Isolation of Stable $(\alpha\beta)_4$-Tetraprotomer from Na^+/K^+-ATPase Solubilized in the Presence of Short-Chain Fatty Acids

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ABSTRACT: Previously, it was demonstrated that acetate anions increase the higher oligomer (H), consuming $(\alpha\beta)_2$-diprotomer (D) and $\alpha\beta$-protomer (P) of solubilized dog kidney Na^+/K^+-ATPase [Kobayashi, T. et al. (2007) *J. Biochem. 142*, 157−173]. Presently, short-chain fatty acids, such as propionate (Prop) and butyrate, have been substituted effectively for acetate. The molecular weight of 6.01×10^5 for H and quantitative Na^+/K^+-dependent interconversion among H, D, and P showed that H was an $(\alpha\beta)_4$-tetraprotomer (T). T was optimally isolated from the enzyme solubilized in aqueous 40 mM K^+Prop at pH 5.6 by gel chromatography performed at 0 °C with elution buffer containing synthetic dioleoyl phosphatidylserine (PS). $K_{0.5}$ values of K^+-congeners constituting K^+Prop for the maximal amount of T were $NH_4^+ \gg Rb^+ \cong K^+ > Tl^+$, while Na^+ had no effect. The oligomers of T, D, and P were simultaneously assayed for ATPase upon elution from the gel column, resulting in a specific activity ratio of 1:2:2. The activity of the chromatographically isolated T increased with an increasing dioleoyl PS, giving a saturated activity of 2.38 units/mg at pH 5.6 and 25 °C, and the active enzyme chromatography of T showed 34% dissociation into D by exposing it at 25 °C. On the basis of these data, the specific ATPase activities of T, D, and P were concluded to be 32, 65, and 65 units/mg, respectively, under the conventionally optimal conditions of pH 7.3 and 37 °C, suggesting an equivalence to a fully active enzyme for D and P but half activity for T. The physiological significance of the stable form of T remains to be investigated.

Na^+/K^+-ATPase, an integral membrane protein, transports three Na^+ ions outward and two K^+ ions inward across the plasma membrane with the consumption of 1 ATP molecule (*1, 2*). The active transport performed by the ATP-hydrolytic enzyme is essential for the proper distribution of Na^+ and K^+ ions, which is necessary for the re-absorption of Na^+ in the kidney, excitation of nerve cells, and transport of sugars and amino acids in many animal tissues. The quaternary structure of Na^+/K^+-ATPase has been studied by solubilizing the membrane-bound enzyme purified from mammalian kidneys with nonionic surfactants, such as $C_{12}E_8$ and Lubrol (*3–6*). These studies consistently demonstrate that two polypeptides, α and β, are noncovalently combined to form a minimal structural unit designated as $\alpha\beta$-protomer (P).[1] However, the enzyme solution contains other oligomers, such as $(\alpha\beta)_2$, $(\alpha\beta)_3$, and $(\alpha\beta)_4$, as shown by chemical cross-linking of the solubilized enzyme (*5*). Conflicting conclusions about the structure necessary for the enzymatic activity have been obtained using the sedimentation equilibrium technique. It has not yet been revealed whether the $\alpha\beta$-protomer, the $(\alpha\beta)_2$-diprotomer (D), or another oligomeric unit is the minimal active unit (*3–5, 7*). Our group devised a high-performance gel chromatography/low-angle laser light scattering (HPGC−LALLS) method to study the minimum functional unit of the quaternary structure (*8*). Using this approach, we determined that P and D are the major protein components and H (a higher oligomer than D) is the minor component of the solubilized enzyme and that they are in equilibrium with respect to association−dissociation at moderately high temperatures (*9*). Recently, our group showed that P associates to form H and D in a K^+-dependent manner, with a $K_{0.5}$ of 64.8 μM, and that H and D dissociate to P in the presence of Na^+ with a $K_{0.5}$ of 6.50 mM (*10*). These findings strongly indicate that the Na^+/K^+-dependent interconversion among the oligomers and the exhibited enzymatic action are closely related to each other. Further-

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[1] Abbreviations: E_1, enzyme form or conformation that carries a higher affinity for Na^+ and can bind ATP with high affinity and accept phosphate from ATP; E_2, enzyme form or conformation that carries a higher affinity for K^+ and can accept phosphate from P_i; HPGC, high-performance gel chromatography; HPGC−LALLS, monitoring of elution from a high-performance gel chromatography column with a low-angle laser light scattering photometer; Prop, propionate; G, aggregates; H, higher oligomer; T, $(\alpha\beta)_4$-tetraprotomer; D, $(\alpha\beta)_2$-diprotomer; P, $\alpha\beta$-protomer; M_p, molecular weight of the protein moiety for solubilized membrane protein; $C_{12}E_8$, octaethyleneglycol *n*-dodecylether; PS, phosphatidylserine; dioleoyl PS, 1,2-oleoyl-*sn*-glycero-3-(phospho-L-serine); dioctanoyl PS, 1,2-octanoyl-*sn*-glycero-3-(phospho-L-serine); EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; UV, ultraviolet; *p*NPPase, *p*-nitrophenyl phosphatase; MES, 2-morpholinoethanesulfonic acid monohydrate; ΔC_{max}, maximum change in the amount of oligomer induced by K^+ or Na^+; $K_{0.5}$, concentration of K^+ or Na^+ for a half-maximum change in the amount of oligomer.

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Table 1: Specific ATPase Activities of Membrane-Bound Enzyme Purified from Dog Kidney under Various Conditions

	temperature (°C)	pH	ATP (mM)	Na^+ (mM)	K^+ (mM)	Mg^{2+} (mM)	EDTA (mM)	buffer (mM/mM)	protein concentration (μg/mL)	ATPase activity (units/mg)
A	37	7.3	4	100 Na^+Cl^-	25 K^+Cl^-	3.9 $MgCl_2$	0.2	30 Imid/30 Glygly[a]	2	41.4
B	37	7.3	4	100 Na^+Prop	25 K^+Prop	3.9 $Mg(CH_3CO_2)_2$	0.2	30 Imid/30 Glygly	2	40.7
C	37	5.6	4	40 Na^+Prop	10 K^+Prop	3.9 $Mg(CH_3CO_2)_2$	0.2	9.4 Imid/50 MES	2	7.61
D	25	5.6	4	40 Na^+Prop	10 K^+Prop	3.9 $Mg(CH_3CO_2)_2$	0.2	9.4 Imid/50 MES	8	2.14 ± 0.08

[a] Imid, imidazole; Glygly, glycylglycine.

more, it has also been demonstrated that the solubilized enzyme is mainly composed of D and P, regardless of the other anions, other than the chloride anion, present in the solubilizing solution (*10*). The exception was acetate, which was observed to behave in a characteristic manner, producing more H (with the addition of K^+, without fixing the oligomeric structure of D) than the other anions (Figure 4B in ref *10*). Furthermore, this interconversion occurred between D and H and P but not between only D and P, suggesting the significant role of H. The formation or deformation of H in the presence of acetate is unequivocally dependent upon K^+ and Na^+ (*10*).

Taniguchi and his co-workers attempted to answer the long-standing question about whether the molecular mechanism described in the Post−Albers scheme functions in the presence of both Na^+ and K^+ and to provide a better understanding of the mechanism of P-type ATPases, such as Na^+/K^+-ATPase (*11–13*) and H^+/K^+-ATPase (*14, 15*). The data on the stoichiometry of reaction intermediates and ligand binding can be consistently explained by a tetraprotomeric hypothesis for Na^+/K^+-ATPase. Accordingly, a working hypothesis was proposed, stating that the tetraprotomeric nature of the enzymes can interpret the experimental results consistently (*16*). In the present study, we have tried to clarify the oligomeric identification, isolation, and characteristics of the higher oligomer of H that we recently reported (*10*). Consequently, short-chain fatty acids, such as propionate and butyrate were found to be more effective than acetate, and H could be identified as an $(\alpha\beta)_4$-tetraprotomer (T) and isolated successfully as stable and active protein fractions in the presence of dioleoyl PS by HPGC at 0 °C. Portions of this work have been described in preliminary reports (*17, 18*).

MATERIALS AND METHODS

Materials. Short-chain fatty acids, including acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, and caproate, were purchased from Tokyo Kasei Kogyo Co. and were used without further purification. Natural phosphatidylserine (PS) purified from ox brain and four kinds of synthetic PS with various lengths of acyl chains, such as dioleoyl (C18:1), dipalmytoyl (C16:0), dimyristoyl (C14:0), and dioctanoyl (C8:0), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Other chemicals used have been described previously (*10*). Special care was taken to minimize contamination by Na^+ and K^+ (*10*).

Membrane-Bound Na^+/K^+-ATPase. The membrane-bound enzyme was purified from microsomes of the outer medulla of frozen dog and pig kidneys using the Jorgensen method (*19*), as described previously (*9, 10*). The purified enzyme was washed to prevent contamination and suspended in 20% (w/v) glycerol, 12 mM imidazole, and 28 mM HEPES at pH 7.1, then frozen, and stored at −80 °C until use. The specific ATPase activities of the dog and pig enzymes ranged from 37−50 and 32−45 μmol of P_i min^{-1} (mg of protein)$^{-1}$ (units/mg; units defined as μmol of P_i/min), respectively, at 37 °C under the optimum conditions described previously (*9*). Apart from the optimal conditions, a portion of the dog enzyme was assayed for ATPase activity under three other conditions. The results as well as the conditions are listed in Table 1.

