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# Change in oligomeric structure of solubilized $Na^+/K^+$-ATPase induced by octaethylene glycol dodecyl ether, phosphatidylserine and ATP

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Membrane-bound $Na^+/K^+$-ATPase purified from dog kidney was solubilized with octaethylene glycol dodecyl ether ($C_{12}E_8$), and the resultant solubilized enzyme was chromatographed on a TSKgel G4000SW$_{XL}$ or G3000SW$_{XL}$ column equilibrated with elution buffers containing various ligands affecting oligomerization of the enzyme. Weight-averaged molecular weight ($\overline{M}_w$) values for the main protein components eluted were estimated by low-angle laser light-scattering photometry. With increasing concentration of $C_{12}E_8$ included in the elution buffer from 0.1 to 5 mg/ml, the $\overline{M}_w$ decreased from 230000 to 153000, indicating that $C_{12}E_8$ induced dissociation of the enzyme. In contrast, the $\overline{M}_w$ of the protein component increased up to $1.44 \cdot 10^6$ as the concentration of phosphatidylserine (PS) added to the elution buffer containing a fixed concentration of 0.3 mg/ml $C_{12}E_8$ was increased to 120 $\mu$g/ml. The association and/or aggregation were reversible by removal of the PS by rechromatography. Addition of PS to the elution buffer also allowed the solubilized enzyme to exhibit ATPase activity comparable to that of the membrane-bound enzyme during passage through the column. This was also the case with phosphatidylglycerol (PG) and phosphatidylinositol, but not with phosphatidylcholine or phosphatidylethanolamine. The specific refractive index increment ($dn/dc_p$) of the solubilized enzyme was increased by addition of exogenous PG or PS, strongly suggesting that the phospholipid became bound to the enzyme, and that it induced association of the enzyme. The association induced by PS was inhibited by ATP and ADP, but not AMP. The concentrations for half-maximal inhibition were 0.44 mM for ATP and 0.88 mM for ADP. The PS-induced associated enzyme isolated by chromatography in the presence of 120 $\mu$g/ml PS was dissociated by ATP with $K_{0.5}$ of 0.16 mM. The dissociating effect of $C_{12}E_8$, ATP and ADP and the associating effect of PS on the solubilized enzyme are consistent with the reports that $C_{12}E_8$ mimics the effect of regulatory ATP at the low-affinity site on the conformational transition from $E_2$ to $E_1$, and that phospholipids are essential for the reverse transition from $E_1$ to $E_2$. The results can be explained by assuming that the enzyme takes the form of a loosely associated diprotomer in the $E_1$ state and a tightly associated one in the $E_2$ state.

## Introduction

$Na^+/K^+$-transporting adenosine triphosphatase ($Na^+/K^+$-ATPase), also known as the sodium pump or sodium-potassium pump, is a typical integral membrane protein which is buried in the plasma membrane of all animal cells. The membrane protein transports $3Na^+$ ions outward and $2K^+$ ions inward across the membrane coupled with hydrolysis of one molecule of intracellular ATP.

According to the Post-Albers reaction scheme for ATP-hydrolysis by the enzyme [1], which has been widely accepted, the membrane protein takes two distinct conformational states designated as $E_1$ and $E_2$ during each turnover. The enzyme has high affinity for $Na^+$ and ATP, but low affinity for $K^+$ in the $E_1$ state. The enzyme with bound $Na^+$ ions is phosphorylated by very low concentrations of ATP ($K_{0.5}$ of ATP for half-maximal phosphorylation; 0.3–4 $\mu$M [2,3]) at the $\beta$-carboxyl group of an Asp residue. The phospho-

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Abbreviations: $C_{12}E_8$, octaethylene glycol n-dodecyl ether; LALLS, low-angle laser light scattering; HPGC/LALLS, monitoring of elution from a high-performance gel chromatography column with a LALLS photometer and supplementary equipment; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LS, light scattering; RI, differential refractive index; UV, ultraviolet; $K_a$, association equilibrium constant; $\overline{M}_w$, weight-averaged value for molecular weights of protein components eluted during a given time in a high-performance gel chromatography.

rylated intermediate of the enzyme in the $E_1$ state, that is, $E_1$-P, changes its conformational state to $E_2$, forming $E_2$-P. In the $E_2$ state the enzyme has high affinity for $K^+$, but low affinity for $Na^+$ and ATP. Then, the conformational change allows the $Na^+$ ions to escape to the cell exterior, and $K^+$ ions bind to the enzyme. The binding of $K^+$ leads to the transfer of the phospho group from the carboxyl group of Asp to water, and this transfer is associated with occlusion of the $K^+$ ions within the newly dephosphorylated enzyme ($E_2$-$K^+$ formation). The binding of ATP at a low-affinity site of the $E_2$ intermediate accelerates a spontaneous conformational change from $E_2$-$K^+$ to $E_1$-$K^+$ [4,5] and allows the occluded ions to escape to the cell interior. Then, the enzyme returns to the starting form of $E_1$. Huang et al. [6] have shown that surfactants such as octaethylene glycol dodecyl ether ($C_{12}E_8$) mimic the effect of ATP at the low-affinity site to accelerate the transition from $E_2$-$K^+$ to $E_1$-$K^+$. Thus, the Post-Albers reaction scheme implies that there are essential structural differences between $E_1$ and $E_2$ which must be investigated to clarify how the enzyme molecule transports the ions.

The enzyme requires phospholipids as well as two kinds of polypeptides for the enzymatic activity. One of the polypeptides is the $\alpha$-chain or catalytic subunit, and the other is the $\beta$-chain, a glycopeptide [7]. The role of the $\beta$-chain itself in the enzymatic reaction is not known yet, but the two polypeptides bind strongly to each other to form $\alpha\beta$-protomer and can not be separated without loss of the enzymatic activity. Wheeler and Whittam [8] have shown that negatively charged phospholipids such as PS and PI are essential for the enzymatic activity. Hegyvary et al. [9] have shown that phospholipids are required for the reaction step from $E_1$-P to $E_2$-P. Therefore, the effects of ATP and $C_{12}E_8$, and phospholipids on the conformational change between $E_1$ and $E_2$ are opposite in direction.

The enzyme was purified in membrane-bound form from mammalian kidneys by sucrose gradient ultracentrifugation in a zonal rotor [10]. The membrane-bound enzyme purified from dog kidney was solubilized with $C_{12}E_8$, without loss of the enzymatic activity. The main protein components of the solubilized enzyme were determined to be $\alpha\beta$-protomer and $(\alpha\beta)_2$-diprotomer with $M_r$ values of $156\,000 \pm 4\,000$ and $302\,000 \pm 10\,000$, respectively, at 0°C by the HPGC/LALLS method [11,12]. $M_r$ values of the $\alpha$- and $\beta$-chains obtained from the enzyme were estimated to be $118\,000 \pm 3\,000$ and $39\,400 \pm 900$, respectively, by the same method [13]. Furthermore, a computer simulation technique showed that the solubilized enzyme was in a dissociation-association equilibrium of 2 protomers $\rightleftharpoons$ diprotomer at 20°C, and the equilibrium for the enzyme in the $E_2$ state was about 50-fold more in favor of the diprotomer than that in the $E_1$ state. Thus, it was strongly suggested that the two protomers associate more tightly in the $E_2$ state than in the $E_1$ state [12].

In the present study we investigated the effects of $C_{12}E_8$, PS and ATP on oligomerization of the solubilized enzyme by estimating weight-averaged molecular weight ($\bar{M}_w$) values for the main protein components fractionated by HPGC. Part of this work has been reported at the 6th International Conference on $Na^+/K^+$-ATPase [14].

## Methods

### *Solubilized $Na^+/K^+$-ATPase*

The membrane-bound enzyme was purified from the outer medulla of frozen dog kidney by the method of Jørgensen [10,15] with slight modifications [12]. The specific ATPase activity of the membrane-bound enzyme ranged from 38 to 48 U/mg protein (units defined as $\mu$mol $P_i$ per min) at 37°C under optimum conditions [12]. The membrane-bound enzyme was incubated in a solution with the final composition of 2 mg $ml^{-1}$ protein/6 mg $ml^{-1}$ $C_{12}E_8$/0.05 M KCl/2 mM dithioerythritol/10% (w/v) glycerol/13 mM imidazole/8 mM Hepes at pH 7.0 and 0°C for 5 min. The solution was centrifuged at $170\,000 \times g$ for 20 min at 0°C. The supernatant was collected and referred to as the solubilized $Na^+/K^+$-ATPase or the solubilized enzyme. The solubilized enzyme was stored at 0°C and used within 3 days. The ATPase activity was fully retained during the storage. Protein concentration of the solubilized enzyme was determined from the absorbance at 280 nm, corrected for light scattering as described by Reddi [16] using an absorption coefficient of 1.22 $mg^{-1}$ ml $cm^{-1}$, as described in detail elsewhere [17]. In the presence of more than 2 mM nucleotide protein concentration was determined from the absorbance at 285 nm using an absorption coefficient of 1.16 $mg^{-1}$ ml $cm^{-1}$.

