Target Size of $(Na^+ + K^+)$-ATPase and Na^+, K^+Pump of Human Erythrocytes*

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=**Abstract**=

Previous biochemical studies indicate that $(Na^+ + K^+)$-ATPase is composed of two subunits, α and β, in a form of $\alpha_2\beta_2$ with a molecular weight of approximately 300,000 daltons. There is also suggestive evidence that the Na^+, K^+ pump in human erythrocytes occurs in a complex with some glycolytic enzymes. We assessed here in situ assembly size of the $(Na^+ + K^+)$-ATPase of human erythrocytes by applying classical target theory to radiation inactivation data of the ouabain-sensitive sodium flux and ATP hydrolysis of intact cells and ghosts. Cells(in the presence of cryoprotective agent) and ghosts were irradiated at $-45°C$ to $-50°C$ with an increasing dose of a 1.5 MeV electron beam, and after thawing, the pump and/or enzyme activities were assayed. Each activity measured was decreased as a simple exponential function of radiation dose, from which a radiation sensitive volume (target size) was calculated. When intact cells were used, the target size of both $(Na^+ + K^+)$-ATPase and Na^+, K^+ pump was found to be approximately 600,000 daltons. This target size of the ATPase was reduced to approximately 325,000 daltons if the cells were pretreated with strophanthidin. When ghosts were used, the target size of the ATPase was again approximately 325,000 daltons.

Our target size measurement suggests that, in intact cells, the $(Na^+ + K^+)$-ATPase/Na^+, K^+ pump exists either as a dimer of $(\alpha\beta)_2$ which is a functional unit or as a monomer of $(\alpha\beta)_2$ but in tight complex with other enzyme or enzymes. The results also suggest that this dimeric or heterocomplex association is dissociated during ghost preparation and strophanthidin treatment.

INTRODUCTION

The molecular weight of protein can be measured by a variety of physical techniques. Among these, ionizing radiation offers several unique advantages in that samples need not necessarily be purified and only minute amounts of enzyme are required. The molecular weights measured by this method for more than 40 enzymes are well agreed with those determined by other physical-chemical methods(Pollard *et al.*, 1955; Kepner and Macey, 1968; Kempner and Schlegel, 1979).

We assessed here in situ assembly size of the Na^+, K^+ pump and $(Na^+ + K^+)$-ATPase of human erythrocytes by applying classical target theory to radiation inactivation data of the ouabain sensitive sodium flux and ATP hydrolysis of intact cells and ghosts.

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Recently, $(Na^+ + K^+)$-ATPase from a variety of sources has been purified and shown by polyacrylamide gel electrophoresis to be composed of two peptide constituents, designed alpha(α) and beta(β), with molecular weight of 100,000 and 60,000, respectively (Uesugi *et al.*, 1971; Jϕrgensen, 1975). Detergent-solubilized preparations of $(Na^+ + K^+)$-ATPase showed an active complex to be 360,000 daltons corresponding subunit structure of $\alpha_2\beta_2$ or $\alpha_2\beta_4$ (Hastings and Reynolds, 1979).

There are some compelling evidences that GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PGK (phosphoglycerate kinase), synthesize ATP at a membrane associated compartment specifically for the Na^+, K^+ pump (Mercer and Dunham, 1981). This suggests that $(Na^+ + K^+)$-ATPase/Na^+, K^+ pump may exist with GAPDH and PGK as a multienzyme complex in membranes.

However, the target size of $(Na^+ + K^+)$-ATPase/Na^+, K^+ pump of human red cell in native state has not been measured although the target size of the ATPase of ghosts is reported by Kepner and Macey (1968).

Here, we report the functional molecular weight of $(Na^+ + K^+)$-ATPase/Na^+, K^+ pump assessed by radiation inactivation data on ^{22}Na efflux in intact cells and $(Na^+ + K^+)$-ATPase hydrolysis function in ghost membranes.

