Biochimica et Biophysica Acta 818 (1985) 267–270
Elsevier

BBA 70235

BBA Report

Stability of [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase solubilized with $C_{12}E_8$

Mutsumi Inaba and Yoshimitsu Maede *

Department of Veterinary Internal Medicine, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060 (Japan)

(Received March 4th, 1985)
(Revised manuscript received June 4th, 1985)

Key words: ($Na^+ + K^+$)-ATPase; Ouabain binding; Enzyme solubilization; (Dog kidney, Dog erythrocyte)

The ($Na^+ + K^+$)-ATPase from dog kidney and partially purified membranes from HK dog erythrocytes were labeled with [^{3}H]ouabain, solubilized with $C_{12}E_8$ and analyzed by HPLC through a TSK-GEL G3000SW column in the presence of $C_{12}E_8$, Mg^{2+}, HPO_4^{2-} and glycerol at 20–23°C. The peaks of [^{3}H]ouabain bound to the enzyme from dog kidney and HK dog erythrocyte membranes corresponded to each other with apparent molecular weights of 470000–490000. In addition, these bindings of [^{3}H]ouabain to the ($Na^+ + K^+$)-ATPase were observed to be stable at 20–23°C for at least 18 h after the solubilization.

Molecular and biochemical studies of ($Na^+ + K^+$)-ATPase have revealed many interesting characteristics of this enzyme (for review, see Ref. 1). In most previous studies, ($Na^+ + K^+$)-ATPase preparations were derived from enzyme rich organs such as mammalian kidney [2–4], shark rectal gland [5–7], brain [8], and brine shrimp embryos [9,10]. However, for determination of the comparative molecular biogenesis and for structural studies of ($Na^+ + K^+$)-ATPase, it seems to be necessary to identify and purify the enzyme from various tissues and cells in which it might be present in small amounts or with a low level of activity.

The present work is part of an attempt at identification and purification of the ($Na^+ + K^+$)-ATPase in such materials. Although, for the purpose of such studies, ouabain seems to be a useful marker of the enzyme, the unstable ouabain-enzyme interactions under analysis conditions requires covallent labeling with affinity analogues such as 2-nitro-5-azidobenzoyl ouabain [11]. In recent years, many investigators have employed a nonionic detergent, $C_{12}E_8$, to preparare soluble, active ($Na^+ + K^+$)-ATPase, not only for the determination of molecular weight and subunit structure [4–6,12,13], but for the purification of ($Na^+ + K^+$)-ATPase [14,15]. This study demonstrates that the binding of [^{3}H]ouabain to the partially purified ($Na^+ + K^+$)-ATPase (from HK dog erythrocytes [16–18] and dog kidney) is stable during solubilization with $C_{12}E_8$ and analysis by HPLC through a TSK-GEL G3000SW column in the presence of $C_{12}E_8$ at room temperature. These results suggest that [^{3}H]ouabain can be used as a specific indicator in the study of the molecular structure, expression, and content of the ($Na^+ + K^+$)-ATPase in various tissues and cells.

* To whom correspondence should be addressed.

Abbreviations: $C_{12}E_8$, octaethyleneglycol mono *n*-dodecyl ether; HPLC, high-performance liquid chromatography; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate; HK and LK, high potassium and low potassium, respectively. HK and LK dog erythrocytes are characterized with their intracellular concentrations of Na^+ and K^+ and the ($Na^+ + K^+$)-ATPase activity. For detailed information, see our previous studies [16–18].

TABLE I

SDS-TREATMENT OF ERYTHROCYTE MEMBRANES FROM HK AND LK DOGS

The $(Ba^+ + K^+)$-ATPase from dog kidney was prepared by the standard method of Jørgensen [22]. Dog erythrocyte membranes were prepared as before [18] using a hypotonic solution consisting of 5 mM Tris-HCl (pH 7.6), 1 mM Na_2EDTA and 0.2 mM phenylmethylsulfonyl fluoride, and further concentration of the $(Na^+ + K^+)$-ATPase activity was carried out by treatment with SDS in the same manner as described previously [22]. The maximal activation of the $(Na^+ + K^+)$-ATPase in HK dog erythrocyte membranes was obtained when the membranes were treated with SDS at a concentration ratio of SDS/protein = 0.4 (0.56 mg SDS/1.4 mg protein per ml); then we employed this ratio to prepare the samples for [^{3}H]ouabain binding experiments. In regard to protein composition in these preparations, no difference was observed between HK and LK dog red cell proteins, both of which were shown to contain bands 3, 4.2 (nomenclature of Steck [23]) and other low molecular weight proteins by analysis with SDS-polyacrylamide gel electrophoresis. Ouabain-dependent phosphorylated 100 kDa polypeptide was observed only in the preparation from HK dog erythrocytes on an acidic pH-polyacrylamide gel electrophoresis [24]. $(Na^+ + K^+)$-ATPase activity was measured as described previously [18], and protein concentrations were estimated by the method of Lowry et al. [25].