### *Molecular weight estimation by the HPGC/LALLS method*

The $M_r$ of the solubilized enzyme was measured as follows by the HPGC/LALLS method, unless otherwise stated. A TSKgel G4000$SW_{XL}$ column (7.8 × 300 mm, Tosoh) equipped with a guard column (TSK guard column $SW_{XL}$, 6.0 × 40 mm) was equilibrated with an elution buffer containing 0.3 mg $ml^{-1}$ $C_{12}E_8$/0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) at a flow rate of 0.40 ml/min and at 20°C. An aliquot of 100 $\mu$l containing 0.16 mg protein of the solubilized enzyme was charged onto the columns and eluted with the same elution buffer. The elution buffer containing phospholipid was prepared as described elsewhere [12]. The eluate from the columns was monitored successively

with the following three kinds of detectors; a LALLS photometer (LS; TSK model LS-8000), a UV spectrophotometer (UV; TSK model UV-8010) and a differential refractometer (RI; TSK model RI-8010). The temperature of the columns, the flow-through cell installed in the LALLS photometer and the lines connecting the columns and the cell was kept constant at the levels stated within $\pm 0.1$°C. Furthermore, to avoid exposure of the enzyme to much higher temperatures than the levels stated, the UV- and RI-monitors were placed in a chamber of which the temperature was controlled within $\pm 1$°C.

The outputs of the detectors thus obtained were read at 6-s intervals from the chart paper record by a computer after correction for the displacement in the pattern due to the differences in positions of the detector cells and recorder pens. $M_r$ of a component $i$ ($M_{ri}$) corresponding to a protein fraction eluted during one 6-s interval was obtained according to the following equation

$$M_{ri} = k_1(dn/dc_p)^{-1}(\text{output})_{LSi}(\text{output})_{RIi}^{-1} \quad (1)$$

where $k_1$ is the instrumental constant; $dn/dc_p$, the specific refractive index increment expressed in terms of weight concentration of the protein moiety of the protein component ($c_p$); $(\text{output})_{LSi}$ and $(\text{output})_{RIi}$, the outputs of LS and RI, respectively, for the component $i$ [11,12]. The value of $(dn/dc_p)$ was determined from the following equation

$$dn/dc_p = k_2 A(\text{output})_{RI}(\text{output})_{UV}^{-1} \quad (2)$$

where $k_2$ is the instrumental constant; $A$, the absorption coefficient expressed in terms of weight concentration of $c_p$ at 280 or 285 nm; $(\text{output})_{RI}$ and $(\text{output})_{UV}$, the outputs at the tops of the protein peaks in the RI-pattern and in the UV-pattern, respectively. It was assumed that $dn/dc_p$ was constant among the protein components eluted under a given set of conditions for the chromatography. The constants $k_1$ and $k_2$ were determined using yeast glutamate dehydrogenase as a standard protein under the same conditions as those used for chromatography of the solubilized $Na^+/K^+$-ATPase, as described elsewhere [11,12]. Weight-averaged values for $M_r$ values, that is $\bar{M}_w$, of the protein components eluted during a given time were calculated according to the equation

$$\bar{M}_w = \sum w_i M_{ri} \quad (3)$$

where the suffix $i$ denotes the value for a component eluted at each 6-s interval and $w_i$ is the weight fraction of each component relative to all the protein components eluted during the measurement time, which was estimated from the UV-pattern.

*Test of phospholipids for effect on restoration of ATPase activity of the solubilized enzyme during passage through HPGC column*

The elution buffer containing 60 $\mu$g/ml phospholipid as well as 0.3 mg $ml^{-1}$ $C_{12}E_8$/0.05 M NaCl/0.05 M KCl/1.33 mM ATP/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) was prepared as described elsewhere [12]. A TSKgel G3000SW column (7.5 × 600 mm) had been equilibrated and was eluted with the elution buffer at a flow rate of 0.50 ml/min and at 20°C. The solubilized enzyme (80 $\mu$g protein) was chromatographed on the column. The eluate was monitored with a UV-spectrophotometer at 280 or 285 nm, and 1-ml aliquots of the eluate were collected in test-tubes pre-loaded with 0.50 ml of 5% (w/v) SDS to stop the enzyme reaction. Concentration of $P_i$ in aliquots of the eluate and specific ATPase activity of the enzyme exhibited during passage through the column were determined as described elsewhere [12].

*Sum of amounts of bound $C_{12}E_8$ and phospholipid*

The solubilized enzyme was chromatographed in the same way as described above except that various concentrations of PG or PS ranging from 0 to 120 $\mu$g/ml were added to the elution buffer. Values of $(\text{output})_{UV}$ and $(\text{output})_{RI}$ were obtained at the top of the main protein peak eluted, and values of $dn/dc_p$ were determined using Eqn. 2. If the solubilized enzyme is composed of protein, $C_{12}E_8$, phospholipids and carbohydrate, its $dn/dc_p$ can be expressed in terms of parameters of the bound substances as follows:

$$dn/dc_p = 0.187 + 0.134(\delta_{CE} + \delta_{PL} + \delta_{CH}) \quad (4)$$

where $\delta_{CE}$, $\delta_{PL}$ and $\delta_{CH}$ are the amounts of bound $C_{12}E_8$, phospholipid and carbohydrate expressed as grams per gram protein, respectively [11]. The sum of $\delta_{CE}$ and $\delta_{PL}$ was evaluated by subtracting $\delta_{CH}$ of 0.073 g/g [11] from the sum of $\delta_{CE}$, $\delta_{PL}$ and $\delta_{CH}$, which was estimated using Eqn. 4.

*Materials*

$C_{12}E_8$ was obtained from Nikko (Japan) and used without further purification. SDS was a specially pure grade from BDH (UK). Glutamate dehydrogenase from yeast was purchased from Oriental Yeast (Japan). PS (bovine brain, P6641), PI (bovine brain, P2517), PC (egg yolk, P2772) and PE (bovine brain, P5531) were purchased from Sigma (USA). PG (egg lecithin, A431) and PI (pig liver, A38) were purchased from Serdary Research Laboratories (Canada).

## Results

*Effect of $C_{12}E_8$ concentration on $\overline{M}_w$ of solubilized $Na^+/K^+$-ATPase*

The solubilized enzyme was chromatographed on a TSKgel G4000SW$_{XL}$ column with the elution buffer containing various concentrations of $C_{12}E_8$. Fig. 1 shows the elution patterns of the solubilized enzyme with the elution buffer containing 0.1, 1 and 5 mg/ml $C_{12}E_8$, obtained by the HPGC/LALLS method. The solubilized enzyme was eluted as a main protein component of which the peak top appeared at a retention time of 23.4 min with the elution buffer containing 0.1 mg/ml $C_{12}E_8$ (Fig. 1A). With the elution buffer containing 1 mg/ml $C_{12}E_8$ the solubilized enzyme was eluted as two nearly equivalent protein components (Fig. 1B). One of them corresponded to the component mentioned above, and the other exhibited the peak top at 24.6 min. When the concentration of $C_{12}E_8$ added was increased to 5 mg/ml, the latter component was increased in amount, leaving the former component as a minor preceding shoulder (Fig. 1C). The shoulder component was clearly detected with the LALLS-photometer because the output of LS is dependent on both $M_r$ and protein concentration.

