268.3

The enzyme was solubilized in the standard solubilizing solution containing various combinations of NaCl and KCl maintaining [NaCl] + [KCl] = 0.1 M, and the resultant enzyme solubilized under the respective conditions was analysed on the HPGC column by the two ways of elution. One of them was that the column of TSKgel G3000SW_{XL} was equilibrated and eluted with elution buffer containing the same composition of NaCl and KCl as that used on solubilization of the enzyme (see Fig. 1A and B on the elution pattern and the graph for the data obtained). The other one was that the column was equilibrated and eluted with the elution buffer containing 0.1 M choline chrolide alone (neither elution pattern nor graph was shown). The dependence of oligomer amount on KCl concentration was analysed by curve-fitting method using the Hill equation, $Y = C_0 + \Delta C_{max}\cdot[KCl]^n/([KCl]^n + K_{0.5}^n)$, where Y (%) is the oligomer amount; C_0, the level of the amount (%) before the KCl addition; ΔC_{max} (%), the maximum change in the amount induced by stepwise substituting KCl for NaCl; [KCl] (M), the concentration of KCl added; $K_{0.5}$ (M), the concentration of K^+ for the half-maximum change in the amount; n, the Hill constant of the K^+-induced oligomeric interconversion. Different lots of the enzyme were used for the two cases.
[a] C_0, $\triangle C_{max}$ and $K_{0.5}$ were at first fitted by fixing n at 2.00 according to Origin Software (OriginLab Corporation), and then n was fitted as described in this table by fixing these three parameters at the values obtained.

The columns were equilibrated and the samples eluted at 0°C and the same pH as was used during pH-treatment. The amounts of oligomeric components in the protein eluted were estimated as described above.

RESULTS

Oligomeric Protein Components Separated Chromatographically from the Enzyme Solubilized with $C_{12}E_8$—The membrane-bound enzyme was solubilized in $C_{12}E_8$ in the presence of various concentrations of NaCl and KCl such that the total sum concentration of Na^+ and K^+ was fixed at 0.1 M. An 100 µl aliquot of the resulting solubilized enzyme was charged onto a TSKgel G3000SW_{XL} column which had been equilibrated with elution buffer containing the same composition of NaCl and KCl as was used during solubilization, and the columns were eluted with the same elution buffer as was used for equilibration. Four protein components in addition to mixed micelles (M), which were comprised of $C_{12}E_8$, lipids and small, non-protein substances (S), were detected by the UV-monitor. The protein components were designated G, H, D and P according to the data acquired by HPGC-LALLS method (*28*, *49*, *52*) (see an inset in Fig. 1A). As shown in Fig. 1A, the retention times for G, H, D and P did not change with changes in the molar ratio of NaCl to KCl, but the relative amounts of H, D and P were altered. The M_p values for the four protein components eluted under conditions similar to those used in the present study have been previously determined (*28*, *49*). G (see Fig. 4 in ref. 49) was too large to permit accurate size estimation and H was larger than D. The values of M_p for P and D were estimated to be 156 000 (±4000) and 302 000 (±10 000), respectively, and their oligomeric structure were unequivocally concluded to be the αβ-protomer and its dimer, i.e. the $(\alpha\beta)_2$-diprotomer (*28*, *52*).

Dependence of Oligomer Amounts on Molar Ratio of Na^+ to K^+ Used during Enzyme Solubilization—The relative amounts of G, H, D and P were plotted against the concentrations of KCl and NaCl. As shown in Fig. 1B, the amounts of H and D relative to the sum of all four protein components, increased from 5.3 to 7.6% and from 27.6 to 54.3%, respectively, with increasing K^+ and decreasing Na^+ concentrations. Whereas the relative amount of P decreased from 66.8 to 37.4%. The amount of G was constant. The dependence of oligomer amount on KCl concentration was analysed by curve-fitting according to the Hill equation:

$$Y = C_0 + \frac{\Delta C_{max}[KCl]^n}{[KCl]^n + K_{0.5}^n} \quad (1)$$

where Y (%) is the amount of oligomer; C_0, the relative amount (%) before KCl addition; ΔC_{max} (%), the maximum change in the amount induced by the stepwise substitution of KCl for NaCl; [KCl] (M), the concentration of KCl added; $K_{0.5}$ (M), the concentration of K^+ for a half-maximum change in the amount of oligomer; n, the Hill constant for the oligomeric interconversion. The fitting was performed for each case of P, D and H by fixing n at 2.00, and the other parameters were then determined. Second, the three parameters of C_0, ΔC_{max} and $K_{0.5}$ were fixed at the values thus obtained and n was best fitted in the same way. Values of n ranging between 1.44 and 2.15 were consistently obtained. The results of the curve-fitting are shown in Table 2 (in rows in the upper half). The sum of ΔC_{max} for D and H was 29.0%—equivalent to the absolute value of ΔC_{max} for P (−29.4%). The $K_{0.5}$ values for the three components ranged between 9 and 12 mM, and showed significantly no difference among them.

Another batch of the membrane-bound enzyme purified from dog kidney was solubilized, and subjected to