Subjects	SDS concn. (mg/1.4 mg protein per ml)	$(Na^+ + K^+)$-ATPase activity (nmol of ATP hydrolyzed/ mg protein per min)
Dog kidney microsomes	0.56	4450–8960
HK dog erythrocytes	0[a]	40.4
	0.42	127.6
	0.49	159.5
	0.56	175.8
	0.70	–[b]
LK dog erythrocytes	0, 0.56 and 0.70	–[c]

[a] Not treated with SDS.
[b] Recovery of proteins was below 0.5% and no significant activity was obtained.
[c] No significant activity was observed.

The preparative procedures of the $(Na^+ + K^+)$-ATPase and enzymatic activities of the preparations used here are shown in Table I.

Fig. 1A shows a typical elution pattern of solubilized $(Na^+ + K^+)$-ATPase from dog kidney microsomes labeled with [^{3}H]ouabain. [^{3}H]-Ouabain was detected as two major peaks (peaks

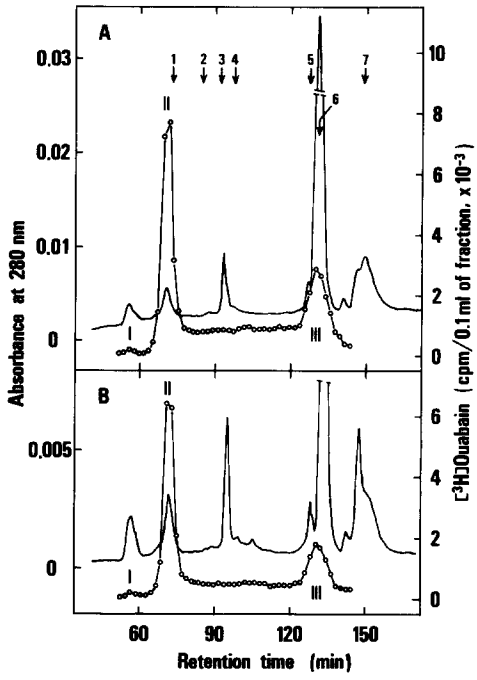

Fig. 1. Elution patterns of the solubilized $(Na^+ + K^+)$-ATPase from dog kidney microsomes labeled with [^{3}H]ouabain from an HPLC column. 50 μg of the enzyme from dog kidney were suspended in 1.0 ml of the buffer solution for HPLC containing 2 μCi of [^{3}H]ouabain (20.9 Ci/mmol, New England Nuclear) with neither $C_{12}E_8$ nor glycerol and incubated for 1 h at 37°C. Membrane proteins were pelleted by centrifugation at 15000 × g for 20 min, then resuspended in the buffer solution for HPLC. The $C_{12}E_8$ (Nikko Chemicals, Tokyo, Japan) was added to the suspension at a final concentration of 1.5 mg $C_{12}E_8$/mg protein. The suspension was vigorously shaken for 1 min, allowed to stand for 5 min at 25°C and then centrifuged for 20 min at 15000 × g. The supernatant obtained was filtered through a Millipore SJHV-type membrane (pore size, 0.45 μm) and aliquots of 100 μl (A) and 80 μl (B) of the solubilized protein were injected into a column of TSK-GEL G3000SW (Toyo Soda, Tokyo, Japan) equipped with a guard column of TSK-GSWP (Toyo Soda) through an HPLC system from Waters Associates. The column had been equilibrated and was eluted with a buffer solution containing 1 mg/ml of $C_{12}E_8$, 150 mM sodium acetate, 3 mM $MgSO_4$, 2 mM Na_2HPO_4, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, 7.5% (v/v) glycerol and 20 mM Mops-Tris (pH 7.2) at a flow rate of 0.2 ml/min at 20–23°C. The eluate was monitored for ultraviolet absorption at 280 nm, fractionated into 0.4 ml portions and counted for radioactivity (○) using Scintisol EX-H (Dojindo Laboratories, Kumamoto, Japan) as a scintillant. The samples were subjected to the column at 30 min (A) and 18 h (B) after the solubilization. The arrows designated as 1–7 indicate the elution positions of standard proteins and others as follows: 1, ferritin (M_r = 450000); 2, aldolase (158000); 3, bovine serum albumin (68000); 4, ovalbumin (45000); 5, cytochrome c (12500); 6, ouabain; 7, β-mercaptoethanol.