$\overline{M}_w$ of the protein component eluted from 21 to 26 min was calculated from $M_r$ values determined at 6-s intervals by the HPGC/LALLS method. Portions of the elution patterns used for the calcuation were indicated by bars with arrowheads in Fig. 1A, B and C. The amount of the protein component taken into the calculation constituted 81% (w/w) of the whole protein eluted. As shown in Fig. 2, the $\overline{M}_w$ decreased from 230 000 to 153 000 with increase in the concentration of $C_{12}E_8$ from 0.1 to 5 mg/ml. The two protein components exhibiting the peak tops at 23.4 and 24.6 min, were judged to be $(\alpha\beta)_2$-diprotomer and $\alpha\beta$-protomer, respectively, because $M_r$ values were estimated to be

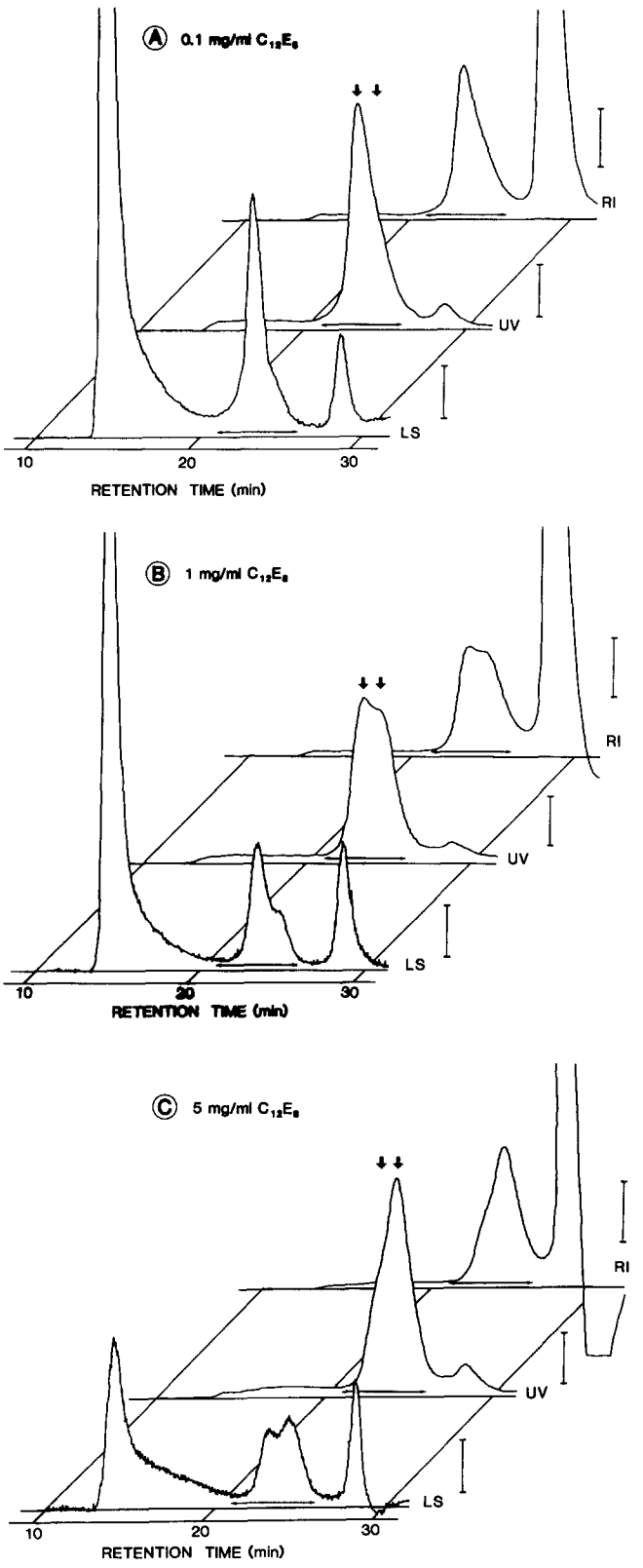



Fig. 1. Dependence of elution patterns of solubilized $Na^+/K^+$-ATPase revealed by HPGC/LALLS method upon concentration of $C_{12}E_8$ included in elution buffer. A TSKgel G4000SW$_{XL}$ column had been equilibrated with the elution buffer containing 0.1 (A), 1.0 (B) or 5 mg/ml (C) $C_{12}E_8$ and in common 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes at pH 7.0 and 20°C. The solubilized enzyme (an aliquot of 100 $\mu$l containing 0.16 mg protein) was charged onto the column and was eluted with the same elution buffer. The eluate from the column was monitored successively with a LALLS photometer (LS), a UV spectrophotometer (UV) and a differential refractometer (RI). The three kinds of elution patterns recorded on chart paper are displayed after correction for the displacement in the patterns due to the differences in the positions of the detector cells and recorder pens. Weight-averaged molecular weight ($\overline{M}_w$) was calculated for the protein components eluted at the positions indicated by bars with arrowheads as described in Methods. Two vertical arrows denote the positions (23.4 and 24.6 min) of the peak tops of the two main protein components. Gain settings for the detectors were 8 for LS, 64 for RI and 0.32 absorbance unit (full-scale) for UV. The lengths of the bars with flags correspond to one-fifth full-scale for the LS-pattern, 0.032 absorbance unit for the UV pattern and $6.4 \cdot 10^{-6}$ refractive index unit for the RI pattern.

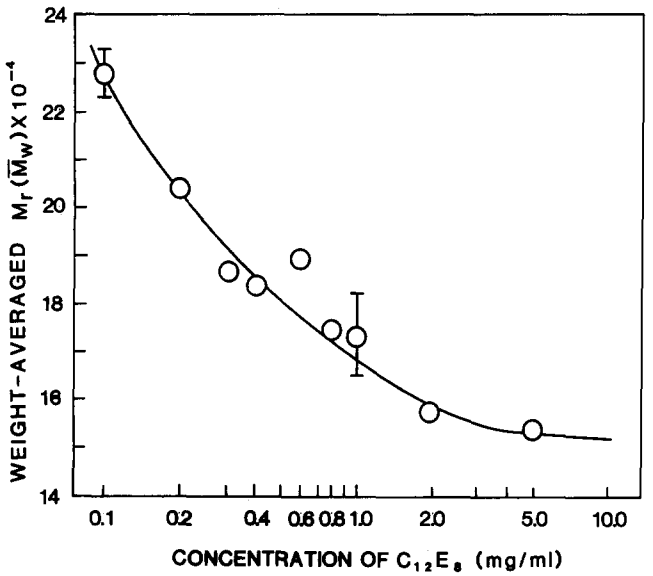
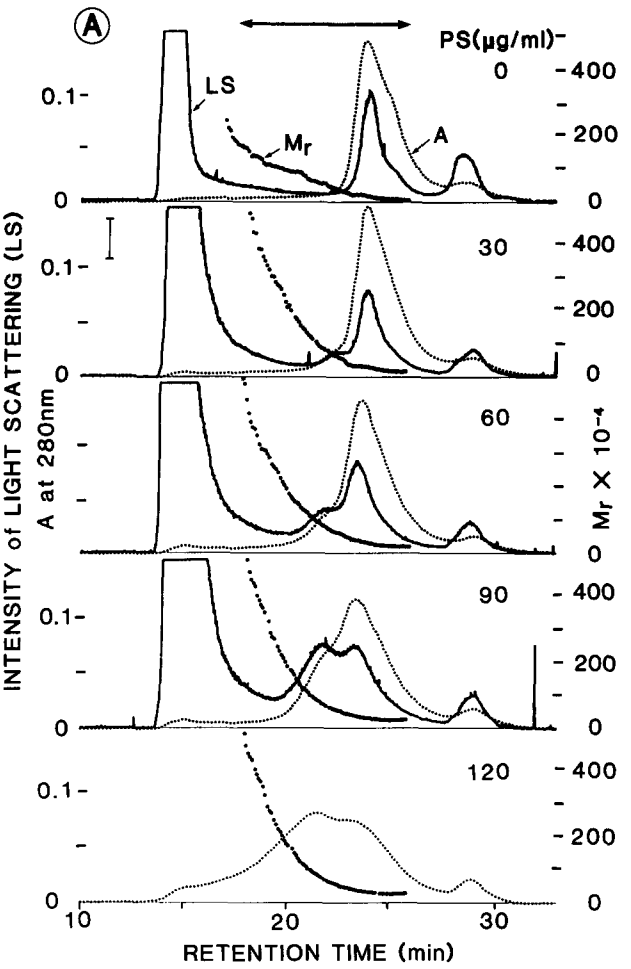



Fig. 2. Dependence of weight-averaged molecular weight ($\bar{M}_w$) of solubilized $Na^+/K^+$-ATPase upon $C_{12}E_8$ concentration. The solubilized enzyme was chromatographed with the elution buffer containing 0.1 to 5 mg/ml $C_{12}E_8$, and the eluate was monitored as described in the legend to Fig. 1. The $\bar{M}_w$ for the protein components eluted between 21 and 26 min was calculated as described in Methods. Open circles with error bars indicate means of three values and other open circles are means of two values.

309 000 to 393 000 around the peak top at 23.4 min and 134 000 to 142 000 around that at 24.6 min. Therefore, it was concluded that the weight ratio of the diprotomer to the protomer was 0.53 : 0.47 in the solubilized enzyme at 0.1 mg/ml $C_{12}E_8$, and that almost all the diprotomer was dissociated into the protomer at 5 mg/ml of $C_{12}E_8$.