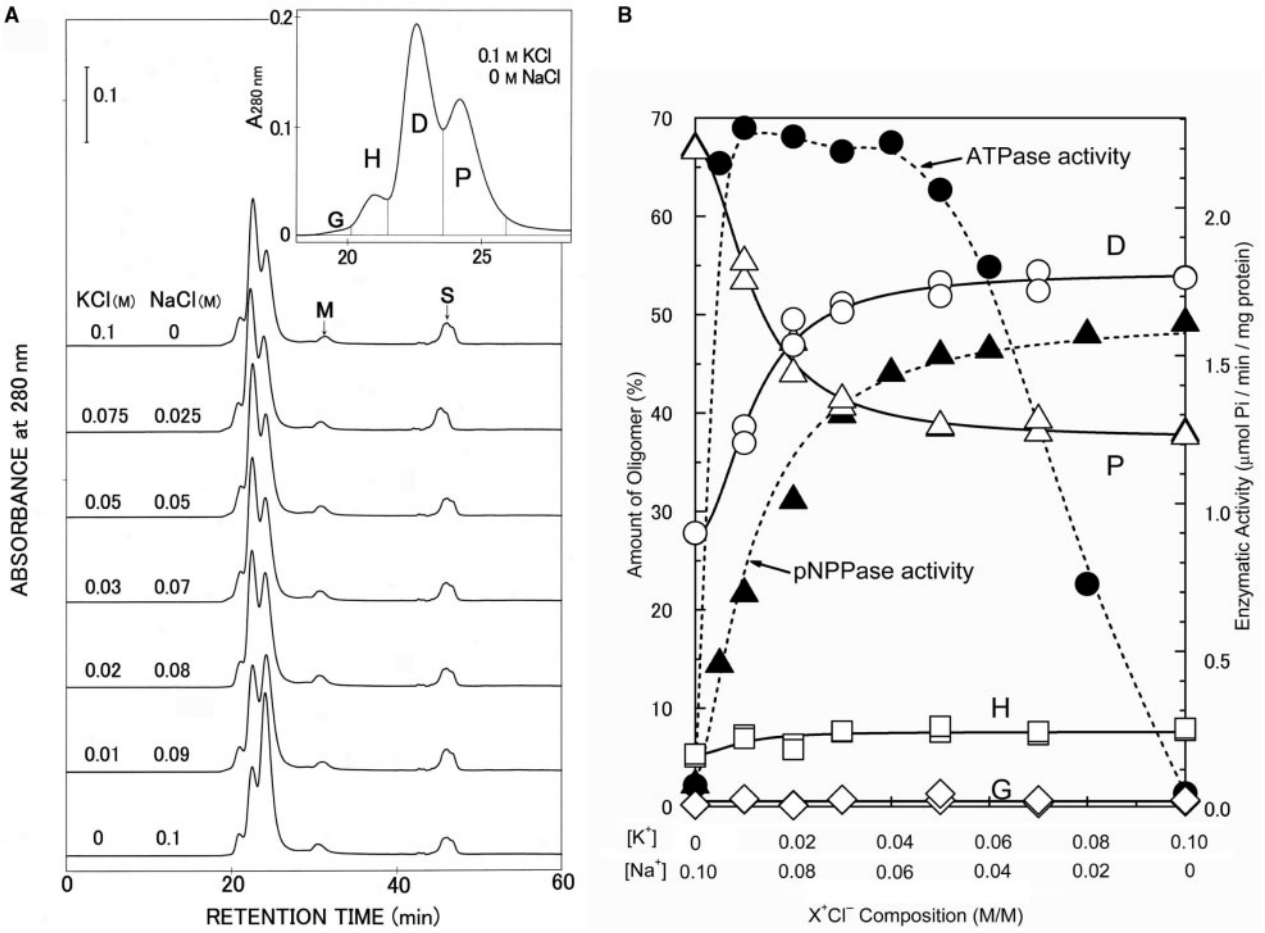

Fig. 1. **Elution patterns of Na^+/K^+-ATPase solubilized with $C_{12}E_8$ in the presence of various combinations of NaCl and KCl (A), and dependence of the amount of oligomer separated upon the combination (B).** A: The membrane-bound enzyme was solubilized with $C_{12}E_8$ at pH 7.0 and at 0°C in a standard solubilizing solution containing various concentrations of NaCl and KCl, as indicated on the horizontal axis. An aliquot of 100 μl of the resulting solubilized enzyme was subjected to HPGC on TSKgel G3000SW_{XL} columns equilibrated and eluted with a buffer containing the same concentrations of NaCl and KCl as those involved in the solubilizing solution at pH 7.0 and at 0°C. As indicated in the inset, the components eluted earlier than about 26 min were divided into four components of G, H, D and P, and after this, mixed micelles (M) and non-protein small substance (S) eluted. G, H, D and P denote aggregate, higher oligomer, $(\alpha\beta)_2$-diprotomer and αβ-protomer, respectively. The bar with flags denotes 0.1 absorbance units at 280 nm. The inset shows an enlarged elution pattern for a part of the top one obtained with 0.1 M KCl and 0 M NaCl. B: The protein amounts of G (open diamond), H (open square), D (open circle) and P (open triangle) were estimated from areas under the respective protein peaks of *A* at 280 nm, and the relative values plotted against the composition of NaCl and KCl adopted in the solubilization. Aliquots of 79 μl of each of the solubilized enzyme (1.19 mg/ml protein) prepared with a given composition of NaCl and KCl was added to 1.50 ml of the reaction mixture for an ATPase assay or for a *p*NPPase assay containing the same composition of NaCl and KCl as that involved in the enzyme at 0°C. The specific activities for ATPase (filled circle) and for pNPPase (filled triangle) were assayed at 20°C, and plotted against the composition of NaCl and KCl.

HPGC as described above except the columns were always equilibrated and eluted with an elution buffer containing 0.1 M choline chloride and neither added NaCl nor KCl. The solubilized enzyme was separated into the same four protein components as obtained above (data not shown). The dependence of oligomer amount on the molar ratio of Na^+ to K^+ also was analysed according to the Hill equation, as described above. As shown in the lower half of Table 2, ΔC_{max} for H, D and P were +5.4, +17.9 and −24.0%, respectively, with increasing K^+ concentrations, while the amount of G was constant. Thus, the sum (23.3%) of the increase in H and D was equivalent to the absolute value of the change in P (−24.0%). The $K_{0.5}$ values ranged between 3 and 9.2 mM for H, D and P. The characteristics of oligomeric interconversion among the four protein components were essentially the same as those observed when KCl and NaCl were used during solubilization and were included in the elution buffer. However, ΔC_{max} for P and D were decreased when choline chloride was used rather than KCl or NaCl. ΔC_{max} for P decreased from -29.4 ± 0.8 to $-24.0 \pm 2.0\%$ and ΔC_{max} for D also decreased from 26.7 ± 0.8 to $17.9 \pm 2.1\%$. The difference was mainly attributed to the specific ATPase activity of the membrane-bound enzyme used, since the difference in oligomeric amount between solubilizing solutions of

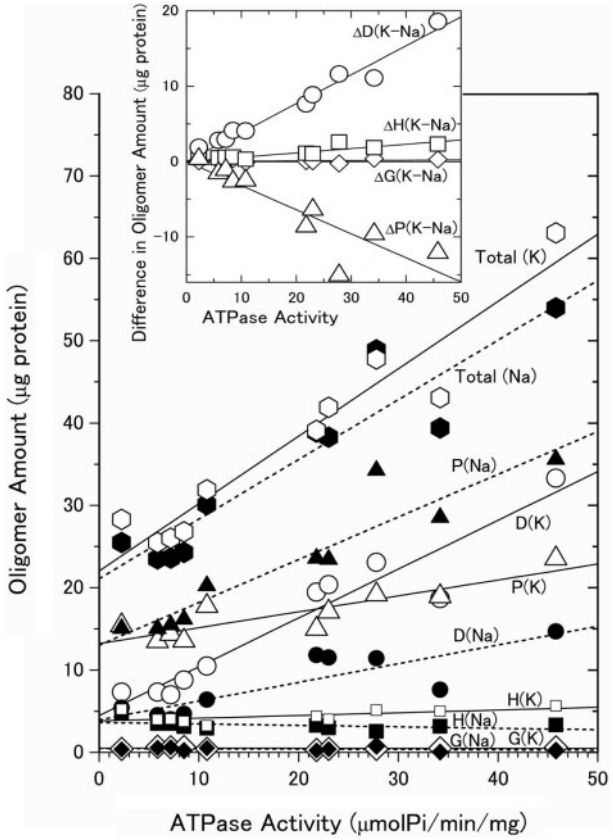

Fig. 2. **Dependence of difference in oligomer amount in the presence of KCl or NaCl upon specific ATPase activity of the enzyme used.** The purified membrane-bound enzyme was stored in 1 mM EDTA/imidazole at pH 6.9 and 0°C for a period of 0 days to 28 months. The enzyme thus stored exhibited ATPase activities ranging from 2.25 to 45.8 μmol/Pi/min/mg at 37°C under optimum conditions. The enzyme thus obtained was solubilized in the presence of 0.1 M KCl or 0.1 M NaCl under standard conditions, and was subjected to HPGC with an elution buffer containing 0.1 M choline chloride at 0°C. The amounts of respective oligomers for the two cases of the enzymes solubilized in the presence of KCl or NaCl were estimated separately, and displayed as open symbols (P(K)open triangle, D(K)open cirlce, H(K)open square, G(K)open diamond) or solid symbols (P(Na)filled triangle, D(Na)filled circle, H(Na)filled square, G(Na)filled diamond), respectively. The total amounts of the respective oligomers for the two cases of KCl or NaCl were displayed as Total (K) open hexagon or Total (Na) filled hexagon, respectively. Inset: The difference (μg) in the amount of P, D and H expressed in ΔP(K-Na) (open triangle), ΔD(K-Na) (open circle), ΔH(K-Na) (open square) or ΔG(K-Na) (open diamond) were obtained by subtracting the amount of the respective oligomer for the enzyme solubilized in the presence of 0.1 M NaCl from that in the presence of 0.1 M KCl, and plotted against the specific ATPase activities of the starting membrane-bound enzyme in the inset. Parameters of linear regression for the data in Fig. 2 are summarized in Table 3.

0.1 M KCl and 0.1 M NaCl was dependent on the specific ATPase activity of the enzyme used, as shown in Fig. 2. Therefore, the relative amounts of the oligomers and the concentration of KCl required to induce oligomeric changes could be measured consistently by HPGC when the columns are equilibrated and eluted at 0°C, regardless of which cation was used with choline alone or the appropriate mixture of Na^+ and K^+ is included in the eluent.

Dependence of the Catalytic Activities of the Solubilized Enzyme on Molar Ratio of Na^+ to K^+ in the Solubilizing Solution—The enzyme was solubilized in solutions containing variable ratios of NaCl to KCl, as described above, and the activities of ATPase and *p*NPPase were then measured at 20°C without changing the ratios of NaCl to KCl used during solubilization, although the concentrations of protein and $C_{12}E_8$ were diluted 20-fold. As shown in Fig. 1B, the curve for ATPase activity plotted against the ratio of NaCl to KCl was bell-shaped and highly skewed, which is the characteristic shape for membrane-bound Na^+/K^+-ATPase (*60*). ATPase activity increased sharply with an increase in KCl on the left part of the curve, and the value of $K_{0.5}$ for K^+ was ~3 mM, where the interconversion in the oligomeric structure was remarkable. On the right part of the curve, ATPase activity increased gradually with increasing Na^+, and $K_{0.5}$ for Na^+ was 27 mM. The dependence of ATPase activity on the ratio of NaCl to KCl observed at 10°C was the same as that at 20°C, although the maximum activity at 10°C was one-eleventh of the activity at 20°C (data not shown). In contrast to ATPase activity, the K^+-dependent *p*NPPase activity increased hyperbolically with an increase in KCl. $K_{0.5}$ for K^+ was 13 mM (Fig. 1B), which was comparable to the $K_{0.5}$ (19 mM) for the membrane-bound enzyme reported by Skou (*61*). Furthermore, $K_{0.5}$ for KCl was almost equivalent to that for the half-maximum increment for D, i.e. 12 mM (Table 2).

