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Minimum enzyme unit for Na^+/K^+-ATPase is the $\alpha\beta$-protomer. Determination by low-angle laser light scattering photometry coupled with high-performance gel chromatography for substantially simultaneous measurement of ATPase activity and molecular weight

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The oligomeric state of canine renal Na^+/K^+-ATPase solubilized by octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$) was studied by means of low-angle laser light scattering photometry coupled with high-performance gel chromatography (HPGC). At around 0°C the solubilized enzyme was separated into the $(\alpha\beta)_2$-diprotomeric and $\alpha\beta$-protomeric protein components with M_r values of 302000 ± 10000 and 156000 ± 4000, respectively, in approximately equal quantities. As the temperature of chromatography was increased toward 20°C, the two protein components converged into a single major component. The M_r of this component depended on the monovalent cation included in the elution buffer, and was 255000 or 300000 in the presence of 0.1 M NaCl or 0.1 M KCl, respectively. A computer simulation technique showed that the solubilized enzyme was in a dissociation-association equilibrium of 2 protomers ⇌ diprotomer at 20°C, and the difference in apparent M_r of the solubilized enzyme between the two species of monovalent cation was interpreted by an association constant (K_a) in the presence of 0.1 M KCl that was about 50-fold larger than in the presence of 0.1 M NaCl. In order to measure ATPase activity and M_r of the solubilized enzyme simultaneously, a TSKgel G3000SW column had been equilibrated and was eluted with an elution buffer containing 0.30 mg/ml $C_{12}E_8$ and 60 μg/ml phosphatidylserine (bovine brain) as well as the ligands necessary for the enzyme to exhibit the activity at pH 7.0 and 20°C. The solubilized enzyme was always eluted as a single protein component irrespective of the amount of the protein applied to the column, ranging between 240 and 10 μg. The M_r of the protein component, however, decreased from 214000 to 158000 with the decrease of the protein amount. The specific ATPase activity, however, remained constant at a level of 64 ± 4% of that of the membrane-bound enzyme even in the range of protein concentration sufficiently low as to allow the enzyme to exist only in the protomeric form. Thus, the $\alpha\beta$-protomer is concluded to be the minimum functional unit for the ATPase activity. The value of K_a obtained from the concentration-dependent dissociation curve was $5 \cdot 10^5$ M^{-1} for the enzyme turning over, and $1.1 \cdot 10^7$ M^{-1} for the enzyme inhibited with ouabain. It was discussed, based on the values of K_a obtained, that the enzyme would exist as the diprotomer or the higher oligomer in the membrane.

Introduction

Na^+/K^+-ATPase is the integral membrane protein that transports 3 Na^+ ions outward and 2 K^+ ions inward across the plasma membrane at the expense of 1 ATP molecule [1]. The protein is mainly composed of two polypeptides, a catalytic subunit and a glycopeptide, which are designated α and β, respectively [2,3]. The M_r values of the α and β subunits of dog

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; LS, light scattering; RI, differential refractive index; UV, ultraviolet; K_a, association equilibrium constant.

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kidney Na^+/K^+-ATPase are 118000 ± 3000 and 39400 ± 900, respectively, determined by the same technique used in the present study [4]. The values are in good agreement with those obtained for the α (112177–112424) and β (34671–34958) subunits of the enzyme from other sources by the cDNA sequencing technique [5–9]. Isolated α and β subunits have never been reconstituted into a functional enzyme.

The quaternary structure of the ATPase has been studied by molecular weight estimation [3,10–12] and chemical cross-linking [13,14] of the enzyme that was solubilized with nonionic surfactants, such as $C_{12}E_8$ and Lubrol, without substantial loss of enzymatic activity. These studies have shown consistently that the two polypeptides, α and β, are noncovalently combined together in a minimum structural unit, that is, an $\alpha\beta$-protomer. On the other hand, the conflicting conclusions on the structure necessary for the enzymatic activity has been obtained by the sedimentation equilibrium technique: the dimer of the minimum unit, that is, the $(\alpha\beta)_2$-diprotomer was the minimum active protein unit of the solubilized enzyme prepared from shark rectal gland [10,11], while the $\alpha\beta$-protomer was the minimum active protein unit of the enzyme prepared from pig kidney [12]. Chemical cross-linking of the solubilized enzyme prepared from dog kidney has, however, demonstrated that the enzyme solution contains different oligomers of the $\alpha\beta$-protomer, such as $\alpha\beta$, $(\alpha\beta)_2$, $(\alpha\beta)_3$ and $(\alpha\beta)_4$ [13], and that an $\alpha\beta$-protomer constituting more than 85% of a population of oligomers can express full enzymatic activity [14]. By the method of low angle laser light scattering photometry coupled with high-performance gel chromatography (HPGC/LALLS method) we have shown that the $\alpha\beta$-protomer and $(\alpha\beta)_2$-diprotomer are the major protein components (more than 82%) in our preparation of the solubilized enzyme prepared from dog kidney [3]. Thus, it remained uncertain which of the two protein components is the minimum functional unit for hydrolysis of ATP. To make clear this point, measurement of molecular weight should be performed on the solubilized enzyme or its protein components while turning over. The HPGC/LALLS method, which has been improved to be applicable to research on membrane protein [3,15–17], seemed suitable for the purpose.

In the present study, we have further refined the HPGC/LALLS method so that not only the molecular weight could be determined for the protomer and its dimer immediately after emergence from the column, but also the ATPase activity exhibited during passage through the column could be monitored. Results thus obtained unambiguously demonstrated that the solubilized enzyme was in an equilibrium of dissociation–association between the protomer and the diprotomer, whether the enzyme turned over or not, and that the $\alpha\beta$-protomer is the minimum functional unit for the enzymatic activity. Furthermore, the equilibrium constant estimated suggests that the membrane-bound enzyme exists as a higher oligomer than the protomer in the plasma membrane of kidney. Preliminary results of M_r measurement at low temperature and of measurement of ATPase activity exhibited during the chromatography have been presented [18,19].

Methods

Membrane-bound Na^+/K^+-ATPase

The enzyme was purified from microsomes of outer medulla of frozen dog kidney by the method of Jørgensen [20] with the following modifications: 2 mM dithioerythritol was included in all the media, and the concentration of original SDS solution to be added to the microsomes was lowered to less than 0.83 mg/ml to minimize denaturation by SDS. Final concentrations of the microsomal protein and SDS were 1.40 and 0.70 mg/ml, respectively. The purified enzyme was washed to exclude contamination of the enzyme preparation by Na^+ and K^+ as described elsewhere [3]. The protein concentration was estimated by the method of Bradford [21], and was corrected by multiplication by a factor of 1.144 determined by quantitative amino-acid analysis as described elsewhere [3]. The specific ATPase activity of the enzyme ranged from 37 to 48 U/mg protein (units defined as $\mu mol\ P_i \cdot min^{-1}$) under the optimum conditions of 100 mM NaCl/25 mM KCl/4 mM Na_2ATP/3.9 mM $MgCl_2$/0.2 mM EDTA/30 mM imidazole/30 mM glycylglycine at pH 7.2 and 37°C.