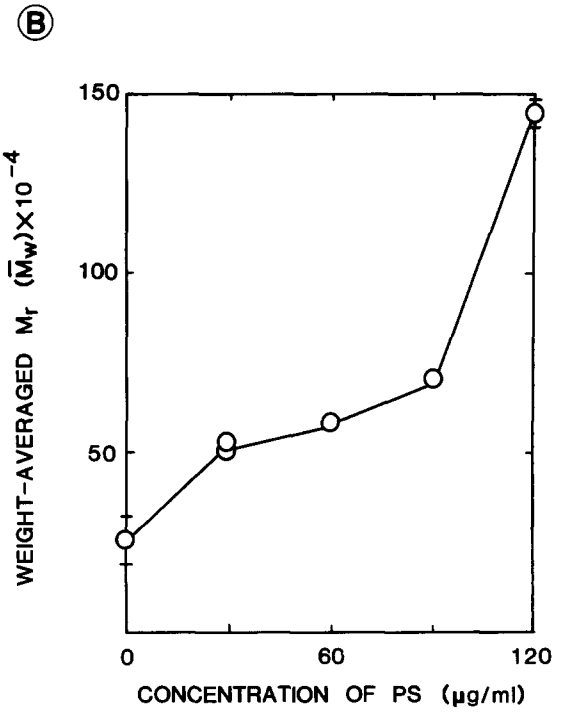




Fig. 3. Dependence of $\bar{M}_w$ of the solubilized enzyme upon concentration of PS added to the elution buffer. A TSKgel G4000$SW_{XL}$ column equipped with a guard column was equilibrated with the elution buffers containing various concentrations of PS and in common 0.3 mg $ml^{-1}$ $C_{12}E_8$/0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) at a flow rate of 0.40 ml/min and at 20°C. Aliquots of 100 μl each of the solubilized enzyme containing 0.16 mg protein were charged onto the columns and eluted with the same elution buffer. $M_r$ values were measured for protein components eluted at 6-s intervals by the HPGC/LALLS method, as described in Methods. (A) The elution patterns obtained with a LALLS-photometer (LS) and a UV-spectrophotometer at 280 nm are displayed by solid (LS) and dotted lines (A), respectively. The $M_r$ values were displayed by closed circles ($M_r$). Gain setting for the LALLS-photometer was 16. The length of the bar with flags corresponds to one-fifth full-scale for LS. (B) The $\bar{M}_w$ values were calculated from the $M_r$ values shown in (A) for the protein components eluted between 17 and 26 min (indicated by the bar with arrowheads in (A)), as described in Methods. The $\bar{M}_w$ values were plotted against the concentration of PS added to the elution buffer. Means of four and three values of $\bar{M}_w$ were plotted at 0 and 120 μg/ml PS, respectively, with open circles with error bars. The other circles were for single values.

*Association of the solubilized enzyme by addition of PS to the elution buffer*

Various concentrations of PS were added to the elution buffer which contained a fixed concentration of 0.3 mg/ml $C_{12}E_8$ and otherwise the same ingredients as those described above. The solubilized enzyme was chromatographed with the elution buffer thus obtained. $\overline{M}_w$ of the protein components eluted between 17 and 26 min was estimated to be 258000 ± 78000 ($n = 4$) with the elution buffer containing no PS. As shown in Fig. 3B, the $\overline{M}_w$ of the protein components increased hyperbolically to 702000 as the concentration of PS added to the elution buffer was increased to 90 μg/ml. With the elution buffer containing 120 μg/ml PS, however, $M_r$ often could not be measured owing to the abnormally large output of light scattering. On the other hand, the relationship between $M_r$ and retention time was nearly independent of the concentration of PS added in the range from 30 to 90 μg/ml, as shown in Fig. 3A. Therefore, the $\overline{M}_w$ for the protein component eluted with the elution buffer containing 120 μg/ml PS was calculated to be 1437000 ± 33000 from the weight fractions ($w_i$) of the protein components observed with 120 μg/ml PS and the relationship of $M_r$-retention time determined with 90 μg/ml PS. The results showed that PS induced the solubilized enzyme to associate into protein components corresponding to oligomers larger than the diprotomer.

*Reversibility of the enzyme association upon exclusion of PS*

The solubilized enzyme was chromatographed with the elution buffer containing 120 μg/ml PS and otherwise the same ingredients as above, and the protein component eluted between 19.8 and 22.4 min was collected (Fig. 4A inset). The $\overline{M}_w$ of the protein component was calculated to be $1.15 \cdot 10^6$. Aliquots of 400 μl containing 0.06 mg protein of the solubilized enzyme thus associated with PS were rechromatographed with the elution buffer containing no PS or 120 μg/ml PS and otherwise the same ingredients as those in the first chromatography (Figs. 4A and 4B). The $\overline{M}_w$ of the protein components eluted between 17 and 26 min were $3.06 \cdot 10^5$ and $2.75 \cdot 10^6$ for the case of no PS and 120 μg/ml PS, respectively. The elution pattern obtained at 17 to 26 min with the elution buffer containing no PS was simulated as a summation of two Gaussian distribution curves, as described elsewhere [17]. Consequently, 26% and 61% (w/w) of the protein eluted in that region were ascertained to be the protomer and the diprotomer, respectively. When no PS was added to the elution buffer in the rechromatography, it was expected that the exogenous PS bound to the enzyme on the first chromatography would be separated from the enzyme. Thus, it was concluded that the association induced by PS was reversible upon removal of PS from the enzyme.

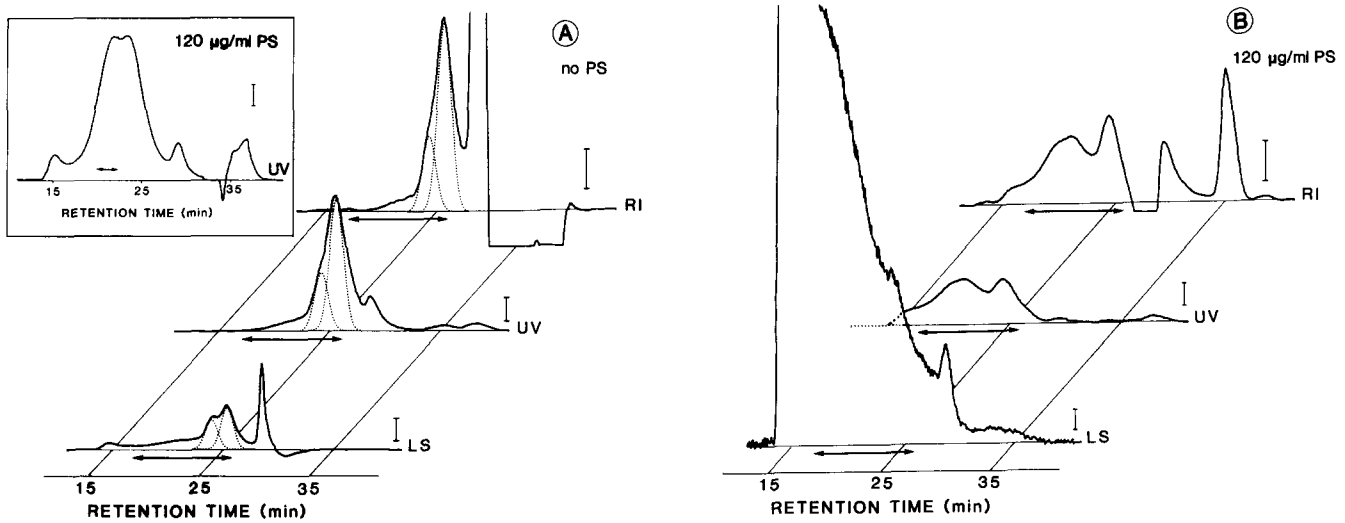



Fig. 4. Reversibility of association of solubilized $Na^+/K^+$-ATPase induced by PS. Several aliquots of 350 μl of the solubilized enzyme containing 0.56 mg protein were chromatographed with the elution buffer containing 120 μg/ml PS and otherwise in the same way as described in the legend to Fig. 3. The protein component eluted between 19.8 and 22.4 min was collected as the PS-associated enzyme. The elution pattern obtained with a UV-spectrophotometer at 280 nm is displayed in the inset of Fig. 4A. The length of the scale bar with flags corresponds to 0.032 absorbance unit. Two aliquots of 400 μl each containing 0.06 mg protein of the PS-associated enzyme were rechromatographed on the column equilibrated with the elution buffer containing (A) no PS or (B) 120 μg/ml PS and in common the same ingredients as those in the first chromatography. See the legend to Fig. 1 for LS, UV, and RI. Gain setting for the detector was 32 for LS. The lengths of the scale bars with flags correspond to one-tenth full-scale for LS, 0.008 absorbance unit for UV and $0.1 \cdot 10^{-6}$ refractive index unit for RI. Dotted lines in (A) represent Gaussian distribution curves corresponding to $(\alpha\beta)_2$-diprotomer (earlier curves) and $\alpha\beta$-protomer (later ones) which were obtained by a computer simulation technique described elsewhere [12,17]. Bars with arrowhesds represent the regions where the protein components used for estimation of $\overline{M}_w$ were eluted.