Separation of Oligomeric Protein Components from the Solubilized Enzyme by HPGC. The membrane-bound enzyme was solubilized with a nonionic surfactant, $C_{12}E_8$, in the presence of various concentrations of KCl or NaCl or salts consisting of the monovalent cation and short-chain fatty acids as described above. The enzyme was then placed either in a conventional solubilizing solution containing 10−14 mM imidazole and 13−23 mM HEPES at pH 7.0 or in an improved solubilizing solution containing 5.4 mM imidazole and 29 mM MES at pH 5.6. Both solutions contained 2 mg/mL protein, 6 mg/mL $C_{12}E_8$, 1 mM EDTA, and 10% (w/v) glycerol and were held at 0 °C for 5 min unless otherwise stated. After incubation for 5 min, the solution was centrifuged at 436000*g* for 5 min at 2 °C using a Beckman TL100 ultracentrifuge (Beckman Coulter, Fullerton, CA). The supernatant was then collected as the solubilized enzyme, stored at 0 °C, and used within 12 h unless otherwise noted.

The solubilized enzyme was subjected to chromatography in a 7.8 × 300 mm TSK gel G3000 SWXL column (Tosoh, Tokyo, Japan) equipped with a 6 × 48 mm TSK SW_{XL} guard column (Tosoh), which had been equilibrated at 0 °C and had a flow rate of 0.350 mL/min. Unless otherwise noted, elution was either with a conventional elution buffer containing 0.2 mg/mL $C_{12}E_8$, 1 mM EDTA, 10 mM imidazole, and 13 mM HEPES at pH 7.0 or with an improved elution buffer containing 0.2 mg/mL $C_{12}E_8$, 1 mM EDTA, and 17 mM MES at pH 5.6. Both buffers were supplemented with various salts consisting of mono- or divalent cations and anions. The relative amounts of the oligomeric components were estimated from the area under protein peaks as previously described (*10*). The protein concentration of the solubilized enzyme and its oligomeric components, such as T (H), D, P, and G was determined from the absorbance at 280 or 285 nm using an absorption coefficient of 1.22 or 1.16 mg^{-1} mL cm^{-1}, respectively, as previously described (*6, 20*).

Determination of Molecular Weight and ATPase Activity for an $(\alpha\beta)_4$-Tetraprotomer. An aliquot of 100 μL of the enzyme solubilized in the conventional solubilizing solution (pH 7.0) supplemented with 0.1 M K^+acetate and 4 mM $MgCl_2$ was applied to a novel 7.8 × 300 mm TSK gel column, which was made by mixing TSK gel G3000 SWXL and TSK gel G4000 SWXL in a weight ratio of 1:1 (Tosoh). The column was equilibrated with the conventional elution

buffer (pH 7.0) at 0 °C and flow rate of 0.350 mL/min. This elution buffer was supplemented with 0.09 M NaCl, 0.01 M KCl, 4 mM $MgCl_2$, and 60 μg/mL natural PS, and the column was eluted with the same elution buffer. The eluate from the column was monitored successively with the following detectors: an LALLS photometer (LS), a differential refractometer (RI), and a UV spectrophotometer. The molecular weight of the protein moieties (M_p) was estimated as previously described (*8, 9*). To confirm the values of M_p, T fractions were collected by HPGC using the conventional TSK gel G3000 SWXL column and the same conventional elution buffer (pH 7.0) as described above, except that no PS was added to the elution buffer. An aliquot of the isolated T was rechromatographed on the same novel column by the same method used to estimate M_p of T.

The substantial simultaneous confirmation of the oligomeric structure and the ATPase activity for T was determined by active enzyme chromatography (*21*), as previously described (*9*). An aliquot of the T fractions was isolated by the first HPGC and applied to the conventional TSK gel G3000 SWXL column, which had been equilibrated with the improved elution buffer (pH 5.6) at 25 °C. This elution buffer was supplemented with 1 mM ATP, 80 μg/mL PS, 0.04 M Na^+Prop, 0.01 M K^+Prop, and 4 mM $Mg(acetate)_2$, and the column was eluted with the same elution buffer.

Specific ATPase Activities of the Oligomeric Components Assayed Immediately after Separation. The same chromatographic technique as described for the active enzyme of the isolated T was performed at 0 °C instead of 25 °C for the solubilized enzyme. The eluate was warmed to 27 (±1) °C immediately after eluting from the column oven, so that the respective separated oligomers could start ATP hydrolysis in the elution buffer during passage inside the steel tube connected to the column. After a flow of 41 s at 27 °C, the eluate was fractionated into microtubes chilled at 0 °C in 15 s intervals. The amount of P_i liberated was determined after the addition of 1.67% (w/v) SDS to the respective fractions using a Technicon Auto Analyzer II, as described previously (*9*). The specific ATPase activity was calculated from the amount of P_i liberated, the time the effluent was exposed to 27 °C, and the amount of protein estimated under the peak area, by monitoring with a UV spectrophotometer at 285 nm.

RESULTS

Effect of Acetate on the Distribution of Oligomeric Protein Components in the Solubilized Enzyme. The enzyme solubilized with $C_{12}E_8$ in the conventional solubilizing solution at pH 7.0 was previously separated by HPGC at 0 °C into two major protein components, D and P, and two minor protein components, H eluted earlier than D and aggregates (G) eluted at the void volume of the column (*6, 9, 10*). Our group replaced the Cl^- anion of the solubilizing solution by various anions of the Hofmeister series. Consequently, only acetate was found to increase H and decrease D and P (*10*). When the effect of acetate was measured as a function of the incubation time after the solubilization, H increased and D and P decreased but G was constant at 0 °C. The changes in the amount of the respective oligomers were saturated at 24 h, as shown in Figure 1. The effect of K^+acetate on the increment of H exhibited a bell-shaped curve and showed a

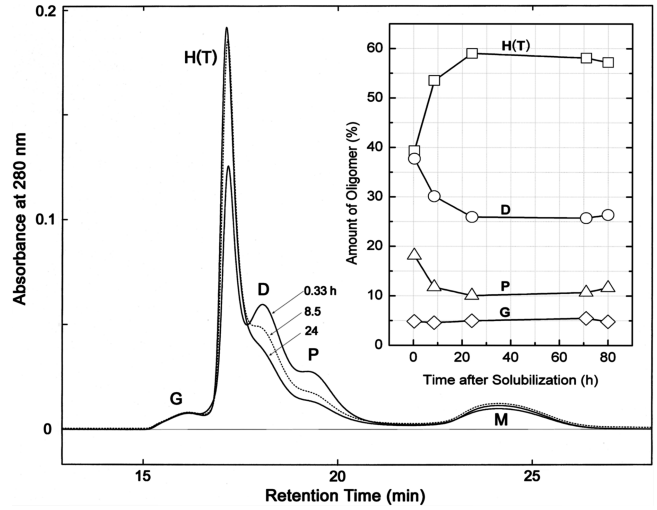

FIGURE 1: Dependence of the amount of the respective oligomers on the incubation time after solubilizing the enzyme in the presence of 0.1 M K^+acetate at pH 7.0. The membrane-bound enzyme (dog kidney) was solubilized with $C_{12}E_8$ in the conventional solubilizing solution (pH 7.0) containing 0.1 M K^+acetate. At various times indicated for incubation at 0 °C after solubilizing, an aliquot of 30 μL of the solubilized enzyme was subjected to HPGC on a standard TSK gel G3000 SWXL column equilibrated and eluted with the elution buffer containing 0.1 mg/mL $C_{12}E_8$, 0.09 M NaCl, 0.01 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole, and 13 mM HEPES at pH 7.0 and 0 °C. (Inset) The amounts of G, H (T), D, and P were quantified from the areas under the respective peaks of absorbance at 280 nm and the relative values plotted against the incubation time. G, H, D, P, and M denote aggregate, higher oligomer, $(\alpha\beta)_2$-diprotomer, $\alpha\beta$-protomer, and mixed micelles, respectively. (T) denotes that H is identified to be $(\alpha\beta)_4$-tetraprotomer (T) later in the text.

maximal level at 0.02−0.06 M (data not shown). The same effect of 0.04 M K^+butyrate, which is a member of the same homologous series as that including acetate, was compared at various pH levels ranging from 4.8−7.5 and was found to exhibit a maximal effect at pH 5.6 (data not shown).

Identification of the Oligomer H by Molecular-Weight Estimation as an $(\alpha\beta)_4$-Tetraprotomer. To estimate M_p for H, the enzyme solubilized in the presence of 0.1 M K^+acetate was subjected to HPGC−LALLS using a novel column of TSK gel made by mixing two types of matrices, TSK gel G3000 SWXL and TSK gel G4000 SWXL, and the conventional elution buffer (pH 7.0) supplemented with 60 μg/mL natural PS purified from bovine brain. Figure 2A shows the elution patterns of the solubilized enzyme from the novel TSK gel column monitored by LS, RI, and UV spectrophotometer. In comparison to the elution patterns obtained with a standard column of TSK gel G3000SWXL, the separation of H from G was improved so that the estimation of M_p for H could be accomplished with the novel column. However, the separation of P from D became worse because P emerged as a shoulder at the latter half of the D peak (compare to Figure 5). According to the HPGC−LALLS method described in previous studies (*8, 9*), the specific refractive index increments, d_n/d_{c_p}, and the values of M_p for H and D were calculated from output values LS, UV, and RI. As summarized in Table 2, M_p values for H and D were estimated to be 6.01 (±0.10) × 10^5 and 2.57 (±0.09) × 10^5, respectively. The value of M_p for D was significantly less compared to values previously reported [3.02 (±0.10) × 10^5 for D and 1.56 (±0.04) × 10^5 for P] (*8, 9*). This can be