Solubilized Na^+/K^+-ATPase

The membrane-bound enzyme was incubated in a solution with the final composition of 2 $mg \cdot ml^{-1}$ protein/6 $mg \cdot ml^{-1}$ $C_{12}E_8$/0.2 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, unless otherwise stated. The solution was centrifuged under 170000 × g for 20 min at 0°C. The supernatant was collected and stored at 0°C. This preparation will be hereafter referred to as the solubilized Na^+/K^+-ATPase or the solubilized enzyme. The ATPase activity of the enzyme was almost fully retained for 48 h at least after the solubilization. Aggregation of protein components constituting the solubilized enzyme was not detected during the storage. The solubilized enzyme was applied to a chromatography column within 3 min (for the experiments shown in Figs. 1–4) or 12 h (for those shown in Figs. 5–7) after the isolation. Concentrations of the solubilized enzyme, $\alpha\beta$-protomer and $(\alpha\beta)_2$-diprotomer were determined by the absorbance at 280 nm using an absorption coefficient of 1.22 $mg^{-1} \cdot ml \cdot cm^{-1}$ [3].

The preparation of the ouabain-treated, solubilized enzyme was made as follows. The membrane-bound enzyme (3.1 mg/ml protein) was incubated with 1.5

mM ouabain in a solution of 3.1 mM $MgCl_2$/3.1 mM dithioerythritol/1.5 mM EDTA/15.4% (w/v) glycerol/20 mM imidazole/16 mM Hepes at pH 7.0 and 37°C. After incubation for 10 min, the solution was cooled to 0°C, and KCl followed by $C_{12}E_8$ were added to the solution so that the final composition became the same as described above with respect to the constituents other than ouabain and $MgCl_2$ (their final concentrations were 1 and 2 mM, respectively). The enzyme thus treated was solubilized as described above, and the resultant enzyme is referred to as the ouabain-treated, solubilized enzyme.

Estimation of M_r by the HPGC / LALLS method

A TSKgel G3000SW column (7.5 × 600 mm, Tosoh Co.) equipped with a guard column (TSK guard column SW, 7.5 × 75 mm) was used for HPGC of the solubilized enzyme, unless otherwise stated. The columns were equilibrated and eluted with an elution buffer (pH 7.0) containing 0.2 mg · ml^{-1} $C_{12}E_8$/0.1 M KCl/1 mM EDTA/10 mM imidazole/13 mM Hepes at a flow rate of 0.30 ml/min at 0–1.6°C or at a flow rate of 0.50 ml/min at 20°C. In some cases, 0.1 M KCl was replaced by 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$ or by 0.1 M NaCl. The eluate from the columns was monitored successively with the following three kinds of detectors: a LALLS photometer (LS; TSK model LS-8000), a differential refractometer (RI; TSK model RI-8011), and a UV spectrophotometer (UV; TSK model UV-8000). The temperature of the columns, the flow-through cell installed in the LALLS photometer, and the lines connecting the column and the cell was kept constant at the levels stated within ± 0.1 C°. The M_r of the protein moiety of the protein component eluted was obtained according to the following equation

$$M_r = k_1(dn/dc_p)^{-1}(\text{output})_{LS} \cdot (\text{output})_{RI}^{-1} \quad (1)$$

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); $(\text{output})_{LS}$, the output of LS; and $(\text{output})_{RI}$, the output of RI [15,22,23]. The value of dn/dc_p reflects the amount of substances bound to the protein, such as surfactant and lipids, and was determined by the following equation

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

where k_2 is the instrumental constant; A, the absorption coefficient expressed in terms of weight concentration of c_p at 280 nm; and $(\text{output})_{UV}$, the output of UV [3,15,16]. The constants k_1 and k_2 were determined using the standard proteins of glutamate dehydrogenase (yeast, M_r = 297 354 [24], dn/dc = 0.187 ml/g *, A = 1.27 mg^{-1} · ml · cm^{-1}) and lactate dehydrogenase (pig heart, 142 000 [25], 0.187 ml/g, 1.38 mg^{-1} · ml · cm^{-1}). The outputs of the three kinds of monitor were obtained as the heights of the elution peaks.

Substantially simultaneous measurement of ATPase activity and M_r during turnover in an HPGC column

An aliquot of a chloroform/methanol solution containing 30 mg of PS from bovine brain with 98% purity (Sigma Chemical) was evaporated to dryness in a stream of nitrogen. Several ml of a solution of 1.5 mg · ml^{-1} $C_{12}E_8$/0.25 M NaCl/0.25 M KCl/20 mM $MgCl_2$/5 mM EDTA/50 mM imidazole/75 mM Hepes (pH 7.0) were added to the residue of PS and sonicated until visible fragments of PS disappeared, leaving a slightly cloudy solution. The resultant suspension was mixed with a large volume of a solution without PS so that the final ingredients were 60 μg · ml^{-1} PS/0.3 mg · ml^{-1} $C_{12}E_8$/0.05 M NaCl/0.05 M KCl/4 mM $MgCl_2$/1 mM EDTA/1.33 mM ATP/10 mM imidazole/15 mM Hepes (pH 7.0). The final solution became clear after incubation overnight at room temperature, and was used as the elution buffer. The same columns as those described above were equilibrated with about 100 ml of the elution buffer at a flow rate of 0.50 ml/min and 20°C. The solubilized enzyme was usually diluted with 1–15 vols of the elution buffer lacking ATP and PS. An aliquot of 100–150 μl containing 10–240 μg protein of the solubilized enzyme was applied to the columns, and the columns were eluted with the elution buffer. The eluate was monitored successively with the three kinds of monitor, 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. The concentration of P_i in each fraction was estimated on a Technicon auto-analyzer by the method of Hegyvary et al. [26] and was plotted against the retention time at which the fraction was taken.

The specific ATPase activity of the enzyme exhibited during passage through the column was calculated from the amount of P_i liberated, the time taken to liberate the P_i, and the amount of protein eluted. The amount of P_i liberated was calculated from the plateau region of the P_i elution pattern located between the elution volume of major protein component and the column volume so that overestimation due to extra liberation of P_i observed around the two volumes would be avoided. The plateau region corresponds to the elution time between 32 and

* Since non-ionic surfactants do not bind to most of the typical water-soluble globular proteins [31,32], the values of dn/dc_p of the standard proteins in the presence of a nonionic surfactant were assumed to be the same as the values for the proteins in the absence of surfactant (dn/dc).