*Reactivation of the solubilized ATPase activity exhibited during passage through the column by addition of phospholipid to the elution buffer*

We showed elsewhere that the solubilized enzyme lost almost all ATPase activity owing to exclusion of endogenous phospholipid from the enzyme during passage through the column, and that the addition of PS to the elution buffer allowed the enzyme to retain the activity [12]. As shown in Fig. 5, the ATPase activity restored increased with increasing PS added, and reached a saturated level with more than 120 $\mu$g/ml PS. The saturated level was nearly equivalent to the ATPase activity of the membrane-bound enzyme that was measured in reaction medium containing neither $C_{12}E_8$ nor PS and otherwise the same ingredients as those in the elution buffer used for the active enzyme chromatography. The ATPase activity of the solubilized enzyme during passage through the column was also restored with the elution buffer containing PG or PI, but not with that containing PC or PE (Table I).

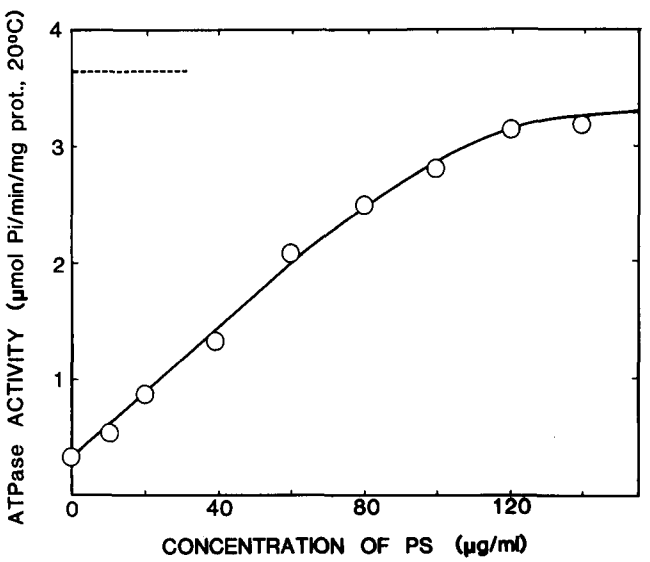



Fig. 5. Dependency of ATPase activity of the solubilized enzyme exhibited during passage through the chromatography column upon PS-concentration added to the elution buffer. A TSKgel G3000SW column (7.5×600 mm) had been equilibrated and eluted with the elution buffer containing various concentrations of PS and in common 0.3 mg ml$^{-1}$ $C_{12}E_8$/0.05 M NaCl/0.05 M KCl/4 mM ATP/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) at a flow rate of 0.50 ml/min and at 20°C. An aliquot of the solubilized enzyme containing 0.09 mg protein was chromatographed on the column. The eluate was monitored with a UV-spectrophotometer at 285 nm and a differential refractometer. 1-ml aliquots of the eluate were collected in test-tubes preloaded with 0.5 ml of 5% SDS to estimate the $P_i$ content. The specific ATPase activity of the solubilized enzyme exhibited during passage through the column was calculated from the protein amount and velocity of $P_i$ liberation, as described elsewhere [12]. The membrane-bound enzyme was assayed for the specific activity in the reaction medium containing the same ingredients as those in the elution buffer except for the absence of $C_{12}E_8$ and PS at 20°C. The resultant activity of 3.64 U/mg protein is shown by a broken line.

TABLE I

*Dependence of ATPase activity exhibited during chromatography upon exogenous phospholipids added to the elution buffer*

A TSKgel G3000SW column had been equilibrated and eluted with an elution buffer containing phospholipid as indicated below and in common 0.3 mg ml$^{-1}$ $C_{12}E_8$/1.33 mM ATP/0.05 M NaCl/0.05 M KCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/15 mM Hepes (pH 7.0) at a flow rate of 0.5 ml/min and at 20°C. An aliquot of 100 $\mu$l of the solubilized enzyme (0.8 mg/ml protein) was chromatographed on the column. The specific ATPase activity of the enzyme exhibited during passage through the column was estimated as described in Methods.

| Phospholipids | Source | Concn. ($\mu$g/ml) | ATPase activity [a] (%) |
|---|---|---|---|
| PS | bovine brain | 60 | 87 |
| PG | egg lecithin | 60 | 74 |
| PI | bovine brain | 60 | 46 |
| | | 100 | 83 |
| | | 100 | 0 [b] |
| PI | pig liver | 60 | 47 |
| PC | egg yolk | 60 | 9.3 |
| PE | bovine brain | 60 | < 0.1 |

[a] The membrane-bound enzyme was assayed for ATPase activity in a reaction medium containing neither $C_{12}E_8$ nor phospholipid and otherwise the same ingredients as those in the elution buffer used at 20°C. The specific ATPase activity was 3.03 U/mg protein. Specific ATPase activity of the solubilized enzyme was represented as the relative value with respect to that of the membrane-bound enzyme thus obtained.

[b] The solubilized enzyme was incubated in a solution with the final composition of 0.8 mg ml$^{-1}$ protein/3 mg ml$^{-1}$ $C_{12}E_8$/1 mM ouabain/2 mM ATP/0.11 M NaCl/0.04 M KCl/1 mM $MgCl_2$/0.2 mM EDTA/7 mM imidazole/4 mM Hepes at pH 7.0 and 0°C for 1 h and 35 min, and then chromatographed in the same way as described above.

*Binding of phospholipids to the solubilized enzyme upon its addition to the elution buffer*

When PS or PG was added to the elution buffer containing all ligands necessary for the enzyme to exhibit ATPase activity, as well as a fixed concentration of 0.3 mg/ml $C_{12}E_8$, the sum of the amount of $C_{12}E_8$ and phospholipid bound to the enzyme, and its specific ATPase activity, were measured as described in Methods. As shown in Fig. 6, the sum of bound $C_{12}E_8$ and phospholipid increased in proportion to the specific ATPase activity of the enzyme as the amount of added PG or PS increased. It is very unlikely that the amount of bound $C_{12}E_8$ would increase with increasing the phospholipid added in presence of a fixed concentration of $C_{12}E_8$. Therefore, it was strongly suggested that the amount of bound phospholipid increased with the increase in the amount of exogenous phospholipid added.

*Inhibition of PS-induced association of the solubilized enzyme by nucleotides*

The solubilized enzyme was chromatographed on the TSKgel G4000SW$_{XL}$ column which had been equilibrated and eluted with the elution buffer containing 0.2 mg/ml $C_{12}E_8$, but neither ATP nor PS at 20°C. $\overline{M}_w$ for the protein components eluted between 14 and 21 min was estimated to be 307000. With the elution buffer containing 60 $\mu$g/ml PS and otherwise the same ingredients as above, the $\overline{M}_w$ was 890000, indicating that PS induced association of the solubilized enzyme. When ATP as well as a fixed concentration of 60 $\mu$g/ml PS was added to the elution buffer, the $\overline{M}_w$ decreased with increasing ATP (Fig. 7). The $\overline{M}_w$ was reduced to 366000 at 2.6 mM ATP, which was approximately equivalent to the $\overline{M}_w$ obtained with the elution buffer containing neither PS nor ATP. Based on the data described in Table I, the specific ATPase activity of the enzyme exhibited during passage through the column was supposed to be 3.0 U/mg protein. Accordingly, it was calculated that 22% at most of ATP amount eluted was hydolyzed during that time, showing effectiveness of ATP on the inhibition of the association. The inhibition of the association was similarly observed when ATP was replaced by ADP, but not AMP (Fig. 7). The concentration required for half-maximal inhibition ($K_{0.5}$) was 0.44 mM for ATP and 0.88 mM for ADP.

