Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 361-367

MONOMERIC AND TRIMERIC STRUCTURES OF ACTIVE Na,K-ATPase IN $C_{12}E_8$ SOLUTION

Toshiko NAKAO*, Tomoko OHNO*, Makoto NAKAO**, Goichi MAEKI*, Sachiko TSUKITA*** and Harunori ISHIKAWA***

*Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo

**Department of Biochemistry, Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo

***Department of Anatomy, Faculty of Medicine, The University of Tokyo, Hongo, Bnkyo-ku, Tokyo, Japan

Received April 21, 1983

SUMMARY: Horse kidney Na,K-ATPase solubilized with dodecyloctaethyleneglycolether was subjected to high performance gel chromatography (HPLC) on TSK G 4000 SW in the presence of 0.01% $C_{12}E_8$. Successive on-line measurements of low angle laser light scattering (LS) and refractive index (RI) were made, and values of refractive index increment (dn/dc) were measured with the same differential refractometer under the same conditions. Two peaks (peak-2 and peak-3) with Na,K-ATPase activity besides that at the void volume were detected in the HPLC effluent fractions, and from their (dn/dc) values and the linear plot of protein standards, the M.W.s of these peaks were calculated to be roughly 535K and 175K respectively. Both peaks showed α and ß, but not γ, bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The activity peak fractions obtained after glycerol density gradient centrifugation to remove detergent micelles showed HPLC peak-2 and peak-3. The ratios of (Output)LS/(Output)RI of peak-2 to that of peak-3 and (Output)LS/(Output)UV 280 of the peak-2 to that of peak-3 were both nearly 3. The above findings and the results of electron microscopy of negatively stained preparations strongly suggested that peak-2 and peak-3 of enzyme activity consist of the trimer $(\alpha ß)_3$ and the monomer $(\alpha ß)$, respectively.

It has been suggested that Na,K-ATPase consists of dimers $(\alpha ß)_2$(1), and the monomer $(\alpha ß)$ is assumed to be the

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LS, low angle light scattering intensity; dn/dc, refractive index increment; RI, refractive index; $C_{12}E_8$, dodecyloctaethyleneglycolether; HPLC,high performance liquid chromatography.

0006-291X/83 $1.50

active form on the basis of indirect evidence (2,3). According to Takagi (4,5), the molecular weights of proteins can be estimated by means of on-line light scattering and refractive index intensity measurements after HPLC. The present paper deals with the application of the above method to Na,K-ATPase solubilized with $C_{12}E_8$.

Materials and Methods

Na,K-ATPase was purified from horse kidney outer medulla essentially according to Jørgensen (6). SDS-treated microsomes (specific activity 34-45 μmol Pi/mg protein/min) were suspended in a medium containing 2 mM histidine-HCl and 1 mM EDTA (pH 7.2), and $C_{12}E_8$ solution was added to the suspension at 15°C to the point where a very slight turbidity remained. The solution was centrifuged at 320,000g for 60 min at 4°C. The activity of the supernatant was 42-51 μmol Pi/mg protein/min as determined according to Esmann et al.(7). Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (8). Protein was assayed according to Bradford (9). An LS-8 low angle light scattering photometer (633 mm laser light, Toyo Soda Ltd.) and an RI-8 differential refractometer (white light, Toyo Soda Ltd.) were connected on-line to the HPLC column(TSK G 4000 SW 7.5 x 600 mm). The column was pre-equilibrated with the elution buffer containing 100 mM Tes-Tris pH 6.8, 25 mM NaCl and 0.01% $C_{12}E_8$, then 50μ of the $C_{12}E_8$-solubilized sample was applied at a flow rate of 30 ml/hr. Standard proteins were purchased from Pharmacia and Sigma. For microscopic observation, 2% uranyl acetate was placed on the sample on a carbon-coated Microgrid. A JEOL model 100 electron microscope was used.

Results and Discussion

As shown in Fig. 1, three peaks of Na,K-ATPase were observed at 22.5 min (void volume), 32.5 min and 40.1 min. They all showed Na,K-ATPase activity (specific activities 10, 20, and 20 mols iP/mg/min respectively, when measured just after HPLC). The activity was completely inhibited by 10^{-4}M ouabain. Peak-1 emerged close to the void volume and showed large light scattering. The areas of peak-2 and peak-3 in terms of RI were usually significant. Only α and β but not γ bands were found on SDS polyacrylamide electrophoresis of peak-2 and peak-3, as well as of the extract before application to the column. According to Takagi (4),