II and III) with retention times of 70 and 130 min, respectively. They corresponded to the enzyme bound form (peak II) and free form (peak III). A minor peak with a higher molecular weight (peak I) seemed to represent oligomer forms of the enzyme. Furthermore, as shown in Fig. 1B, the same elution profiles were obtained when the solubilized $(Na^+ + K^+)$-ATPase was kept at 23°C for 18 h and then applied to the column. These results indicate that the $(Na^+ + K^+)$-ATPase solubilized with $C_{12}E_8$ was stable for at least 18 h at room temperature and that the environmental conditions adopted here were adequate for the enzyme to maintain equilibrium conditions for binding with ouabain.

In the $(Na^+ + K^+)$-ATPase-rich preparations from HK dog erythrocyte membranes (treated with SDS), we observed similar elution patterns for [^{3}H]ouabain (Figs. 2A and 2B). However, the radioactive counts of [^{3}H]ouabain in the peaks of ouabain-enzyme complexes were very low as compared to those of the solubilized dog kidney microsomes. This difference in ouabain binding capacity appears to be derived from the discrepancies in the enzyme activities, as shown in Table I. The minor peak, which might reflect the formation of an oligomer of the enzyme, as observed in Figs. 1A and 1B, was not detected.

Moreover, no peak of [^{3}H]ouabain was detected when solubilized membrane proteins from LK dog erythrocytes were subjected to an HPLC column by the same procedures. This observation is in good agreement with our previous studies, which demonstrated that mature LK dog erythrocytes lacked the $(Na^+ + K^+)$-ATPase activity and ouabain binding sites [16,18]. In addition, this result strongly supports the conclusion that the major peak of [^{3}H]ouabain detected in both the solubilized dog kidney microsomes (Fig. 1), and HK dog erythrocyte membranes (Fig. 2) represents the true [^{3}H]ouabain bound to the $(Na^+ + K^+)$-ATPase.

[^{3}H]Ouabain-$(Na^+ + K^+)$-ATPase complex corresponding to the major peak of [^{3}H]ouabain showed an apparent molecular weight of 470 000–490 000, (as shown in Figs. 1 and 2) which agreed with the value (500 000) of the $C_{12}E_8$-solubilized $(Na^+ + K^+)$-ATPase with high activity obtained by Sepharose CL-4B column chromatography reported by Esmann and his co-workers [5]. They analyzed the 500 kDa particles, which included protein, lipid, carbohydrate and detergent, using sedimentation equilibrium, and suggested that the soluble $(Na^+ + K^+)$-ATPase consisted of $\alpha_2\beta_2$-oligomer with a molecular weight of 270 000. This seems to indicate that the $(Na^+ + K^+)$-ATPase solubilized with $C_{12}E_8$ was present in $\alpha_2\beta_2$-form in our study. They also reported that increasing the amount of detergent ($C_{12}E_8$) caused $\alpha\beta$-protomer formations and lack of enzymatic activity [6]. Recently, Hayashi et al. [13] using an HPLC system with the same type of column as in our study (TSK-GEL G3000SW), estimated that the solubilized $(Na^+ + K^+)$-ATPase from dog kidney microsomes consisted mainly of $\alpha\beta$-protomer and its dimer. These solubilized enzymes, however,

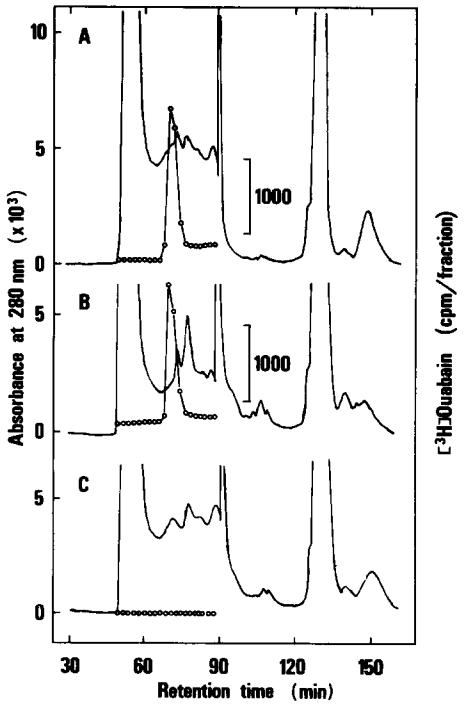

Fig. 2. Elution patterns of the solubilized HK (A and B) and LK (C) dog erythrocyte membranes treated with SDS and labeled with [^{3}H]ouabain. Dog erythrocyte membranes treated with SDS were incubated with [^{3}H]ouabain as described in the legend for Fig. 1. Membranes were solubilized with $C_{12}E_8$ at a concentration of 3 mg/mg protein and aliquots of 100 μl (A and C) or 80 μl (B) of the supernatant were subjected to HPLC at 30 min (A and C) and 18 h (B) after the solubilization. The eluate was fractionated and counted for radioactivity (○).

