ELSEVIER

Biochimica et Biophysica Acta 1240 (1995) 55–64

BBA Biochimica et Biophysica Acta

Quantification of the Na^+/K^+-pump in solubilized tissue by the ouabain binding method coupled with high-performance gel chromatography

Masaru Asami [a], Tadashi Sekihara [a], Tateo Hanaoka [a], Tomoyuki Goya [a], Hideo Matsui [b], Yutaro Hayashi [b,*]

[a] *Second Department of Surgery, Kyorin University School of Medicine, Mitaka, Tokyo 181, Japan*
[b] *First Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181, Japan*

Received 2 February 1995; revised 29 April 1995; accepted 24 May 1995

Abstract

Membrane-bound Na^+/K^+-ATPase purified from dog kidney outer medulla was solubilized with octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$) and incubated with [^{3}H]ouabain in the presence of NaCl, ATP and $MgCl_2$ for 10 min at 0°C. The resulting enzyme was separated, by high-performance gel chromatography executed at 0.2°C, mainly into its $(\alpha\beta)_2$-diprotomer and $\alpha\beta$-protomer, which both bound stoichiometrically to [^{3}H]ouabain. The amounts of ouabain that bound to the tissue itself and its microsomes could be estimated in the same way, as [^{3}H]ouabain was found to bind only to the diprotomer and protomer they possessed. The amounts of ouabain that bound to them in the solubilized state were at least 5-times higher than those that did so when they were non-solubilized, suggesting that the surfactant rendered the enzyme accessible to ouabain. When the solubilized tissue (138 mg ml^{-1} wet tissue) was reacted with ouabain in the presence of 0.1 M NaCl and 4.8 mM $MgCl_2$ for 10 min at 0°C, maximal ouabain binding was attained in the presence of 18.3 μM [^{3}H]ouabain, 1.2 mM ATP and 3 to 5 mg ml^{-1} $C_{12}E_8$, which was common to the outer medulla and human colon cancer cells. The present method enabled the pump number in protein and tissue samples in the range $7.2 \cdot 10^{-9}$ (purified pump) to $1.5 \cdot 10^{-12}$ (cancer tissue) mol/mg protein to be estimated within 2 h.

Keywords: ATPase, Na^+/K^+-; Sodium/potassium pump; Sodium ion; Ouabain; Solubilization; Octaethylene glycol *n*-dodecyl ether

1. Introduction

Na^+/K^+-transporting adenosine triphosphatase (Na^+/K^+-ATPase), also known as the Na^+-pump or Na^+/K^+-pump, is a typical integral membrane protein, which controls the intra- and extracellular ionic environment. Cardiotonic steroid inhibitors, such as ouabain and digoxin, bind specifically to the pump with quite high affinities [1–3] and many variants of the ouabain binding method have been devised to quantify exactly the pumps present in cells and tissues (see reviews [4,5] on this subject). The ouabain binding site is generally considered to be located on the extracellular portion of the catalytic subunit, designated the α-subunit, of the enzyme, which corresponds to the reverse side of the ATP binding and phosphorylation sites. Crude membrane fragment preparations of Na^+/K^+-ATPase are liable to form vesicles with right-side-out or in-side-out structures and/or to aggregate, which hide the ATP and/or ouabain binding sites. Surfactants such as SDS [6] and deoxycholate [7,8] degraded the vesicles and/or aggregates and activated several-fold the ATPase activity. Therefore, both the ouabain binding and the enzymatic assay methods without using surfactants might result in underestimation of the enzyme density in such crude preparations.

The enzyme assumes two distinct conformational states, designated E_1 and E_2, during each turnover [9]. The enzyme with bound Na^+ is phosphorylated by ATP to produce E_1-P and then changes its conformational state to E_2, forming E_2-P. Ouabain binds primarily to the E_2-P form and inhibits ATPase turnover by forming the stable ouabain · E_2-P complex [1,10–12]. A third phosphorylated intermediate has been shown to be present between E_1-P

Abbreviations: $C_{12}E_8$, octaethylene glycol *n*-dodecyl ether; HPGC, high-performance gel chromatography; E_1-P, ADP-sensitive phosphoenzyme intermediate; E_2-P, K^+-sensitive phosphoenzyme intermediate; UV, ultraviolet; DTE, dithioerythritol; Hepes, 2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

* Corresponding author. Fax: +81 422 41 6865.

SSDI 0005-2736(95)00146-8

and E_2-P and to be one of the active intermediates for ouabain binding [13–15]. Thus, the third intermediate would be expected to be involved in 'E_2-P' described in this study as the target intermediate of ouabain. Furthermore, E_2-P is formed in the reverse reaction from P_i in the presence of Mg^{2+} [16,17]. Therefore, the combinations of ligands most effective for promotion of ouabain binding are (1) ATP + Mg^{2+} + Na^+, and (2) P_i + Mg^{2+}. Vanadate facilitated ouabain binding in the same way as the P_i + Mg^{2+} system [18].

The purified membrane-bound form of Na^+/K^+-ATPase has been solubilized with nonionic surfactants, such as $C_{12}E_8$ and Lubrol, without substantial loss of enzymatic activity [19–23]. We have shown, by means of low-angle laser light-scattering photometry coupled with HPGC, that the enzyme solubilized with $C_{12}E_8$ is in a dissociation-association equilibrium between its α β-protomer ($M_r = 1.5 \cdot 10^5$) and $(\alpha\beta)_2$-diprotomer ($M_r = 3.0 \cdot 10^5$) at 20°C [23–25] and that the two components can be separated from each other by HPGC executed at 0°C. Furthermore, the results of active enzyme chromatography [26] suggested strongly that ouabain does not dissociate from the solubilized enzyme preincubated with ouabain during HPGC [24]. In this study, the enzyme and tissues solubilized with $C_{12}E_8$ were subjected to the [^{3}H]ouabain binding reaction and HPGC to estimate the Na^+/K^+-ATPase population present in the enzyme and tissue preparations. The optimal conditions for this estimation were sought and yielded a population range of 10^{-8} to 10^{-12} mol/mg protein.

2. Methods

2.1. Preparation of membrane-bound Na^+/K^+-ATPase and human colon cancer cells

The preparation of microsomes [8] and purification of the ATPase [24] from dog kidney outer medulla are described elsewhere. The purified enzyme was solubilized with $C_{12}E_8$ using the method described elsewhere [24] with minor modifications, which were as follows: the enzyme was solubilized with $C_{12}E_8$ in the presence of 0.1 M NaCl, unless stated otherwise, and centrifuged with a Beckman TL-100 ultracentrifuge at 460 000 × g for 5 min at 2°C. Human colon cancer cells, DLD-1 [27], ($1 \cdot 10^6$ cells per mouse) were transplanted subcutaneously into male BALB/cA Julnu mice (5 weeks old; Clea Japan). When the solid tumors reached 1 cm in diameter (within about 3 weeks) the tumor tissue was extracted from each mouse. On average, 150 mg tissue per mouse was isolated.