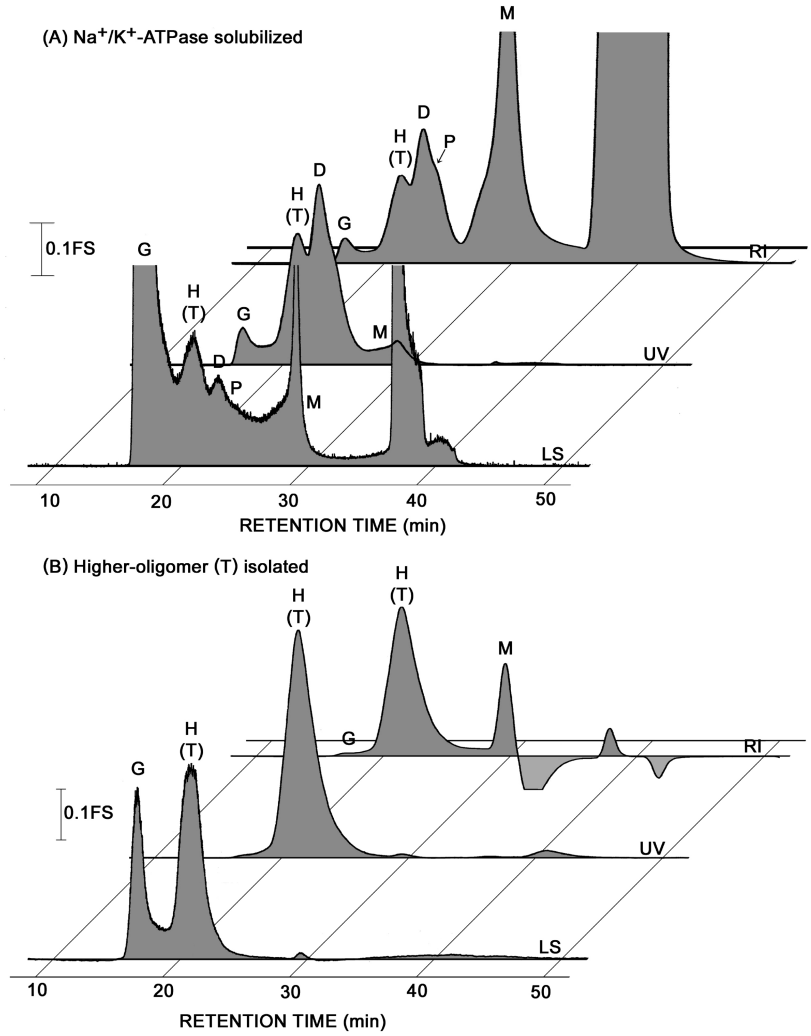

FIGURE 2: Molecular-weight estimation of the higher oligomer (H) by the HPGC−LALLS method carried out at 0 °C. (A) Enzyme purified from dog kidney was solubilized with $C_{12}E_8$ in a solubilizing solution containing 0.1 M K^+acetate and 4 mM $MgCl_2$ at pH 7.0 and 0 °C. A novel column of TSK gel (7.8 × 300 mm), made by mixing the two matrices of TSK gel G3000SWXL and TSK gel G4000SWXL in a weight ratio of 1:1, had been equilibrated with the elution buffer containing 0.20 mg/mL $C_{12}E_8$, 60 μg/mL natural PS (bovine brain), 0.09 M NaCl, 0.01 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole, and 13 mM HEPES at pH 7.0 at a flow rate of 0.350 mL/min and 0 °C. An aliquot of 100 μL of the solubilized enzyme was applied to the column and eluted in the same manner as described in the equilibrium. The eluate from the column was successively monitored with a LALLS photometer (LS), a UV spectrophotometer (UV), and a differential refractometer (RI). (B) First, HPGC of the solubilized enzyme (200 μL) was performed using the same method as described above, except a conventional column of TSK gel G3000SWXL (7.8 × 300 mm) was used instead of the novel column, no PS was included in the elution buffer, and fractions of H were collected without exposing the protein fractions to a temperature above 2 °C. Second, an aliquot of 145 μL of the H fractions was charged on the same novel column and chromatographed by the HPGC−LALLS method in the same way as described in A, with the elimination of PS in the elution buffer. Gain settings for the detectors in the LALLS system for A and B were 16 and 32 for LS, 0.2 and 0.1 absorbance units (full scale) for UV, and 32 and 32 for RI, respectively. The length of the bars with flags corresponds to one-tenth full-scale. See the caption for Figure 1 on H(T), D, P, G, and M.

Table 2: Estimation of Specific Refractive Indices (d_n/d_{c_p}) and Molecular Weights for Protein Moiety (M_p) for H and D by the HPGC−LALLS Method[a]

oligomers	parameters	results			average ± SE (n = 3)
H	d_n/d_{c_p} (mL/g)	0.262	0.264	0.271	0.266 ± 0.004
	M_p	6.14×10^5	5.95×10^5	5.93×10^5	$6.01 \pm (0.10) \times 10^5$
D	d_n/d_{c_p} (mL/g)	0.294	0.296	0.297	0.296 ± 0.002
	M_p	2.65×10^5	2.60×10^5	2.45×10^5	$2.57 \pm (0.09) \times 10^5$

[a] The membrane-bound enzyme (dog kidney) was solubilized, and the resultant-solubilized enzyme was subjected to HPGC with the three kinds of detectors: a LALLS photometer (LS), a differential refractometer (RI), and a UV spectrophotometer, as described in the caption for Figure 2. The value of d_n/d_{c_p} for the respective oligomers of H and D were calculated from the absorption coefficient at 280 nm, (Output)RI and (Output)UV, and those of M_p from the d_n/d_{c_p}, (Output)LS and (Output)RI, according to the HPGC−LALLS method (*8*).

attributed to an incomplete separation of D from P because of the adoption of the novel column. On the other hand, to confirm the M_p of H obtained above, the protein component of H was isolated by HPGC using a standard column of TSK gel G3000 SWXL and then rechromatographed by the same HPGC−LALLS method described above. These two HPGC procedures were performed in the absence of any added PS to examine the effect of PS on the stability of H in HPGC. Although G was still present in 2.3% of all the proteins eluted, a nearly single peak of protein for H emerged as

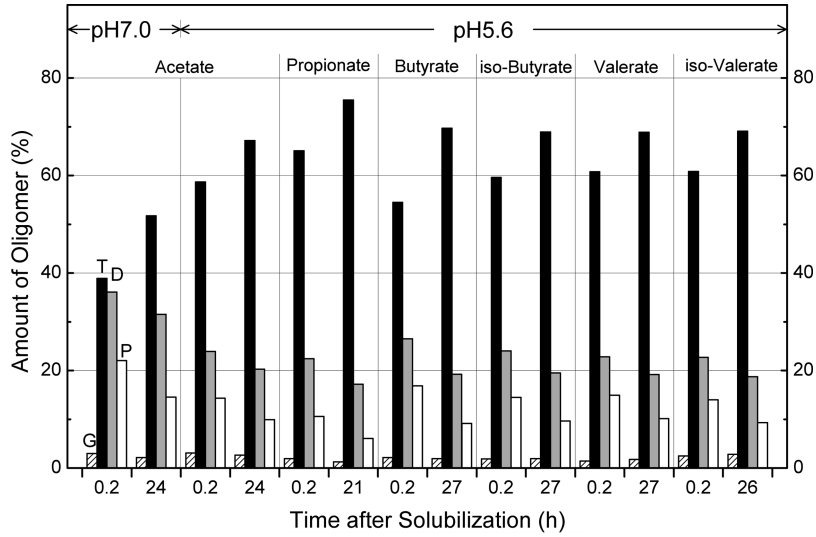

FIGURE 3: Effect of the short-chain fatty acids on the formation of T (H) in the solubilized enzyme. The membrane-bound enzyme (dog kidney) was solubilized with $C_{12}E_8$ in the improved (pH 5.6) or conventional (pH 7.0) solubilizing solution containing 0.04 M salt constituting K^+ and the respective short-chain fatty acids mentioned in the figure. At 0.2 or 21−27 h after solubilization, the respective solubilized enzyme was subjected to HPGC with the standard column of TSK gel G3000 SWXL, which had been equilibrated, and was eluted with the conventional elution buffer supplementing with 0.09 M NaCl, 0.01 M KCl, and 4 mM $MgCl_2$ at pH 7.0 and 0 °C. The amount of the respective oligomers, G, T, D, and P, was estimated from the area under the respective oligomer peaks and plotted against the time after solubilization and with the fatty acids presented during solubilization.

shown in the UV pattern (Figure 2B). This likely indicated that H became very stable and/or scarcely exhibited an association−dissociation equilibrium, unlike P and D. The values of d_n/d_{c_p} and M_p were 0.258 mL/g and 6.14×10^5, respectively, confirming the M_p of H reported in Table 2.

Our group has recently shown that K^+ induces the conversion of P into D and/or H and Na^+ has the opposite effect, regardless of the anion present (*10*). Furthermore, the oligomeric interconversion among P, D, and H have been quantitatively estimated showing unequivocally that H is formed by association of P and/or D and that P and/or D are formed by dissociation of H. Therefore, it could be concluded that H is the $(\alpha\beta)_4$-tetraprotomer (T) from the values of M_p, $6.01-6.14 \times 10^5$ and $1.56\ (\pm 0.04) \times 10^5$ for H and P, respectively.

Effect of Short-Chain Fatty Acids on the Oligomerization. Acetate in the conventional (pH 7.0) or improved (pH 5.6) solubilizing solution was replaced by various acetate homologues having the longer alkyl chain, i.e., short-chain fatty acids, such as Prop, butyrate, isobutyrate, valerate, and isovalerate. The resulting solubilized enzyme was subjected to HPGC to estimate the amount of the respective oligomers. As shown in Figure 3, other fatty acids produced T more effectively than acetate and the solubilization in the presence of 0.05 M K^+Prop at pH 5.6 brought about the highest proportion of H among the oligomers.