Dependence of Oligomer Amounts on Specific ATPase Activity—To confirm the significance of the oligomeric interconversion described above, the relation of ΔC_{max} to the ATPase activity was investigated using the enzyme preparations having various levels of specific ATPase activity. Ten preparations of the purified membrane-bound enzyme were washed to remove any Na^+ or K^+ contamination, held at 0°C (on ice) in a solution containing 1 mM EDTA/imidazole, but no glycerol, at pH 6.9 for periods of 0 days to 28 months. Specific ATPase activity, measured after various holding periods, ranged from 2.25 to 45.8 U/mg at 37°C just before being solubilized for elution by HPGC. Thus, enzyme preparations having the same protein homogeneity, although possessing different values for specific activities, could be used in the experiment. It was not clear why there was a loss of enzymatic activity, but the damage was such that storage of the enzyme in the presence of 20% glycerol at −80°C overcame the loss of activity. The enzyme preparations were solubilized in the presence of 0.1 M KCl or NaCl under standard conditions: 2 mg protein/ml of the membrane-bound enzyme was incubated with 6 mg/ml $C_{12}E_8$. A 50 μl aliquot of the solubilized enzyme was then subjected to HPGC. The four protein components—G, H, D and P—were detected at specific retention times, as described for the untreated enzyme (Fig. 1A). The estimated quantity of the oligomers was plotted against the specific ATPase activity for the starting membrane-bound enzyme. As shown in the main portion of Fig. 2, the amounts of respective oligomers, as well as the sum [Total (K) or

Table 3. **Parameters of linear regression for the relationship between oligomer amount and specific ATPase activity, drawn in Fig. 2.**

Salt added (Data symbol)	Oligomers	*A* (Intercept) (μg)	*B* (Slope) [μg·(U/mg)$^{-1}$]	Correlational coefficient
KCl (Open in Fig. 2)	P	13.208 ± 0.879	0.193 ± 0.038	0.8734
	D	4.477 ± 1.480	0.593 ± 0.064	0.9562
	H	3.822 ± 0.351	0.033 ± 0.015	0.6106
	G	0.527 ± 0.075	−0.001 ± 0.003	−0.1117
	P + D + H + G(sum)[a]	22.035 ± 1.758		
	Total protein	22.037 ± 1.934	0.818 ± 0.084	0.9605
NaCl (Solid in Fig. 2)	P	13.016 ± 1.518	0.519 ± 0.066	0.9413
	D	3.961 ± 1.178	0.227 ± 0.051	0.8436
	H	3.639 ± 0.291	−0.018 ± 0.013	−0.4476
	G	0.489 ± 0.095	−0.004 ± 0.004	−0.3523
	P + D + H + G(sum)[a]	21.104 ± 1.946		
	Total protein	21.049 ± 2.208	0.725 ± 0.096	0.9368
Difference[c] Δ(K-Na) (Inset in Fig. 2)	P	0	−0.319 ± 0.034	−0.8844
		(−1.546 ± 1.779)[b]	(−0.201 ± 0.069)[b]	(−0.6616)[b]
	D	0	0.384 ± 0.014	0.9841
		(0.896 ± 0.617)[b]	(0.344 ± 0.024)[b]	(0.9748)[b]
	H	0	0.057 ± 0.006	0.8805
		(0.290 ± 0.227)[b]	(0.043 ± 0.009)[b]	(0.8274)[b]
	G	0	0.005 ± 0.003	0.2385
		(0.024 ± 0.090)[b]	(0.004 ± 0.004)[b]	(0.3118)[b]
	P + D + H + G(sum)[a]	0		–
		(−0.336 ± 1.899)[b]		
	Total proteins	0	0.126 ± 0.032	0.5196
		(0.778 ± 1.327)[b]	(0.099 ± 0.058)[b]	(0.5196)[b]

The respective membrane-bound enzymes with a specific ATPase activity ranging between 2.25 and 45.8 μmol Pi/min/mg (U/mg) at 37°C were solubilized in the presence of 0.1 M KCl or NaCl, and the oligomer amount for P, D, H and G were estimated, as described in the legend for Fig. 2. The relationship between the amount of protein in units of μg (*Y*) for the respective oligomers and the specific activity (*X*) was fitted by the linear regression of $Y = A + BX$, where *A* is the intercept (μg protein) and *B* is the slope [μg(U/mg)$^{-1}$]. The difference in protein amount of P, D, H and G were obtained by subtracting the respective oligomer amounts for the enzyme solubilized in the presence of 0.1 M NaCl from that in the presence of 0.1 M KCl, and also fitted on the relationship to the specific activity in the same manner as above.
[a]S.D. for the sums of P, D, H and G were calculated according to propagation of the respective errors. [b]Results obtained by the linear regression without restriction of going through the original point. [c]Linear regressions were done with restriction of going through the original point, except the lines attached with footnote b.

Total (Na)] calculated by adding the components, increased in proportion to the ATPase activity. The relationship could be described by the expression, $Y = A + BX$, where *Y* is the amount of solubilized oligomer (μg); *X* is the specific ATPase activity (U/mg) of the starting membrane-bound enzyme; *A* is the intercept (μg protein) on the ordinate axis, i.e. the amount of solubilized enzyme with zero activity; B is the proportional constant as the slope of line. H, however, was not increased in the presence of NaCl, and G remained constant. Because a fixed amount of the enzyme (100 μg = 50 μl × 2 mg/ml, see below), regardless of the specific ATPase activity, was used for the solubilization, the relationship described above indicated that amount of protein insoluble in $C_{12}E_8$ was inversely related to specific ATPase activity. The relationships between the amounts of P and D and ATPase activity of the starting enzyme were linear, although some amount was not associated with any ATPase activity, i.e. $B > 0$ and $A \neq 0$ in the equation, $Y = A + BX$ (Table 3). The amount of protein obtained by extrapolating the relationship to zero activity, i.e. determination of A, were calculated to be 22.0 ± 1.9 and 21.0 ± 2.2 μg protein when solubilized in the presence of 0.1 M KCl and NaCl, respectively [see intercepts for Total (K) and Total (Na) in the main portion of Fig. 2, and *A* values as the intercepts in Table 3]. Thus, the amount of solubilized enzyme present at zero activity was the same (average 21.5 μg) regardless of whether KCl or NaCl was used in the solubilizing solution. Accordingly, the amount solubilized enzyme denatured to zero activity was equivalent to 39.2% of total protein [54.8 μg = 22.04 μg + 0.818 μg·(U/mg)$^{-1}$ × 40 U/mg, see KCl-lines in Table 3] solubilized from the enzyme with a specific activity of 40 U/mg, and 21.5% of the (100 μg = 50 μl × 2 mg/ml) starting enzyme protein. This result strongly suggested that even untreated enzyme with a specific activity as high as 40 U/mg contained some enzyme lacking ATPase activity. The solubilization thus done revealed the content to be 39.2% of the total solubilized protein.

The amounts of the four oligomeric proteins obtained by solubilizing the enzyme of known specific ATPase activity in the presence of 0.1 M NaCl was subtracted from the quantities of the four oligomers observed in the

Table 4. **Confirmation that the K^+-induced interconversion of Na^+/K^+-ATPase oligomers occur after the solubilization.**

Experiments	Salt compositions adopted (M)				Amounts of oligomer (%)			
	In solubilization		In treatment after solubilizing		P	D	H	G
	NaCl	KCl	NaCl	KCl				
A	0.020	0	0.019	0	71.4	18.8	8.4	1.1
			0.099	0	65.0	25.2	8.8	1.0
			0.019	0.080	42.9	48.6	6.3	2.2
B	0	0.020	0	0.019	40.8	42.3	13.0	3.9
			0	0.099	40.6	48.0	7.4	4.0
			0.080	0.019	46.1	42.4	7.9	3.5

The enzyme was solubilized in a solubilizing solution containing 0.02 M NaCl (for Exp. A) or 0.02 M KCl (for Exp. B). One or two aliquots of 2 M KCl, 2 M NaCl or H_2O was added to the resultant solubilized enzyme at 0°C so that the salt composition included in the enzyme was changed to those shown in the table. After 8 min, the enzyme thus treated was subjected to HPGC with the standard elution buffer containing 0.1 M choline chloride to estimate the oligomer amount.

presence of 0.1 M KCl. The differences in oligomer quantities were plotted against the specific activity. As shown in the insert of Fig. 2 and in Table 3, the differences in the amounts of D, P and H in the presence of KCl or NaCl were directly proportional to the specific activity, according to the expression, $Y = BX$; although the difference for G was always zero. The results described above and shown in Fig. 2 indicated that KCl induced the interconversion of P into D and/or H, and that NaCl reversed the conversion. Furthermore, the Na^+- and K^+-dependent interconversion among P, D and H was coupled to the activity to carry out ATP hydrolysis.

Oligomeric Conversion in the Solubilized State of the Enzyme—The enzyme was solubilized in the presence of 0.02 M NaCl but no KCl was present. An aliquot of 2 M KCl, 2 M NaCl and/or H_2O was added to the resulting solubilized enzyme at 0°C, resulting in a change in the composition to 0.019 M NaCl, 0.099 M NaCl or 0.019 M NaCl + 0.080 M KCl. After 8 min the enzyme preparations were subjected to HPGC with an eluent containing 0.1 M choline chloride alone to measure the amounts of oligomer present. As shown in Table 4 (Exp. A in rows in the upper half), the addition of 0.080 M KCl, while maintaining the concentration of NaCl + KCl constant at 0.099 M, increased the amount of D by 23.4% (from 25.2 to 48.6% of all the four protein components), and, on the contrary, decreased that of P by 22.1% (from 65.0 to 42.9%). The addition of 0.08M KCl to the solubilized enzyme containing 0.019 M NaCl brought about both an increase in D and a decrease in P by about 30% (from 18.8 to 48.8% on D and from 71.4 to 42.9% on P) of the total protein amounts, although no significant change was detected in the amounts of H and G. In another experiment (Exp. B in rows in the lower half of Table 4), the enzyme was solubilized in the presence of 0.02 M KCl but no NaCl was present, and then, in the same as above, the resulting solubilized enzyme was incubated under the three different salt compositions, 0.019 M KCl, 0.099 M KCl or 0.080 M NaCl + 0.019 M KCl. The oligomer amounts were not substantially different among the three compositions. The inclusion of KCl at 19 mM to the solubilized enzyme resulted in a higher amount of D (42.3–48.0%), regardless of the presence or absence of 0.08 M NaCl, as shown in Table 4, Exp. B. These results show that KCl, in preference to NaCl, converted the oligomeric structure of solubilized Na^+/K^+-ATPase from P to D, and that, the presence of NaCl alone, a higher amount of P (71.4%) and a lower amount of D (18.8%) resulted. The preference of KCl to NaCl in the interconversion between D and P of the solubilized enzyme is consistent with that the affinity of the membrane-bound enzyme for K^+ is much higher than for Na^+ (*6*, *62*).

