MOLECULAR WEIGHT OF NA, K-ATPASE APPROXIMATED BY THE RADIATION INACTIVATION METHOD

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The Na, K-ATPase has not yet been purified as a single molecular species, mainly because of its insolubility. Nevertheless, an approximate molecular weight of the dried enzyme can be obtained by the radiation inactivation technique introduced by Hutchinson and Pollard (1961). Using this procedure Kepner and Macey (1966) reported a value of about one million as the molecular weight for the ATPase of freeze-dried human erythrocyte ghosts. Their preparation contained both the ouabain sensitive and insensitive ATPase. A re-investigation of this problem seemed worthwhile in that our preparation of Na, K-ATPase from brain microsomes is highly specific, essentially free of the ouabain-insensitive enzyme (Nakao et al., 1965). This preparation showed that the phosphorylated protein, obtained in a reaction with ATP^{32} requiring both Na and Mg ions, consisted of a single chemical species as indicated by the time course of hydrolysis and by its sensitivity towards hydroxylamine. In this report we have estimated the target molecular weight of the enzyme involved in the phosphorylation reaction with ATP and in the overall hydrolysis of ATP.

MATERIALS AND METHODS

Partially purified Na, K-sensitive ATPase was obtained from pig brain microsomes by NaI-treatment which was previously reported (Nakao et al., 1965).

The enzyme suspension containing 0.4 mg protein in 50 µl of 0.005 M EDTA was taken into a flat-bottomed test tube (13mm X 30mm), frozen and dried. While kept in dry ice, the preparation covered with a plastic stopper was irradiated by 6 MeV electron beam produced by the linear accelerator (Varian, model V-7705) at a dose rate of 10^6rad/min. Dosimetry was performed by the Fricke's solution in vessels of the same size (Fricke,1966). After irradiation the protein was suspended in 1 ml of 0.25 M sucrose solution containing 0.005 M Tris buffer pH 7.4 and thoroughly mixed. Two aliquots of 50 µl and one of 0.7 ml of the suspension were taken for the determination of ATP hydrolysis and phosphorylation, respectively.

The assay method of ATPase was described previously (Nakao et al., 1963). The amount of phosphorylation was determined according to the previous paper with the following slight modifications. To a mixture containing enzyme, 140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris HCl buffer pH 7.4 in a total volume of 0.9 ml, 100 µl of ATP^{32} X10^{-4}M(13cpm./µµmole) was added and incubated in an ice bath under constant stirring for 15 seconds. Then, 5 ml of cold 10% TCA was added rapidly using a syringe. The mixture was centrifuged at 20,000 g and the residue was washed with 5 ml of cold 10% TCA containing carrier 0.2mM ATP and 10 mM inorganic phosphate. The residue was taken into a planchet with a small amount of 2% sodium dodecyl sulfate (SDS) for counting.

DISCUSSIONS

The two-fold discrepancy in the molecular weights estimated by Kepner and Macey's result and by the present authors may be due to differences in the enzyme preparations. Kepner and Macey (1966) obtained the activity of the Na-K ATPase of the human red-cell membrane from the difference in ATPase activity observed in the presence and absence of ouabain. The Na-K insensitive ATPase activity is labile towards freezing and thawing (Tashima et al., 1965) and thus the activity may have varied from experiment to experiment. On the other hand, our partially purified pig brain enzyme is highly specific for

sodium and potassium ions, as demonstrated by the fact that the ouabain completely inhibits the ATPase activity both before and after irradiation.

Since Post et al. (1965) proposed a labile phosphorylated intermediate theory, the possibility whether the phosphorylated enzyme involves an acyl phosphate has been extensively examined (Nagano et al., 1965, Hokin et al., 1965). There are some contradictory results(Schoner et al., 1966, Chignell et al., 1966), but more recent investigations seem to support this possibility (Nagano et al., 1967, Kanazawa et al., 1967, Bader et al., 1967). At any rate, the phosphorylated intermediate obtained in the present study is probably closely related to the true intermediate, although the exact nature of the intermediate is still unknown.

The target size of the phosphorylation unit appears much smaller than that of the overall reaction. Assuming that only one enzyme is involved for both reactions, one obvious explanation is that the ATPase consists of subunits. Since the molecular weight of the phosphorylation unit of 30×10^5 is about half as large as that of the overall reaction, the enzyme may consists of two subunits or a several subunits of heterogenous size.