Effect of Cations on the Oligomerization. K^+ cation of K^+acetate included in the solubilizing solution was replaced by the congeners of K^+, such as Tl^+, Rb^+, NH_4^+, and Na^+, and the dog kidney enzyme was solubilized to compare the distribution of the oligomers. As shown in Figure 4, the same maximum amount of T was obtained at 0.1 M X^+acetate, except that Na^+ did not show any effect on the T increment at all. The dependence of the T amount on the X^+acetate concentration was analyzed by curve-fitting according to the Hill equation

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

where Y (%) is the amount of T, C_0, the amount (%) before X^+acetate addition, ΔC_{max} (%) is the maximum change in the amount induced by the addition of X^+acetate, [X^+acetate] (M) is the concentration of X^+acetate added, $K_{0.5}$ (M) is the concentration of X^+acetate for a half-maximum change in the amount of T, and n is the Hill constant for the oligomeric interconversion. $K_{0.5}$ values of the congeners for the maximal increment in the amount of T were $NH_4^+ \gg Rb^+ \cong K^+ > Tl^+$, which increased the affinity for the enzyme in that order. This order was consistent with the order of the affinity in the X^+ binding to the membrane-bound enzyme purified from the dog kidney (*22*). Values of n ranging between 2.1 and 3.2 were obtained for Rb^+, K^+, and Tl^+. When the pig kidney enzyme was used instead of the dog kidney enzyme, a similar increment could be detected in the amount of T, with the improved solution containing K^+Prop at pH 5.6 compared to that detected using the dog kidney enzyme (Figure 5).

A previous study of the conventional solution containing Cl^- at pH 7.0 showed that K^+ induces the conversion of P into D and/or H and that Na^+ has the opposite effect, regardless of the anion present (*10*). Thus, the effect of K^+ and Na^+ on the amount of T was compared in the presence of Prop at pH 5.6. The replacement of K^+ by Na^+ in the solubilizing solution decreased the amount of T from 47.4 to 14.6% (expressed as $\Delta C_{max} = -32.8\%$) and increased D and P from 31.1 to 43.9% ($\Delta C_{max} = +12.8\%$) and from 17.6 to 36.7% ($\Delta C_{max} = +19.1\%$), respectively, although G did not change, as shown in Figure 6. Accordingly, the absolute value of ΔC_{max} for T was equivalent to that of the sum of D and P, indicating that Na^+ induced the dissociation of T into

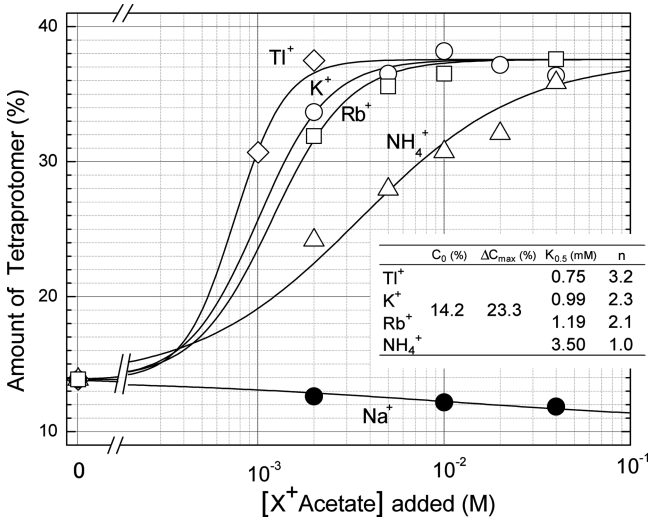

FIGURE 4: Effect of K^+ congeners on the formation of T. The enzyme (dog kidney) was solubilized with $C_{12}E_8$ in the conventional solubilizing solution (pH 7.0) containing various concentrations of the acetate salts constituting Tl^+, K^+, Rb^+, NH_4^+, and Na^+, of which monovalent cation concentrations were adjusted to 0.1 M with choline chloride. An aliquot of 30 μL of the solubilized enzyme was subjected to HPGC at 24 h after solubilizing in the same ways as the caption for Figure 1, except that the conventional elution buffer containing 0.2 mg/mL $C_{12}E_8$, 0.09 M NaCl, 0.01 M KCl, and no $MgCl_2$ was used. The amounts of G, T, D, and P were quantified from the areas under the respective peaks of absorbance at 280 nm, and the relative values of T were plotted against the concentrations of cations. Data for Tl^+ are partially plotted because it was not soluble at the concentrations higher than 2×10^{-3} M in the solubilizing solution used. The data were analyzed by curve-fitting according to the Hill equation of $Y = C_0 + \Delta C_{max}[X^+acetate]^n/([X^+acetate]^n + K_{0.5}{}^n)$, where Y (%) is the amount of T, C_0 is the amount (%) before X^+acetate addition, ΔC_{max} (%) is the maximum change in the amount induced by the addition of X^+acetate, [X^+acetate] (M) is the concentration of X^+acetate added, $K_{0.5}$ (M) is the concentration of X^+acetate for a half-maximum change in the amount of T, and n is the Hill constant for a T increment, obtaining the parameters indicated in the figure (Origin Software, OriginLab Corporation, $R^2 > 0.977$). The solid curves were drawn using the parameters, with the exception of Na^+.

D and P under the new improved conditions, where T was present as a major oligomeric component of the solubilized enzyme.

Effect of Synthetic PSs on the Distribution of the Oligomers. The enzyme was solubilized in the conventional solubilizing solution (pH 7.0) containing either 0.1 M K^+acetate or $Na^+NO_3^-$ to make the enzyme more associated or dissociated, respectively, according to previously reported data (*10*). The K^+acetate-solubilized enzyme was chromatographed with the conventional elution buffer (pH 7.0) supplemented with 80 μg/mL of various types of synthetic PS or natural PS, 0.09 M NaCl, 0.01 M KCl, and 4 mM $MgCl_2$ at 0 °C. As shown in Figure 7A, the characteristic composition of the greatest amount of T(H) was common to all of the synthetic PSs used, with the exception of dioctanoyl PS, and was observed even without the addition of PS. When the $Na^+NO_3^-$-solubilized enzyme was chromatographed as described above, P and not T was found to be the most abundant component in all of the cases (Figure 7B). When compared to the amount of T formed both with and without PS, the first and second highest amounts of T were produced with the natural PS and dioleoyl PS, respectively, for both the $Na^+NO_3^-$- and the K^+acetate-solubilized enzymes (Figure 7). Dioctanoyl PS produced the

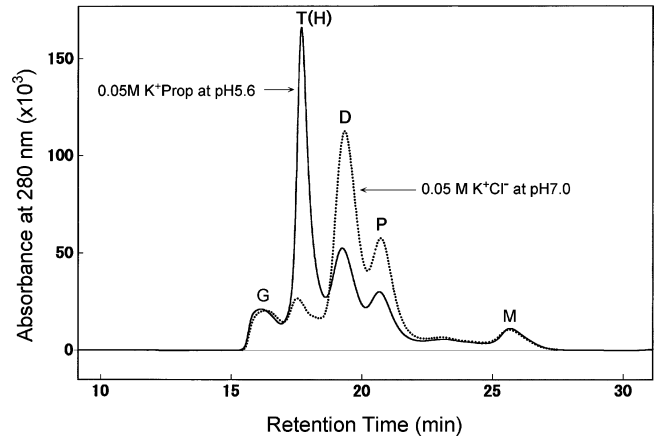

FIGURE 5: Marked differences in the distribution of oligomers between the enzymes solubilized in the presence of K^+Prop at pH 5.6 and that in the presence of K^+Cl^- at pH 7.0. The membrane-bound enzyme purified from pig kidney was solubilized in two contrasting solutions: one containing 0.05 M K^+Prop, 5.4 mM imidazole, and 29 mM MES at pH 5.6; another containing 0.05 M K^+Cl^-,14 mM imidazole, and 22 mM HEPES at pH 7.0; and, in common, containing 2.0 mg/mL protein, 6.0 mg/mL $C_{12}E_8$, 1 mM EDTA, and 10% glycerol. The respective solubilized enzyme placed in common for 0.2 h was subjected to HPGC after solubilization with the standard TSK gel G3000 SWXL column, which had been equilibrated and was eluted with 0.2 mg/mL $C_{12}E_8$, 0.05 M K^+Prop, 4 mM $Mg(acetate)_2$, 1 mM EDTA, and 17 mM MES at pH 5.6 and 0 °C. The elution patterns for the enzyme solubilized in 0.05 M K^+Cl^- at pH 7.0 or in 0.05 M K^+Prop at pH 5.6 are shown with a dotted or solid line, respectively. See the caption for Figure 1 on G, T(H), D, P, and M.

least amount of T among all of the PSs used and produced a lesser amount of T when no PS was added, as in the case of the K^+acetate-solubilized enzyme (Figure 7A). Therefore, while the synthetic PSs carrying alkyl chains longer than C14 had comparable functions to the natural PS, dioleoyl PS was the most similar to the natural PS among the synthetic PSs with respect to T production.

ATPase Activities of the Respective Oligomers Supported with Various PSs. To estimate the specific ATPase activity for T, D, and P, the $Na^+NO_3^-$- and K^+acetate-solubilized enzymes were subjected to HPGC at 0 °C with the conventional elution buffer (pH 7.0) containing 1 mM ATP, 0 or 80 μg/mL PS, 0.09 M NaCl, 0.01 M KCl, and 4 mM $MgCl_2$. The respective oligomers of G, T, D, and P were separated by HPGC at 0 °C and then warmed to 27 °C immediately after eluting out of the gel column, so that they can be assayed for ATPase activity during passage inside the outlet steel tube connected to the column. The elution patterns thus obtained for eluted protein and liberated P_i were piled up and displayed in Figure 8 separately in the cases where the elution buffer contained dioleoyl PS (Figure 8A) or no PS (Figure 8B). On the basis of the elution patterns obtained, the specific ATPase activities of the four kinds of the oligomers (G, T, D, and P) were estimated with various kinds of the synthetic PSs, natural PS, and no PS, which were included in the elution buffer. The activities of the respective oligomers with the synthetic PSs and no PS were plotted against those with natural PS (Figure 9). The linear regression curves whose respective slopes and correlation coefficients were very close to 1 could be obtained with dioleoyl PS, dimyristoyl PS, and natural PS, showing that the three kinds of PSs were very similar to one another with respect to the activation of ATPase.