Effect of Hofmeister Anions on the Oligomer Amounts in the Presence of K^+ or Na^+—Figure 3 shows typical elution patterns for the enzyme solubilized in the presence of the strong chaotropic anion of thiocyanate, the moderate acetate kosmotropic anion or the strong citrate kosmotropic anion. These elution patterns were the same as those obtained for the enzyme solubilized in the presence of KCl and/or NaCl containing chloride anion in that the four protein components were detected at their respective specific retention times (see the inset in Fig. 1A). This was true for all the other anions tested such as sulphate, tartrate, fluoride, bromide, nitrate or iodide (data not shown). Furthermore, in the same manner as the presence of Cl^- described above, the co-presence of K^+ increased the amount of H as well as D, while decreasing that of P, in the presence of every anion, and the co-presence of Na^+ instead of K^+ resulted in the reverse effect. As shown in Fig. 4A, when a weaker chaotropic anion ranging from thiocyanate to fluoride was present in the solubilizing solution, the amount of P was less, and the amounts of H and D were more, regardless of which cation of K^+ or Na^+ was present, with the only exception being that the amount of H in the presence of Na^+ was not changed. Accordingly, these findings clearly demonstrated that a greater amount of the dissociated state P of the enzyme was obtained in the presence of stronger chaotropic anions, and that a greater amount of the associated states such as D and H was favoured by weaker chaotropic anions. However, the effect of kosmotropic anions stronger than acetate (citrate, sulphate, tartrate and acetate) on oligomerization is difficult to explain for anions weaker than fluoride.

The amount of each oligomer obtained with the various anions in the presence of Na^+ was subtracted from that produced by replacing Na^+ with K^+, and the difference is plotted against the anions of the Hofmeister series in

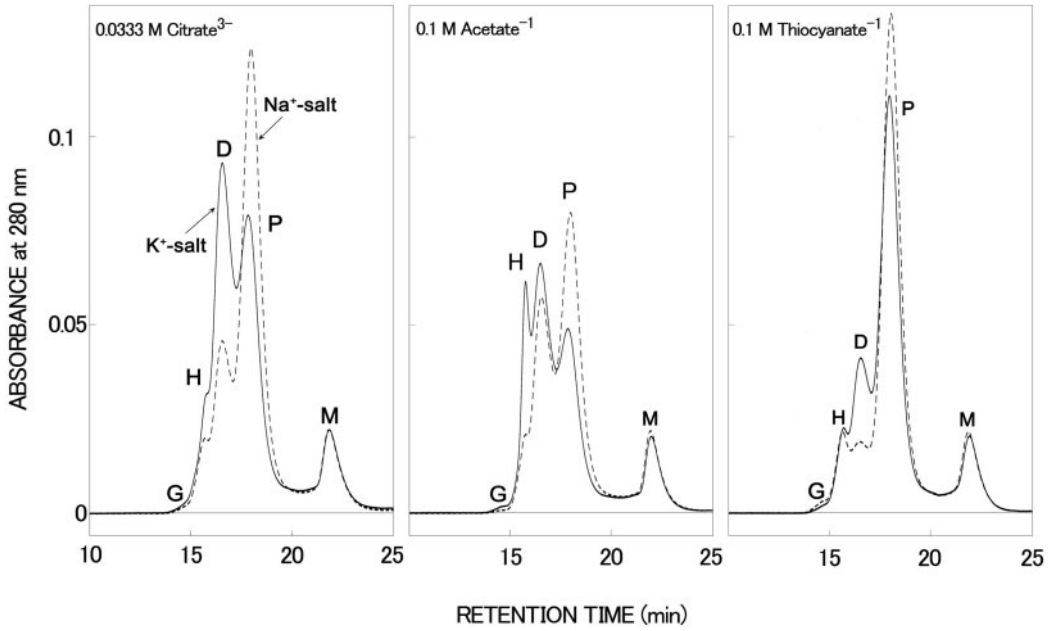

Fig. 3. **Elution patterns obtained by HPGC of Na^+/K^+-ATPase solubilized in the presence of 0.033 M citrate, 0.1 M acetate or 0.1 M thiocyanate accompanying by 0.1 M K^+ or Na^+ as counter cation.** The enzyme was solubilized and chromatographed in the same manner as described in the legend for Fig. 1A, except that the chloride in the solubilizing solution was replaced with the indicated anions, and that 0.1 M choline chloride was included in the elution buffer whatever salt was used in solubilizing the enzyme. The patterns for the solubilized enzyme obtained in the presence of K^+ or Na^+ were displayed by solid or broken lines, respectively. See the legend for Fig. 1A on G, H, D, P and M.

Fig. 4B. The sum ($\Delta H + \Delta D$) of the increases in the amount of D and H, and the decrease (ΔP) in that of P were mirror images of each other around the horizontal axis. This implies that the addition of K^+ causes P to associate into D and/or H without any conversion to other oligomers, regardless of the nature of the anion present. Acetate behaved in a characteristic manner, producing more H without fixing the oligomeric structure at D than did the other anions. The difference in the amount of G is approximately zero, suggesting that G is not sensitive to either K^+ or Na^+, and/or that G is an entirely different entity from native Na^+/K^+-ATPase. On the other hand, the amount of H was sensitive to the anions as well as K^+ and Na^+, in spite of the fact that the amount was 21% at most (Fig. 4A) of all the four protein components under the experimental conditions adopted here.

Dependence of the Oligomeric Conversion on KCl and NaCl Concentration—The concentration of KCl added to the solubilizing solution was increased from 0 to 0.1 M, with no added NaCl, and the relative amounts of the oligomers were estimated by HPGC. As shown in Fig. 5 by the open symbols, the amounts of D and H increased from 24.6 to 44.9% and from 6.7 to 14.9% of total oligomeric protein, respectively. The relative amount of P decreased from 66.9 to 39.9% and that of G remained constant. These data were fitted to Equation 1 and the parameters obtained are summarized in Table 5. The sum (27.1%) of the increases in D and H was equivalent to the decrease in P (−27.3%), suggesting that K^+ induced the conversion of P into D and H. The $K_{0.5}$s were 68, 50 and 108 μM for P, D and H, respectively. The K^+-dependence for oligomeric interversions among P, D and H were best fitted when the Hill constant (n) = 2, but not when n = 1 or 3 (see dotted and broken lines in Fig. 5 and Table 5).

When the concentration of NaCl was increased from 0 to 0.1 M, with no added KCl, there was no change in the amount of any of the oligomers (see the solid symbols in Fig. 5). Therefore, Na^+ did not cause either D or H to dissociate into P. It is, however, likely that choline$^+$ and $C_{12}E_8$ have a dissociating capacity comparable to that of Na^+. When 0.3 mM KCl was added to solubilizing solution, the amount of P decreased from 64.7 to 44.8%, D increased from 26.3 to 39.0%, and H increased from 8.0 to 14.9% of total oligomeric protein. The amount of G remained almost unchanged (Fig. 6). The respective changes in oligomer amount were equivalent to ~80% of the maximum changes induced by addition of 0.1 M KCl. When NaCl was increased from 1 to 100 mM in the presence of 0.3 mM KCl, the amount of P increased from 44.8 to 70.4%, D decreased from 41.2 to 24.1%, and H decreased from 14.9 to 4.7%, as shown in Fig. 6. The final amounts were nearly equal to those obtained without addition of KCl. Thus, it can be concluded that Na^+ reversed the association induced by K^+. The $K_{0.5}$s for Na^+ for the reversal were 5.5, 6.2 and 3.9 mM for P, D and H, respectively. The Na^+-dependence for the conversion of D and P demonstrated cooperativity with n = 2, but not with n = 1 or 3 (see broken and dotted lines in Fig. 6 and Table 5).

