SEPARATION OF TWO ADENOSINE TRIPHOSPHATASES FROM ERYTHROCYTE MEMBRANE

Toshiko Nakao, Kei Nagano, Kenji Adachi, and Makoto Nakao

Department of Biochemistry, Tokyo Medical and Dental University
School of Medicine, Yushima, Bunkyo, Tokyo, Japan

Received October 7, 1963

Post et al. (1960), Dunham and Glynn (1961), and Whittam (1962) have presented evidence that ouabain-sensitive adenosine triphosphatase (ATPase) in erythrocytes participates in the active transport of sodium and potassium ion in erythrocytes.

In previous papers, our collaborators and some of the present authors (M. Nakao et al., 1960a, 1960b, and 1961) reported on the reversible shape transformation of erythrocytes depending upon ATP level inside the cells. These results may indicate the presence of an ATPase which controls shape transformation of erythrocytes. Onishi (1962) suggested the presence of actin-like and myosin-like proteins in erythrocytes in his preliminary experiments. Furthermore, evidence that ouabain does inhibit the ATPase activity partially but not completely (Dunham and Glynn, 1961), strongly suggests the presence of a different ATPase (i.e. ouabain-insensitive ATPase) other than the ouabain-sensitive ATPase. However, it has not been proved whether the ouabain-sensitive and ouabain-insensitive components of the ATPase are two separate enzymes or not (Dunham and Glynn, 1961). In the present paper, separation of two ATPases from erythrocyte membranes and some of their properties will be presented.

METHODS

Assay of ATPase activity: The reaction mixture containing, in a final volume of 1 ml., 20 μmoles of cysteine, 80 μmoles of NaCl, 40 μmoles of KCl, 3 μmoles of ATP, 5 μmoles of magnesium ion and 40 μmoles of Tris buffer at pH 7.5 was incubated for 20 to 60 min. Veronal buffer

and acetate buffer were also used, however, no essential difference was observed among them. After incubation, 0.03 ml. of 5% albumin solution was added to the reaction mixture prior to deproteinization with 2 ml. of cold trichloroacetic acid. Orthophosphate was determined by the method of Fiske and Subbarow (1925). Protein was determined by the method of Lowry et al., (1951)

Partial purification of enzymes: Human erythrocyte ATPase was prepared according to the method of Caffrey et al. (1956). As Post et al. (1960) pointed out, this preparation yielded insoluble ATPase, which under the microscope appeared to be a part of the membrane. In some experiments, a crude ATPase (erythrocyte membrane) was prepared according to the method of Post et al. (1960). However the results were essentially the same. The erythrocyte membrane was centrifuged four times with 0.005 M Tris buffer, pH 8.0. The resulting pellet was suspended in physiological saline and subjected to further fractionation with sodium iodide to obtain 0.6 M and 2 M fractions. Human erythrocytes used for each experiment were approximately 30 ml in packed volume.

a) 0.6 M fraction: To 15 ml of the pellet suspension, 5 ml of NaI solution mixture, containing 1.8 ml of 6.6 M NaI (pH 8.0 adjusted with Tris buffer), 1 ml of 50 mM cysteine, and 2 ml of 40 mM ATP, was added, the final concentration of NaI being 0.6 M. After the suspension was kept at 0°C for 15 min., it was centrifuged at 20,000g for 20 min. The supernatant solution was diluted with 20 volumes of cold water and recentrifuged. The small amount of precipitate obtained was washed twice with cold water by centrifugation, and suspended in 2 to 3 ml of cold water for ATPase assay.

b) 2 M fraction: The first residue of 0.6 M NaI extraction was suspended in 10 ml of cold water, and retreated with 10 ml of NaI solution mixture, containing 6 ml of 6.6 M NaI (pH 8.0, adjusted with Tris buffer), 1 ml of 50 mM cysteine, 1 ml of 100 mM magnesium chloride, 2 ml of 40 mM ATP, and 20 µl of 100 mM EDTA, for 10 min. at 0°C. This suspension was

centrifuged at 20,000g for 20 min., and the resultant supernatant solution was diluted with cold water to the final concentration of 0.6 M NaI solution. It was again centrifuged at 20,000g for 20 min. at 0°C, yielding a relatively large amount of precipitate. The precipitate was washed twice with cold water by centrifugation and suspended in 5 to 10 ml of cold water for the enzyme assay.

RESULTS AND DISCUSSION

Results of a typical purification are shown in Table I.