2.2. Standard method to estimate the amount of ouabain bound to tissue homogenates

The dog kidney and tumor tissues were excised, minced with scissors, 150 mg was suspended in a 300 μl aliquot

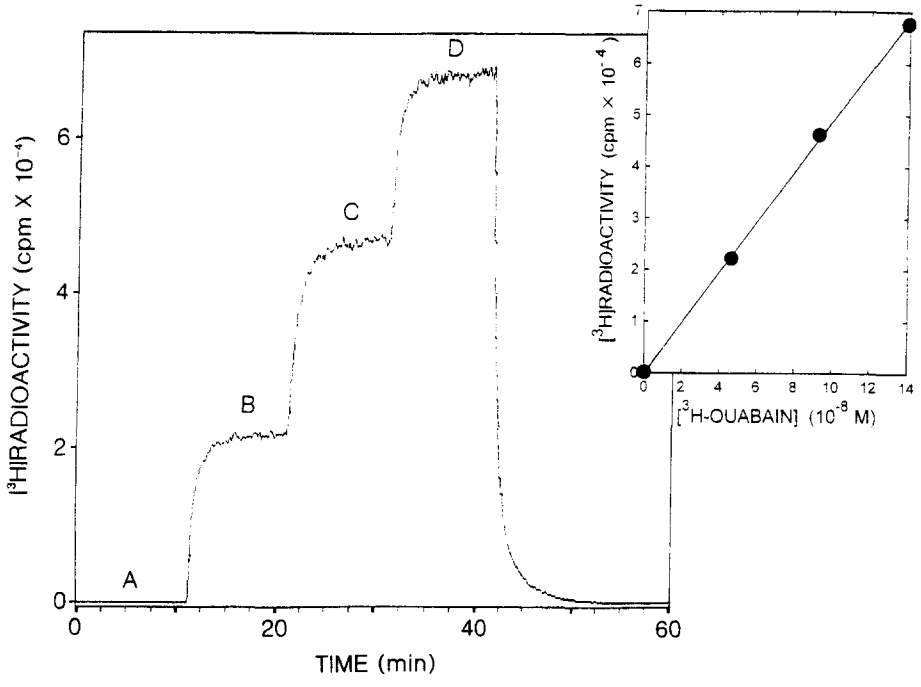


Fig. 1. Determination of the [^{3}H]ouabain concentration in the eluate from the HPGC column. [^{3}H]Ouabain solutions of various concentrations were prepared by mixing the diluted stock [^{3}H]ouabain solution with a scintillator cocktail (Scintisol 500) at a flow rate ratio of elution buffer (0.30 ml/min) to scintillator cocktail (1.80 ml/min) of 1 to 6 (v/v). The mixed solution was pumped to the radiomonitor at a flow rate of 2.10 ml/min and the radioactivity was monitored by scintillation counting. A, B, C and D denote the elution patterns of the mixed solutions containing 0, $4.64 \cdot 10^{-8}$, $9.28 \cdot 10^{-8}$ and $1.39 \cdot 10^{-7}$ M [^{3}H]ouabain, respectively. These concentrations correspond to those in the elution buffer before mixing with the cocktail. The inset panel shows the plot of the counts against the [^{3}H]ouabain concentration.

of a solution comprising 0.25 M sucrose/2 mM EDTA/1 mM DTE/31 mM imidazole/30 mM Hepes (pH 7.1) and homogenized two times with a Polytron (PT-35, Kinematica) at 20 000 rpm for 1 min at 0°C. The resulting tissue homogenate was incubated with $C_{12}E_8$ in a solution with the final composition of 138 mg ml^{-1} tissue homogenate/3 mg ml^{-1} $C_{12}E_8$/0.1 M NaCl/2 mM DTE/1 mM EDTA/10% (w/v) glycerol/13 mM imidazole/15 mM Hepes (pH 7.0) at 0°C and then sonicated with an ultrasonicator (Branson 2200) for 15 min at 0°C. After adding 1.2 mM ATP and 4.8 mM $MgCl_2$ (final concentrations) and standing for 5 min at 0°C, the tissue homogenate was incubated with 18.3 μM [^{3}H]ouabain for 10 min at 0°C and then centrifuged at 460 000 × g for 5 min at 2°C with a Beckman TL-100 ultracentrifuge. The supernatant referred to as the tissue homogenate solubilized protein, was collected and chromatographed, as described below.

2.3. HPGC to detect Na^+/K^+-pump bound to [^{3}H]ouabain

A TSKgel G3000SW_{XL} column (7.8 mm × 300 mm, Tosoh) equipped with a guard column (TSKgel guard column SW_{XL}, 6 mm × 40 mm) was equilibrated with elution buffer comprising 0.3 mg ml^{-1} $C_{12}E_8$/0.1 M NaCl/1 mM EDTA/10 mM imidazole/15 mM Hepes (pH 7.0) at a flow rate of 0.30 ml/min at 0.2°C. The columns were charged with 50 or 100 μl aliquots of the tissue homogenate solubilized protein obtained from the dog kidney or tumor tissues, respectively, and eluted under the equilibration conditions described above. The eluates were monitored successively with a UV spectrophotometer (TSK model UV-8010) and a radiomonitor (Ramona-90, Raytest). After emerging from the spectrophotometer at a flow rate of 0.30 ml/min, the eluate was mixed on-line with a scintillator cocktail (Scintisol 500, Dojindo Laboratories) at a flow rate of 1.80 ml/min and the ^{3}H radioactivity was monitored with the radiomonitor, which was equipped with a flow cell of the liquid cell type. The amounts of protein eluted and [^{3}H]ouabain bound were estimated from the peak area under the elution curve of the UV absorbance at 280 nm between 15 and 37.5 min and the radioactivity of [^{3}H]ouabain eluted between 17 and 25 min, respectively. The amount of [^{3}H]ouabain bound to the tissue protein was calculated by dividing the amount of [^{3}H]ouabain bound by that of protein eluted, and expressed as mol [^{3}H]ouabain/mg protein.

2.4. Determination of the concentration of [^{3}H]ouabain eluted from the column

A 1 ml aliquot of an ethanolic solution of [^{3}H]ouabain with a specific radioactivity of 758.5 to 777 GBq/mmol (NET-211, New England Nuclear) was evaporated to dryness in a stream of nitrogen. The vessel containing the [^{3}H]ouabain was placed in vacuo (200 to 300 mmHg) for 10 min to remove any traces of ethanol and then a 200 μl aliquot of H_2O was added to the vessel. The resulting aqueous solution of [^{3}H]ouabain was stored at 4°C until required for use as a stock solution. A 10 μl aliquot of this stock solution was diluted to 100 μl with H_2O and the concentration of ouabain in this diluted solution was determined from its absorbance at 220 nm using a molar absorption coefficient of 14 500 ($cm^{-1} \cdot l \cdot mol^{-1}$), which was based on the value of 14 800 at 218.5 nm [28]. [^{3}H]Ouabain solutions of various concentrations were prepared by adding less than 0.005 volumes of the diluted [^{3}H]ouabain solution to 1 volume of the elution buffer, mixed at a ratio of 1:6 (v/v) with the scintillator cocktail, which contained dioxane, pumped to the radiomonitor at a flow rate of 2.10 ml/min and the radioactivity was determined as described above. Each [^{3}H]ouabain count was plotted against the concentration of ouabain in the elution buffer before mixing with the scintillator cocktail. As shown in Fig. 1, the count increased in direct proportion to the concentration and the specific count for the stock [^{3}H]ouabain solution was calculated from the proportional constant of the linear relationship between them. The specific count for 1 μM ouabain was ranged from $4.39 \cdot 10^7$ to $4.81 \cdot 10^7$ cpm. The stock [^{3}H]ouabain solution was used within 60 days of mixing the dried aliquot with H_2O.