EXPERIMENTAL PROCEDURE

Preparation of samples: Human red cells were obtained from blood bank. The cells were washed four or five times at room temperature with about five volumes of iso-NaCl. The buffy coat of the cells was discarded. In order to measure sodium flux and $(Na^+ + K^+)$-ATPase in intact cell, one part of washed cells was prepared in same volume of cryoprotective agent (180 ml of glycerol + 28 g of sorbitol + 720ml of 0.9% NaCl) and transferred into aluminum trays for irradiation. Another part of cells was treated with strophanthidin (10^{-6}M) for 30 minutes at 37°C and subjected to the same procedure. These samples were equilibrated for 30 minutes at 0~4°C, and frozen in liquid nitrogen refrigerator until being irradiated. Ghosts prepared by the method of Dodge *et al.* (1963) were also transferred into aluminum trays and frozen at same temperature.

Radiation inactivation procedure: The samples were irradiated in a air-free chamber in the frozen state (−45° to −50°C) with a Van de Graff generated 1.5 MeV electron beam (Western New York Nuclear Research Center, Inc., Buffalo, New York, U.S.A.). Radiation dose was measured at the sample irradiation temperature using the transmittance change of blue cellophane calibrated against a chemical dosimeter (Fricke and Hart, 1966). The radiation chamber was cooled with a stream of liquid nitrogen and the sample temperature (−45° to −50°C) was monitored as described previously (Saccomani *et al.*, 1981). The sample holder was on a chain conveyor system which could move around the beam in a uniform way. At each pass the sample received doses about 0.675 M rad. After irradiation, cells were subjected to quick thaw procedure and then measured ^{22}Na transport in resealed cell preparation. Ghosts were thawed and $(Na^+ + K^+)$-ATPase activities were measured promptly.

Quick thaw procedure: The freeze-irradiated cells were thawed in water bath at 37°C, mixed with an equal volume of 3.5% NaCl and centrifuged at 1,000×g for 10 minutes. The cells were treated consecutively with 10

volumes of 1.8% and 0.9% NaCl by the same procedure. The packed cells then resuspended in incubation medium with 5 mM glucose and stood in ice bath until being used. For preparing ghost membranes, the cells were subjected to direct hemolysis avoiding above procedure.

^{22}Na efflux measurement: In order to study net outward Na movements, ^{22}Na was introduced into the cells by reversal of haemolysis (Schatzmann and Vincenzi, 1969). The freeze-thawed cells were hemolyzed at room temperature with mild stirring in a three fold volume of "lysing solution" containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris buffer with 0.1 μCi/ml of ^{22}Na. After 135 seconds a sufficient amount of 3M KCl solution was added to make the mixture isotonic. The cells thus resealed while taking up ^{22}Na with KCl as the major salt. After 5 minutes at room temperature, or immediately after adding the KCl solution, the suspension was diluted with one or two parts of ice-cold "incubation medium" containing 140 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, and 10 mM Tris buffer, pH 7.4. The mixture was centrifuged under refrigeration for 5 minutes at 18,000×g. The cells were then washed with the same solution once at 0°C and were finally suspended in a volume of the same ice-cold solution, such that the hematocrit was 0.2~0.3.

The experiment was started by warming the cells to 37°C in thin walled Erlenmeyer flasks in a water-bath. Samples were taken at various times and cooled rapidly. The cells were separated from the medium by centrifugation at 24,000×g for 7 minutes under refrigeration. The net movement of Na was calculated by counting radioactivity of ^{22}Na transferred to supernatant.

$(Na^+ + K^+)$-ATPase assay: The $(Na^+ + K^+)$-ATPase was assayed by measuring the amount of inorganic phosphate liberated from ATP either colorimetrically (Dunham and Glynn, 1961) or by detecting radioactivity of ^{32}Pi released from γ-^{32}P-ATP (Blostein, 1968). As procedure 0.5 ml of ghost membrane were added to 4.25 ml of incubation medium containing 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 1 mM $CaCl_2$ with or without ouabain (5×10^{-5}M) and equilibrated for 3 minutes at 37°C The mixture was then incubated for 30 minutes after adding 0.1 ml of ATP (2×10^{-3}M).

The reaction was stopped by the addition of 1 ml of ice-cold 10% trichloroacetic acid. After centrifugation at 30,000×g for 20 minutes, the inorganic phosphate in the supernatant was determined by the method of Fiske and Subbarow (1925) and the ouabain sensitive $(Na^+ + K^+)$-ATPase was calculated. Procedure for using γ-^{32}P-ATP is basically same except using γ-^{32}P-ATP.