Using the results shown in Fig. 5 and Fig. 6, the sum of the oligomeric changes in H, D and P upon the addition KCl or NaCl was calculated, and plotted against

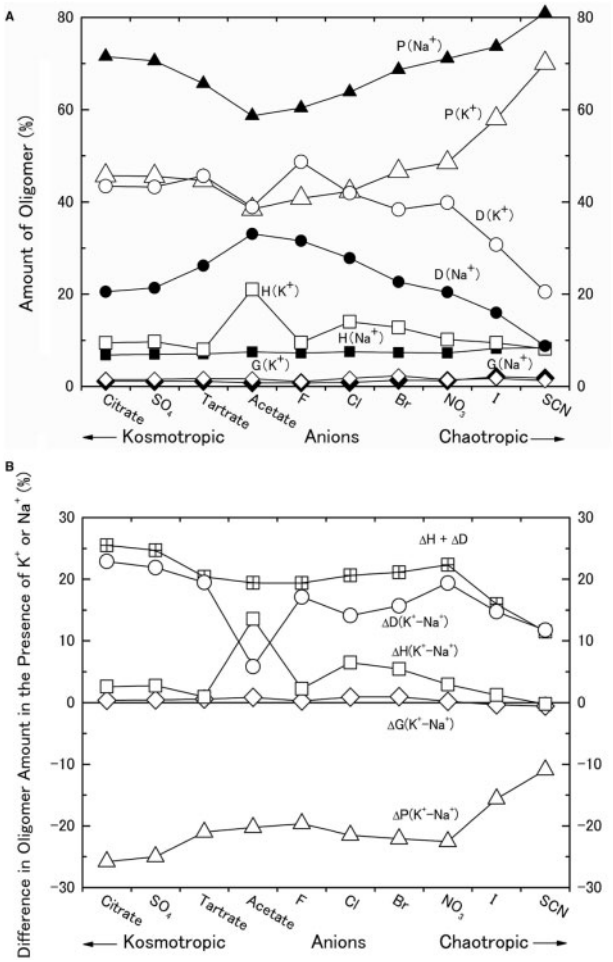

Fig. 4. **Oligomer amount for the enzyme solubilized in the presence of 0.1 N kosmotropic and chaotropic anions of Hofmeister series accompanying by 0.1 M K^+ or Na^+ as counter cation.** The enzyme was solubilized and chromatographed in the presence of various anions of the Hofmeister series by the procedures described in the legends for Fig. 3. A: The amount of the respective oligomers of P (open triangle, filled triangle), D (open circle, filled circle), H (open square, filled square) and G (open diamond, filled diamond) separated for the enzyme solubilized in the presence of K^+ (open symbols) or Na^+ (solid symbols) as the counter cation were plotted against the anions used. B; The amount of respective oligomers for the enzyme solubilized in the presence of 0.1 M Na^+ was subtracted from that for it solubilized in the presence of 0.1 M K^+ for each anion used, and the respective difference in the oligomer amount expressed in $\Delta P(K^+\text{-}Na^+)$(open triangle), $\Delta D(K^+\text{-}Na^+)$(open circle), $\Delta H(K^+\text{-}Na^+)$(open square) and $\Delta G(K^+\text{-}Na^+)$(open diamond), and the sum of the two differences for H and D expressed in $\Delta H + \Delta D$(grid square) were plotted against the anions.

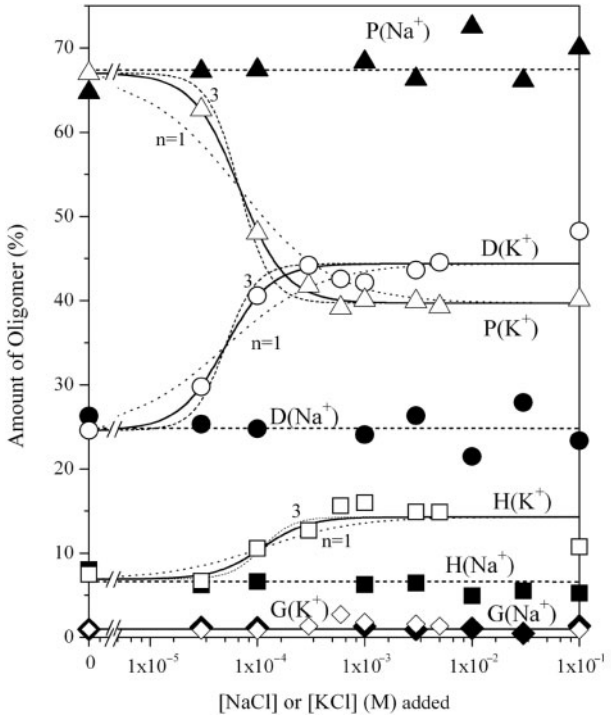

Fig. 5. **Dependencies of changes in oligomer amount on KCl or NaCl concentration.** The enzyme was solubilized with $C_{12}E_8$ in a solution containing 0 to 0.1 M KCl or NaCl at pH 7.0 and 0°C. The solubilized enzyme was subjected to HPGC, and separated into an aggregate (G, open diamond, filled diamond), a higher oligomer (H, open square, filled square), a $(\alpha\beta)_2$-diprotomer (D, open circle, filled circle) and a αβ-protomer (P, open triangle, filled triangle). The relative amounts of the oligomers were plotted against the concentrations of KCl (open triangle, open circle, open square, open diamond) or NaCl (filled triangle, filled circle, filled square, filled diamond) with open or solid symbols, respectively. The solid lines for the data of $P(K^+)$, $D(K^+)$ and $H(K^+)$ were drawn according to the Hill equation of $Y = C_0 + \Delta C_{max}\,[KCl]^n/([KCl]^n + K_{0.5}^n)$, where Y(%) is the oligomer amount; C_0, the level of the amount (%) before KCl addition; ΔC_{max} (%), the maximum change in the amount; [KCl] (M), concentration of KCl added; $K_{0.5}$ (M), concentration of K^+ or Na^+ for the half-maximum change in the oligomer amount; n, the Hill constant of the K^+- or Na^+-induced oligomeric interconversion. The dashed and dotted lines for the same data were drawn with the Hill constants (n) of 1 and 3, respectively, while the other parameters of C_0, ΔC_{max} and $K_{0.5}$ were fixed to the values described in the rows of the upper half in Table 5. The broken lines for the data of $P(Na^+)$ (filled triangle), $D(Na^+)$ (filled circle) and $H(Na^+)$ (filled square) were drawn in parallel to the abscissa.

KCl and NaCl concentration. The plots for KCl and NaCl were analysed using Equation 1 with C_0 fixed at 0 (as shown in Fig. 7): $n = 2.0$ and $K_{0.5} = 64.8\,\mu M$ for the change caused by KCl; and $n = 2.52$ and $K_{0.5} = 6.5\,mM$ for the change induced by NaCl.

pH-Dependence of Oligomer Amount and ATPase Activity of the Solubilized Enzyme in the Presence of K^+ or Na^+—The solubilized enzyme was treated at pH values ranging from 5.5 to 9.4 for 5 min at 0°C in the presence of 0.1 M KCl or NaCl, and was then analysed by HPGC, performed at 0°C and at the same pH and with the eluent containing the same monovalent cation concentration as were used during pH-treatment of the enzyme. The solubilized enzyme was separated into D, P, H and G at each pH, as occurred at pH 7.0 described above (data not shown). The difference in oligomer amounts in the presence of K^+ or Na^+ plotted against pH showed that Na^+- and K^+-dependent interconversion among D, P and H occurred at pHs ranging from 5.5 to 8.0, but not at pH >8.5 (Fig. 8). The sum ($\Delta D + \Delta H$) of the differences in the amounts of D and H, and the

Table 5. **Curve-fitting the dependencies of oligomerization of solubilized Na^+/K^+-ATPase on KCl or NaCl concentration by the Hill equation.**

XCl varied	Parameters	Protomer (P)	Diprotomer (D)	Higher oligomer (H)	D + H
KCl	C_0 (%)	67.0 ± 0.6	24.6 ± 2.2	7.0 ± 1.7	–
	ΔC_{max} (%)	−27.3 ± 0.7	19.8 ± 2.4	7.3 ± 2.0	27.1 ± 3.1
	n	2.0 ± 0.2	2.0 ± 0.8	2.5 ± 3.4	–
	$K_{0.5}$ (μM)	68 ± 4	50 ± 14	108 ± 53	–
	R^2	0.998	0.951	0.796	–
NaCl[a]	C_0 (%)	44.8 ± 1.0	40.1 ± 1.1	14.6 ± 1.7	–
	ΔC_{max} (%)	24.7 ± 1.5	−15.9 ± 1.8	−10.5 ± 3.4	−26.4 ± 3.8
	n	2.0 ± 0.4	2.0 ± 0.7	0.9 ± 0.7	–
	$K_{0.5}$ (μM)	5.5 ± 0.7	6.2 ± 1.5	3.9 ± 3.7	–
	R^2	0.996	0.985	0.930	–

The membrane-bound enzyme was solubilized in standard solubilizing solution containing either various concentrations of KCl but with no NaCl added, or various amounts of NaCl but constant one of 0.3 mM KCl, and the oligomer amounts were estimated as described in the legend for Figs. 5 and 6, and the data were illustrated in the figures. The Hill equation of $Y = C_0 + \Delta C_{max}[XCl]^n/([XCl]^n + K_{0.5}^n)$ was used, and the data were analyzed by curve-fitting in the same as described in the legend for Table 2.
[a]0.3 mM KCl always coexisted.