Table 1
Protein contents per g tissue (wet weight) of whole tissue homogenates and supernatant and precipitate fractions

Tissues	mg protein/g tissue (wet weight) (%)		
	whole	supernatant	precipitate
Dog kidney			
outer medulla	84.6 ± 8.7 (100)	54.2 ± 4.0 (64.1)	24.8 ± 2.5 (29.3)
inner medulla	42.1 ± 1.6 (100)	29.7 ± 1.0 (70.5)	11.7 ± 2.9 (27.8)
cortex	128.4 ± 2.7 (100)	88.0 ± 2.7 (68.5)	29.4 ± 0.1 (22.9)
Human colon cancer	79.1 ± 6.3 (100)	45.9 ± 3.4 (58.0)	26.8 ± 2.9 (33.9)

The tissues stated below were homogenized in medium containing $C_{12}E_8$ by the standard method described in Section 2, except that neither ATP nor [^{3}H]ouabain were included, and separated into supernatant and precipitate fractions by centrifugation. The whole tissue, supernatant and precipitate were resuspended in 1% (w/v) SDS solution and the protein concentrations were determined as described in Section 2. The data represent means of three values ± S.E. The percentages in parentheses represent the sample protein content relative to that of the whole tissue homogenate.

2.5. Estimation of the protein contents of solubilized tissue, microsome and purified Na^+/K^+-ATPase preparations

The tissues isolated from dog kidney outer medulla and transplanted colon cancer tumors were solubilized and each tissue homogenate solubilized protein was collected using the standard method described above. The solubilized protein was diluted 10-fold with H_2O, the protein concentration of this solution was measured using the standard bicinchoninic acid method assay procedure [29] and calibrated using bovine serum albumin as the standard. Various amounts of solubilized protein were subjected to HPGC and the absorbance of each eluate at 280 nm was monitored. The peak areas under the elution curves between the retention times of 15 and 37.5 min were plotted against the amount of protein with which the column was charged and the proportional constant of the linear relationship between these two parameters was obtained. In the experiments to estimate the amount of ouabain bound to the solubilized tissue, the peak area under each elution curve, between the retention times described above was converted into the content protein using this proportional constant. The protein content of solubilized microsomes was determined in the same way as that of solubilized tissue. The protein concentration of solubilized Na^+/K^+-ATPase eluted from the column was determined from its absorbance at 280 nm using the absorption coefficient of 1.22 $mg^{-1} \cdot ml \cdot cm^{-1}$ [23].

Each homogenized tissue (dog kidney outer medulla, inner medulla and cortex and human colon cancer tumor) was incubated with $C_{12}E_8$ (138 mg ml^{-1} tissue), sonicated and separated into supernatant and precipitate by centrifugation, as described above. A sample of each whole tissue homogenate before centrifugation and the supernatant and precipitate obtained were resuspended in 1% (w/v) SDS solution, their protein concentrations were measured by the bicinchoninic acid method and their protein contents per g tissue (wet weight) are shown in Table 1.