47 min and was indicated by a bar with arrows in the P_1 pattern of Fig. 6A. The protein amount was estimated from the area under the peak of RI using the ratio of RI to protein concentration, that is, the value of the dn/dc_p described below. The M_r of the major protein component eluted was calculated as described above. In the calculation of M_r, the value of dn/dc_p for the enzyme hydrolyzing ATP was assumed to be the same as that determined for the solubilized enzyme having no ATPase activity, i.e., the ouabain-treated, solubilized enzyme.

Computer simulation of the elution pattern

The elution patterns obtained with a UV spectrophotometer in HPGC carried out at 0.3 and 20°C were simulated according to the method of Stevens and Schiffer [27], which has been developed for a single-protein system exhibiting dissociation–association equilibrium of 2 monomers ⇌ dimer. The columns used were divided into 1500 imaginary cells in our program of simulation. The parameters required for the simulation were (a) migration rates of the monomer and the dimer, (b) K_a, (c) sample volume, (d) original protein concentrations in sample, (e) dispersion factor and (f) the number of repetitions of the dispersion cycle. The migration rates (a) of the $\alpha\beta$-protomer and the $(\alpha\beta)_2$-diprotomer were determined from the elution pattern observed at 0.3°C. The relative rates were satisfied by setting the migration rate of the protomer at 2.284 cells and that of the diprotomer at 2.434 cells per each migration cycle. The relative rate was set at 1 cell per cycle for an imaginary standard substance assumed to be able to occupy all available space in the unit cell. The elution pattern obtained at 0.3°C was simulated first. In this case, the dissociation–association between the protomer and the diprotomer was assumed to have been frozen. The sample volume (c) and the concentrations of the protomer and the diprotomer in the sample (d) were varied keeping the original total protein concentration constant at the experimentally determined value, so that the simulated pattern could fit to the observed pattern. Dilution and band spreading were accomplished by varying the parameters (e) and (f). In the case of the simulation of the elution pattern observed at 20°C, the two protein components were assumed to be in a rapid equilibrium instantaneously reestablished with progress of the migration. Simulation to find the best-fitted curve was carried out by variation of the parameters (b), (e) and (f) while keeping the other parameters constant at the values determined in the simulation of the pattern obtained at 0.3°C. It took about 16 h to simulate a pattern with the use of a personal computer (NEC Co., model PC-9801F2).

Other methods

Phospholipid content of protein component isolated by HPGC was determined by measuring organic phosphorus content according to the ultramicro method of Bartlett [28], as described elsewhere [3].

Results

$\alpha\beta$-Protomer and $(\alpha\beta)_2$-diprotomer as main protein components of solubilized Na^+/K^+-ATPase

Fig. 1 shows the elution patterns of the solubilized enzyme from the TSKgel column monitored by the three kinds of detector: a LALLS photometer, a differential refractometer and a UV spectrophotometer. The chromatography was performed with an elution buffer of 0.2 mg·ml^{-1} $C_{12}E_8$ containing 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/13 mM Hepes at 0.4°C and pH 7.0. The first peak emerged around 36 min (approximately corresponding to the void volume of the column) and was rather large in the tracing of the LALLS photometer (Fig. 1, LS), but very small in the tracings of the differential refractometer and the UV spectrophotometer (Fig. 1, RI and UV). Judging from these facts, the

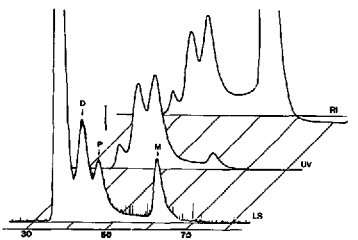

Fig. 1. Separation of $(\alpha\beta)_2$-diprotomer and $\alpha\beta$-protomer of solubilized Na^+/K^+-ATPase by the HPGC/LALLS method carried out at 0.4°C. The membrane-bound enzyme was solubilized with the $C_{12}E_8$ solution containing 0.1 M NaCl instead of 0.2 M KCl and otherwise the same ingredients as described in Methods at 0°C. The solubilized enzyme (an aliquot of 200 μl containing 0.32 mg protein) was chromatographed immediately on a TSKgel G3000SW column equipped with a guard column. The column had been equilibrated and was eluted with an elution buffer of 0.20 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 0.30 ml/min at 0.4°C. The eluate from the column was monitored successively with a LALLS photometer (LS), a differential refractometer (RI) and a UV spectrophotometer (UV). The three kinds of elution patterns recorded on chartpaper are displayed after correction for the displacement in the patterns due to the differences in positions of the detector cells and recorder pens, and are indicated by LS, RI and UV. Gain settings for the detectors were 8 for LS, 4 for RI and 0.32 absorbance units (full-scale) for UV. The length of the bar with flags corresponds to one-tenth full-scale for the UV pattern. D and P denote the protein components of $(\alpha\beta)_2$-diprotomer and $\alpha\beta$-protomer, respectively, and M denotes mixed micelles mainly constituted of $C_{12}E_8$ and lipids.

peak was assigned to minor component(s) (in terms of amount) having very high M_r values. Two peaks designated 'D' and 'P' in Fig. 1 (LS) were assigned to major protein components as judged from the tracings of the differential refractometer and the UV spectrophotometer, also shown in Fig. 1 (RI and UV). A peak designated 'M' in Fig. 1 (LS) was attributed to mixed micelles mainly composed of $C_{12}E_8$ and phospholipids by the criteria described previously [3]. The same characteristics of the chromatographic patterns as those described above were observed when the conditions for the solubilization of the enzyme and for the chromatography were varied as described in Table I. Under the conditions investigated, the M_r values of the two major protein components were estimated to be 156 000 ± 4000 and 302 000 ± 10 000 ($n = 4$), and their specific refractive index increments to be 0.302 ± 0.004 and 0.279 ± 0.002 ml/g, respectively, by the HPGC/LALLS method (Table I).

We have previously shown that each of the two major protein components of the solubilized enzyme was composed of both α and β subunits in an equimolar ratio, even under less favourable conditions for association of the two subunits than the present ones, that is, at higher concentration of $C_{12}E_8$, i.e., 1 mg/ml, in the elution buffer and at a higher temperature of the chromatography, i.e., 23°C [3]. Thus, we may conclude that the two components separated under the present conditions are composed of the α and β subunits in the molar ratio of 1 to 1. We have shown that M_r values of the α and β subunits are 118 000 ± 3000 and 39 400 ± 900, respectively, by the HPGC/LALLS method in the presence of SDS [4]. The sum of these two M_r values is in good agreement with the M_r of the smaller component, and half as large as that of the larger one of the two protein components revealed by the present chromatography. Therefore, the protein components, 'P' and 'D', can be identified unambiguously as the $\alpha\beta$-protomer and $(\alpha\beta)_2$-diprotomer, respectively.