RESULTS

Target size of Na^+, K^+ pump in intact cell

Inactivation of the sodium transport led by Na^+, K^+ pump across the resealed cell membrane was measured as a function of radiation dose. The net ^{22}Na efflux was decreased in a simple exponential fashion, giving straight line on a semilog plot (Fig. 1). The molecular weight of approximately 600,000 daltons was calculated by the equation (2). This value is about twice as high as that reported for $(Na^+ + K^+)$-ATPase molecular weight (Kepner and Macey, 1968; Ellory *et al.*, 1979). This implies that Na^+, K^+ pump functions as either dimeric form of $(Na^+ + K^+)$-ATPase or a hetero-multienzyme complex with other protein(s) in the cell.

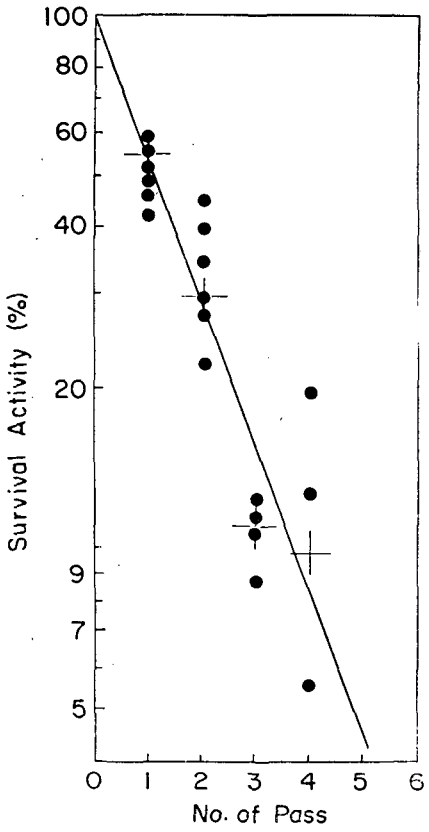

Fig. 1. Radiation inactivation of Na^+, K^+ pump in intact cell. Inactivation data for ^{22}Na efflux function were plotted in semilogarithmic scale as a function of radiation dose. One pass equivalent with 0.675 M rad. A molecular weight of 600,000 daltons was calculated by the equation(2).

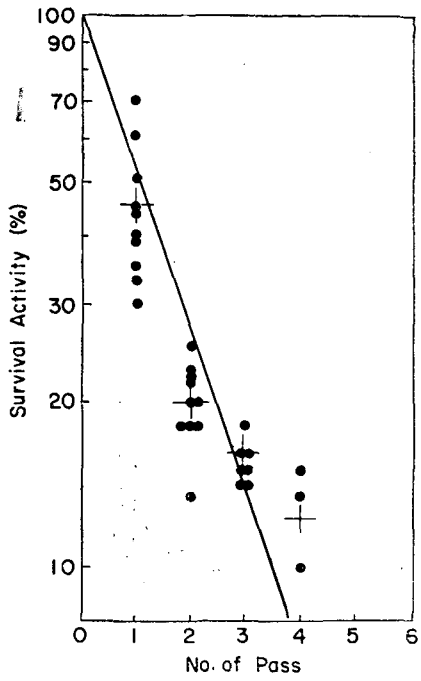

Fig. 2. Radiation inactivation of $(Na^+ + K^+)$-ATPase in red cell ghosts prepared from irradiated cells. Inactivation of $(Na^+ + K^+)$-ATPase as ATP hydrolysis function was plotted in semilogarithmic scale as a function of radiation dose. The line appeared to be straight showing D_{37} of 1.55 passes. The calculated target size was approximately 600,000 daltons which represents the functional molecular weight of $(Na^+ + K^+)$-ATPase in intact cell.