RESULTS

The inactivation curve of ATP hydrolysis as indicated in Figure 1 corresponds with a single exponential predicted from the one-hit target theory, as described by Kepner and Macey (1966). All samples after irradiation at each dose were inhibited completely by 10^{-5}M ouabain.

As shown in Figure 2, the inactivation of the phosphorylation reaction by irradiation also follows a single exponential curve. The level of intermediate recovered should not be taken as the rate of phosphorylation, but rather as the total amount of active enzyme which had been phosphorylated.

From these radiation inactivation curves the target molecular weight can be estimated for the enzyme involved in both the phosphorylation and hydrolytic reaction.

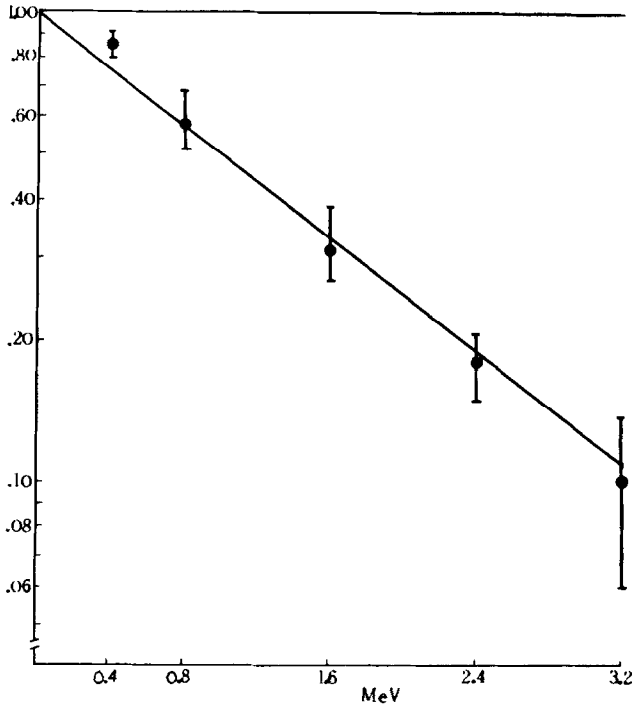

Fig. 1 ATPase activity after irradiation

Mean values and standard deviations of 8 experiments are shown in the figure except points at 0.4 and 3.2 MeV (4 experiments).

Ordinate: relative activity Condition: see text.

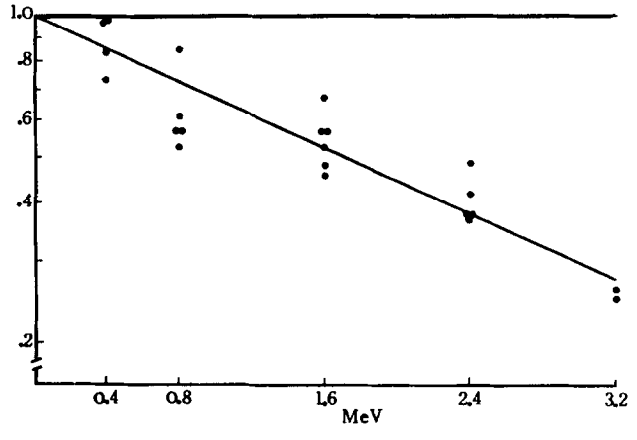

Fig. 2 Amount of phosphorylation by ATP^{32} after irradiation

Ordinate:relative amount of phosphorylation

Condition:see text.

Analyzing the data presented in Figure 1 and 2 by the least square procedure, the dose required for 37% inactivation was determined. These values (D37) of 1.44 and 2.44 MeV, was then used to calculate the molecular weights according to Hutchinson's equation, assuming the energy released per inactivating event is 75 eV (Hutchinson and Pollard 1961). The results of these calculations are shown in Table 1.

Table 1 Calculations from the data of Figures 1 and 2.

	ATP hydrolysis	Phosphorylation
a*	0.3230±0.0403(95% confidence limit)	0.1726±0.0718(95% confidence limit)
b*	0.0335±0.0414(95% confidence limit)	1.9887±0.0747(95% confidence limit)
D37	1.44	2.44
Molecular weight**	50×10^4	29.3×10^4

* a and b are defined as follows

$$y = ax + b,$$

where x and y represent the dose (Mrad) and the logarithm of the fraction of Na, K-sensitive ATPase activity after irradiation respectively.

** M.W. = $\frac{0.72 \times 10^{12}}{D37}$

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