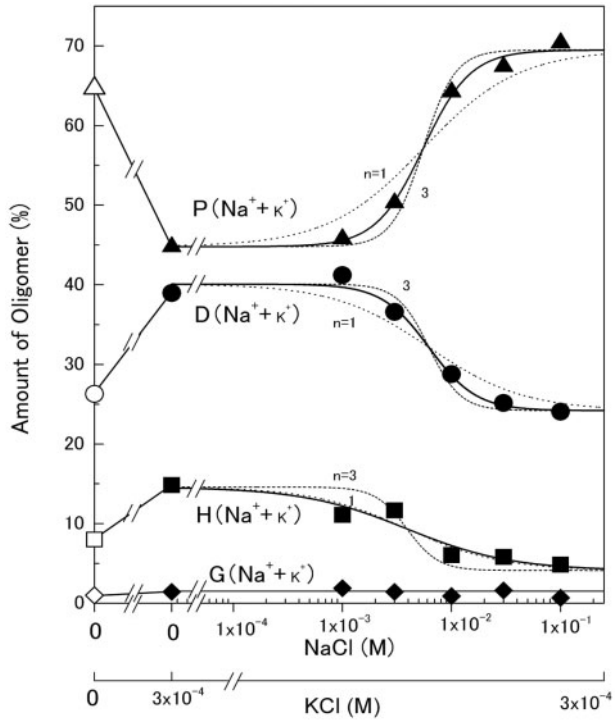

Fig. 6. **Dependencies of changes in the oligomer amounts upon NaCl concentration in the presence of 0.3 mM KCl.** The membrane-bound enzyme was solubilized in a solution containing 0 to 0.1 M NaCl with a constant concentration of 0.3 mM KCl, except for the experiment for the data plotted on the ordinate (open symbols) where neither KCl nor NaCl was added. The oligomers of P, D, H and G were separated by HPGC in the same manner as described in the legend for Fig. 5, and their amounts estimated. The data for the exceptional solubilization were plotted in open triangle for P, open circle for D, open square for H and open diamond for G on the ordinate. The solid lines for the data of P (Na^++K^+) (filled triangle), D (Na^++K^+) (filled circle) and H (Na^++K^+) (filled square) were drawn in the same ways as described in the legend for Fig. 5, using the parameters of C_0, ΔC_{max}, n and $K_{0.5}$ described at rows of lower half in Table 5. The solid line for G (Na^++K^+) (filled triangle) was drawn in parallel to the abscissa.

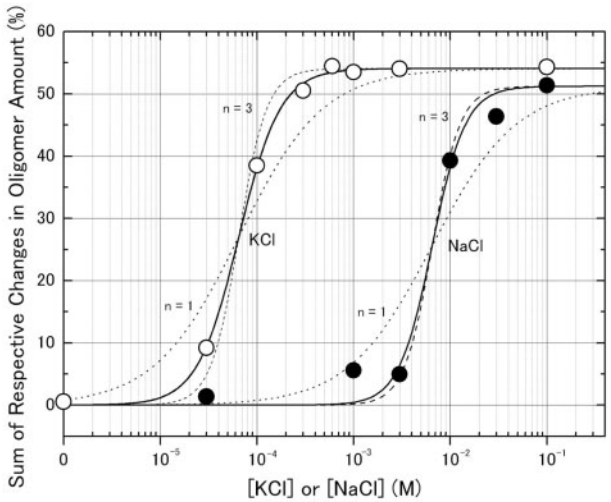

Fig. 7. **KCl- or NaCl-dependencies of the sum of oligomeric change occurring in the respective oligomers of H, D and P.** The changes in oligomer amount caused by the addition of KCl (see Fig. 5) or NaCl (see Fig. 6) from the level before salt addition were added up with respect to the three oligomers of H, D and P. In the calculations, the amount of P changed by the addition of KCl and those of H and D changed by the addition of NaCl were added using their absolute values. The sum of the oligomeric changes was plotted against the concentrations of KCl (open circle) or NaCl (filled circle), and the plots were best fitted by the Hill equation described in the legend for Fig. 5. K^+- or Na^+-dependencies of the sum of oligomeric changes were best fitted with the following parameters: $\Delta C_{max} = 54.1 \pm 0.4$ or 51.2%, $K_{0.5} = 64.8 \pm 2.0$ μM or 6.50 mM, $n = 2.02 \pm 0.10$ or 2.52 ± 0.48, respectively. The best-fitted results are shown as the solid lines. The dotted and dashed lines for the respective results were drawn with $n = 1$ and 3, respectively, while the other parameters were fixed to the values described above.

difference in P (ΔP) were also mirror images of each other around the horizontal axis, as described above for the relation between the difference in oligomer amount and Hofmeister anions.

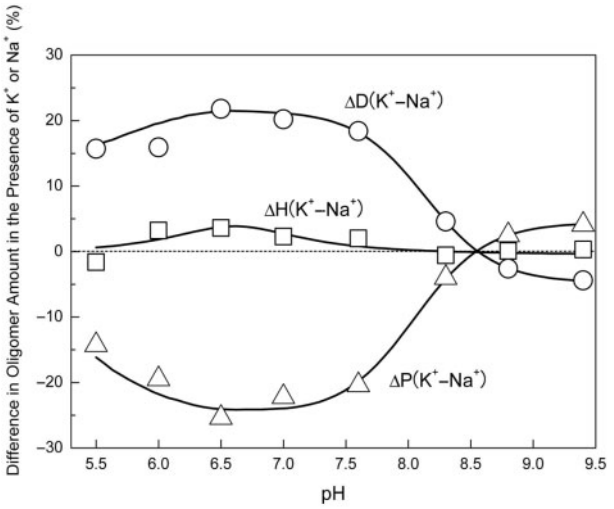

Fig. 8. **pH-Dependencies for the difference in oligomer amount in the presence of KCl or NaCl**. The membrane-bound enzyme was solubilized in the presence of 0.1 M KCl or NaCl under standard conditions, except that the buffer concentration in the solubilizing solution was reduced to one-third. A 50 μl aliquot of the solubilized enzyme was added to 10 μl of various buffers such as 170–70 mM HEPES/100–380 mM MES (pH 5.5 to 7.0), 500 mM imidazole/380–750 mM HEPES (pH 7.0 to 7.6), 500 mM Tris/300 mM HCl (pH 8.3), 500 mM TEA/50-330 mM CHES (pH 8.8 to 9.4), so that the solubilized enzyme was treated at the pH's indicated on the abscissa. After 5 min at 0°C, the enzymes thus treated at pH's from 5.5 to 7.0 or from 7.0 to 9.4 were subjected to HPGC with a TSKgel G3000SW$_{XL}$ or a Superdex 200HR 10/30 column, respectively. The columns had been equilibrated and eluted at 0°C and at the same pHs as those adopted for the pH-treatment. The amounts of P, D, H and G for the enzyme solubilized in the presence of 0.1 M KCl or NaCl were obtained at the respective pH's. The differences in the respective amount of P, D and H in the presence of KCl or NaCl expressed as $\Delta P(K^+\text{-}Na^+)$ (open triangle), $\Delta D(K^+\text{-}Na^+)$ (open circle) and $\Delta H(K^+\text{-}Na^+)$ (open square), respectively, were obtained as described in the legend for Fig. 2, and the differences expressed in units of percentage, instead of the microgram adopted for Fig. 2, were plotted against the pHs.

The enzyme solubilized at pH 7.0 was treated at 0°C and at various pH values in the presence of 0.1 M KCl or NaCl, as described above. After 5 min, the ATPase activity of the resulting enzyme preparations were assayed at the same pH values as those used during pH-treatment or at pH 7.0, at 20°C. The curves for pH versus ATPase activity were bell-shaped when the activities were measured at the same pH values as those used in the treatment, regardless of whether 0.1 M KCl or NaCl was present (data not shown). On the other hand, the ATPase activities of the enzymes treated at pH <8, assayed at pH 7.0, were equivalent to that of the enzyme treated at pH 7.0. Therefore, the solubilized enzyme was not irreversibly denatured at pHs ranging from 5.5 to 8.0, regardless of whether KCl or NaCl was present (data not shown).

DISCUSSION

Validity of the Determination of Oligomer Amount by HPGC Conducted at 0°C—The enzyme solubilized in solutions containing variable concentrations of KCl and NaCl was applied to an equilibrated HPGC column and was eluted at 0°C with eluent containing either the same composition of KCl and NaCl as that used in the solubilization or 0.1 M choline chloride. The results obtained for the dependence of oligomer composition on KCl or NaCl concentration were essentially the same, regardless of whether KCl, NaCl, or choline chloride was included in the eluent (Table 2). We have previously shown that the solubilized enzyme is in dissociation-association equilibrium of $D \rightleftharpoons 2P$ when it is chromatographed on a column equilibrated with elution buffer containing exogenous PS at 20°C, and that the interconversion between P and D is completely suppressed when the column is chilled to 0°C (*28*). Therefore, it is likely that the change in oligomeric structure initially occurs when the molecules are bound to K^+ or Na^+, the partitioning between $E_1{\cdot}Na^+$ and $E_2{\cdot}K^+$ takes place in the membrane, and the converted molecules are dispersed within seconds by the detergent. If this is the case, then there is no interconversion among oligomeric forms during elution on the HPGC column when it is chilled to 0°C. On the other hand, the enzyme solubilized in the presence of NaCl with a high amount of P was converted to a composition rich in D by addition of KCl prior to HPGC (Table 3). Therefore, the oligomeric state of the solubilized enzyme remains be practically unchanged in the column due to the low temperature, low protein concentration and lack of phospholipids—conditions that are required so that HPGC can run successfully at 0°C—and the amounts of the oligomers can be estimated under the given solubilization conditions.