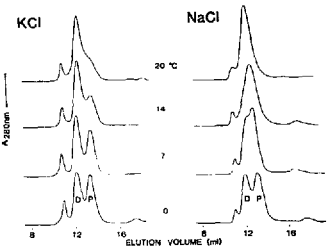

Fig. 2. Temperature-dependences of elution patterns of solubilized Na^+/K^+-ATPase revealed by HPGC in the presence of KCl or NaCl. The solubilized enzyme (each aliquot of 220 µl containing 0.35 mg protein) was charged onto a TSKgel G3000SW column. The columns had been equilibrated and were eluted with two kinds of elution buffer containing either 0.1 M KCl or 0.1 M NaCl and otherwise the same ingredients of 0.20 mg ml^{-1} $C_{12}E_8$/1 mM EDTA/10 mM imidazole/13 mM Hepes (pH 7.0) and at a flow rate of 0.35 ml/min. The temperature of the columns was set as indicated. The eluate was monitored with a UV spectrophotometer. The length of the bar with flags corresponds to 0.128 absorbance units at 280 nm. See the legend to Fig. 1 for 'D' and 'P'.

Dependence of the separation between the diprotomer and the protomer on temperature and cations

Fig. 2 shows the elution patterns of the solubilized enzyme obtained using two kinds of elution buffer containing either 0.1 M KCl or 0.1 M NaCl and otherwise the same constituents at temperatures ranging from

TABLE I

Molecular weights of two main protein components of solubilized Na^+/K^+-ATPase, $(\alpha\beta)_2$-diprotomer and $\alpha\beta$-protomer, separated by HPGC performed at temperatures near 0°C

The purified membrane-bound enzymes were solubilized with $C_{12}E_8$ solution, and then the solubilized enzymes were chromatographed as described in the legend to Fig. 1. The cations to be included in the $C_{12}E_8$ solution for the solubilization and in the elution buffer for the chromatography were varied as indicated in the first and the second column of the table below, respectively. The eluate was monitored successively with the three kinds of detector (see Fig. 1). M_r and specific refractive index increments (dn/dc_p) of the protein components separated were determined from the outputs of the detectors by the HPGC/LALLS method as described in Methods.

Cations in solubilization	Cations in chromatography	Temperatures in chromatography (°C)	$(\alpha\beta)_2$-Diprotomer dn/dc_p (ml/g)	$(\alpha\beta)_2$-Diprotomer M_r	$\alpha\beta$-Protomer dn/dc_p (ml/g)	$\alpha\beta$-Protomer M_r
0.2 M KCl	0.1 M KCl	0.0	0.281	303 000	0.305	151 000
0.2 M KCl	0.1 M KCl	1.6	0.279	297 000	0.303	157 000
0.2 M KCl	0.1 M KCl	1.6	0.278	291 000	0.303	161 000
0.1 M NaCl	0.05 M KCl, 0.05 M NaCl, 4 mM $MgCl_2$	0.4	0.276	315 000	0.295	155 000
		Averages ± S.E.	0.279 ± 0.002	302 000 ± 10 000	0.302 ± 0.005	156 000 ± 4000

0 to 20 °C. The elution patterns at 0 °C were almost the same, regardless of which monovalent cation, K^+ or Na^+, was included in the elution buffer (see the bottom of Fig. 2). In contrast, as the temperature increased to 20 °C, the diprotomer increased at the expense of the protomer in the case of the elution buffer containing KCl (see left-hand side of Fig. 2). The M_r of the resultant main component obtained at 20 °C was estimated to be 300 000 ($dn/dc_p = 0.253$ ml/g) confirming that the component was the $(\alpha\beta)_2$-diprotomer. When an elution buffer containing 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$ instead of 0.1 M KCl was used, the elution pattern and the M_r of the main protein component (295 000, $dn/dc_p = 0.258$ ml/g) coincided with those obtained with the elution buffer containing 0.1 M KCl alone at 20 °C. On the other hand, when the elution buffer containing 0.1 M NaCl was used, the diprotomer and the protomer seemed to converge into a new protein component with the increase of temperature (right-hand side of Fig. 2). The converged protein component appeared at a position intermediate between the positions of the diprotomer and the protomer as indicated by the chromatography carried out at 0 °C. The M_r of the intermediate component was 255 000 ($dn/dc_p = 0.236$ ml/g), showing that the protein component was neither the diprotomer nor the protomer itself.

The contents of phospholipid were estimated to be 4.7 ± 1.5 ($n = 8$) mol phospholipid per $1.5 \cdot 10^5$ g protein for the major protein components obtained with the three kinds of elution buffer described above at 20 °C. In contrast, the diprotomer and the protomer obtained with the elution buffer containing 0.1 M KCl at 0 °C contained much more phospholipid, specifically, 13.8 ± 0.4 and 14.6 ± 6.3 ($n = 4$) mol per $1.5 \cdot 10^5$ g protein, respectively. Thus, the content of phospholipid in the protein component isolated depended somehow on the temperature of the chromatography column, but not on the ligands included in the elution buffer. These contents of phospholipid for the protein components thus isolated, however, were far less than that (40–50 mol per $1.5 \cdot 10^5$ g protein) required for the solubilized enzyme to exhibit the maximum activity [29,30].

Computer simulation of the gel chromatography behavior of the solubilized enzyme

Fig. 3 shows a simulation of the elution patterns obtained under conditions similar to those in Fig. 2 according to the procedure developed for a reversibly associating protein in 'small-zone-gel-chromatography' by Stevens and Schiffer [27]. The elution pattern having two separated protein peaks could be simulated by assuming that the solubilized enzyme comprised a mixture of equal weights of the diprotomer and the protomer, and that the two protein components passed through the column at a migration rate determined by

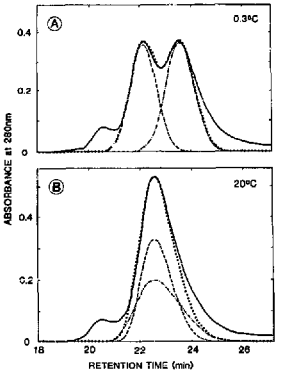

Fig. 3. Computer simulation of the elution patterns with two separated protein peaks observed at 0.3 °C and with a single protein peak observed at 20 °C in the presence of 0.1 M NaCl. Elution profiles indicated by solid lines were experimentally obtained as follows. An aliquot of 200 μl each of the solubilized enzyme (protein concentration: 1.6 mg/ml) was chromatographed on a TSKgel G3000SW_{XL} column (7.8 × 300 mm) equipped with a TSK guard column SW_{XL} (6.0 × 40 mm). The column had been equilibrated and was eluted with the same elution buffer as that containing 0.1 M NaCl described in the legend to Fig. 2 at a flow rate of 0.287 ml/min and at 0.3 (A) or 20 °C (B). The eluate from the column was monitored with a UV spectrophotometer at 280 nm. The elution profiles thus obtained were simulated as described in Methods using the following parameters: sample volume, 140 μl; total protein concentration, 1.60 mg/ml (10.7 μM in terms of molar protomer concentration); concentration of the diprotomer and the protomer in the sample before the chromatography, 0.760 (5.07 μM) and 0.840 mg/ml (5.60 μM), respectively. (A) Simulation of the elution profile observed at 0.3 °C. The diprotomer and the protomer were assumed to pass through the column at the respective rates of migration (0.700 and 0.657 ml/min, respectively) deduced from the elution pattern experimentally observed (the solid line). The dispersion factor and the number of dispersion cycle were 0.20 and 4, respectively. Elution profiles thus simulated for the whole protein, the diprotomer and the protomer were represented by dotted, broken and dashed-and-dotted line, respectively. (B) Simulation of the elution profile observed at 20 °C. Complete equilibration of reversible association between the protomer and the diprotomer ($2\ \alpha\beta \rightleftharpoons (\alpha\beta)_2$) was assumed to occur between migration steps. The association constant of $2 \cdot 10^6\ M^{-1}$ was adopted. The dispersion factor was 0.38, but the other parameters were assumed to be identical to those used above.