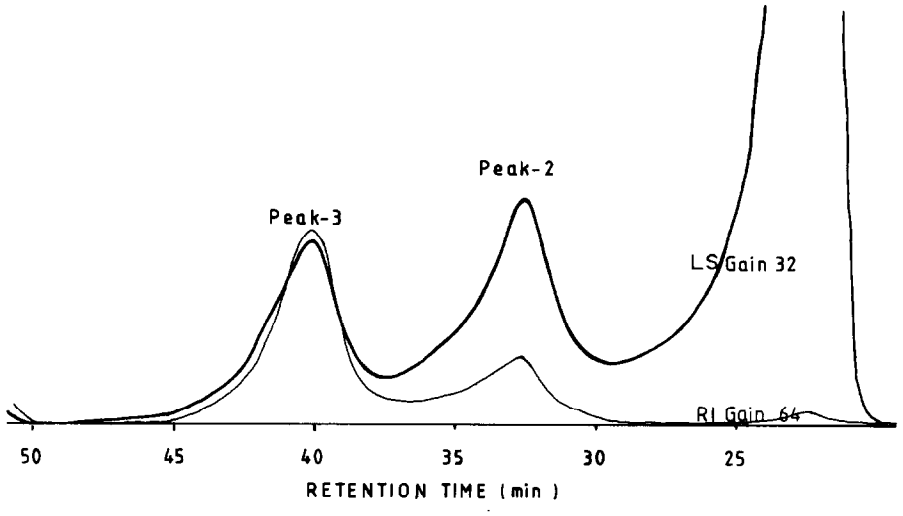


Fig.1
Low angle light scattering and refractive index of Na,K-ATPase fractions measured on-line after HPLC on a Toyo Soda TSK G 4000 SW column
Eluent: 100 mM Tes-Tris pH 6.8, 25 mM NaCl and 0.01% $C_{12}E_8$. Flow rate: 30 ml/hr at 25°C
top; refractive index, light scattering intensity

$$\frac{(\text{Output})\ LS}{(\text{Output})\ RI} = \frac{dn}{dc} MK$$

where LS, RI, dn/dc, M and K are low angle light scattering intensity, refractive index, refractive index increment, molecular weight and a constant respectively when the same instrument and solvent are used. For standard proteins, a plot based on this equation gave an almost linear relationship (Fig. 2). (dn/dc)s of bovine thyroglobulin and BSA were measured with the same differential refractometer under the same conditions as used for HPLC (eluent $C_{12}E_8$; temperature, 25°C; same instruments). The values obtained were 0.144 for BSA, 0.141 for thyroglobulin, and 0.135 for $C_{12}E_8$. According to Armstrong et al. (10), 10 serum proteins or protein fractions including human serum albumin, fibrinogen, globulin and lipoprotein containing 75% lipid had very similar (dn/dc)s within the range of 0.188 - 0.171

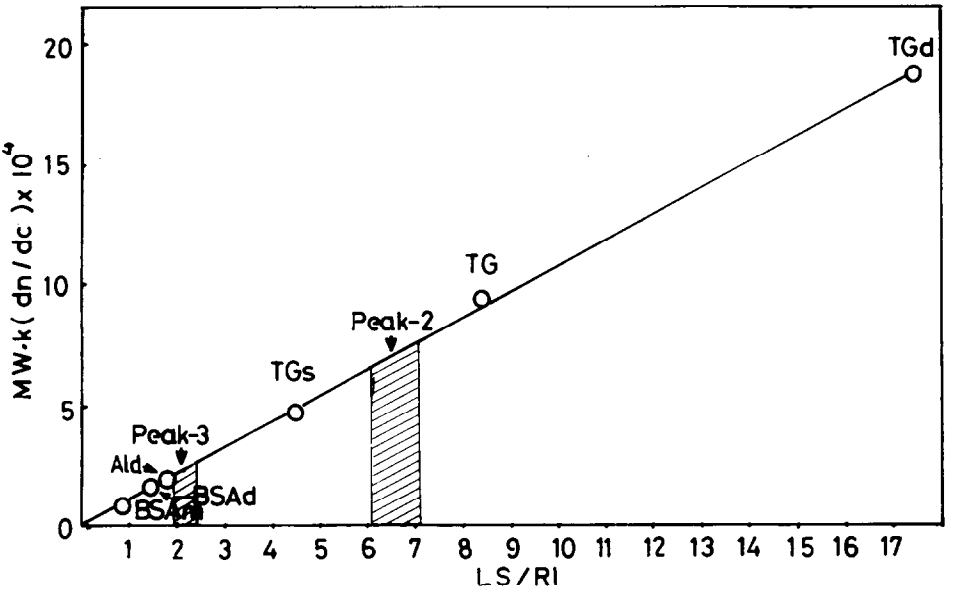


Fig.2
Relation between [molecular weight x k (dn/dc] and [output of light scattering/output of refractive index]
BSA, bovine serum albumin; TG, TGs, TGd, thyroglobulin, thyroglobulin subunit, thyroglobulin dimer, respectively
Values of output of light scattering per output of refractive index of peak-2 and peak-3 were 2.2 ± 0.3 and 6.6 ± 0.5 (n=5) respectively.