Target size of $(Na^+ + K^+)$-ATPase as ATP hydrolysis function in intact cell

In order to measure the target size of $(Na^+ + K^+)$-ATPase as ATP hydrolysis function in native state, the frozen cell was irradiated before being made ghost. Then, the activity of $(Na^+ + K^+)$-ATPase of the ghost membrane was measured as a function of irradiating dose. Shown in Fig. 2 is representative radiation-inactivation curve given by the $(Na^+ + K^+)$-ATPase prepared from the irradiated cells. The calculated target size from 6 experiments appeared to be about 600,000 daltons, which is identical with that obtained for the Na^+, K^+ pump activity in intact cells. Above two results imply that ATP production function of $(Na^+ + K^+)$-ATPase could conceivably be coupled to the flux of sodium out of the cell.

Target size of $(Na^+ + K^+)$-ATPase as ATP hydrolysis function in ghost

One question arises here why target size of $(Na^+ + K^+)$-ATPase in intact cell is twice as large as that reported previously from ghost membranes. One possibility is that the fun-

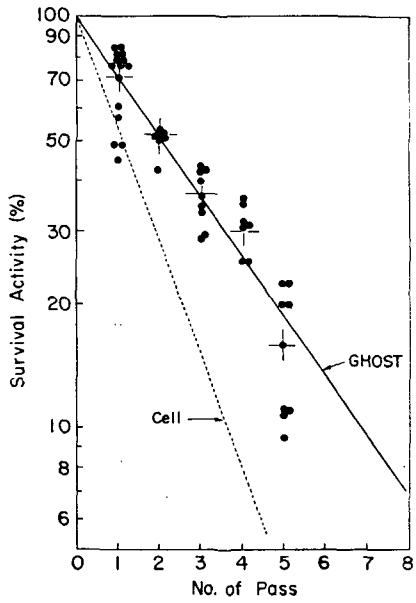

Fig. 3. Radiation inactivation of $(Na^+ + K^+)$-ATPase in red cell ghosts. The solid line represents the radiation inactivation curve for $(Na^+ + K^+)$-ATPase in ghosts showing a target size of approximately 325,000 daltons. Radiation inactivation data(dotted line) for $(Na^+ + K^+)$-ATPase in intact cell were plotted to compare with the ATPase in ghosts.

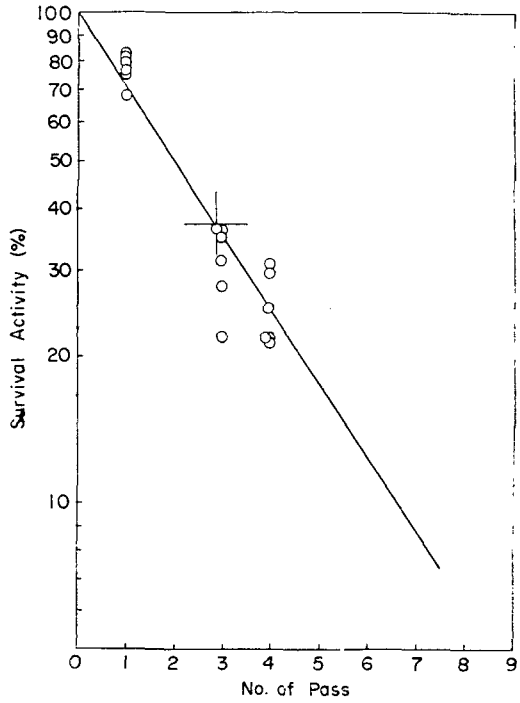

Fig. 4. Radiation inactivation of $(Na^+ + K^+)$-ATPase in ghosts prepared from the cells pretreated with strophanthidin (10^{-6}M). The calculated molecular weight was about 325,000 daltons which corresponds to a half size of the ATPase in intact cell.

ctional unit of $(Na^+ + K^+)$-ATPase could be dissociated during ghost preparation. To inquire this, ghost membranes were prepared prior to irradiation and then assayed for the ATP hydrolysis function. Fig. 3 contains the results of 16 experiments and shows the fractional surviving $(Na^+ + K^+)$-ATPase activity as a function of irradiating dose: this activity also follows monoexponential decay and the target size of approximately 325,000 daltons was calculated, which corresponds to the molecular weight reported by other investigators. This indicates that the preparation of ghosts creates a significant disruption of the normal distribution of the red cell proteins.