the chromatography carried out at 0.3 °C (see the solid line in Fig. 3A). The best fit of the simulated patterns was obtained by assuming that the weight ratio of the diprotomer to the protomer was 1.104, and by additionally assuming that a volume of the sample solution corresponding to 70% of that actually charged was

applied to the columns. Since recovery of protein was 93% in the chromatography, 23% of the sample protein was not accounted for by the simulation. Some of the protein was eluted as the minor component(s) with very high M_r (7%) and as a trailing portion behind the simulated peak (12%).

On the other hand, the elution pattern having a single protein peak could be simulated by assuming that the diprotomer and the protomer were in an equilibrium of dissociation–association with a very fast rate as compared with the elution time. The position of the protein peak obtained by the simulation varied between the elution positions of the diprotomer and the protomer depending on the K_a adopted. By assuming the K_a to be $2 \cdot 10^6$ M^{-1} and other parameters being the same as those used in the simulation of the elution pattern observed at 0.3°C, except that the dispersion factor was changed from 0.20 to 0.38, the best fit could be obtained for the elution pattern observed at 20°C (Fig. 3B). The ratio of weight concentration of the diprotomer to that of the protomer was found to be 0.628 : 0.372 at the top of the protein peak in the best fitting pattern. A weight-averaged M_r was calculated from this ratio to be 248 000 at the top of the peak, which agreed with the M_r of 255 000 experimentally estimated for the protein component under the same conditions as those adopted here by the HPGC/LALLS method, as already described in Fig. 2. The main protein peak with the M_r of about 300 000, which was observed in the elution pattern obtained with the elution buffer containing 0.1 M KCl at 20°C *, could be simulated by assuming the K_a to be greater than $1 \cdot 10^8$ M^{-1} and otherwise the same parameters as those used for the simulation of the pattern in the presence of 0.1 M NaCl at 20°C. Thus, the difference of the chromatographic behaviour of the solubilized enzyme in M_r and elution position between the elution pattern obtained in the presence of NaCl and that obtained in the presence of KCl can be attributed to the difference in the K_a of the equilibrium between the protomer and the diprotomer.

Chromatography of the solubilized active enzyme in the presence of exogenous PS

A gel-chromatography procedure was designed so that the solubilized enzyme turned over during its passage through the column at 20°C: the elution buffer was made to contain the same components as those in an ATPase assay medium, i.e., 4 mM ATP/0.05 M Na^+/0.05 M K^+/4 mM Mg^{2+} (1 mM EDTA), as well as 0.2 mg/ml $C_{12}E_8$ at pH 7.0. Immediately after the solubilization, the enzyme was applied to the column, and eluted with the above buffer. ATPase activity exhibited during passage through the column was evaluated by measurement of the amount of P_i liberated for each fraction of the eluate. Based on the results described above of the lack of endogenous phospholipids, exogenous PS was added to the elution buffer so that it could keep the enzyme active replacing the endogenous phospholipids. As shown in Fig. 4, the rate of P_i liberation in the final phase (at a retention time of 22–28 min) of the passage through the column was increased as the concentration of PS was increased. At 45 μg/ml PS, the rate in the final phase reached almost the same level as that in the initial phase (at a retention time of 48–54 min), showing that the enzyme exhibited constantly the ATPase activity during passage through the column. When the solubilized enzyme was chromatographed with the elution buffer additionally con-

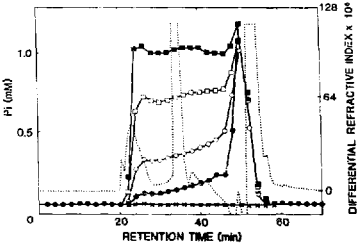

Fig. 4. Steady ATP hydrolysis of the solubilized Na^+/K^+-ATPase maintained by exogenous PS during passage through the HPGC column. The elution buffers containing 0 (●), 15 (○), 30 (□), 45 (■) or 60 μg/ml (×) of PS, and in common 0.20 mg·ml^{-1} $C_{12}E_8$/4 mM ATP/0.05 M NaCl/0.05 M KCl/4 mM $MgCl_2$/1 mM EDTA/10 mM imidazole/15 mM Hepes (pH 7.0) were prepared as described under Methods. The same column as that described in the legend to Fig. 1 had been equilibrated with the elution buffers at a flow rate of 0.493 ml/min and at 20°C. An aliquot of 230 μl containing 0.37 mg protein of the solubilized enzyme was charged onto the columns, which were eluted with the elution buffers under the same conditions as above. In one experiment (×), the ouabain-treated, solubilised enzyme (see Methods) was chromatographed. The eluate was monitored by a differential refractometer which was set up in a box under temperature control at 20°C. 1 ml aliquots of the eluate were collected in testtubes pre-loaded with 0.5 ml of 5% SDS. The dotted line shows the pattern of RI obtained when no PS was included in the elution buffer. Every protein component was eluted earlier than 30 min under all the conditions. The peak eluted at around 34 min corresponds to mixed micelles composed of $C_{12}E_8$ and phospholipids, and peaks eluted between 50 and 55 min correspond to substances with low molecular weight, such as salts.

* In this case, a shoulder remained behind the main peak (see the top left part of Fig. 2). It may be attributable to that some of the protein is not convertible from the protomeric form into the diprotomeric one owing to denaturation. We can not, however, exclude the possibility that rate of the conversion is not fast enough to reestablish the equilibrium with progress of the migration of the protein.