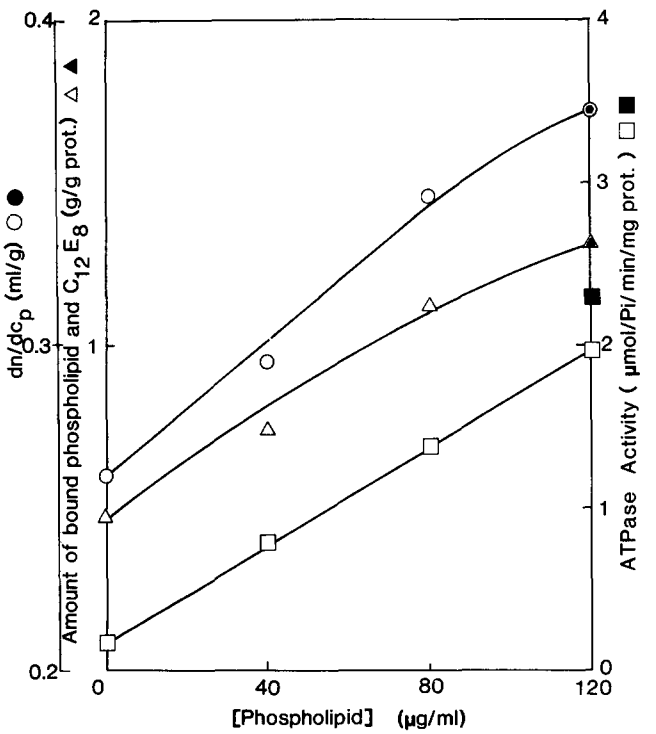



Fig. 6. Binding of phospholipids to the solubilized enzyme upon reactivation of ATPase activity by addition of exogenous phospholipid to the elution buffer. Reactivation of the solubilized ATPase activity by PG or PS was done under the same conditions as described in the legend for Fig. 5 except that an aliquot of the solubilized enzyme containing 0.16 mg protein was chromatographed on a TSKgel G3000SW$_{XL}$ column at a flow rate of 0.40 ml/min. The value of $dn/dc_p$ (○, ●) at the top of the main protein peak and the sum of the amounts of bound $C_{12}E_8$ ($\delta_{CE}$) and phospholipid ($\delta_{PL}$) (△, ▲) were evaluated as described in Methods. The specific ATPase activity of the enzyme (□, ■) exhibited during passage through the column was determined as described in the legend for Fig. 5. Clear and solid symbols denote the data for PG and PS, respectively.

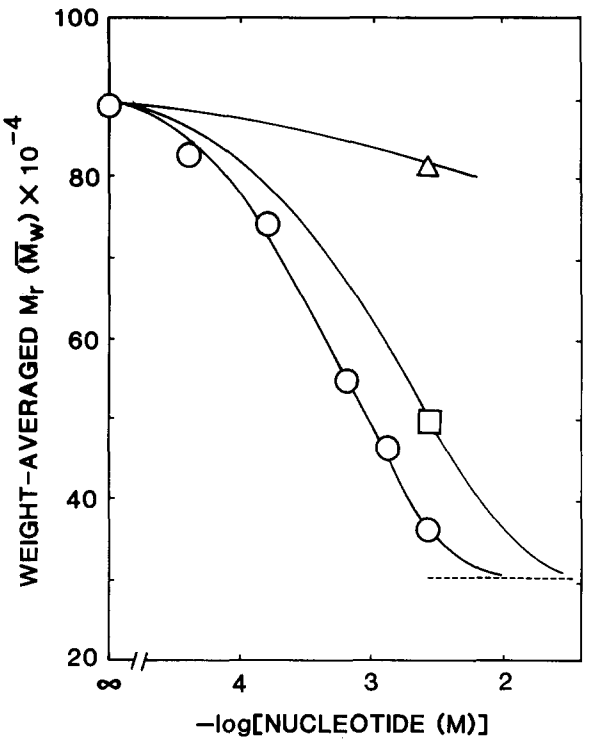



Fig. 7. Inhibition of PS-induced association of the solubilized enzyme by nucleotides. A TSKgel G4000SW$_{XL}$ column had been equilibrated and eluted with the elution buffer containing (○) various concentrations of ATP, (□) 2.56 mM ADP or (△) 2.56 mM AMP and in common 60 $\mu$gml$^{-1}$ PS/0.2 mgml$^{-1}$ $C_{12}E_8$/0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) at a flow rate of 0.50 ml/min and at 20°C. An aliquot of 120 $\mu$l of the solubilized enzyme containing 0.20 mg protein was chromatographed on the column. The $\overline{M}_w$ for the protein components eluted between 14 and 21 min was estimated as described in the legend to Fig. 1 and in Methods. The $\overline{M}_w$ obtained with the elution buffer containing neither PS nor nucleotide and otherwise the same ingredients as those described above is shown by a broken line.

*Dissociation of the PS-induced associated enzyme by ATP*

The associated enzyme was isolated by chromatography with the elution buffer containing 0.3 mg/ml $C_{12}E_8$ and 120 $\mu$g/ml PS in the same way as described in the legend for Fig. 4A (inset). Then, it was rechromatographed using the elution buffer containing 80 $\mu$g/ml PS and various concentrations of ATP, and otherwise the same ingredients as in the first chromatography. $\overline{M}_w$ for the protein component eluted between 17 and 27 min was estimated to be 458000

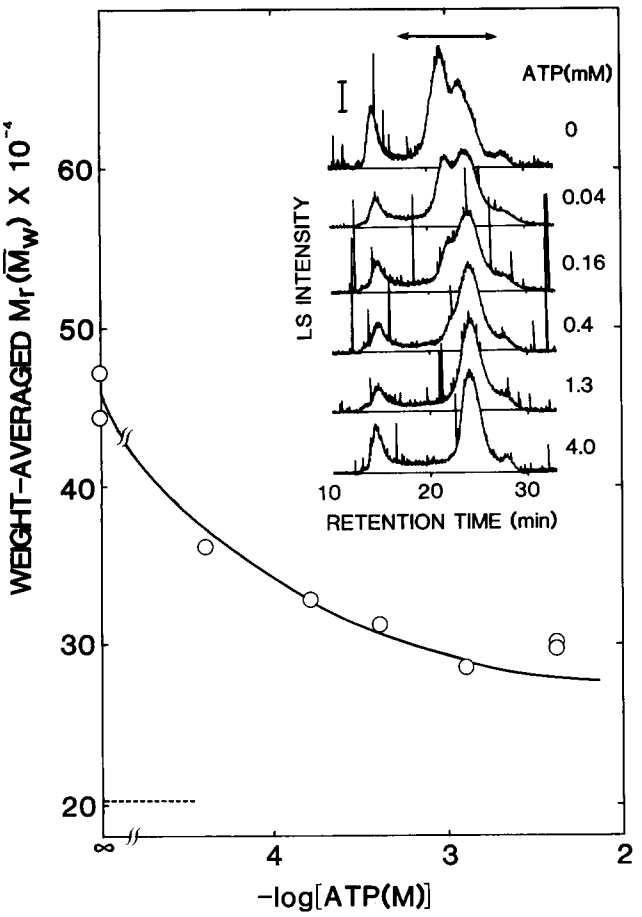



Fig. 8. Dissociation of the PS-induced associated enzyme by ATP. The associated enzyme was isolated in the same way as described in the legend to Fig. 4. Aliquots of 400 $\mu$l each of the associated enzyme containing 0.082 mg protein were rechromatographed with the elution buffer containing various concentrations of ATP and in common 80 $\mu$g/ml PS and otherwise the same ingredients as those in the first chromatography at 20°C. Insets represent the elution patterns monitored by the LALLS photometer on rechromatography. The photometer was used on a gain setting of 8, and a bar with flags corresponds to one-eighth full-scale. $\bar{M}_w$ values were calculated for the protein components eluted during the retention times indicated with a bar with arrowheads, and plotted against ATP concentration. The broken line shows the $\bar{M}_w$ of 202000 obtained with the elution buffer containing neither PS nor ATP on rechromatography.

with the elution buffer containing no ATP. As shown in Fig. 8, the $\bar{M}_w$ decreased with increasing ATP. The concentration of ATP for half-maximal decrease of $\bar{M}_w$ was 0.16 mM. When the rechromatography was done with the elution buffer containing neither PS nor ATP, the $\bar{M}_w$ was 202000. The protein component was shown to be composed of the diprotomer and the protomer in a weight ratio of 0.40 to 0.60 by computer simulation. The results showed that ATP dissociated the PS-induced associated enzyme into the protomer.