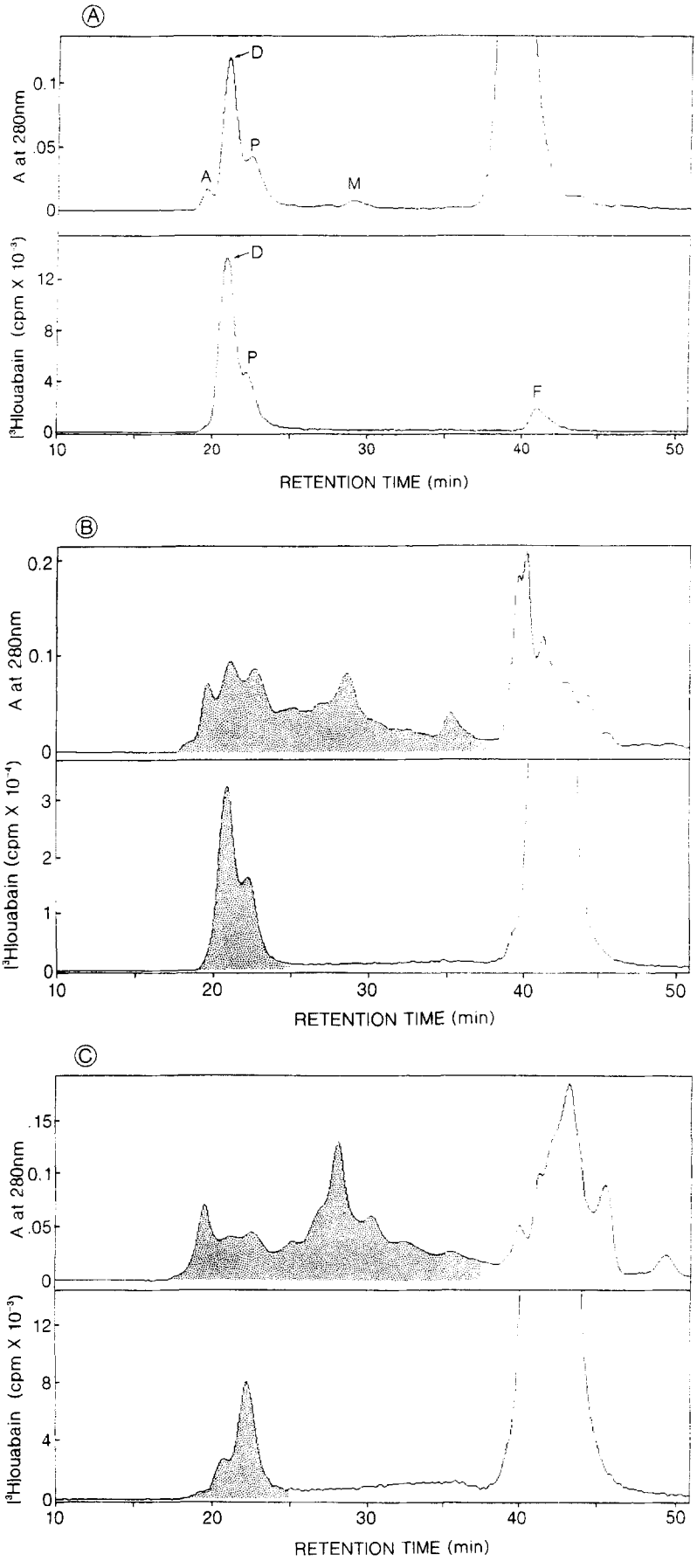
3. Results

3.1. Detection of [^{3}H]ouabain bound to purified Na^+/K^+-ATPase and dog kidney outer medullary microsomes by the HPGC

The solubilized, purified enzyme was incubated with 4.8 mM ATP and 4.8 mM $MgCl_2$ for 5 min at 0°C and then incubated with 9.4 μM [^{3}H]ouabain for 2 min at 0°C. The elution patterns of the resulting enzyme detected with a UV spectrophotometer and a radiomonitor are shown in Fig. 2A. The enzyme eluted as two major protein components, the diprotomer and protomer, and a minor one with a M_r higher than that of the diprotomer [24]. These protein components were designated D, P and A, respectively, and they accounted for 68, 25 and 6% (w/w) of the total protein eluted, respectively. The minor component A would be aggregates that contained partially denatured Na^+/K^+-ATPase and/or contaminants of the enzyme specimen. Peak M was attributed to mixed micelles of $C_{12}E_8$, phospholipids and other materials that absorbed UV light of the wavelength used [22]. The amounts of [^{3}H]ouabain bound to the diprotomer and protomer were 1.00 and 1.01 mol/150000 g protein, respectively. The ouabain binding had virtually reached saturation after incubation for 10 min, and the saturation level was maintained even after incubation for over 29 h, showing no substantial denaturation of the enzyme during that period.

The dog kidney outer medullary microsomes were solubilized and incubated with 23.4 μM [^{3}H]ouabain under conditions similar to those used for the purified enzyme described above. When the resulting solubilized microsomes were chromatographed, two peaks of protein-bound [^{3}H]ouabain were eluted at the same retention times as those of the diprotomer and protomer of the purified enzyme (Fig. 2B).

Fig. 2. Detection of [^{3}H]ouabain bound to purified Na^+/K^+-ATPase, dog kidney outer medullary microsomes and the outer medulla itself by HPGC. A TSKgel G3000SW$_{XL}$ column was equilibrated with an elution buffer comprising 0.3 mg ml^{-1} $C_{12}E_8$/0.1 M NaCl/1 mM EDTA/10 mM imidazole/15 mM Hepes (pH 7.0) at a flow rate of 0.30 ml/min at 0.2°C. (A) The solubilized enzyme (1.7 mg ml^{-1} protein) was incubated with 9.4 μM [^{3}H]ouabain in the presence of 4.8 mM ATP and 4.8 mM $MgCl_2$ and then a 100 μl aliquot of the reaction mixture was charged to the column. Two HPGC elution patterns of A at 280 nm and [^{3}H]radioactivity were obtained and are shown after correction for the displacement due to the different positions of the detector cells. The protein components designated D, P and A correspond to the $(\alpha\beta)_2$-diprotomer, $\alpha\beta$-protomer and a minor component with a M_r higher than that of the diprotomer, respectively. F and M denote free [^{3}H]ouabain and mixed micelles composed mainly of $C_{12}E_8$ and lipids, respectively. (B) The microsomes (2 mg ml^{-1} protein) were solubilized as described for the purified enzyme, incubated with 15.5 μM [^{3}H]ouabain in the presence of 1.2 mM ATP and 4.8 mM $MgCl_2$ for 10 min at 0°C and a 50 μl aliquot was chromatographed. The hatched areas under the elution curves of A at 280 nm and radioactivity of [^{3}H]ouabain represent the peak areas used to estimate the amounts of protein eluted and protein-bound [^{3}H]ouabain, respectively. (C) The outer medullary tissue was reacted with [^{3}H]ouabain by the standard method as described in Section 2 and a 50 μl aliquot of the solubilized protein was chromatographed. See above regarding the hatched areas.


Ⓐ
A at 280nm
0.1
.05
0
D
P
A
M
[³H]ouabain (cpm X 10⁻³)
12
8
4
0
D
P
F
10
20
30
40
50
RETENTION TIME (min)
Ⓑ
A at 280nm
0.2
0.1
0
[³H]ouabain (cpm X 10⁻⁴)
3
2
1
0
10
20
30
40
50
RETENTION TIME (min)
Ⓒ
A at 280nm
.15
0.1
.05
0
[³H]ouabain (cpm X 10⁻³)
12
8
4
0
10
20
30
40
50
RETENTION TIME (min)

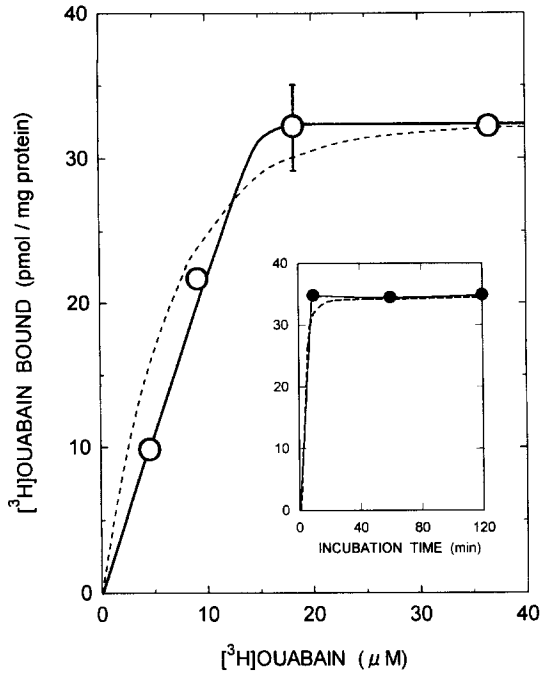


Fig. 3. Dependence of the amount of ouabain bound to the dog kidney outer medullary tissue homogenate on [^{3}H]ouabain concentration. The amount was measured by the standard method, except that incubation was carried out in the presence of 4.6, 9.2, 18.3 and 36.6 μM [^{3}H]ouabain for 10 min. The amount bound was also measured by the standard method, except that incubation was carried out for 10, 70 and 120 min in the presence of 18.3 μM [^{3}H]ouabain. The latter result is displayed in the inset. The open circle with error bars represents the mean of four values and the other circles denote one value each. The amount of ouabain bound at 10 min in the pre-steady state for ouabain·enzyme complex formation was calculated as a function of ouabain concentration by assuming that the association- and dissociation-rate constants and initial concentration of ouabain binding sites (a_0) were $0.25 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, negligible ($< 0.28 \cdot 10^{-5}$ s^{-1}) and $2.87 \cdot 10^{-7}$ M, respectively. The results were plotted and are shown by the broken line. Each of these values as a function of time in the presence of 18.3 μM [^{3}H]ouabain was calculated by assuming the same parameters as above, except $a_0 = 3.08 \cdot 10^{-7}$ M, and are displayed in the inset.