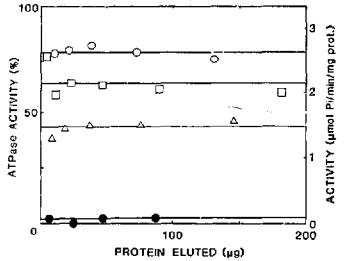

Fig. 5. Specific ATPase activities of the solubilized enzyme exhibited during the gel chromatography, and dependences of the activities on the amount of protein applied and concentrations of $C_{12}E_8$ used. The chromatographies were done in the same way as previously described in the legend to Fig. 4 with the following changes: the concentrations of $C_{12}E_8$ in the elution buffer were 0.20 (○, ●), 0.30 (□) or 0.40 mg/ml (△), and no PS (●) or 60 μg/ml PS (○, □, △) was added to the elution buffers. The protein amounts charged were varied between 10 and 240 μg. Specific ATPase activities of the protein component eluted were estimated as described in Method. The membrane-bound enzyme from which the solubilized enzyme was prepared was assayed for ATPase activity in a reaction medium of 4 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) by incubation for 4 min at 20 °C. 100% corresponds to the resultant specific activity (3.34 ± 0.17 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$).

taining 0.1 mM ouabain, P_i was liberated in the early phase, but not in the late phase of passage of the protein through the column (data not shown). That is, a lag was observed in the appearance of the inhibition by ouabain owing to the slow binding of ouabain to the solubilized enzyme. When the solubilized enzyme prepared from the membrane-bound enzyme had been treated with ouabain, i.e. the ouabain-treated, solubilized enzyme, was applied to the columns in the absence of ouabain, P_i liberation could not be observed at all during the chromatography even in the presence of PS (Fig. 4). This suggests that ouabain does not dissociate from the enzyme during the chromatography under conditions adopted. Evidently, the ATPase activity of the solubilized enzyme supported by the exogenous PS is like the ouabain-inhibitable activity observed in the membrane-bound enzyme and in situ.

The specific ATPase activity of the solubilized enzyme exhibited during passage through the column was estimated with various amounts of the protein charged onto the column between 4.5 and 182 μg and with the elution buffer containing 60 μg/ml PS, 0.2 mg/ml $C_{12}E_8$ and otherwise the same constituents as those described above. As shown by the open circles in Fig. 5, the resultant activities did not depend on the protein amount, but were constant at a value of 2.64 ± 0.08 U/mg protein ($n = 5$). The membrane-bound Na^+/K^+-ATPase from which the solubilized enzyme was prepared was assayed for ATPase activity in the absence of PS and $C_{12}E_8$ under otherwise the same conditions as those in the column. The activity was 3.34 ± 0.17 U/mg protein ($n = 3$), showing that the activity of the solubilized enzyme in the column was equivalent to 79% of that of the membrane-bound enzyme. When the concentration of $C_{12}E_8$ was increased to 0.3 to 0.4 mg/ml, keeping the concentration of PS constant at 60 μg/ml, the activities of the solubilized enzyme decreased to 64 and 43% of that of the membrane-bound enzyme, respectively, while the activities were not dependent on the amount of protein charged (Fig. 5). The ouabain-treated, solubilized enzyme did not hydrolyze ATP at any concentrations of $C_{12}E_8$ adopted above in the presence of 60 μg/ml PS (data not shown). The results strongly suggest that PS competed with $C_{12}E_8$ for hydrophobic regions of the enzyme, and that the higher retention of PS to the enzyme occurred in the lower concentration of $C_{12}E_8$ to bring about the higher ATPase activity in the column.

Substantially simultaneous measurement of M_r and ATPase activity

The M_r of the solubilized enzyme exhibiting ATPase activity during the passage through the columns was measured by the HPGC/LALLS method using an elution buffer containing 0.3 mg/ml $C_{12}E_8$, 60 μg/ml PS/and 1.33 mM ATP and otherwise the same constituents as those described above (see the legend to Fig. 4). Fig. 6A shows a set of four elution patterns of LS, RI, UV and P_i obtained when 43 μg protein of the enzyme was charged onto the columns. The enzyme protein was eluted as a major peak. When the amount of protein applied to the columns was varied between 10 and 240 μg, elution patterns with one major protein peak were consistently obtained. The specific refractive index increment could not be correctly determined owing to underestimation of the UV absorption (see the fall-off of the elution curve of UV at 30–50 min in Fig. 6A) *. Next, the ouabain-treated, solubilized enzyme was chromatographed under conditions identical to those used for the untreated solubilized enzyme. As shown in Fig. 6B, a single protein component was observed in the same way as in the cases of the untreated enzyme, but P_i liberation was not detected at all. The specific refractive index increment of the ouabain-treated, solubilized en-

* The underestimation occurred only when the enzyme turned over in the column. The depth of the fall-off seemed to be inversely proportional to P_i concentration liberated, and the fall-off disappeared behind the column volume. Thus, product(s) of the ATPase reaction may cause the fall-off.

zyme was estimated to be constant at 0.301 ± 0.002 ml/g ($n = 4$) independent of the protein amount applied. The same value (0.302 ± 0.008 ml/g, $n = 3$) was obtained when the untreated solubilized enzyme was chromatographed with the elution buffer containing 0.1 M NaCl, no KCl, no $MgCl_2$ and otherwise the same

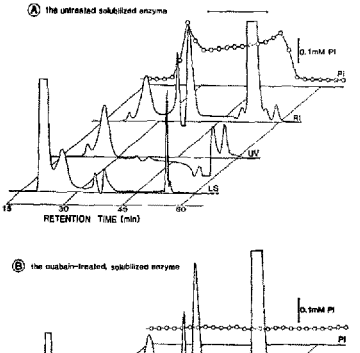

Fig. 6. A set of four elution patterns of solubilized Na^+/K^+-ATPase for substantially simultaneous measurements of M_r and ATPase activity. The same columns as those described in the legend to Fig. 1 were equilibrated with an elution buffer containing 0.30 mg/ml $C_{12}E_8$, 1.33 mM ATP, 60 μg/ml PS and otherwise the same constituents as those described in the legend to Fig. 4 at a flow rate of 0.50 ml/min and at 20 °C. The untreated solubilized enzyme (A) or the ouabain-treated, solubilized enzyme (B) prepared as indicated in Methods was diluted with 3 vol of the elution buffer depleted of ATP and PS. Immediately, an aliquot of 100 μl of the diluted enzyme containing 0.043 mg protein was chromatographed on the column. The eluate was monitored successively with a LALLS photometer (LS), a differential refractometer (RI) and a UV spectrophotometer (UV), and finally 1 ml aliquots of the eluate were collected to assay for P_i (○). Every protein component was eluted earlier than 30 min. The amount of P_i to be used for calculation of specific activity of the enzyme exhibited during passage through the column was determined at the positions indicated by a bar with arrows. The M_r values and the specific ATPase activities of the major protein component were estimated as indicated in Methods to be (A) 158000 ($d_n/dc_p = 0.301$ ml/g) and 2.03 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$, and (B) 224000 ($d_n/dc_p = 0.300$ ml/g) and 0 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$, respectively.