(488 nm, 25°C). Since the differences in dn/dc values of Na,K-ATPase [in 0.1% $C_{12}E_8$ protein, lipid, carbohydrate, $C_{12}E_8$ ratio = 100:27:15:78 (11)] and standard proteins may be less than 10%, we tentatively estimated the molecular weights of peak-2 and peak-3 to be 1.71×10^5 (±10%) and 50×10^5(±10%). In order to remove $C_{12}E_8$ micelles from peak-3 completely, the $C_{12}E_8$-solubilized enzyme was centrifuged through a linear gradient of 10-30% glycerol according to Craig (3), then various fractions (Nos. 3,10,15 and 19) were subjected to the HPLC on TSK G 4000 SW (Table I). Peak-2 and peak-3 appeared in HPLC only when fractions Nos. 10 and 15 (which showed ATPase activity) from the gradient were applied. The ratios of (Output)LS/(Output)RI of peak-2 to that of peak-3 and (Output)LS/(Output)UV 280 of

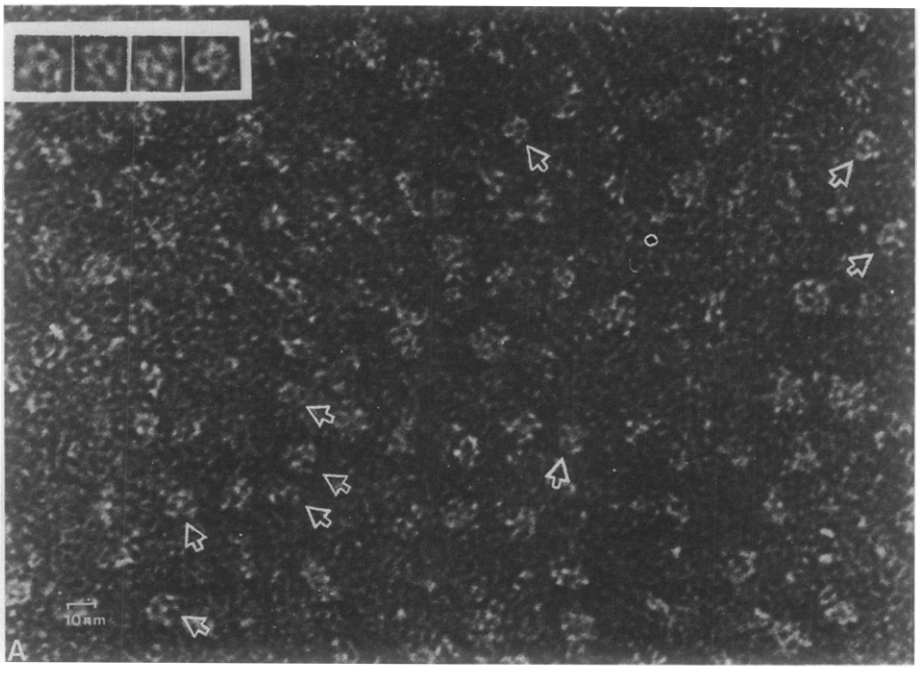
Table I

Ultraviolet absorption at 280 nm, refractive index and low angle laser light scattering intensity of peaks obtained by HPLC of fractions from glycerol gradient centrifugation

Fraction number in glycerol gradient centrifugation		HPLC Peak-2	HPLC Peak-3	Peak-2/peak-3
10	UV	5.0	9.7	
	RI	6.0	12.0	
	LS	13.0	9.0	
	LS/UV	2.6	0.92	2.83
	LS/RI	2.2	0.75	2.93
15	UV	4.8	9.0	
	RI	7.0	12.0	
	LS	12.0	6.8	
	LS/UV	2.5	0.75	3.33
	LS/RI	1.71	0.57	3.05

One-fifth ml of the $C_{12}E_8$-solubilized Na,K-ATPase was centrifuged on a linear glycerol gradient (10-30%) to remove $C_{12}E_8$ micelles according to Craig (3). Nos. 3, 10, 15 and 19 of the total of 35 fractions were subjected to HPLC on a G4000 SW column equipped with light scattering, ultraviolet absorption (280 nm) and differential refraction monitors. Only Nos. 10 and 15 (which showed ATPase activity) gave two HPLC peaks corresponding to peak-2 and peak-3. Most of the $C_{12}E_8$ was found in fractions No.1 - 7. Outputs of these monitors are expressed in arbitrary units.