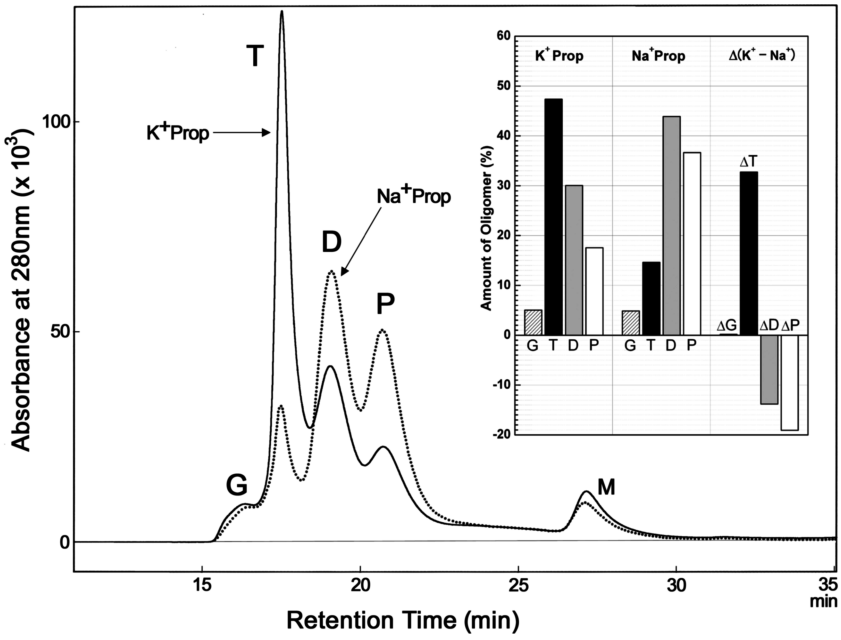

FIGURE 6: Na^+/K^+-dependent interconversion among T, D, and P in the presence of propionate at pH 5.6. The membrane-bound enzyme (pig kidney) was solubilized in the improved solubilizing solution (pH 5.6) containing 0.05 M K^+Prop or Na^+Prop at 0 °C. An aliquot of 50 μL of the resulting solubilized enzyme was subjected to HPGC with a standard column of TSK gel G3000 SWXL, which had been equilibrated and eluted with the elution buffer containing 0.2 mg/mL $C_{12}E_8$, 0.05 M K^+Prop, 4 mM $Mg(acetate)_2$, 1 mM EDTA, and 17 mM MES at pH 5.6 and 0 °C. The solid and dotted lines indicate the elution patterns obtained for the enzyme solubilized in the presence of K^+Prop and Na^+Prop, respectively. (Inset) The amount of the respective oligomers, G, T, D, and P, was obtained from the area under the respective elution peaks and displayed as a column having various heights above G, T, D, and P drawn on the ordinate, respectively, in the inset. The amount of respective oligomers obtained in the presence of Na^+Prop was subtracted from that obtained in the presence of K^+Prop, and the respective differences in the oligomer amount expressed in ΔG , ΔT , ΔD, and ΔP were displayed below the term of $\Delta(K^+-Na^+)$. See the caption for Figure 1 on G, T, D, P, and M.

Because the ionic conditions adopted above were not optimum for the ATPase reaction, the ATPase activity of the membrane-bound enzyme was compared at 37 °C between the above-mentioned conditions and the optimum ones described at the A line in Table 1. Thus, the activity under the present conditions was found to be 75.6 ± 1.4% of that under the optimum conditions, and the ratio was adopted to calculate the values at the B line in Table 3. Using both the ratio (75.6%) thus obtained and the temperature dependence of ATPase activity (data not shown), the average value of the ATPase activities for T, D, and P estimated at 27 °C with these three kinds of PSs (A line in Table 3) was converted to those expected at 37 °C under the optimum conditions. The resulting converted values for T, D, and P were 41.0 ± 1.9, 66.1 ± 1.1, and 75.9 ± 3.4 units/mg at 37 °C (B line in Table 3), respectively, which showed ratios of 1.00 ± 0.07, 1.61 ± 0.08, and 1.85 ± 0.12 (C line in Table 3), respectively. The specific activity of the original membrane-bound enzyme was determined to be 41.4 units/mg under the same optimum conditions (A line in Table 1). The dog kidney enzyme with nearly 100% pure ouabain binding very likely contains 39% inactive oligomers (*10*). Thus, a 100% active enzyme would have a specific activity of 67.9 units/mg (B line in Table 3), showing that the activity of the membrane-bound enzyme is nearly equivalent to those of P and D but to twice that of T.

Dioleoyl PS was very similar to natural PS on aspects such as distribution of the oligomers (Figure 7) and activation of ATPase (Figure 9). Accordingly, dioleoyl PS was prominently comparable to natural PS with respect to the activation of ATPase and the formation of T.

Re-activation of ATPase Activity with Dioleoyl PS for Isolated T. To isolate T in the oligomeric state in a stable form, the membrane-bound enzyme (pig kidney) was solubilized in the improved solubilizing solution (pH 5.6) supplemented with 0.05 M K^+Prop. The resulting solubilized enzyme was chromatographed by HPGC with the improved elution buffer (pH 5.6) supplemented with 0.04 M Na^+Prop, 0.01 M K^+Prop, and 4 mM $Mg(acetate)_2$ at 0 °C. After the collected fractions of T were concentrated with a Vivaspin ultrafiltration device, T was assayed for ATPase activity in the reaction mixture made by adding 4 mM ATP and 0−80 μg/mL dioleoyl PS to the same elution buffer as above at 25 °C. Correlation coefficients of the time course in P_i liberation were higher than 0.999 for almost all of the cases, indicating that any change in the specific activity of T would not occur during the assay for 0.5−6.0 min, as shown in the inset of Figure 10. The specific ATPase activity of T increased with an increasing dioleoyl PS and saturated to 2.38 units/mg with more than 64 μg/mL dioleoyl PS (Figure 10), of which the maximum activity of T was completely inhibited by 0.29 mM ouabain (Figure 10). The activation of T induced by dioleoyl PS occurred cooperatively with n = 3.6 (Figure 10). The reversibility of ouabain-sensitive ATPase activity for T before and after isolation by HPGC was very important from the point of physiological significance as well as availability of the preparation for active and stable T. However, at this stage, it was not known whether the oligomeric structure of T was retained during the assay.

Active Enzyme Chromatography of T. To investigate whether any oligomeric change of T occurs in the improved

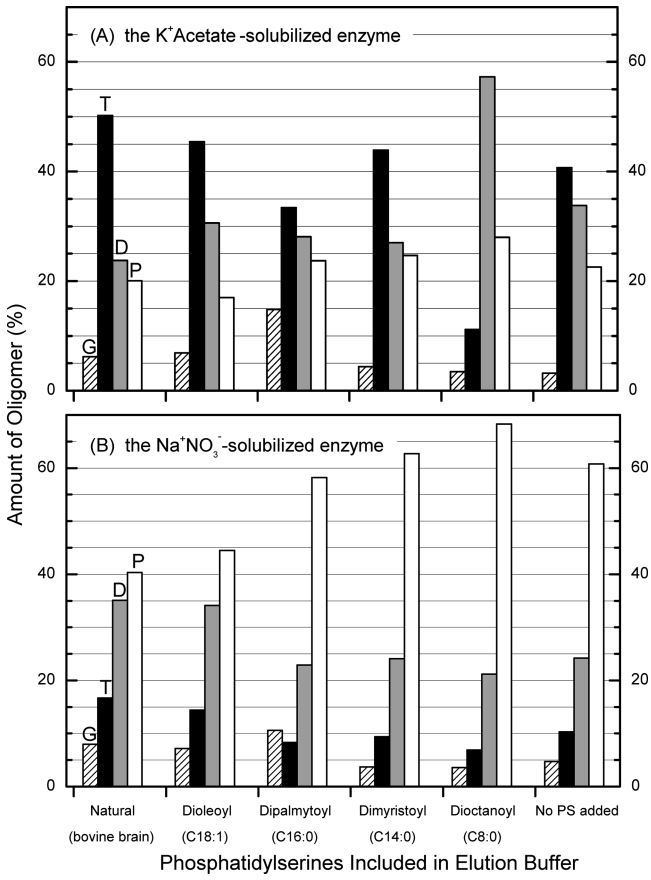

FIGURE 7: Effects of various kinds of PS included in the elution buffer for HPGC on the distribution of the oligomers. The membrane-bound enzyme (dog kidney) was solubilized in the conventional solubilizing solution (pH 7.0) containing either 0.1 M K^+acetate or $Na^+NO_3^-$. The solubilized enzyme was subjected to HPGC with a standard column of TSK gel G3000 SWXL, which had been equilibrated and was eluted with the elution buffer containing 0.2 mg/mL $C_{12}E_8$, 0 or 80 μg/mL the respective PSs indicated on the ordinate, 0.09 M NaCl, 0.01 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole, and 13 mM HEPES at pH 7.0 and 0 °C to estimate the amount of the respective oligomers. Amounts of the oligomers for the solubilized enzyme obtained in the presence of (A) K^+acetate or (B) Na^+NO_3 were plotted against the PS included in the respective elution buffers.

ionic environment with propionate at pH 5.6, the oligomeric structure and the ATPase activity of T were determined simultaneously by active enzyme chromatography using the improved elution buffer (pH 5.6) supplemented as described below at 25 °C. First, T was isolated by HPGC at 0 °C on a preparative 21.5 × 300 mm column of TSK gel G3000 SWXL with the improved elution buffer (pH 5.6) supplemented with 80 μg/mL dioleoyl PS, 0.04 M Na^+Prop, 0.01 M K^+Prop, and 4 mM $Mg(acetate)_2$. It was then incubated with 4 mM ATP at 0 °C, followed by active enzyme chromatography with the same elution buffer used for the preparative column, additionally supplemented with 1 mM ATP at 25 °C. As shown in Figure 11A, T was observed to be dissociated into D by 37% during the experimental processes. A similar amount (31%) of T conversion was detected when T was previously incubated in the elution buffer additionally supplemented with 0.2 mM ouabain and 4 mM ATP, when not exhibiting ATP hydrolysis (Figure 11B). On the other hand, as shown in Figure 11C, T was scarcely dissociated when T treated with neither ATP nor ouabain was subjected to the same active enzyme chromatography performed at 0 instead of 25 °C. Therefore, the dissociation of T into D by 31−37% (an average of 34%) could be attributed to the exposure of the enzyme to 25 °C in the pre-incubations but not to ATP hydrolysis. Furthermore, T was eluted at the same position when chromatographed at 25 °C (parts A and B of Figure 11) or 0 °C (Figure 11C). Therefore, it was feasible that the remaining portion (66%) of T was not in a dissociation−association equilibrium among T, D, and P during the passage through the column at 25 °C whichever exhibited ATP hydrolysis or not, indicating that the portion of T was very stable or rigid.