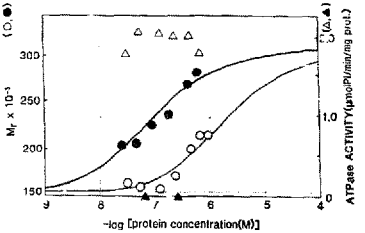

Fig. 7. Relationship between M_r and ATPase activity of solubilized Na^+/K^+-ATPase revealed by their substantially simultaneous measurement. Various amounts of the solubilized enzyme were chromatographed, and the sets of four elution patterns were obtained in the same way as described in the legend to Fig. 6. The M_r values (○) and the ATPase activities (△) of the untreated solubilized enzyme were estimated from each set of the elution patterns (except the open circle plotted in right-hand edge) and are plotted against the protein concentrations expressed in terms of molar protomer concentration. The concentrations refer to those at the peak of the protein curve where the outputs of LS, RI and UV were obtained to calculate M_r. The M_r values of the ouabain-treated, solubilized enzyme were measured in the same way as above, and are also plotted against the protein concentrations (●). The ATPase activities (▲) during the chromatography were measured in another experiment at two values of protein concentrations with the same elution buffer as described above except that the concentration of ATP was 4 mM instead of 1.33 mM. Assuming that the solubilized enzyme is in a self-association equilibrium of $2\ \alpha\beta \rightleftharpoons (\alpha\beta)_2$, regardless of whether the enzyme was treated with ouabain or not, and assuming that the M_r of the solubilized enzyme is given as the weight-averaged value of M_r of the protomer and the diprotomer, the K_a of the equilibrium reaction was obtained by computer fitting from the M_r-dependences on the protein concentrations. K_a values of $5.0 \cdot 10^8$ and $1.1 \cdot 10^7\ M^{-1}$ were obtained for the untreated (○) and ouabain-treated, solubilized enzyme (●), respectively, as shown by solid lines.

ingredients as above under the identical conditions with those above. Thus, the value of 0.301 ml/g was adopted as the specific refractive index increment of the solubilized enzyme turning over in the column.

In Fig. 7, M_r values as well as ATPase activities measured for the untreated and the ouabain-treated, solubilized enzyme are plotted against protein concentrations (for details, see the legend to the figure). The M_r values were dependent on the protein concentration, irrespective of whether the enzyme was treated with ouabain or not. As shown in Fig. 7, the M_r of the untreated solubilized enzyme decreased from 214000 to 158000 with a decrease in the protein concentration from 0.937 to 0.125 μM (expressed in terms of molar protomer concentration), and remained constant at protein concentrations of less than 0.125 μM. The protein concentrations refer to those at the peak of the protein curve. Evidently, the solubilized enzyme

existed as the $\alpha\beta$-protomer around the lower limit of the protein concentration adopted. On the other hand, the specific ATPase activities were approximately constant at a value of 1.94 ± 0.13 U/mg protein ($n = 6$) regardless of the changes in protein concentration. The activity was $64 \pm 4\%$ of that of the membrane-bound enzyme assayed in the absence of $C_{12}E_8$ and PS under otherwise the same conditions as those in the column. These results show that the solubilized enzyme can exhibit a specific ATPase activity almost equivalent to that of the enzyme in situ, no matter which oligomeric state (protomer and diprotomer) is assumed by the enzyme. Therefore, it is concluded that the $\alpha\beta$-protomer is the minimum functional unit for the ATPase activity.

Effect of ouabain treatment on the dissociation–association equilibrium between the diprotomer and the protomer

As shown by the solid circles in Fig. 7, the dependence of M_r on protein concentration obtained for the ouabain-treated, solubilized enzyme was much different from that obtained for the control solubilized enzyme (open circles). On the basis of the assumption of a dissociation–association equilibrium between the protomer and diprotomer, the K_a was estimated from the dependences of M_r on protein concentrations by computer fitting. The values of $5.0 \cdot 10^5$ and $1.1 \cdot 10^7$ M^{-1} were obtained for the solubilized enzyme turning over and the solubilized enzyme inhibited with ouabain, respectively, under the conditions investigated (see the solid lines in Fig. 7). Evidently, ouabain modified the enzyme so that the association between the protomers became 22-fold stronger.

Discussion

Factors determining K_a of the 2 protomers ⇌ diprotomer equilibrium

At 20°C, the solubilized enzyme was eluted as a single peak for which the M_r varied depending on the cation included in the elution buffer used. The M_r values of the protein component obtained with the elution buffers containing 0.1 M NaCl and 0.1 M KCl were 255000 and 300000, respectively. The M_r of the protein component obtained with the elution buffer containing 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$ was the same as that obtained with 0.1 M KCl alone. The result of computer simulation suggests that the difference of M_r of the protein component was brought about by a difference in K_a of the dissociation–association equilibrium. The enzyme takes two distinct conformations of E_1 or E_2 in the presence of Na^+ alone or K^+ alone, respectively, in the solubilized form [33,34] as well as in the membrane-bound form [35–38]. Since K^+ preferentially binds to the enzyme under the conditions where the same concentrations of Na^+ and K^+ coexist and the binding of K^+ is not affected by Mg^{2+} at a sufficiently high concentration of K^+ [39,40], the solubilized enzyme probably takes the E_2 conformation in the solution of 0.05 M KCl/0.05 M NaCl/4 mM $MgCl_2$. Thus, the above results can be interpreted if the two protomers in the E_2 conformation associate more strongly than in the E_1 conformation. That is, the E_2 form of the solubilized enzyme is likely to have higher value of the K_a than the E_1 form. The working hypothesis will have to be examined by determination of M_r of the solubilized enzyme under various conditions where the conformation of the E_1 or E_2 is expected to be dominant.

The K_a of the solubilized enzyme inhibited with ouabain was 22-fold greater than that of the enzyme turning over. According to the reaction scheme of Post and Albers [41–43], the enzyme conformation changes cyclically from E_1 to E_2, and then from E_2 to E_1 during each turnover. It has been shown that ouabain prevents the turnover by fixing the conformation in E_2 state phosphorylated by ATP (E_2-P) [44,45]. Karlish and Yates [36] and Suzuki et al. [46] have demonstrated by measurement of fluorescence intensity that the dominant conformational form of the membrane-bound enzyme was E_2 during turnover at a low concentration of ATP. On the other hand, the rate of transition from the E_2 form bound by K^+ (E_2-K) to the initial conformation of E_1 is significantly accelerated by a higher concentration of ATP [46–48]. Furthermore, Huang et al. [49] have shown that $C_{12}E_8$ mimics the effect of ATP. Therefore, it is not yet clear in the case of the enzyme solubilized with $C_{12}E_8$ which conformational state of E_1 or E_2 is dominant during turnover at the relatively high concentration of ATP (1.33mM) used in the present study. If the dominant conformation of the solubilized enzyme is E_1, the difference in the K_a between the untreated enzyme turning over and the ouabain-inhibited enzyme not turning over could be attributable also to the difference in conformational state of E_1 and E_2. However, since Hegyvary and Jørgensen [50] have shown a distinct conformational change induced by binding of ouabain to the enzyme, the possibility cannot be excluded that ouabain binding to the enzyme itself enhances the association between the protomers.