Relationship of Oligomeric Interconversion to ATPase and pNPPase Activities—Skou (*60*) showed that the $K_{0.5}$ values for KCl and NaCl for membrane-bound Na^+/K^+-ATPase were 2 mM and 33 mM, respectively, in the presence of 150 mM (KCl + NaCl) (see Fig. 10 in ref. 60). In the present study, the $K_{0.5}$ values for KCl and NaCl for the solubilized enzyme were 4 mM and 27 mM, respectively (see Fig. 1B). Thus, the dependence of ATPase activity of the solubilized enzyme on the molar ratio of K^+ to Na^+ was similar to that of the membrane-bound enzyme reported by Skou (*60*). This suggests that partitioning between $E_1{\cdot}Na^+$ and $E_2{\cdot}K^+$ is maintained in the same ionic environment as for 20°C, even after solubilization. With respect to the solubilized enzyme, the $K_{0.5}$ values for the dramatic changes in oligomeric form (increase in D and decrease in P) and in ATPase activation induced by KCl were 10 mM and 4 mM, respectively. This result suggested that the association of P into D, induced by KCl, is an essential component of the molecular mechanism of ATP hydrolysis. However, when the ratio of Na^+ to K^+ was decreased from 1.5 (0.06 M NaCl to 0.04 M KCl) to 0 (0 M NaCl to 0.10 M KCl), no change in oligomer amount occurred, while ATPase activity decreased ($K_{0.5}$ for NaCl: 33 mM) (Fig. 1B). The apparent discrepancy between oligomeric conversion and ATPase activity can be interpreted as follows: the KCl-induced association of P to D ($K_{0.5}$ for KCl: 7 mM) was maintained when the concentration of NaCl was decreased from 60 to 0 mM due to preferential K^+-binding to the enzyme in this region, stabilizing the

Table 6. **Simulation of the difference in oligomer amounts in the presence of 0.1 M KCl or NaCl based on an association-dissociation equilibrium of D⇌2P.**

			0.1 M KCl		0.1 M NaCl		Difference (KCl-NaCl)		Sum	
			P(%)	D + H(%)	P(%)	D + H (%)	Δ(D + H)	ΔP	Δ(D + H) + |ΔP|	
Experiments	#1	Fig. 1B (Table 2)	37.4	61.9	66.8	32.9	29.0	−29.4	58.4	
	#2	Fig. 5 [Table 5(upper)]	39.7	58.7	67.0	31.6	27.1	−27.3	54.4	
		Unconvertible oligomers included (%)			K_d adopted for D⇌2P					
		P	D + H		1.6×10^{-7} M		8.0×10^{-6} M			
Simulations	#3	29.1	10.1		36.4	63.4	65.9	34.0	29.4	−29.5	58.9
	#4	24.1	15.1		31.5	68.3	60.5	39.2	29.1	−29.0	58.1
	#5	0	0		10.0	90.1	52.1	48.0	42.1	−42.1	84.2

The enzyme with specific ATPase activity of 40 U/mg would be expected to yield 54.8 μg of the solubilized protein in an aliquot of 50 μl (7.03×10^{-6} M in terms of P) to be eluted by HPGC under the experimental conditions described in the legend of Fig. 2. The simulations were done on the assumptions that the solubilized protein included un-convertible oligomers by 39.2% (w/w), that is, 21.5 μg (#3 and #4) or no such oligomers at all (#5). The distributions of the un-convertible proteins among P, D and H were deduced from the experimental data shown in Fig. 2 or Table 3 (see the intercepts of the linear regression line for the relationship between the amount of oligomer and the activity), and those were applied to the simulation as it is (#4) or after being modified (#3). The dissociation constants (K_d's) of the equilibrium were assumed to be 1.6×10^{-7} M or 8.0×10^{-6} M in the presence of 0.1 M KCl or NaCl, respectively, and H was regarded as D, and their amounts were grouped together as D, since H was a minor constituent and the sum of the oligomeric interconversion in D and H were usually equivalent to that for P.

enzyme in the $E_2 \cdot K^+$ conformation. However, ATP hydrolysis can be exhibited through the cyclic transition between two enzyme conformations, E_1 and E_2; both the rates of ATP hydrolysis and of the transition between E_1 and E_2 depend on the molar ratio of Na^+ to K^+. Thus, the inhibition of ATPase activity would be greater when the enzymes is held in the E_2 conformation at by preferential K^+-binding as the ratio of Na^+ to K^+ was decreased. On the other hand, *p*NPPase activity should remain constant without conformational changes between E_1 and E_2 during the reaction sequence. That is, *p*NPPase remains in the E_2 conformation, and, thus, enzymatic activity increases monophasically with increasing KCl, and becoming saturated, as was observed for D. Consequently, the results obtained for changes in ATPase and *p*NPPase activities, and in oligomeric structures at low K^+- and high Na^+-concentrations strongly suggest that enzymatic activation and oligomeric conversion are closely related to each other.

Affinities of K^+ and Na^+ for the Oligomeric Interconversion—The $K_{0.5}$ values for K^+ for oligomeric interconversion for P, D and H were 68 ± 4, 50 ± 14 and 108 ± 53 μM, respectively (see Fig. 5 and Table 5). In contrast, no oligomeric changes were detected with increasing Na^+ using the methods described above for the addition of K^+ (see solid symbols in Fig. 5). The oligomeric changes brought about by the addition of 0.3 mM KCl, were, however, reversed by the increase in Na^+ (Fig. 6). Na^+-binding to the enzyme caused D and H to dissociate into P, leading to a decrease in D and H, and an increase in P. The $K_{0.5}$'s values for Na^+ for the maximal reversal were 5.5 ± 0.7, 6.2 ± 1.5 and 3.9 ± 3.7 mM for P, D and H, respectively (Table 5). To integrate the oligomeric changes induced by KCl or NaCl, the sum of the changes in H, D and P induced by the addition of KCl and NaCl was plotted against concentration (Fig. 7). The best-fit plots clearly show, that KCl- and NaCl-induced oligomeric interconversions, with $K_{0.5}$ values of 64.8 μM and 6.50 mM and *n*s of 2.02 and 2.52, respectively. Thus, the oligomers, H, D and P, were dependent on one another with respect to oligomeric change. Accordingly, K^+-binding to the enzyme strengthened protein-protein interactions, such as P-P and/or D-D, and Na^+-binding antagonized the effect of K^+ to dissociate D and H into P and/or D. We have previously shown that the solubilized enzyme is in a dissociation-association equilibrium of D⇌2P at 20°C, and that an association constant (K_a) for the equilibrium in 0.1 M KCl was about 50-fold greater than that observed in 0.1 M NaCl (*29*). The results of the present study, demonstrating dependence of oligomeric change on K^+ and Na^+, are consistent with the dependence of association-dissociation equilibria on monovalent cation concentration, although the presence of the oligomer H previously went unrecognized.

The specific binding of $^{42}K^+$ and $^{22}Na^+$ to the membrane-bound enzyme isolated dog kidney were determined by centrifugation to be 2.0 and 2.7 mol per mol of bound ouabain, respectively (*62*). The apparent K_d's for K^+- and Na^+-binding were 6 μM and 0.34 mM, respectively, with positive co-operativities of $n = 1.72$ and 1.23, respectively. The $K_{0.5}$ values for oligomeric conversion were about 10-fold greater than the apparent K_d for specific binding, while the cooperativity of K^+, but not Na^+, -binding to the enzyme, was the same. The discrepancy between the K_d's for the binding of K^+ and Na^+ to the enzyme and the $K_{0.5}$ values for the oligomeric interconversion can be attributed to substances present in the solubilized enzyme preparation, such as 0.1 M choline chloride and 6 mg/ml $C_{12}E_8$, which have Na^+-like and ATP (with a low affinity)-like effects, respectively, and therefore decrease the K^+- and Na^+-binding affinities for the solubilized enzyme. The binding of $^{204}Tl^+$ and $^{86}Rb^+$ to the solubilized enzyme were measured using the Hummel–Dreyer method (*63*) with the eluent containing

0.3 mg/ml $C_{12}E_8$, 0.1 M choline chloride at pH 7.0 and 3°C and either 16 μM $^{204}Tl^+$ or 20 μM ^{86}Rb (*64*). The amounts of Tl^+ bound for D and P were 1.9 [95% saturation binding with 2 mol/mol of P (*65*)] and 1.2 mol/mol (60%) of P, respectively, and those of Rb^+ were 1.2 (60%) and 0 (0%) in the same units for D and P, respectively. Taking into consideration that the order of binding affinity of the K^+-analogue to the membrane-bound enzyme was $Tl^+ > Rb^+ \geq K^+$ (*66*), it can be concluded that the $K_{0.5}$ for K^+ for the half-maximal oligomeric conversion, 64.8 μM, (Fig. 7) is equivalent to the K_d for K^+-binding to the solubilized enzyme under the present conditions.