The specific ATPase activity of the isolated T was estimated by active enzyme chromatography to be 2.09 units/mg at 25 °C, although the apparent T behaved as T by 66% and as D by 34% during the assay. Assuming that the ratio of T/D/P in the specific ATPase activity was 1:2:2 as described above, the specific activities of T and D were calculated to be 1.56 and 3.12 units/mg, respectively, in which the latter activity of D was consistent with the value (3.31 ± 0.06 units/mg) estimated by another method described above (D line in Table 3). The membrane-bound enzyme in which the ATPase activity was 41.4 units/mg, under optimal conditions at 37 °C, showed activity of 2.14 ± 0.08 units/mg (D line in Table 1) under the same conditions used in the active enzyme chromatography, except that neither $C_{12}E_8$ nor any PS was involved in the reaction mixture. Assuming that the enzyme purified from dog kidney may contain 39% inactive peptide (*10*), the 100% pure and active membrane-bound enzyme would have a specific activity of 3.51 ± 0.13 units/mg under active enzyme chromatography (F line in Table 3). This value was almost twice that of T and almost equivalent to that of D and P (G line in Table 3).

Stability of a Tetraprotomer Isolated by HPGC. T was isolated by HPGC on the preparative column chilled at 0 °C with the same elution buffer as that used for the active enzyme chromatography, except that neither dioleoyl PS nor ATP was included. After being treated in various ways, T was subjected to rechromatography by the same HPGC as that adopted in the isolation, except that the column was replaced by an analytical one. The isolated T was shown to be about 80% pure at least 41 h after standing at 0 °C without any treatment, and neither D nor P increased even 10 days later. When T was incubated at −80 °C or in liquid N_2 for 3 h and 1 min, respectively, without any glycerol or trehalose added, 68% of T was converted into D and P. The addition of 21% glycerol completely protected T from the conversion, while 30% trehalose was somewhat effective but not equivalent to the effect of glycerol.

DISCUSSION

Effect of the Short-Chain Fatty Acids on the Increase of the Amount of T. In our previous study, the dog kidney enzyme solubilized with $C_{12}E_8$ in the conventional solubilizing solution containing a salt constituting K^+ or Na^+ and Cl^- at pH 7.0 had D and P as the major protein components and T(H) as the minor one (*9*). D and P were shown to be in an association−dissociation equilibrium of $D \rightleftharpoons 2P$ at 20 °C under conditions where all of the ligands necessary for the enzyme to exhibit ATPase activity coexist with natural PS and the respective oligomers of D and P had the capacity

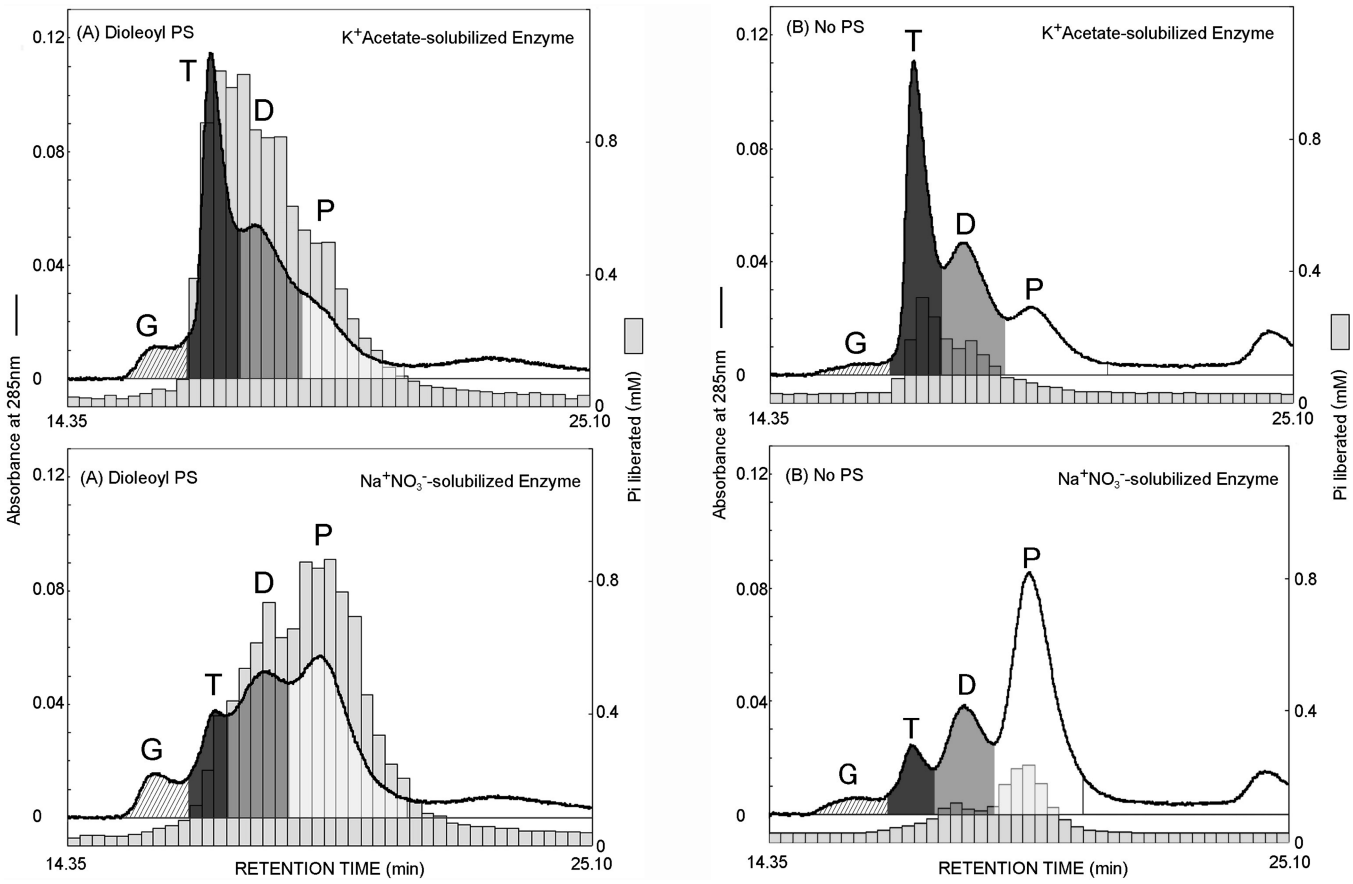

FIGURE 8: Estimation of ATPase activity for the respective oligomers immediately eluted from the HPGC column. The membrane-bound enzyme (dog kidney) was solubilized in the same way as described in the caption of Figure 7, and 40 μL aliquot each of the K^+acetate- or the $Na^+NO_3^-$-solubilized enzyme was subjected to the same HPGC as that described in Figure 7, except the elution buffer was supplemented additionally with 1 mM ATP and only dioleoyl PS or no PS was used in place of various kinds of PS. The effluent was warmed to 27 ± 1 °C immediately after eluting out the column and then passing the column oven for the respective oligomers to start hydrolyzing ATP inside the steel tube connected to the column. After the effluent flowed for 41 s, it was fractionated at 15 s intervals into micro test tubes chilled at 0 °C, and the amount of P_i was determined after 1.67% SDS (final concentration) was added to the effluent. Concentrations of P_i were piled onto the elution pattern detected by absorbance at 285 nm against retention times. (A) Elution patterns of the K^+acetate- (upper panel) and $Na^+NO_3^-$-solubilized enzyme (lower panel) obtained with the elution buffer containing dioleoyl PS, and (B) those of the K^+acetate- (upper panel) and $Na^+NO_3^-$-solubilized enzyme (lower panel) obtained with that containing no added PS.

for ATP hydrolysis (*9*). We indicated a higher possibility of the enzyme to exist in the form of D in the membrane because the protein and phospholipids are sufficient in the membrane. Undergoing a remarkable change in this study, T was shown to occupy a major oligomeric component in the presence of K^+ and short-chain fatty acids at a pH of about 6. As it was strongly suggested in our study (*10*), K^+ binding to the active site of the enzyme with $K_{0.5}$ of 64.8 μM shifted the equilibrium between E_2 (the state of strong subunit−subunit interaction) and E_1 (that of the weak interaction) to E_2, while Na^+ with $K_{0.5}$ of 6.50 mM did the opposite. Taking the Na^+/K^+-dependent interconvertible properties of Na^+/K^+-ATPase and the artificial dissociation effect on the enzyme by $C_{12}E_8$ (*20*) into consideration, the sizable increment of T can be attributed to the action of the short-chain fatty acids. The fatty acids work as a kind of detergent, because they are amphiphiles with both hydrophobic alkyl chains and hydrophilic acetate ions in the same molecule. The fatty acids and $C_{12}E_8$ should bind competitively to some hydrophobic region of the enzyme in themselves. Thus, the addition of propionate may exclude some $C_{12}E_8$ from the sites so that the excess dissociation or degradation of the enzyme because of $C_{12}E_8$ would be recovered and/or suppressed. On the other hand, long-chain fatty acids, such as lauric acid (C12) and oleic acid (C18), and their derivatives activate the ATPase activity of the enzyme at a suboptimal ATP concentration (50 μM), and the amphiphiles might bind specifically to a highly hydrophobic segment of the α-subunit intracellular domain to bring about the activation (*23*). Thus, it cannot be excluded that the fatty acids may bind specifically to the enzyme in the membrane in the absence of $C_{12}E_8$ or other detergents and that they would lead the structure to a stable tetraprotomer.