3.2. Dependence of ouabain binding to tissue homogenates on the concentrations of [^{3}H]ouabain, ATP, tissue homogenate and $C_{12}E_8$

The dog kidney outer medullary tissue homogenate was subjected to the [^{3}H]ouabain binding reaction under the standard conditions, except the $C_{12}E_8$ concentration was 6 mg ml^{-1}, centrifuged and the supernatant was collected. As shown in Table 1, the respective protein yields of the supernatant and precipitate were 64.1 and 29.3% of the whole homogenate protein. Two peaks of protein-bound [^{3}H]ouabain corresponding to the diprotomer and protomer of the purified enzyme were eluted by HPGC of the supernatant (Fig. 2C). The homogenate was incubated with various concentrations of [^{3}H]ouabain for 10 min and, as shown in Fig. 3, the [^{3}H]ouabain binding was judged to be saturated in the presence of 18.3 μM [^{3}H]ouabain, as it increased no further after longer incubation for up to 120 min (Fig. 3, inset). The dependence of ouabain binding on [^{3}H]ouabain concentration could be calculated in the same way as described by Erdmann and Schoner [35] by assuming that the ouabain-enzyme complex was in a pre-steady state at 10 min in the presence of less than 18.3 μM ouabain and that the association and dissociation rate constants were $0.25 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ and negligible, respectively (Fig. 3, broken lines). [^{3}H]Ouabain binding was saturated in the presence of 1.2 mM ATP and 18.3 μM [^{3}H]ouabain and the concentration of ATP for half maximal ouabain binding was 0.41 mM. When the tissue concentrations were varied from 9.52 to 345 mg ml^{-1} in the presence of a fixed concentration of $C_{12}E_8$ (6 mg ml^{-1}), the amounts of ouabain bound ranged from 0.39 to 32.3 pmol/mg protein and maximal binding was observed with 138 mg ml^{-1} tissue homogenate, when the weight ratio of tissue to $C_{12}E_8$ was 23.

Homogenized dog kidney outer medulla and transplanted human colon cancer tissue were treated with various concentrations of $C_{12}E_8$ and then bound by [^{3}H]ouabain, under the standard conditions. As shown in Fig. 4, maximal ouabain binding to both tissues was observed at 3 to 6 mg ml^{-1} $C_{12}E_8$ and the amounts of

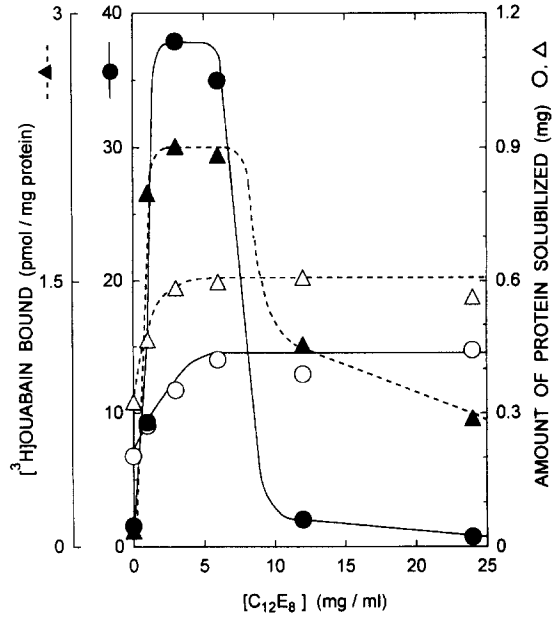


Fig. 4. Dependence of the amounts of protein solubilized and ouabain bound to two types of tissue on $C_{12}E_8$ concentration. Dog kidney outer medulla (●, ○) and transplantable human colon cancer (▲, △) tissues were isolated and homogenized, as described in Section 2. Both tissue homogenates were incubated with various concentrations of $C_{12}E_8$ under the conditions described in Section 2, followed by 18.3 μM [^{3}H]ouabain in the presence of 1.2 mM ATP and 4.8 mM $MgCl_2$, then ultracentrifuged and the supernatants were collected. Aliquots (50 μl, dog kidney outer medulla; 100 μl, transplanted human colon cancer) of the supernatants were subjected to HPGC, the amounts of protein eluted from the outer medulla (○) and colon cancer (△) and [^{3}H]ouabain bound to them (●) and (▲), respectively, were estimated, as described in Section 2, and plotted against the concentration of $C_{12}E_8$ used.

protein solubilized were almost saturated at about 5 mg ml^{-1} $C_{12}E_8$. The results show that the Na^+/K^+-pumps involved in these two types of tissue behaved in the same manner with respect to the dependence of solubilization and stability on the $C_{12}E_8$ concentration. The optimum weight ratios of tissue to $C_{12}E_8$ for the maximum ouabain binding and the saturated level of protein solubilized, which were common to the two types of tissue, ranged between 46 and 23. The ratio was consistent with that (23) obtained above when concentration of tissue was varied at the fixed concentration of $C_{12}E_8$ (6 mg ml^{-1}) with dog kidney outer medulla.

3.3. Effect of solubilization with $C_{12}E_8$ on the amount of [^{3}H]ouabain bound to tissue homogenate

The dog kidney outer medullary tissue homogenate was subjected to the [^{3}H]ouabain binding reaction in two different states, solubilized with $C_{12}E_8$ and non-solubilized. In the solubilized state, the amount of [^{3}H]ouabain bound was measured using the standard method. The amounts of solubilized protein in and [^{3}H]ouabain bound to the tissue homogenate (21 mg tissue) were 1.14 ± 0.04 ($n = 3$) mg and 20.6 ± 0.4 ($n = 3$) pmol/mg protein, respectively. The non-solubilized homogenate was incubated first with [^{3}H]ouabain in the absence of $C_{12}E_8$ under the same conditions as those for the solubilized state, ultracentrifuged and the resulting supernatant and precipitate were collected separately. The supernatant was subjected to HPGC to estimate the amounts of protein and [^{3}H]ouabain bound, which, for 21 mg tissue, were 0.996 mg and negligible (maximum of 0.0263 pmol/mg protein), respectively. The precipitate was solubilized with the solution containing 3 mg ml^{-1} $C_{12}E_8$ used to solubilize the tissues, ultracentrifuged, as described above, and this supernatant was chromatographed. The amounts of protein eluted and [^{3}H]ouabain bound were 0.208 ± 0.008 ($n = 4$) mg and 16.4 ± 3.0 ($n = 4$) pmol/mg protein for 21 mg tissue, respectively. With the non-solubilized tissue, any soluble proteins present before adding $C_{12}E_8$ had been excluded, as they remained in the supernatant from the first ultracentrifugation, and were not taken into account when the amount of protein was estimated chromatographically. Therefore, the soluble proteins, the amount of ouabain bound to which and capacity for ouabain binding (0.996 mg and 0.0263 pmol/mg protein, respectively) were taken into consideration and the final amount of ouabain bound to the tissue homogenate in the non-solubilized state was calculated to be 2.94 ± 0.49 pmol/mg protein. Therefore, the amount of [^{3}H]ouabain bound to the tissue in the solubilized state was 7.0-times higher than that in non-solubilized state.

Ouabain binding to microsomes prepared from dog kidney outer medullary tissue in two different states, solubilized with $C_{12}E_8$ and non-solubilized, was also compared. The amounts of ouabain bound to solubilized and non-solubilized microsomes were 230 ± 12 and 46.3 ± 6.4 ($n = 3$) pmol/mg protein, respectively, after incubation for over 10 min. The amount of ouabain bound to the solubilized microsomes was 5-times higher than that to the non-solubilized ones.