Dissociation of $(Na^+ + K^+)$-ATPase complex by strophanthidin

Since strophanthidin is known to dissociate the $(Na^+ + K^+)$ ATPase complex (Fossel and Solomon, 1977), it was attempted to measure the target size of $(Na^+ + K^+)$-ATPase of the ghost membranes prapared from the cells pretreated with strophanthidin (10^{-6}M) for 30 minutes at 37°C and then followed by the freeze-irradiation procedure. As shown in Fig. 4, the target size of $(Na^+ + K^+)$-ATPase in intact cell was reduced to approximately 325,000 daltons after the strophanthidin treatment. This gives a strong evidence that na-

Table 1. Target sizes of Na^+, K^+ pump/(Na^++K^+)-ATPase under different set of conditions.

Enzymes	Functions	Intact cells	Ghosts
(Na^++K^+)-ATPase	ATP hydrolysis	600,000 daltons	325,000 daltons
(Na^++K^+)-ATPase	Na flux	600,000 daltons	—
Strophanthidin treated ATPase	ATP hydrolysis	325,000 daltons	—

Table 2. The molecular weights of (Na^++K^+)-ATPase determined by radiation inactivation from a variety sources.

Target Size	System	Assays	References
190 K daltons	microsomes(kidney)	ATP hydrolysis	Kepner & Macey(1968)
260 K daltons	microsomes(kidney)	ATP hydrolysis	Ottolenghi & Ellory(1983)
300 K daltons	ghosts	ATP hydrolysis	Kepner & Macey(1968)
500 K daltons	microsomes(brain)	ATP hydrolysis	Nakao et al.(1967)
500 K daltons	microsomes(brain)	Gel filtration	Mizuno et al.(1968)
660 K daltons	microsomes	ATP hydrolysis	Kaniike et al.(1976)
1 million	ghosts	ATP hydrolysis	Kepner & Macey(1966)
600 K daltons	intact cell	Na flux	Hah et al.(1984)
600 K daltons	intact cell	ATP hydrolysis	Hah et al.(1984)
320 K daltons	ghosts	ATP hydrolysis	Hah et al.(1984)

tive form of Na^+, K^+ pump/(Na^++K^+)-ATPase exists either as a dimeric form of (Na^++K^+)-ATPase or as a multienzyme complex with other proteins like glycolytic enzymes. The calculated target sizes of (Na^++K^+)-ATPase/Na^+, K^+ pump under the four set of conditions are summarized in Table 1.

DISCUSSION

The target size of (Na^++K^+)-ATPase/Na^+, K^+ pump was reported from a variety of sources. As shown in Table 2, molecular weight varies as tissues. In ghosts the taget size of (Na^++K^+)-ATPase was found to be approximately 325,000 daltons in this study, which corresponds with that reported by Kepner and Macey(1968). However, the target size of both (Na^++K^+)-ATPase and Na^+, K^+ pump was appeared to be approximately 600,000 daltons when intact cells were used. The 600,000 daltons target size is twice as big as that of ghosts. This 600,000 daltons target size also roughly corresponds to the estimated multiple enzyme complex of ATPase-GAPDH-PGK. There is evidence that strophanthidin dissociates ATPase from this complex(Fossel and Solomon, 1977). When the cells were pretreated with strophanthidin, this target size of the ATPase reduced to approximately 325,000 daltons. The 325,000 daltons target size corresponds with the structure of $(\alpha\beta)_2$ suggested as a functional unit of ATPase based on electron microscopy (Hebert et al., 1982), low angle neutron scattering (Pachence et al., 1983), cross linking (Kyte, 1975; Huang and Askari, 1981), and kinetic analysis of ligand binding (Ottolenghi and Ellory, 1983).

Our target size measurement thus suggests

that, in intact cells, the $(Na^+ + K^+)$-ATPase exists either as a dimer of $(\alpha\beta)_2$ which is a functional unit or a monomer of $(\alpha\beta)_2$ in tight complex with other enzymes particularly GAPDH and PGK. The results also suggest that this dimeric or heterocomplex association is dissociated during ghost preparation and strophanthidin treatment.