## Discussion

*Dissociating effect of $C_{12}E_8$*

It was shown by the HPGC/LALLS method that the $(\alpha\beta)_2$-diprotomer was dissociated into the $\alpha\beta$-protomer as the $C_{12}E_8$ content in the elution buffer was increased (Fig. 2). The dissociating effect of $C_{12}E_8$ was consistent with the results reported by Brotherus et al. [18] and Esmann [19]. They measured $s_{20,w}$ and the Stokes radius of the solubilized enzyme at various weight ratios of $C_{12}E_8$ to protein after chromatography on Sepharose 6B [18] and Sephacryl S-300 [19] columns. What effect on the kinetic properties is brought about by the solubilization of the membrane-bound $Na^+/K^+$-ATPase with $C_{12}E_8$? Esmann and Skou [20] showed that $K_m$ of ATP for the ATPase was 30 $\mu$M for the solubilized enzyme in contrast with 100 $\mu$M for the membrane-bound enzyme. Huang et al. [6] showed that $C_{12}E_8$ at concentrations that were insufficient to solubilize the membrane-bound enzyme mimicked the regulatory effect of ATP at the low-affinity site, and observed similar effects with many other surfactants such as SDS, Lubrol WX, Triton X-100, octyl glucoside, digitonin and sodium deoxycholate. They concluded that the surfactants have an ATP-like enhancing effect on the conversion from $E_2$-$K^+$ to $E_1$-$K^+$. Esmann [21] showed, using a sensitive, non-covalently bound fluorescent probe, 6-carboxyeosin, that $Na^+/K^+$-ATPase favors the $E_1$ form more in the solubilized state than in the membrane-bound state. We proposed elsewhere that the enzyme would operate in the structural form of the $(\alpha\beta)_2$-diprotomer in the membrane, and that the enzyme consists of a loosely associated diprotomer and a tightly associated diprotomer in the $E_1$ and $E_2$ states, respectively [12]. Therefore, we concluded that $C_{12}E_8$, whether it can solubilize the membrane or not, loosens the interactions of protomer with protomer, tending to switch the enzyme toward the $E_1$ state, so that $C_{12}E_8$, like ATP with low affinity, would shift the equilibrium between $E_1$ and $E_2$ towards $E_1$.

*Effect of PS on oligomerization and ATPase activity*

Some endogenous phospholipids bound to the enzyme were released upon chromatography of the solubilized enzyme with elution buffer containing $C_{12}E_8$ [17–19]. The more $C_{12}E_8$ was included in the elution buffer, the less phospholipid was detected in the enzyme eluted [19]. The content of endogenous phospholipid was also dependent upon the temperature at which the chromatography was done, but not upon which monovalent cation favoring $E_1$ or $E_2$ state is included in the elution buffer (Hayashi, Y., unpublished data). The eluted enzyme retained $15 \pm 6$ and $6 \pm 2$ mol phospholipid/150000 g of protein at 0 to 1.6°C and 20°C, respectively. Thus, it was attributable to the exclusion of endogenous phospholipids that

scarcely any ATPase activity of the solubilized enzyme was exhibited during passage through the column at 20°C. Addition of exogenous PS to the elution buffer allowed the solubilized enzyme to recover the ATPase activity comparable to that of the membrane-bound enzyme. The restoration of activity of the solubilized enzyme was also accomplished by PG and PI, but not by PC and PE (Table I). Wheeler and Whittam [8] showed, using microsomes from ox brain, that $Na^+/K^+$-ATPase activity was almost completely removed by delipidation of the microsomes with deoxycholate, and that the activity was restored by addition of PS, PI or phosphatidic acid, but not by PC or PE, to the delipidated microsomes. Our result obtained with the purified enzyme was almost completely consistent with that of Wheeler and Whittam.

The amount of phospholipid bound to the solubilized enzyme increased with the increasing amount of PG or PS added to the elution buffer (Fig. 6). It is possible that the addition of exogenous phospholipid might have suppressed the liberation of endogenous phospholipid from the enzyme. However, reactivation of the solubilized ATPase activity was accomplished with PS, PG or PI, but not with PC and PE. This difference in reactivation effects among the phospholipids excludes the possibility that exogenous phospholipid suppresses the liberation of endogenous phospholipids from the enzyme. Furthermore, it also excludes the possibility that the association of the enzyme induced by exogenous phospholipid is attributable to a decrease in the effective $C_{12}E_8$ concentration to below the level required for dissociation of ATPase oligomers. If the effective concentration of $C_{12}E_8$ were decreased similarly by every phospholipid, then reactivation would occur similarly upon addition of each phospholipid. Thus, it was strongly suggested that exogenous phospholipid became bound to the enzyme, followed by enzyme association.

Esmann [19], and Jørgensen and Andersen [22] showed that heat-treatment of the solubilized enzyme at 40°C to 60°C was accompanied by aggregation of $\alpha\beta$-protomer to higher oligomers. The association of the solubilized enzyme to the diprotomer and higher oligomers induced by PS was reversible by removal of PS from the enzyme. Therefore, the association induced by PS would not be caused by the same mechanism as that of heat-induced aggregation. In the case of PS-induced association, positive charges distributed on the protein moiety of the enzyme around the periphery of the lipid bilayer may be covered with headgroups of phospholipids with negative net charge, diminishing electrostatic repulsion between two protomers carrying positive charges. In contrast with the above mechanism, the heat-induced aggregation would involve exposure of hydrophobic residues of the enzyme to the solvent.

Hegyvary et al. [9] showed that phospholipids were essential for the conformational transition of $E_1$-P to $E_2$-P. Their result can be explained by assuming that phospholipids tighten the protomer-with-protomer interaction following the conformational change from $E_1$ to $E_2$.

*Dissociation by ATP*

The PS-induced association of the enzyme was inhibited by ATP and ADP, but not by AMP (Fig. 7). The $K_{0.5}$ of ATP for the inhibition was 0.44 mM. ATP dissociated the associated enzyme with $K_{0.5}$ of 0.16 mM (Fig. 8). ATP participates in the ATP-hydrolysis not only as a substrate with apparent $K_m$ of 0.3 to 4 $\mu$M, but also as a regulatory factor with apparent $K_m$ of 0.3 to 0.5 mM [2,3]. The regulatory ATP accelerates the step of the conformational change from $E_2$-$K^+$ to $E_1$-$K^+$ [4–6,28]. Therefore, the $K_{0.5}$ of ATP for dissociation of the solubilized enzyme was almost equivalent to that for the conformational transition. It is also consistent with effectiveness of ADP for the conformational transition [5,28] that ADP dissociated the associated enzyme like ATP.

Suzuki et al. [28] reported that their enzyme preparation, with a specific activity of 10 to 33 U/mg protein, contained adenylate kinase as an impurity, the activity of which was 94 nmol ATP per h per mg $Na^+/K^+$-ATPase protein at 25°C in the presence of 3.2 mM ADP. If our enzyme preparation contained the same adenylate kinase activity as that in their preparation, then 0.64 $\mu$M ATP at most would be produced in the eluate during one chromatography run. This concentration of ATP is negligibly small because it is about three orders of magnitude below the $K_{0.5}$ of ATP (0.44 mM) for the dissociation. Furthermore, since the $M_r$ of adenylate kinase (24 000) is much lower than that of the solubilized $Na^+/K^+$-ATPase, the kinase is eluted very far from the ATPase using the same kind of chromatography column as that employed in the present study (see Fig. 2 in Ref. 11). Thus, ATP produced by the kinase would not make contact with the ATPase. However, as much as 5 $\mu$M ATP at most may be present as a contaminant in the 2.6 mM ADP used for the dissociation experiment, according to the catalogue of Boehringer Mannheim. A concentration of ADP as much as three orders of magnitude greater than that of putative ATP would be expected to occupy the ATP-binding sites of the ATPase. Therefore, it is unlikely that $E_1$-P and/or $E_2$-P are made when the ATPase is incubated with 2.6 mM ADP.

Harada et al. have shown that ATP or ADP dissociates the $\alpha_3\beta_3$ complex of ATP synthase obtained from a thermophilic bacterium into the $\alpha\beta$ complex, and that the dissociation would be related to ATPase activity of the ATP synthase [23,24].