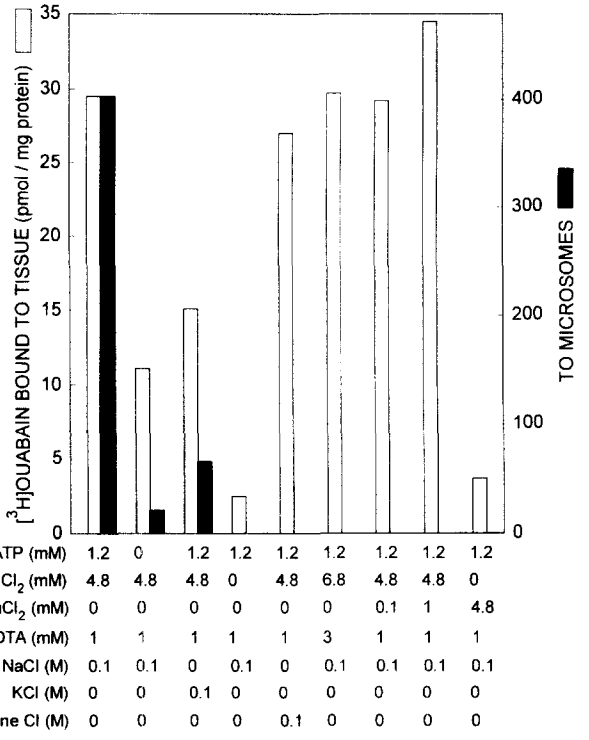


Fig. 5. Effects of ligands on ouabain binding to the dog kidney outer medullary tissue homogenate and the microsomes prepared from this tissue. The amount of [^{3}H]ouabain bound to the tissue homogenate (empty column) was measured by the standard method described in Section 2 and the result is displayed on the far left side of the figure. The ligands indicated in the figure were replaced, added or omitted from the reaction mixture used for the standard method and the amounts of [^{3}H]ouabain bound to the tissue were measured, as described in Section 2. The microsomes were solubilized with $C_{12}E_8$ in the presence of 0.1 M NaCl or KCl under the conditions described in Section 2 for the purified enzyme, then incubated with 23.4 μM [^{3}H]ouabain under the conditions indicated in the figure and the amounts bound (solid column) to the protein were evaluated, as described in Section 2.

3.4. Effects of ligands on ouabain binding to the tissue homogenate and its microsomes

The standard method was used to estimate the amount of ouabain bound to the dog kidney outer medullary tissue: it was 29.5 pmol/mg protein. As shown in Fig. 5, when $MgCl_2$ was excluded from the standard system, ouabain binding decreased to 8.4% of that measured by the standard method and substitution of $CaCl_2$ for $MgCl_2$ reduced it to a similar extent. Replacement of NaCl by KCl decreased ouabain binding to 51.2% of that measured by the standard method, whereas when NaCl was replaced by choline chloride or when 1 mM $CaCl_2$ was added to the

Table 2
The amounts of ouabain bound to various specimens

Specimens		[^{3}H]Ouabain bound	
		mol/mg protein	mol/g tissue (wet weight)
Purified enzyme	diprotomer	$(7.21 \pm 0.37) \cdot 10^{-9}$ ($n = 3$)	
	protomer	$(7.11 \pm 0.29) \cdot 10^{-9}$ ($n = 3$)	
Microsomes	outer medulla	$(1.91–4.03) \cdot 10^{-10}$	
	colon cancer	$(1.66 \pm 0.16) \cdot 10^{-11}$ ($n = 5$)	
Tissues	outer medulla	$(2.03–3.44) \cdot 10^{-11}$	$(1.10–1.86) \cdot 10^{-9}$
	outer medulla [a]	$(1.66 \pm 0.13) \cdot 10^{-11}$	$(0.875 \pm 0.068) \cdot 10^{-9}$ ($n = 4$)
	inner medulla [a]	$3.74 \cdot 10^{-12}$	$1.11 \cdot 10^{-10}$
	cortex [a]	$3.58 \cdot 10^{-12}$	$3.15 \cdot 10^{-10}$
	colon cancer	$(1.54–3.36) \cdot 10^{-12}$	$(0.835–1.54) \cdot 10^{-10}$

The amounts of [^{3}H]ouabain bound to the oligomers of the purified enzyme and microsomes prepared from the tissues stated below were measured, as described in the legend to Fig. 2A and B, respectively. The amounts bound to dog kidney outer and inner medullary, dog kidney cortical and human colon cancer tissues were measured by the standard method, as described in Section 2. When one batch of specimen was used as a sample, the values are presented as mean values ± S.E. and the number of measurements (n). Otherwise, the lowest, highest or a single value is shown. The values expressed as mol/mg protein were recalculated as mol/g tissue (wet weight) using the tissue protein contents presented in Table 1.

[a] These tissues were excised from one kidney, homogenized, as described in Section 2, and frozen separately. After 2 days they were thawed and used.

medium for ATPase phosphorylation, the ouabain binding hardly changed.

The effects of excluding and replacing ligands on ouabain binding to solubilized microsomes prepared from the tissue described above were also investigated. Exclusion of ATP reduced the amount of ouabain bound to 5.2% of that (403 pmol/mg protein) obtained under the standard conditions (Fig. 5) and replacement of NaCl by KCl reduced it to 16% of that before replacement (Fig. 5).

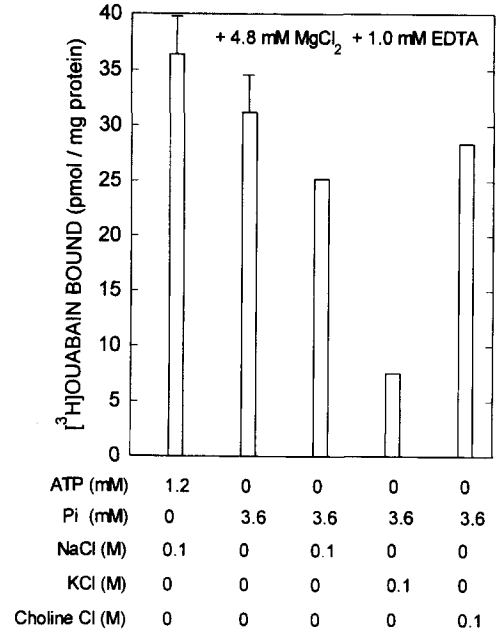


Fig. 6. Comparison of the standard method using ATP, Na^+ and Mg^{2+} with the method using Mg^{2+} and P_i in terms of the amount of ouabain bound to dog kidney outer medullary tissue homogenate. The amount of ouabain bound to the tissue homogenate was measured using the standard method. The result is shown on the far left side of the figure. In the system using Mg^{2+} and P_i, the same tissue homogenate as that used for the standard system was ultrasonicated and incubated with 3 mg ml^{-1} $C_{12}E_8$ in the absence of monovalent cation or in the presence of 0.1 M NaCl, 0.1 M KCl or 0.1 M choline chloride under otherwise the same conditions as those used for the standard method. After adding 3.6 mM P_i and 4.8 mM $MgCl_2$ (final concentrations) and allowing to stand for 5 min at 0°C, the tissue homogenate was incubated with 18.3 μM [^{3}H]ouabain and the amount of ouabain bound was measured as described for the standard method in Section 2. Bars indicate the standard deviation from three experiments and other data are for single values each.