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APPENDIX

Target theory: From atomic physics, it is known that the passage of radiation through matter is characterized by transfer of energy to the target material (Lea, 1955; Pollard et al., 1955). For high energy radiation this occurs in discrete bursts called "primary ionization". In each primary ionization, about 66 eV of energy are transferred, corresponding to approximately 1,500 Kcal/mol.

If a biological material is irradiated, this leads to inactivation of function due to destruction of the molecular structure (Kempner and Schlegel, 1979). At optimal conditions, the chance of a molecule which will be "hit" will be proportional to its size. Because the energy deposition is so large, function is completely destroyed by a single hit: there are no partially damaged target. Therefore, the surviving biological activity, A, after irradiation will be a single exponential function of radiation dose, D, from Poisson formula.

$$A = A_0 e^{-KD} \qquad (1)$$

Where A_0 is the activity prior to the irradiation and K is a constant. At low LET (X-ray, γ-ray or high energy electrons), K has the units of volume (Lea, 1955; Dertinger and Jung, 1955; Andrews, 1974). Adopting the empirically determined value of the energy requirement for a primary ionization (66 eV) and the density of protein, the following relationship will be driven from equation (1)

$$Mr = \frac{6.4 \times 10^{11}}{D_{37}(\text{in rads})} \qquad (2)$$

where Mr is molecular weight and D_{37} is the dose responsible for reducing the measured activity to 37% of that found in the unexposed controls. The molecular weight, therefore, will be easily calculated by determining D_{37} through a functional assay of biological system.

—Target Size of $(Na^+ + K^+)$-ATPase and Na^+, K^+ Pump of Human Erythrocytes—

=국문요약=

사람 적혈구막의 $(Na^+ + K^+)$-ATPase/Na^+, K^+ Pump의 Target Size

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하 종 식

$(Na^+ + K^+)$-ATPase은 α와 β의 두 subunits로 구성되어 있으며, 분자량이 약 300,000 daltons 정도되는 것으로 보아 $\alpha_2\beta_2$의 형태로 존재할 것으로 알려져 왔다. 한편, 사람 적혈구막에 있는 Na^+, K^+ pump는 glycolytic enzymes과 complex를 이루고 있으리라는 보고도 있다.

우리는 이 실험에서 in situ 상태의 사람 적혈구막$(Na^+ + K^+)$-ATPase의 분자량을 측정하기 위하여, 소위 말하는 "target theory"를 radiation에 의한 ouabain sensitive한 Na^+이동과, intact한 cells과 ghosts에서의 ATP 가수분해능력의 inactivation data에 적용하였다. Intact한 cells은 cryoprotective agent의 존재하에서, ghosts는 직접적으로 액화질소의 용기속에 담고 온도를 −45°C에서 −50°C로 유지시키면서 1.5 MeV의 electron beam으로 조사한 후에 pump의 기능내지 효소의 활성도를 측정하여 radiation에 따르는 inactivation의 정도를 측정하였다. 이들 활성도는 radiation의 양에 따라 simple exponential function으로 inactivated되었으며, 이로부터 radiation sensitive volume(target size)를 계산하였다. Target size는 intact한 cells을 사용하였을 경우$(Na^+ + K^+)$-ATPase나 Na^+, K^+ pump 모두 600,000 daltons으로 계산되었으며, 이 값은 만약 cells을 strophanthidin으로 먼저 처치하고 측정하면 약 325,000 daltons으로 감소하였다. Ghosts를 사용했을 경우에도$(Na^+ + K^+)$-ATPase의 target size는 역시 약 325,000 daltons이었다.

이상의 결과로 미루어 보아 intact한 cells에서는 $(Na^+ + K^+)$-ATPase/Na^+, K^+ pump가 $(\alpha\beta)_2$의 dimer 상태로 존재하거나 혹은 $(\alpha\beta)_2$의 monomer에 glycolytic enzymes과 같은 다른 enzymes이 붙어 functional한 구조를 이루고 있는 것이 아닌가 사료된다. 또한 실험성적은 이러한 dimeric association 혹은 heterocomplex association은 ghost를 만드는 과정에서나 strophanthidin의 처치로 부서질 수 있음을 암시하고 있다.