Hastings and Skou (*67*) measured the binding of K^+ to an enzyme purified from spiny dogfish with a K^+-sensitive valinomycin electrode, and showed a K_d of 59 μM in the presence of 97 mM choline chloride. The dependence of Rb-occlusion upon concentration was determined with enzymes purified from mammalian kidney or shark rectal glands, and the $K_{0.5}$ values for Rb^+ were reported to be 35 μM (*68*), 100 to 200 μM (*69*) and 15 to 30 μM (*70*). The fluorescence intensity of the FITC-labelled enzyme was decreased or increased depending on whether KCl or NaCl, respectively, was added. The $K_{0.5}$ values for K^+ were 83 and 220 μM for the purified enzyme and enzyme reconstituted in phospholipids vesicles, respectively. The $K_{0.5}$ values for Na^+ were 23 and 6.0 mM for the purified enzyme and enzyme reconstituted in vesicles, respectively (*71*). The intensity of Trp in the intact enzyme also was altered by addition of K^+, and the $K_{0.5}$ was 50 μM (*72*). Post (*73*) indicated $K_{0.5}$ values of Na^+ (intracellular side) and K^+ (extracellular side) ions for reversible in ⇌ out exchange reactions to be 1 mM and 100 μM, respectively. Mardh and Post (*74*) estimated Na^+-dependence of the initial rate of phosphorylation, and the affinity (K_m) for Na^+ to be 8 mM (*74*). Thus, there was no discrepancy between the published binding affinities of K^+ and Na^+ to the active or functional sites and those obtained in this study for oligomeric interconversion. It has yet to be determined which elementary reaction(s) for the active transport of Na^+ and K^+ by the enzyme are coupled with oligomeric interconversion.

Relationship of Oligomeric States to the Conformational States of E_1 and E_2—The solubilized enzyme has been shown to be in an equilibrium of D⇌2P at 20°C; the equilibrium is shifted to P and D by NaCl and KCl, respectively (*28*). This observation strongly suggests that the conformational states, E_1 and E_2 correspond to protomeric (more dissociated) and diprotomeric (more associated) forms of the oligomeric enzyme, respectively. On the other hand, Post and Suzuki (*53*, *54*) have shown that the equilibrium between the two reactive states of a phosphoenzyme, E_1-P⇌E_2-P, is shifted to E_1-P and E_2-P by chaotropic and kosmotropic anions, respectively. Taking into account the correspondence between conformational state and oligomeric form observed in this study and the anion-dependence of the conformational state shown by Post and Suzuki (*53*, *54*) into consideration, it is hypothesized that the equilibrium among the oligomeric forms would be shifted to the dissociated and associated oligomeric forms by chaotropic and kosmotropic anions, respectively. As shown in the right half (F to SCN) of Fig. 4A, the stronger chaotropic anions, ranging from acetate$^-$ to SCN^-, increased P and decreased D and H to a greater extent in terms of oligomer amount. Indeed, acetate appeared to have a unique function, as more H was produced by consuming D. Therefore, the effect of inorganic monovalent anions on the phosphoenzyme shift between E_1-P and E_2-P, found by Suzuki and Post (*53*, *54*), was consistent with the anion effect on the oligomeric conversion between the dissociated (P) and associated oligomeric forms (D and H). Other anions, however, such as tartrate, sulphate and citrate, kosmotropic anions, did not show a Hofmeister effect, as described above. Differences in ionic strength between mono- and polyvalent anions may account for the lack of Hofmeister effect.The dependence of oligomer amount on K^+ and Na^+ in the of various Hofmeister anions was obtained by subtracting the amount of the respective oligomer obtained with each anion in the presence of Na^+ from that obtained by replacing Na^+ with K^+. As a result, the sum of the K^+-induced increments in the amount of D and H and the K^+-induced decrement in that of P were mirror images of each other around the horizontal axis (Fig. 4B). This result implies that the addition of K^+ caused P to associate into D and/or H, and that the addition of Na^+ induced the reconversion. It has also been clearly shown that the effects of K^+ and Na^+ on the interconversion among P, D and H occur regardless of which anion is present. Thus, the effect of Hofmeister anions is separate from and additive to the effects of occupancy of monovalent cation transport sites, as shown in Fig. 4B. The relationship between oligomeric state and conformational states, i.e., E_1 and E_2, can now be revised as follows: E_1 corresponds to the state of weak subunit-subunit interaction such that oligomer dissociates; the E_2 conformation has strong subunit–subunit interactions and oligomers associate as consequence, e.g. D and H.

How Much Oligomeric Change is Adequate?—The enzyme isolated from dog kidney, solubilized with $C_{12}E_8$, is in an equilibrium of D⇌2P at 20°C. The dissociation constant, K_d, in the presence of 0.1 M KCl is about 50-fold lower than in the presence of 0.1 M NaCl (*28*, *29*). The K_ds obtained were 2.0×10^{-6} M for the enzyme turning over, and 9.1×10^{-8} M for the enzyme inhibited with ouabain—one of the lowest values estimated so far. Based on the data obtained in this study, the amount of oligomeric interconversion was simulated with the following assumptions. Initially, the equilibrium of D⇌2P was assumed by adding the amount of H to that of D, and neglecting that of G, as H was a minor, but significant, component under the present conditions. The values of K_d used were 1.6×10^{-7} M and 8.0×10^{-6} M in the presence of 0.1 M KCl and 0.1 M NaCl, respectively. Furthermore, 39.2% of all the solubilized enzyme was assumed to maintain an oligomeric state—fixed as either P or D—even after 0.1 M KCl was added. As shown in Table 6, the results from the simulation were in agreement with the experimental values in Fig.1B (Table 2) and Fig. 5 (Table 5, upper portion). If the assumption that 39.2% of oligomers are immune from interconversion was not entered into the simulation, the calculated amounts of P in 0.1 M KCl and NaCl would be

10.0 and 52.1% (see line #5 in Table 6), respectively. Thus, there would be a large discrepancy between the calculated and experimental values of 38.6 and 66.9% (averages of the data described in #1 and #2 in Table 6) in 0.1 M KCl and NaCl, respectively. The sum of oligomeric changes described in Fig. 7, 54.1 and 51.2% for KCl and NaCl, respectively, were nearly equivalent to 58.1 to 58.9% in the simulation by adopting 39.2% insensitivity to interconversion (#3 and #4 in Table 6). If the oligomeric components incapable of interconverting were assumed not to exhibit ATPase activity, the rest of the enzyme (60.8% of all the solubilized enzyme) would have a specific activity of 66 U/mg at 37°C, a value that nearly equivalent to the activity of the enzyme purified from nasal salt glands of salt-adapted ducks (*75*).

Thus, the results obtained in this study indicate that Na^+/K^+-ATPase isolated from dog kidney, with a specific activity as high as 40 U/mg, is heterogeneous by as much as 39% with respect to oligomeric interconversion. Using the same enzyme and HPGC method described in the present study, we previously reported that the amounts of [^{3}H]ouabain bound to D and P per 1.56×10^5g protein (mol of P) were 1.12 ± 0.06 and 1.11 ± 0.05 mol, respectively (see Table 2 in ref. *76*), which was stoichiometric, but not heterogeneous. On the other hand, Xie and Askari (*77*) reported that Na^+/K^+-ATPase has non-pumping functions, such as signal transduction and acting as a receptor for endogenous ouabain-like hormones, in addition to its pumping function (*77*). The issue of whether a relationship exists between the inconvertible oligomers of P and D found here and the non-pumping functions, remains unknown at the present time.

What is the Higher Oligomer?—The amount of H was at most 6% of the oligomers under the usual conditions where the solubilizing medium contained 0.1 M KCl at pH 7.0 (see Fig. 1B). Preliminary determinations of M_p of H by HPGC-LALLS method for the enzyme solubilized in 0.1 M CH_3COOK gave a value of 5.96 to 6.14×10^5 (*78*). Since H would, as shown here, be reversibly converted between the D and P forms, it should now be considered to be a $(\alpha\beta)_4$-tetraprotomer (T). Mimura *et al*. (*29*) showed that the solubilized enzyme reversibly associated into oligomers higher than D with additional PS. Shinji *et al*. (*79*) preliminarily reported that the amount of H increased to 45% of all oligomers by improving the conditions with inclusion of a synthetic dioleoyl PS. Taniguchi and colleagues have strongly suggested that Na^+/K^+-ATPase (*31*, *32*, *34*, *80*) and H^+/K^+-ATPase (*81*, *82*) function in the presence of both Na^+ (or H^+) and K^+, as well as MgATP, in the tetraprotomeric form of the respective enzymes. Currently, studies are underway to characterize H (T) isolated under the improved conditions.

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