The results obtained by Blanco et al. (*24*), Ganjeizadeh et al. (*25*), and Kaplan et al. (*26, 27*) disagree with the assumption that the αα interaction in the molecule of D is due to nonspecific collisions of αβ-protomers. As described above, our group has recently shown that T (formerly designated as H) as well as D and P are interconvertible among the oligomers (*10*). The Na^+- and K^+-dependent conversion of T into D and P has also been quantitatively confirmed in this study (Figure 6). Accordingly, T may be formed by the αα interaction in the same way as D. T formation is optimum at pH 5.5−6 (data not shown), and the isoelectric point (pI) of α constituting the pig kidney

Table 3: Specific ATPase Activities of the Respective Oligomers Separated by HPGC and the Membrane-Bound Na^+/K^+-ATPase (MB-E)

	processes	conditions	PS added	ATPase activity [μmol of P_i min^{-1} (mg of protein)$^{-1}$ or relative ratio] G	T	D	P	MB-E	data
A	measured[a] by HPGC	at 27 °C and pH 7.0 with Cl^-	dioleoyl, dimyristoyl, or natural	3.17 ± 0.38	15.5 ± 0.73	25.0 ± 0.42	28.7 ± 1.3		Figure 8
B	converted[b] from A	the optimum[c] at 37 °C and pH 7.3 with Cl^-	dioleoyl, dimyristoyl, or natural	8.39 ± 1.01	41.0 ± 1.9	66.1 ± 1.1	75.9 ± 3.4	(67.9)[d,e]	(measured)[e]
C	ratios[f]			0.20 ± 0.01	1.00 ± 0.07	1.61 ± 0.08	1.85 ± 0.12	1.66 ± 0.08	
D	converted[g] from B	at 25 °C and pH 5.6 with Prop	dioleoyl, dimyristoyl, or natural	0.42 ± 0.05	2.05 ± 0.10	3.31 ± 0.06	3.80 ± 0.17	3.40	
E	measured by a batch method	dioleoyl		1.78,[h] 1.56[h]					Figures 10 and 11
F			none					3.51 ± 0.13[d]	D line in Table 1
G	ratios[f]		various ones or none[i]	0.23 ± 0.04	1.00 ± 0.16[j]	1.84 ± 0.21	2.11 ± 0.25	1.95 ± 0.23[k]	

[a] An HPGC column was equilibrated and eluted with the elution buffer containing 0.2 mg/mL $C_{12}E_8$, 1 mM ATP, 0.09 M NaCl, 0.01 M KCl, 4 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole, 13 mM HEPES, and 80 μg/mL of the respective PS at a flow rate of 0.350 mL/min at pH 7.0 and 0 °C. The effluent was warmed to 27 ± 1 °C immediately after eluting out the column to leave the protein fractions exhibiting ATP hydrolysis (cf. Figure 8). [b] The values in the A line were multiplied by 2.00 (ratio of activity at 37 °C to that at 27 °C, data not shown) and 1.323 (ratio of activity under the optimum conditions to that under the suboptimum conditions, as described in the text), of which factors were determined with the membrane-bound enzyme. [c] This was the same as that listed in the A line in Table 1. [d] MB-E was assumed to include inactive oligomers by 39% (w/w) (*10*), and the specific ATPase activity was calculated by dividing the measured value by 0.61. [e] The original datum was from the A line in Table 1. [f] Errors were calculated under consideration of their propagation. [g] Conversions were performed using the ratio (0.0517) of 2.14 units/mg (D line) to 41.4 units/mg (A line) shown in Table 1. [h] Specific activities were calculated from the values estimated for isolated T assuming the following: T dissociated into D by 34%, and the ratio of specific ATPase activities for T/D/P was 1:2:2. [i] Dioleoyl, dimyristoyl, and natural PSs were used for G, T, D, and P, and no PS was used for MB-E. [j] The value averaged (1.80 ± 0.20, $n = 3$) activities for T listed in D and E lines in this table was used for normalization. [k] The value of 3.51 ± 0.13 units/mg was used for normalization.

enzyme is 5.5 (*28*), also contributing to the αα interaction in T formation.

Abe et al. (*14*) have found T in pig gastric H/K-ATPase solubilized with *n*-octylglucoside by total internal reflection fluorescence microscopy, while there were few T in the $C_{12}E_8$-solubilized enzyme. However, $C_{12}E_8$ solubilization of

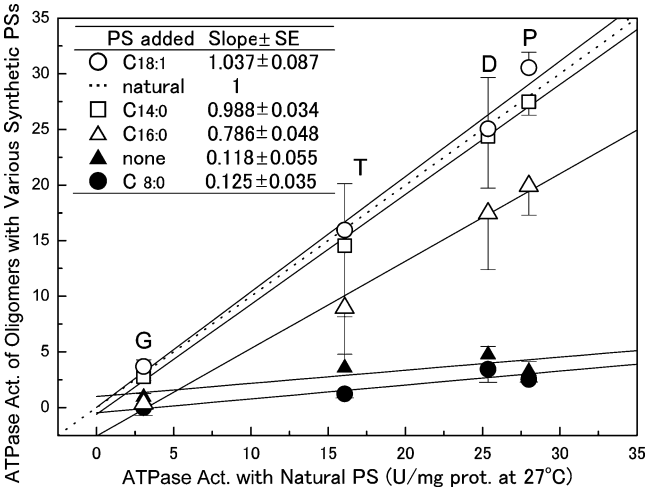

FIGURE 9: Correlations of the activation in ATPase activity of the respective oligomers with various kinds of synthetic PSs to that with natural PS. The membrane-bound enzyme (dog kidney) was solubilized and chromatographed in the same ways as described in the caption of Figure 8, except that the six kinds of the elution buffer supplemented with the respective synthetic PSs of dioleoyl (C18:1), dipalmytoyl (C16:0), dimyristoyl (C14:0), or dioctanoyl (C8:0) PS and with natural PS or no PS were used. Specific ATPase activities of G, T, D, and P were calculated from the protein amount estimated under the peak area, amount of P_i liberated, and the reaction time (41 s) that the effluent was exposed to 27 °C. The respective activities obtained for G, T, D, and P with natural PS were plotted on the ordinate, and the values obtained with the other PSs were plotted against the respective values with natural PS. Slopes of linear lines obtained by the linear regression method for the respective plots were described in the figure, and correlation coefficients for the regression lines ranged between 0.994 and 0.999 for open symbols and 0.931 and 0.836 for C8:0 and no PS, respectively.

H/K-ATPase in the presence of K^+acetate increases the amount of T with $K_{0.5}$ of 27 mM (*15*), consistent with the present results. The K^+acetate concentration dependence of *p*NPPase activity ($K_{0.5}$ of 30 mM for E−P formation) is comparable to that of T formation, leading to the conclusion that the catalytic unit of $C_{12}E_8$-solubilized H/K-ATPase is a tetraprotomer (*15*).

When D or P isolated by HPGC with the conventional elution buffer at pH 7.0 at 0 °C was rechromatographed by the same HPGC as the first one, the respective oligomers of D and P used to be separated again into D and P in a weight ratio of approximately 1:1, indicating that D and P would be easily interconvertible (Y. Hayashi, unpublished data). Therefore, our group was not able to characterize independently and separately D and P. In striking contrast to D and P, the oligomeric component of T, isolated under the improved conditions for solubilization and HPGC, was rather stable even during exhibition of ATP hydrolysis at 25 °C (parts A and B of Figure 11) as well as during passage through the HPGC column at 0 °C (Figure 11C and Figure 2B). Accordingly, T, bearing such a stable property, can be available to investigate properly the structure−function relationship for Na^+/K^+-ATPase.

Specific ATPase Activities of T, D, P, and the Membrane-Bound Enzyme. The respective oligomers were assayed at 27 °C for ATPase activity immediately after separating by HPGC (Figure 8). The ATPase activities of T, D, and P thus obtained (A line in Table 3) were converted to those under the optimum conditions with Cl^- at pH 7.3 and 37 °C to be 41.0, 66.1, and 75.9, respectively, indicating ratios of 1.00:1.61:1.85 (C line in Table 3). The similar ratio of T/D/P was also obtained by the batch method for ATPase assay to be 1.00:1.84:2.11 under the conditions with Prop at pH 5.6 and 25 °C (G line in Table 3). Our group has preliminarily reported that the amount of [γ-^{32}P]ATP bound to the respective oligomers, estimated by Hummel−Dreyer's method, is 0.40 ± 0.04, 0.71 ± 0.03, and 0.74 ± 0.02 mol of ATP/