3.5. Comparison of ouabain binding using the standard system ($ATP + Na^+ + Mg^{2+}$) and the $P_i + Mg^{2+}$ system

The outer medullary tissue homogenate was bound by [^{3}H]ouabain in individual reaction mixtures containing no monovalent cation, 0.1 M NaCl, 0.1 M KCl or 0.1 M choline chloride and in common 3.6 mM P_i and 4.8 mM $MgCl_2$ under otherwise the same conditions as those for the standard method in the $ATP + Na^+ + Mg^{2+}$ system. As shown in Fig. 6, the maximum amount of ouabain bound was observed in the reaction mixture containing no monovalent cation among the other mixtures with the $P_i + Mg^{2+}$ system, but it was a little less than (85.7% of) that with the standard system of $ATP + Na^+ + Mg^{2+}$.

3.6. Summary of the amounts of ouabain bound to various specimens

The amounts of [^{3}H]ouabain bound to the diprotomer and protomer of purified Na^+/K^+-ATPase, the microsomes obtained from dog kidney and transplanted human colon cancer tissues and the tissues themselves are summarized in Table 2. The amounts of ouabain bound to the tissues expressed as mol/mg protein were converted to values expressed as mol/g tissue (wet weight) using the solubilized protein contents of the tissues presented in Table 1.

4. Discussion

4.1. Ouabain binding to purified Na^+/K^+-ATPase

Using the present method, the enzyme solubilized with $C_{12}E_8$ was found to bind 7.2 nmol ouabain/mg protein, irrespective of whether it was in its $(\alpha\beta)_2$-diprotomer or $\alpha\beta$-protomer oligomeric form (Table 2). This value was consistent with the amount of ATP (7.5 nmol/mg protein) bound to the enzyme purified from pig kidney and solubilized with $C_{12}E_8$ determined, using the rate-dialysis technique, by Jensen and Ottolenghi [30]. Jørgensen and Andersen [31] reported that the enzyme solubilized with $C_{12}E_8$ was inclined to aggregate from the protomer to the diprotomer and higher oligomers by thermal inactivation. In this study, the minor component (A) of the aggregate did not appear to bind to [^{3}H]ouabain, which agrees with their results, but the diprotomer did, in disagreement with them (Fig. 2A), although the solubilized enzyme was not exposed to a high temperature, but was kept at 0°C throughout the present study.

We reported our preliminary finding that ouabain bound to the enzyme in its diprotomer oligomeric form phosphorylated with ATP [32]. The present results suggest that the diprotomer bound to [^{3}H]ouabain partially dissociated into the protomer without releasing ouabain during its passage through the HPGC column. We measured the amount of ouabain bound to the purified enzyme in the membrane-bound form by centrifugation methods [33] and obtained a value of 6.3 nmol/mg protein with a specific activity of 45 U/mg protein (units defined as μmol P_i per min) at 37°C [34]. This value was almost equivalent to that obtained in the present study. Therefore, we concluded that the solubilized enzyme retained its ouabain binding capacity, even when it passed through the HPGC column in the presence of $C_{12}E_8$ at 0.2°C, and its binding affinity was so high that ouabain did not dissociate from the enzyme during passage through the HPGC column.

4.2. Affinity of ouabain for the enzyme in the tissue homogenate solubilized protein

The dissociation constant (K_d) for ouabain · enzyme $\rightleftharpoons$ ouabain + enzyme in microsomal fractions prepared from mammalian organs has been determined to be 1 to 5 nM using the reaction systems of ATP + Na^+ + Mg^{2+} and P_i + Mg^{2+} at 37°C [35–37]. The K_d at 0°C is about one third of that at 37°C [36]. In the present study, the concentration of ouabain necessary for half- and full-maximal binding to dog kidney outer medullary tissue homogenate was 7.3 and 18.3 μM, respectively, in the ATP + Na^+ + Mg^{2+} system at 0°C (Fig. 3). However, when the tissue was reacted with ouabain for 10 min under the present conditions, the reaction had not reached equilibrium in the presence of less than 18.3 μM [^{3}H]ouabain, but was in a pre-steady state of ouabain binding. Therefore, the concentration (7.3 μM) for half-maximal binding does not equate to K_d. As shown in Fig. 3 by broken lines, the dependence of the amount of ouabain bound on the concentration of ouabain or reaction time could be calculated by assuming that the association rate constant was $0.25 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, which is one tenth of the reference value for ouabain binding to erythrocytes at 0°C[36]. We assumed the dissociation rate constant was $0.28 \cdot 10^{-5}$ s^{-1} [36] and calculated that the release of bound [^{3}H]ouabain was suppressed to less than 1% of its saturation level after passage through the HPGC column. Therefore, under the conditions adopted in this study, the maximum K_d was presumed to be $1.1 \cdot 10^{-8}$ M.

4.3. Effect of $C_{12}E_8$-solubilization on ouabain binding to microsomes and tissue homogenates

The amounts of ouabain binding to the microsomes and tissue homogenate estimated under solubilized conditions were at least 5-times higher than those under non-solubilized conditions, respectively. The differences in the amount of ouabain binding between the two conditions might have been attributable to an increase in accessibility of the enzyme, to the ligands necessary for formation of phosphorylation intermediate such as Mg^{2+}, Na^+ and ATP, as well as to ouabain which was induced by solubilization with $C_{12}E_8$. Therefore, the possibility that the absolute pump number may have been underestimated by the estimation of ouabain-binding without use of any surfactant cannot be ruled out. Likewise, in the present study, it was not possible to rule out that ouabain, once bound without any addition of $C_{12}E_8$, became dissociated upon solubilization with $C_{12}E_8$. Since no trials without addition of surfactant could be performed using the present method, this possibility could not be investigated. Furthermore, 23 to 34% of whole tissue homogenate protein could not be solubilized with $C_{12}E_8$, showing that it was impossible to estimate the amount of ouabain bound to the precipitate fraction.

The ouabain-sensitive ATPase activity of the microsomes prepared from the dog kidney outer medulla was 0.57 to 2.5 U/mg protein at 37°C and it was activated to 3.3 to 4.2 U/mg protein by treatment with the surfactant deoxycholate (see Fig. 1 of [8]). The activities correspond to 3.5 to $5.1 \cdot 10^{-10}$ mol site of ouabain binding/mg protein, as the turnover number of the enzyme would be 8300 to 9300 min^{-1} at 37°C [38]. This number is consistent with the amount of ouabain bound (1.91 to $4.03 \cdot 10^{-10}$ mol/mg protein) shown in Table 2, suggesting that the values estimated using the present method are reasonable.