lacked $(Na^+ + K^+)$-ATPase activity because of the delipidation which occurred during chromatography and fractionation. It is well known that lipids have important roles in the activation of $(Na^+ + K^+)$-ATPase [1,5,6,12,19]. On the other hand, from the study of ligand binding to the enzyme, it was demonstrated that $\alpha\beta$-protomer had an ouabain binding site [1]. It has also been suggested by Taniguchi and Iida [20] that the binding of ouabain to the $(Na^+ + K^+)$-ATPase does not require phospholipid directly. These obsevations indicate that the protomer, or its oligomer formation, of the $(Na^+ + K^+)$-ATPase in a soluble form might be detected as [^{3}H]ouabain content if the binding of [^{3}H]ouabain to the enzyme could be stabilized. In HPLC analysis, we used a buffer solution containing Mg^{2+} and HPO_4^{2-} for the optimal binding of ouabain [21], and glycerol for stabilizing the enzyme [5]. When these three elements were omitted from the elution buffer, we could not observe the stable binding of [^{3}H]ouabain to the enzyme (data not shown). In this case, [^{3}H]ouabain bound to the $(Na^+ + K^+)$-ATPase seemed to dissociate during chromatography.

Thus, we concluded that the binding of [^{3}H]ouabain to the $(Na^+ + K^+)$-ATPase solubilized with $C_{12}E_8$ was stable in the presence of Mg^{2+}, HPO_4^{2-} and glycerol (in the presence of sodium) during chromatography through an HPLC column. This indicates the usefulness of $C_{12}E_8$ and [^{3}H]ouabain for identifying the $(Na^+ + K^+)$-ATPase from small amounts of material and for revealing the molecular structure of this enzyme.

We thank Dr. K. Taniguchi for his valuable comments and Professor S. Namioka for his contribution to this study. This study was supported in part by grants from Scientific Research Fund of the Ministry of Education, Science, and Culture of Japan.

References

1 Jørgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68
2 Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366–380
3 Kyte, J. (1971) J. Biol. Chem. 246, 4157–4165
4 Craig, W.S. (1982) Biochemistry 21, 2667–2674
5 Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim. Biophys. Acta 567, 410–420
6 Esmann, M., Christiansen, C., Karlsson, K.-A., Hansson, G.C. and Skou, J.C. (1980) Biochim. Biophys. Acta 603, 1–12
7 Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817–821
8 Nakao, T., Nakao, M., Mizuno, N., Komatsu, Y. and Fujita, M. (1973) J. Biochem. (Tokyo) 73, 609–619
9 Peterson, G.L., Churchill, L., Fisher, J.A. and Hokin, L.E. (1982) J. Exp. Zool. 221, 295–308
10 Fisher, J.A., Baxter-Lowe, L.A. and Hokin, L.E. (1984) J. Biol. Chem. 259, 14217–14221
11 Forbush, B., Kaplan, J.H. and Hoffman, J.F. (1978) Biochemistry 17, 3667–3676
12 Brotherus, J.R., Møller, J.V. and Jørgensen, P.L. (1981) Biochem. Biophys. Res. Commun. 100, 146–154
13 Hayashi, Y., Takagi, T., Maezawa, S. and Matsui, H. (1983) Biochim. Biophys. Acta 748, 153–167
14 Spector, M., O'Neal, S. and Racker, E. (1980) J. Biol. Chem. 255, 5504–5507
15 Morohashi, M. and Kawamura, M. (1984) J. Biol. Chem. 259, 14928–14934
16 Maede, Y., Inaba, M. and Taniguchi, N. (1983) Blood 61, 493–499
17 Inaba, M. and Maede, Y. (1984) J. Biol. Chem. 259, 312–317
18 Maede, Y. and Inaba, M. (1985) J. Biol. Chem. 260, 3337–3343
19 Roelofsen, B. and Van Deenen, L.L.M. (1973) Eur. J. Biochem. 40, 245–257
20 Taniguchi, K. and Iida, S. (1971) Biochim. Biophys. Acta 233, 831–833
21 Tobin, J. and Sen, A.K. (1970) Biochim. Biophys. Acta 198, 120–131
22 Jørgensen, P.L. (1974) in Methods in Enzymology, vol. 32 (Fleischer, S. and Packer, L., eds.), pp. 277–290, Academic Press, New York
23 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
24 Resh, M.D. (1982) J. Biol. Chem. 257, 6978–6986
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275