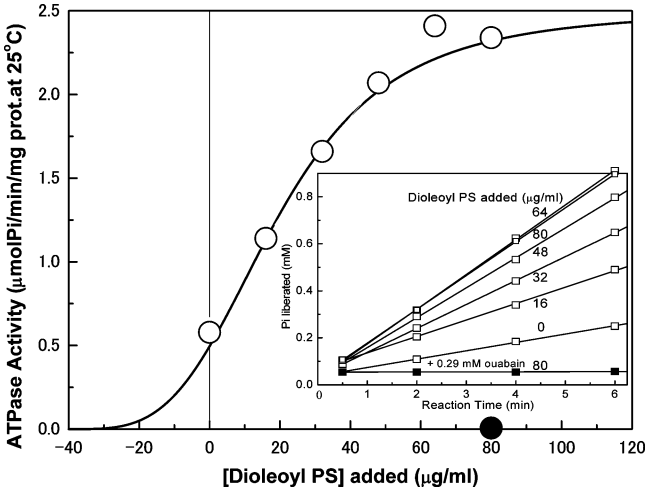

FIGURE 10: Re-activation of ATPase activity for T by the addition of dioleoyl PS after isolation by HPGC. The enzyme (pig kidney) was solubilized in the improved solubilizing solution (pH 5.6) containing 0.05 M K^+Prop at 0 °C, and an aliquot of 1.5 mL of the resulting solubilized enzyme was subjected to HPGC with the preparative column of TSK gel G3000 SWXL, which had been equilibrated and was eluted with the elution buffer containing 0.2 mg/mL $C_{12}E_8$, 0.05 M K^+Prop, 4 mM Mg(acetate)$_2$, 1 mM EDTA, and 17 mM MES at pH 5.6 and 0 °C to isolate T. T fractions (8.3 mL) collected were concentrated 12-fold by diafiltration under a centrifugal force of 1920*g* for 40 min with an ultrafiltration of Vivaspin 20 (molecular weight cutoff of 100 000). T, thus isolated and concentrated, was assayed for ATPase activity in the reaction mixture with the final composition of 0.0615 mg/mL protein, 4.0 mM ATP, 0.04 M Na^+Prop, 0.01 M K^+Prop, 4 mM Mg(acetate)$_2$, 1 mM EDTA, 0−80 μg/mL dioleoyl PS, and 17 mM MES at pH 5.6 and 25 °C. The specific activities estimated in the absence of ouabain were plotted against the concentrations of dioleoyl PS added (○), and those estimated in the presence of ouabain as well as 80 μg/mL dioleoyl PS were plotted against the dioleoyl PS concentration (●). The data (○) were analyzed by curve-fitting according to the Hill equation of $Y = V_{max}[X + 40]^n/([X + 40]^n + [K_{0.5} + 40]^n)$, where Y (units/mg) is the ATPase activity of T, V_{max} (units/mg) is the maximum activity induced by the addition of dioleoyl PS, X (μg/mL) is the concentration of dioleoyl PS added, $K_{0.5}$ (μg/mL) is the concentration of dioleoyl PS for a half-maximum activation in the ATPase activity of T, and n is the Hill constant for the activation. The solid line was drawn using the parameters of V_{max} = 2.50, n = 3.6, and $K_{0.5}$ = 19. (Inset) The concentrations of P_i liberated in the assay of T described above were estimated with a Technicon Auto Analyzer and plotted against the reaction time. Correlation coefficients of the time course were higher than 0.999 for all of the cases, except in the case of ouabain.

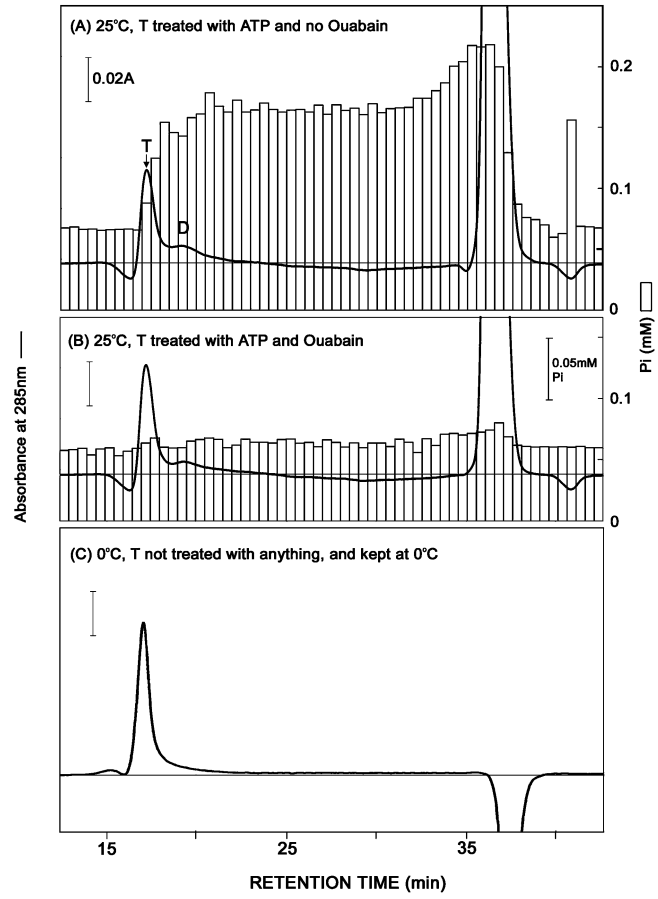

FIGURE 11: Active enzyme chromatography of T treated without and with ouabain. The enzyme (dog kidney) was solubilized with $C_{12}E_8$ in the improved solubilizing solution (pH 5.6) containing 0.05 M K^+Prop at 0 °C. The resulting solubilized enzyme was subjected to HPGC with a preparative column of TSK gel G3000 SWXL equilibrated and eluted with the elution buffer containing 0.2 mg/mL $C_{12}E_8$, 80 μg/mL dioleoyl PS, 0.04 M Na^+Prop, 0.01 M K^+Prop, 4 mM Mg(acetate)$_2$, 1 mM EDTA, and 17 mM MES at pH 5.6 and a flow rate of 1.20 mL/min at 0 °C. (A and B) Aliquots of T isolated carried out by the above HPGC were incubated with 4 mM ATP for 30 s at 0 °C and subsequently (A) without or (B) with 0.20 mM ouabain for 3 min at 25 °C in the solution with the final composition of 0.118 mg/mL protein of T and 4 mM ATP in the elution buffer diluted consequently to 93.7% of the original composition. The respective resulting aliquots of 250 μL of the isolated T were subjected to the active enzyme chromatography, where a standard column of TSK gel G3000 SWXL had been equilibrated with the same elution buffer as above, with the exception of 1 mM ATP supplemented and eluted at 0.35 mL/min and 25 °C. Aliquots of 175 μL (0.50 min fraction) each of the eluate were collected in test tubes containing 175 μL each of 3.33% SDS to stop the ATPase reaction and to assay for P_i. (C) Another aliquot of T isolated, without the incubation with ATP and/or ouabain, was subjected to the active enzyme chromatography in the same way as A and B, with the exception of performance at 0 instead of 25 °C.

mol of protomer in T, D, and P, respectively, at 1 μM ATP (*17*). Thus, considering the stoichiometry for the ATP-binding site, it can be reasonably concluded that the specific activity ratio of T, D, and P is 1:2:2.

The specific ATPase activity of the original membrane-bound enzyme was measured to be 41.4 and 2.14 ± 0.08 units/mg under the conditions with Cl^- at pH 7.3 and 37 °C and with Prop at pH 5.6 and 25 °C, respectively (A and D lines in Table 1, respectively). As shown recently by our group, the solubilized dog kidney enzyme brought about Na^+- and K^+-dependent oligomeric interconversion among P, D, and T (T is described in terms of H in ref *10*). It has been strongly suggested on the basis of the $K_{0.5}$ values of 64.8 μM and 6.5 mM for K^+ and Na^+, respectively, that the interconversion is coupled with Na^+/K^+ binding to their active transport sites (*10*). On the other hand, the purest preparation of the enzyme used in this investigation contained 39% unconvertible oligomers of the eluted protein, strongly suggesting that as much as 39% was inactive in terms of ATP hydrolysis. If this is true, the specific activity for the full active enzyme can be 67.9 and 3.51 ± 0.13 units/mg under the conditions with Cl^- at pH 7.3 and 37 °C and with Prop at pH 5.6 and 25 °C, respectively (B and F lines in Table 3). The activity of T was estimated to be 41.0 ± 1.9 and 1.78 ± 0.32 units/mg under the above conditions of 37 and 25 °C, respectively, indicating that the ratio of the membrane-bound enzyme to T in the specific activity ranged between 1.66 and 1.95 under both conditions (C and G lines in Table 3).

In summary, the corrected activities of T, D, P, and the membrane-bound enzyme are 1.67, 3.34, 3.34, and 3.51 units/mg at 25 °C. These values could be converted by multiplying by 19.3 (=41.4/2.14 units/mg) to those under the optimal conditions at 37 °C, yielding 32.3, 64.6, 64.6, and 67.9 units/mg, respectively. These results suggest that D and P are equivalent to a full active enzyme, but T is only half of it. The activities deduced for those of D, P, and the full active enzyme are nearly equivalent to the activity of the enzyme purified from nasal glands of salt-adapted ducks (*29, 30*). The preparation of Na^+/K^+-ATPase from the nasal salt glands has a phosphorylation capacity and specific ATPase activity (66 ± 2 units/mg at 37 °C) near the theoretical maxima (*29, 30*).

Our group previously reported that the solubilized dog kidney enzyme with a specific activity of 37−48 units/mg at 37 °C is composed of 98% D and P, which bound stoichiometrically to [^{3}H]ouabain (1.12 ± 0.06 and 1.11 ± 0.05 mol/mol of the protomer for D and P, respectively) (*31*). Accordingly, almost all of the subunits of P and/or D constituting the T molecule should be able to bind to ouabain with a stoichiometry of 1 mol/mol of protomer before constructing the T molecule. However, T could exhibit the specific ATPase activity half as much as P or D, suggesting that heterogeneity with regard to ATPase site is induced by T formation. Xie and Askari (*32*) and Liang et al. (*33*) have shown that Na^+/K^+-ATPase contains a nonpumping enzyme in addition to a pumping one, both of which can bind to ouabain. The heterogeneity of Na^+/K^+-ATPase cannot yet be ascribed to any differences in structure and function between the respective enzymes.

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