Minimum functional unit of the enzyme

It was concluded from the present substantially simultaneous measurement of ATPase activity and M_r that the $\alpha\beta$-protomer and the $(\alpha\beta)_2$-diprotomer exhibited comparable ATPase activity in the presence of exogenous PS at 20°C (Fig. 7). Craig, however, has insisted that only the $\alpha\beta$-protomer shows ATPase activity [14]. He fractionated the solubilized enzyme into $\alpha\beta$-protomer and its oligomer, $(\alpha\beta)_n$ ($n = 1$–5), using density gradient centrifugation to find that only the slowest sedimenting component showed ATPase activ-

ity. The component was assigned to the $\alpha\beta$-protomer by SDS-polyacrylamide gel electrophoresis after cross-linking with glutaraldehyde according to the method developed by Craig [13]. What is the cause for the inconsistency between Craig's result and ours? No attention was paid in his experiments to the indispensability of phospholipid for the solubilized enzyme, which has been pointed out in this paper as well as by Esmann [30]. If only the $\alpha\beta$-protomer sedimenting most slowly remained in the region where phospholipids were concentrated as the result of flotation, the inconsistency might be interpreted properly. It has been shown in the present study that the solubilized enzyme was in an equilibrium of dissociation–association between the protomer and the diprotomer. We wonder if the equilibrium is perturbed by the cross-linking reaction.

The method of active enzyme centrifugation [51] has been the only available means to determine the active oligomeric form of an enzyme. It generally permits one to estimate the sedimentation constant of the enzyme in its catalytically active form. In the investigation on a minimal active unit of sarcoplasmic reticulum Ca^{2+}-ATPase solubilized with $C_{12}E_8$, the diffusion constant as well as the sedimentation constant of the active particles were determined from the data of active enzyme centrifugation [52]. The molecular weight of the solubilized particle of Ca^{2+}-ATPase constituted mainly of $C_{12}E_8$, lipid and protein can be calculated from the two constants thus obtained by the Svedberg equation, provided the buoyant density factor of the particle is known. This factor has been determined from the following parameters: amounts of $C_{12}E_8$ and lipid bound to the protein, partial specific volumes of those constituents and solution density. Thus, the monomeric protein component with M_r of 104000 ± 2300 was concluded to be the minimal ATPase active unit of Ca^{2+}-ATPase. Knowing the buoyant density factor is also crucial for the M_r estimation for solubilized membrane protein by the sedimentation equilibrium technique [53].

In contrast to the active enzyme centrifugation as well as the sedimentation equilibrium technique, the HPGC/LALLS method allows one directly and simply to estimate M_r of solubilized membrane protein knowing only the absorption coefficient at 280 nm of the protein moiety itself of the solubilized protein. Furthermore, the novel coupling of 'active enzyme chromatography' with the HPGC/LALLS method as developed in the present study made it possible to estimate simply the M_r of the solubilized enzyme turning over at a rate comparable to that of the membrane-bound enzyme. The substantially simultaneous measurement of M_r and ATPase activity was accomplished by addition of exogenous PS to the elution buffer. It is likely to occur by an involvement of PS such that endogenous phospholipids of the solubilized enzyme are replaced by exogenous PS, and that all of the resultant molecular species are in 'dialysis' equilibrium with the elution buffer containing given ingredients during the passage through the HPGC column. The present procedure permitting substantially simultaneous measurement of M_r and enzymatic activity is strongly recommended as a powerful technique for studying structure–function relationships of enzymes consisting of loosely combined subunits. The technique is very efficient for such study of membrane enzyme solubilized by surfactant as exemplified in the present study.

Oligomeric state in situ

Oligomer structure of the enzyme in situ has been investigated by means of chemical cross-linking [54,55] and electron microscopy [56–58] with the use of the membrane-bound enzyme purified from mammalian kidney, and also by means of radiation inactivation with the use of human red cell [59–61]. It is concluded from these studies that the enzyme exists only in the form of $(\alpha\beta)_2$-diprotomer and/or the higher oligomer. In contrast, Karlish and Kempner [62], and Jensen and Nørby [63] have concluded by the radiation inactivation method that the minimum functional unit for the ATP hydrolysis of the enzyme isolated from pig kidney is $\alpha\beta$-protomer.

In the present study it has been shown that the solubilized enzyme was in an equilibrium of dissociation–association between the diprotomer and the protomer with a K_a of $5 \cdot 10^5$ M^{-1} at least. Thus, we can calculate how many populations of the enzyme protein exist as $(\alpha\beta)_2$-diprotomer in the membrane if the protein concentration of the enzyme there is evaluated. According to the electron micrographs of Na^+/K^+-ATPase in the plasma membrane of rat kidney, particles corresponding to an $\alpha\beta$-protomer appeared as a cylindrical protein with a height of 18 nm and a diameter of 4 nm at a density of about 19000 per μm^2 [56]. The distribution permits one to estimate the concentration of the protomer to be 0.26 g/ml (1.7 mM in terms of molar protomer concentration). The resultant concentration coincided approximately with that calculated from the computer-reconstituted image of the two-dimensional crystal of the enzyme [57], and that calculated from both an equiweight ratio of protein to lipid and their densities (1.3 and 1.0 g/cm^2, respectively). By assuming the K_a and the protein concentration described above, at least 97% on a weight basis of the enzyme can be expressed to exist as the diprotomer in the membrane. In the intact state, the enzyme is surrounded by lipid bilayer naturally lacking exogenous surfactant. The protomers in a pair are presumed to interact with much higher affinity in such a situation than in the environment provided with the surfactant and minor amount of added PS.

The present result that the $\alpha\beta$-protomer can hydrolyze ATP by itself is consistent with the results [64] of

our previous ligand-binding study obtained by the centrifugation method, which showed that the membrane-bound enzyme has binding sites for 1 mol each of ouabain and ATP, and 2 mol of K^+ or 3 mol of Na^+ per less than 160000 g of the enzyme protein. Taking these results and the conclusion described above into consideration it is likely that the enzyme is oligomerized into the diprotomer or the higher oligomer in the membrane, while each protomeric unit can hydrolyze ATP and bind Na^+ or K^+. The result obtained here shows that the ATPase activity of the solubilized enzyme is not affected by the degree of association. The result, however, does not necessarily mean that the protomer transports actively Na^+ and K^+ in the membrane. Vilsen et al. [65] have shown that the solubilized enzyme could occlude Rb^+ or Na^+ in the form of $\alpha\beta$-protomer with the same stoichiometry as the membrane-bound enzyme. Their result suggests that one unit of $\alpha\beta$-protomer has the capacity for transporting Na^+ and K^+ as well as hydrolyzing ATP.

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