4.4. Dependence of ouabain binding on weight ratio of tissue to $C_{12}E_8$ and added ligands

Maximal ouabain binding to solubilized tissues was observed at the weight ratio of tissue to $C_{12}E_8$ of 46 to 23

(Fig. 4). The lower binding at ratios higher than 46 and lower than 23 was attributed to solubilization of other proteins in preference to the Na^+/K^+-pump and to denaturation of the pump due to excess $C_{12}E_8$, respectively. The optimum weight ratio of tissue to $C_{12}E_8$ was common to the two quite different types of tissue, dog kidney and colon cancer, and, therefore, this procedure would appear be applicable to other animal tissues. The optimal ratio expressed as the weight ratio of tissue to $C_{12}E_8$ corresponds to 0.45 to 0.23 expressed as the weight ratio of membrane protein to $C_{12}E_8$, as 21 mg outer medullary tissue contained 0.208 mg protein, which was insoluble without, but soluble with, $C_{12}E_8$ (see Section 3.3). This optimal value was equivalent to the weight ratio (0.33) of the protein to $C_{12}E_8$ with which the purified membrane-bound enzyme could be solubilized without substantial loss of the ATPase activity [22,39].

Replacement of 0.1 M Na^+ by 0.1 M K^+ reduced the amount of ouabain bound to the solubilized microsomes of dog kidney outer medulla to 17% of that without replacement in the presence of both ATP and Mg^{2+} (Fig. 5). However, when the outer medullary tissue itself was used, the K^+ for Na^+ replacement reduced the ouabain binding to 51% (Fig. 5). The lesser degree of Na^+-dependency of ouabain binding to the tissue than to the microsomes may be attributable to endogenous Na^+ in the tissue. NaCl could be replaced almost completely by choline chloride in ouabain binding to the outer medullary tissue in the both systems of $Na^+ + ATP + Mg^{2+}$ (Fig. 5) and $Mg^{2+} + P_i$ (Fig. 6). This result was consistent with the finding that choline had sodium-like effects on the time course of ATPase activity inactivation by tryptic digestion [40]. Ouabain binding to the tissue in the absence of Mg^{2+} was reduced to 8.4% of that in the presence of 4.8 mM Mg^{2+} and both ATP and Na^+ (Fig. 5). The amount of endogenous Mg^{2+} in the tissue may not have been high enough and the enzyme may not have turned over and/or not formed E_2-P in the absence of exogenous $MgCl_2$. Therefore, the presence of Mg^{2+} was found to be essential for ouabain binding in the solubilized system.

Acknowledgements

We are grateful to Mr. Takayuki Kobayashi for his cooperation at the outset of this study and to Misses Masumi Ishihira and Nobuko Shinji for their technical assistance with the preparation of the purified enzyme.

References

[1] Matsui, H. and Schwartz, A. (1968) Biochim. Biophys. Acta 151, 655–663.
[2] Tobin, T. and Sen, A.K. (1970) Biochim. Biophys. Acta 198, 120–131.
[3] Hootman, S.R. and Ernst, S.A. (1988) Methods Enzymol. 156, 213–229.
[4] Hansen, O. (1984) Pharmacol. Rev. 36, 143–163.
[5] Hansen, O. and Clausen, T. (1984) Am. J. Physiol. 254, C1–C7.
[6] Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
[7] Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366–380.
[8] Hayashi, Y., Kimimura, M., Homareda, H. and Matsui, H. (1977) Biochim. Biophys. Acta 482, 185–196.
[9] Glynn, I.M. (1985) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.), Vol. 3, pp. 35–114, Plenum Press, New York.
[10] Charnock, J.S. and Post, R.L. (1963) Nature 199, 910–911.
[11] Sen, A.K., Tobin, T. and Post, R.L. (1969) J. Biol. Chem. 244, 6596–6604.
[12] Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s–326s.
[13] Nørby, J.G., Klodos, I. and Christiansen, N.O. (1983) J. Gen. Physiol. 82, 725–759.
[14] Lee, J.A. and Fortes, P.A.G. (1985) in The Sodium Pump (Glynn, I.M. and Ellory, J.C., eds.), pp. 277–282, The Company of Biologists, Cambridge.
[15] Yoda, S. and Yoda, A. (1987) J. Biol. Chem. 262, 103–109.
[16] Post, R.L., Toda, G. and Rogers, F.N. (1975) J. Biol. Chem. 250, 691–701.
[17] Schuurmans Steckhoven, F.M.A.H., Swarts, H.P.G., De pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 100–111.
[18] Hansen, O. (1979) Biochim. Biophys. Acta 568, 265–269.
[19] Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817–821.
[20] Esmann, M., Christiansen, C., Karlsson, K.A., Hansson, G.C. and Skou, J.C. (1980) Biochim. Biophys. Acta 603, 1–12.
[21] Brotherus, J.R., Jacobsen, L. and Jørgensen, P.L. (1983) Biochim. Biophys. Acta 731, 290–303.
[22] Hayashi, Y., Takagi, T., Maezawa, S. and Matsui, H. (1983) Biochim. Biophys. Acta 748, 153–167.
[23] Hayashi, Y., Matsui, H. and Takagi, T. (1988) Methods Enzymol. 156, 514–528.
[24] Hayashi, Y. Mimura, K., Matsui, H. and Takagi, T. (1989) Biochim. Biophys. Acta 983, 217–229.
[25] Mimura, K., Matsui, H., Takagi, T. and Hayashi, Y. (1993) Biochim. Biophys. Acta 1145, 63–74.
[26] Jones, M.N., Ogilvie, J.W. and Ackers, G.K. (1976) Biophys. Chem. 5, 339–350.
[27] Tibbetts, L.M., Chu, M.Y., Hager, J.C., Dexter, D.L. and Calabresi, P. (1977) Cancer 40, 2651–2659.
[28] Jones, J.B. and Middleton, H.W. (1970) Can. J. Chem. 48, 3819–3826.
[29] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[30] Jensen, J. and Ottolenghi, P. (1983) Biochim. Biophys. Acta 731, 282–289.
[31] Jørgensen, P.L. and Andersen, J.P. (1986) Biochemistry 25, 2889–2897.
[32] Hayashi, Y., Kobayashi, T., Nakajima, T. and Matsui, H. (1994) in The Sodium Pump: Structure Mechanism, Hormonal Control and its Role in Desease (Bamberg, E. and Schoner, W., eds.), pp. 453–456, Steinkopff, Darmstadt.
[33] Matsui, H. and Homareda, H. (1988) Methods Enzymol. 156, 229–236.
[34] Matsui, H., Hayashi, Y., Homareda, H. and Taguchi, M. (1983) Curr. Top. Membr. Transp. 19, 145–148.
[35] Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 307, 386–398.
[36] Erdmann, E. and Hasse, W. (1975) J. Physiol. 251, 671–682.
[37] Wallick, E.T. and Schwartz, A. (1988) Methods Enzymol. 156, 201–213.
[38] Esmann, M. (1988) Methods Enzymol. 156, 105–119.
[39] Esmann, M. (1988) Methods Enzymol. 156, 72–79.
[40] Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415.