peak-2 that of peak-3 were both nearly 3. These results strongly suggested that peak-3 is a monomer (α ß)and peak-2 is a trimer $(\alpha ß)_3$. Electron micrographs of negatively stained peaks-2 and 3 are shown in Fig. 3 AB. Peak-3 showed scattered, isolated, small polygonal circles with a diameter of 37° $\pm$ 7 (n=19) and peak-2 showed larger polymorphic forms with a diameter of 70A $\pm$ 10 consisting of small circles which corresponded to those in peak-3. Triangular trimer-like structures were often observed and quadramer-, pentamer- and hexamer-like structures were also seen, but arrays resembling dimeric form were seldom seen. These results support the view that peak-3 is the monomer (α ß) and peak-2 is the trimer $(\alpha ß)_3$, while peak-1 represents aggregates.



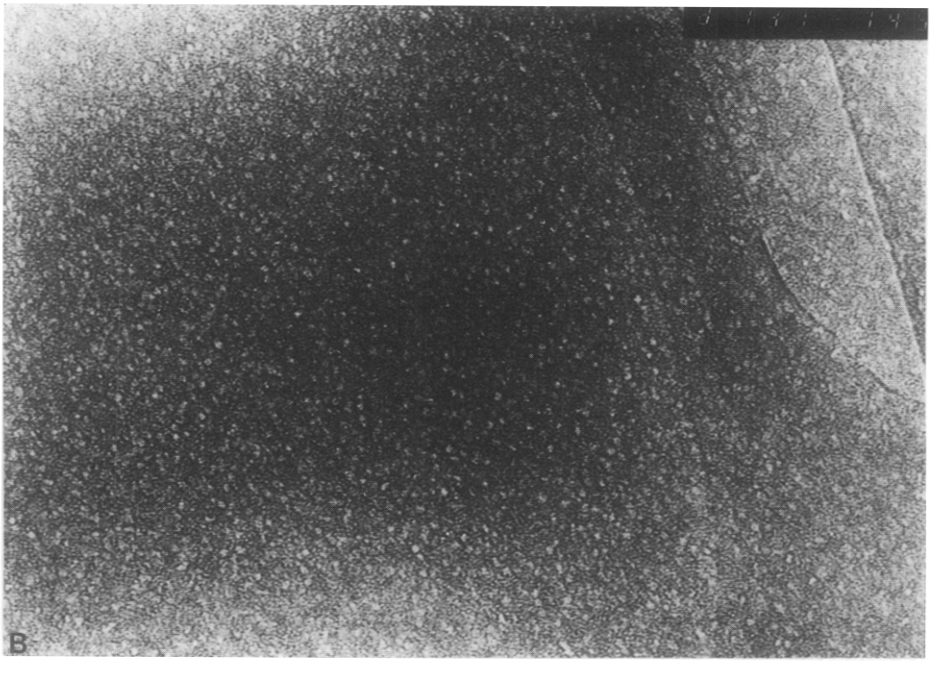


Fig.3
Electron micrographs of effluent peak-2 and peak-3 from HPLC (see Fig. 1)
Peak-2 (A) and peak-3 (B) were observed after negative staining with 2% uranyl acetate. Magnification x 300,000 (B), x 420,000 (A) and x 560,000 (insertions)

Acknowledgments

This study was supported by a grant in aid for Scientific Research (5712205) from the Ministry of Education, Science and Culture of Japan.

References

1. Jørgensen, P.L. (1982) Biochim.Biophys.A. 694, 27-68.
2. Brotherus, J.R., Moller, J.V. and Jørgensen, P.L. (1981) Biochem.Biophys.Res.Commun. 100, 146-154.
3. Craig, W.S. (1982) Biochem. 21, 5707-5717.
4. Takagi, T. (1981) J.Biochem. 89, 363-368.
5. Kameyama, K., Nakae, T., and Takagi, T. (1979) Biochim.Biophys.A. 706, 19-26.
6. Jørgensen, P.L. and Skou, J.C. (1969) Biochem.Biophys.Res.Commun. 37, 39-46.
7. Esmann, M., Christiansen, C., Karlsson, K., Hansson, K.C. and Skou, J.C. (1980) Biochim.Biophys.A., 603, 1-12.
8. Laemmli, U.K., (1975) Nature 256, 495-497.
9. Bradford, M.M. (1976) Anal.Biochem. 72, 248-254.
10. Armstrong, S.H., Budka, M.J.E., Morrison, K.C. and Hasson, M. (1947) J.Am.Chem.Soc. 69, 17746-1753.
11. Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim.Biophys.A. 567, 410-420.