*Hypothesis concerning oligomeric structure difference between $E_1$ and $E_2$*

Craig [25], Brotherus et al. [18], and Vilsen et al. [26] have claimed that the minimum functional unit of $Na^+/K^+$-ATPase is the $\alpha\beta$-protomer. We concluded from the substantially simultaneous measurement of ATPase activity and $M_r$ by the HPGC/LALLS method that the solubilized enzyme could exist as $\alpha\beta$-protomer exhibiting ATPase activity comparable to that of the membrane-bound enzyme only at less than $10^{-7}$ M in terms of molar protomer concentration in the presence of 60 $\mu$g/ml PS as well as 0.3 mg/ml $C_{12}E_8$ at 20°C [12]. It could also be inferred from the association constant obtained for the self-association equilibrium of $2\alpha\beta \rightleftharpoons (\alpha\beta)_2$ that the solubilized enzyme would exist only as $(\alpha\beta)_2$-diprotomer in situ, where the enzyme is surrounded by a lipid bilayer in the absence of exogenous surfactant [12]. Thus, the enzyme probably works in the form of $(\alpha\beta)_2$-diprotomer or higher oligomer in the membrane, even though it could hydrolyze ATP in the range of protein concentration sufficiently low as to allow the enzyme to exist only in the protomeric form. On the other hand, the association constant for $2\alpha\beta \rightleftharpoons (\alpha\beta)_2$ in the $E_2$ state was 50 times that in the $E_1$ state (Table II). From the results obtained before and in the present study we can propose

TABLE II

*$M_r$ values and association constants of solubilized $Na^+/K^+$-ATPase in the two conformational states of $E_1$ and $E_2$ revealed by the HPGC/LALLS method*

A TSKgel G3000SW column (7.5×600 mm) had been equilibrated and eluted with the elution buffer containing ingredients as indicated below and in common 0.2 mg $ml^{-1}$ $C_{12}E_8$/1 mM EDTA/10 mM imidazole/16 mM Hepes at pH 7.0 and 20 C. An aliquot of 220 $\mu$l containing 0.35 mg protein of the solubilized enzyme was chromatographed on the column. $M_r$ values of the protein component, eluted as a single peak in every case, were measured by the HPGC/LALLS method as described elsewhere [12]. Association constants for $2\alpha\beta \rightleftharpoons (\alpha\beta)_2$ were obtained by computer simulation as described elsewhere [12].

| Ligands in elution buffer | Conformational state | $M_r$ | Association constant ($M^{-1}$) | Ref. |
|---|---|---|---|---|
| 0.1 M NaCl | $E_1$ | 255000 | $2\cdot10^{-6}$ | 12 |
| 0.1 M NaCl + 9.8 $\mu$g/ml oligomycin | $E_1$ | 247000 | $2\cdot10^{-6}$ | [a] |
| 0.1 M NaCl + 4 mM $MgCl_2$ | $E_1$ | 258000 | $2\cdot10^{-6}$ | [a] |
| 0.1 M KCl | $E_2$ | 300000 | $\geq 1\cdot10^{-8}$ | 12 |
| 0.05 M KCl + 0.05 M NaCl | $E_2$ | 298000 | $\geq 1\cdot10^{-8}$ | 12 |
| 0.05 M KCl + 0.05 M NaCl + 4 mM $MgCl_2$ | $E_2$ | 295000 | $\geq 1\cdot10^{-8}$ | 12 |

[a] Hayashi, Y., unpublished data

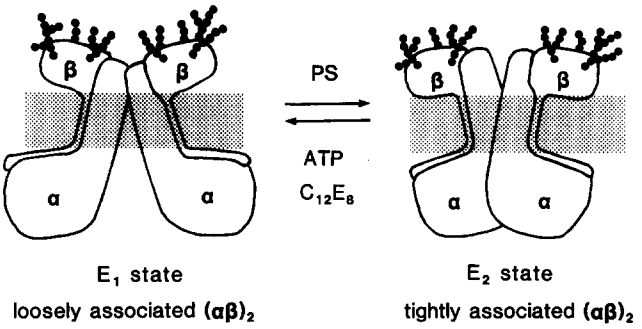



Fig. 9. A model to explain the effects of $C_{12}E_8$, PS and ATP on oligomeric structure of $Na^+/K^+$-ATPase molecule and the difference between the conformational states of $E_1$ and $E_2$. The enzyme operates as the $(\alpha\beta)_2$-diprotomer structural unit in the membrane. The conformational states of $E_1$ and $E_2$ correspond to a loosely associated diprotomer and a tightly associated diprotomer, respectively. PS shifts the equilibrium of $E_1 \rightleftharpoons E_2$ toward $E_2$, and $C_{12}E_8$ and low-affinity ATP shift it toward $E_1$. The shaded area corresponds to the position of the lipid bilayer. Protein masses distributed in the extracellular space, the lipid bilayer and the intracellular space were calculated to be 11, 18 and 71%, respectively, for $\alpha$-subunit, and 80, 9 and 11%, respectively, for $\beta$-subunit from amino acid sequence data [27]. Areas of portions of $\alpha\beta$-protomer corresponding to the three spaces mentioned above were drawn in proportion to the protein masses.

a hypothetical model for the $E_1$ and $E_2$ states of the enzyme, as shown in Fig. 9. The $E_1$ state and $E_2$ state of the enzyme would correspond to loosely associated diprotomer and to tightly associated diprotomer, respectively. $C_{12}E_8$ and ATP with low affinity loosen the interaction of protomer with protomer, and PS tightens the interaction. The solubilized enzyme bound by ouabain showed a $K_a$ of 20-times that of the enzyme exhibiting turnover [12]. It is known that ouabain traps the enzyme in the $E_2$ state, which is consistent with the above model.

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## References

1 Glynn, I.M. (1985) in The Enzymes of Biological Membranes (Martonosi, A.N. ed.), Vol. 3, pp. 35–114, Plenum Press, New York.
2 Post, R.L., Sen, A.K. and Rosenthal, A.S. (1965) J. Biol. Chem. 240, 1437–1445.
3 Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. 67, 693–711.
4 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.

5 Glynn, I.M. and Richards, D.E. (1982) J. Physiol. 330, 17–43.
6 Huang, W.-H., Kakar, S.S. and Askari, A. (1985) J. Biol. Chem. 260, 7356–7361.
7 Kyte, J. (1971) J. Biol. Chem. 246, 4157–4165.
8 Wheeler, K.P. and Whittam, R. (1970) J. Physiol. 207, 303–328.
9 Hegyvary, C., Chigurupati, R., Kang, K. and Mahoney, D. (1980) J. Biol. Chem. 255, 3068–3074.
10 Jørgensen, P.L. (1988) Methods Enzymol. 156, 29–43.
11 Hayashi, Y., Matsui, H. and Takagi, T. (1989) Methods Enzymol. 172, 514–528.
12 Hayashi, Y., Mimura, K., Matsui, H. and Takagi, T. (1989) Biochim. Biophys. Acta 983, 217–229.
13 Takagi, T., Maezawa, S. and Hayashi, Y. (1987) J. Biochem. 101, 805–811.
14 Hayashi, Y., Mimura, K., Matsui, H. and Takagi, T. (1991) in The Sodium Pump: Recent Developments (Kaplan, J.H. and Weer, P.D., ed.), pp. 281–284, The Rockefeller University Press, New York.
15 Jørgensen, P.L. (1974) Methods Enzymol. 32, Part B, 277–290.
16 Reddi, K.K. (1957) Biochim. Biophys. Acta 24, 238–241.
17 Hayashi, Y., Takagi, T., Maezawa, S. and Matsui, H. (1983) Biochim. Biophys. Acta 748, 153–167.
18 Brotherus, J.R., Jacobsen, L. and Jørgensen, P.L. (1983) Biochim. Biophys. Acta 731, 290–303.
19 Esmann, M. (1984) Biochim. Biophys. Acta 787, 81–89.
20 Esmann, M. and Skou, J.C. (1984) Biochim. Biophys. Acta 787, 71–80.
21 Esmann, M. (1991) Biochem. Biophys. Res. Commun. 174, 63–69.
22 Jørgensen, P.L. and Andersen, J.P. (1986) Biochemistry 25, 2889–2897.
23 Harada, M., Sato, M., Aono, O., Ohta, S. and Kagawa, Y. (1991) J. Biol. Chem. 266, 11455–11460.
24 Harada, M., Ohta, S., Sato, M., Ito, Y., Kobayashi, Y., Sone, N., Ohta, T. and Kagawa, Y. (1991) Biochim. Biophys. Acta 1056, 279–284.
25 Craig, W.S. (1982) Biochemistry 21, 5707–5717.
26 Vilsen, B., Andersen, J.P., Petersen, J. and Jørgensen, P.L. (1987) J. Biol. Chem. 262, 10511–10517.
27 Jørgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
28 Suzuki, K., Taniguchi, K. and Iida, S. (1987) J. Biol. Chem. 262, 11752–11757.