Ouabain-insensitive ATPase: ATPase activity of 0.6 M fraction is quite insensitive to ouabain (1×10^{-5} M) in the presence or absence of Na^+ and K^+. As shown in Table II, however, the addition of either sodium or potassium ion activates the reaction slightly in contrast to the 2 M fraction, which is not activated by either sodium or potassium ion. The pH-activity curve of 0.6 M fraction shows a rather sharp peak at about pH 7.2, while the pH optimum of 2 M fraction enzyme is found to be about 7.7 in contrast to the optimum pH of 7.2 - 7.6 of untreated membrane (Caffrey et al., 1956). Calcium (5×10^{-4} M) or magnesium (5×10^{-3} M) ion activates the reaction approximately 70% and 130% respectively. In the presence of both ions 170% increase of activity is observed.

TABLE I PURIFICATION DATA

Exp. No.	Specific activity (μmoles iP/hr/mg protein)		Percent recovery of total activity
	Ouabain(-)	Ouabain(+)	
1. Membranes	0.87	0.80	100
0.6 M fraction	0.19	0.19	2.4
2.0 M fraction	0.26	0.039	10.6
2. Membranes	0.91	0.80	100
0.6 M fraction	2.03	2.03	8.6
3. Membranes	1.06	0.81	100
2.0 M fraction	0.81	0.08	7.0

Enzyme suspension (0.1 ml) was incubated with the reaction mixture, as described in text, for 20 min. at 37°C.

TABLE II EFFECT OF SODIUM AND POTASSIUM ION, AND OUABAIN ON THE ACTIVITY OF TWO ATPASES FROM ERYTHROCYTE MEMBRANE

Addition	2 M fraction (μmole iP/hr/mg protein)		0.6 M fraction (μmole iP/hr/mg protein)	
	Ouabain(-)	Ouabain(+)	Ouabain(-)	Ouabain(+)
None	0.06	0.05	0.45	0.44
80 mM Na^+	0.06	0.05	0.62*	0.60*
40 mM K^+	0.07	0.07	0.55	0.57
80 mM Na^++40 mM K^+	0.29	0.05	0.75*	0.75*

The conditions of incubation were the same as for Table I, except for the addition of Na^+ and K^+ as specified in the table. (* Na 120 mM)

Ouabain-sensitive ATPase: When the membrane preparation or the residue of 0.6 M fraction is treated with 2.0 - 2.2 M sodium iodide, the activity is greatly decreased, but the enzyme becomes extremely sensitive to ouabain 1×10^{-5} M. The addition of both sodium and potassium ions simultaneously increases the activity several times, and, in some cases, more than ten times, although the addition of only one of these ions has no effect on the activity. When magnesium ion is omitted, the activity is reduced to below one-tenth. Various concentrations of calcium ion inhibit the activity, but sensitivity to ouabain is eliminated at 5×10^{-4} M calcium concentration in the presence of 5×10^{-3} M of magnesium ion.

These results may be taken to indicate the presence at least two different ATPases in the erythrocyte membrane.

As far as the effectiveness of the addition of sodium or potassium ion, respectively, to red cell membrane is concerned, some conflicting results have been reported. Post et al. (1960) observed no activation in the presence of either sodium or potassium ion, while a slight activation with potassium ion was described by Dunham and Glynn (1961), and a rather marked acceleration by the addition of either sodium or potassium ion was reported by Askari et al., (1963). Such a difference might be due to varying content of 0.6 M fraction in the membrane preparations.

REFERENCES

Askari, A., and Fratau, J. C., Biochem. Biophys. Acta, 71, 232 (1963).

Caffrey, R. W., Tremblay, A., Gabrio, B. W. and Huennekens, F. M., J. Biol. Chem., 223, 1 (1956).

Dunham, E. T. and Glynn, I. M., J. Physiol., 156, 274 (1961).

Fiske, C. H., and Subbarow, Y., J. Biol. Chem., 66, 375 (1925).

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

Nakao, M., Nakao, T., Tachibana, M., and Yoshikawa, H., J. Biochem. (Japan), 47, 661 (1960a).

Nakao, M., Nakao, T., and Yamazoe, S., Nature, 187, 945 (1960b).

Nakao, M., Nakao, T., Yamazoe, S., and Yoshikawa, H., J. Biochem. (Japan), 49, 487 (1961).

Onishi, T., J. Biochem. (Japan), 52, 307 (1962).

Post, R. C., Merritt, C. R., Kinsolving, C. R. R. and Albright, C. D., J. Biol. Chem., 235, 1796 (1960).

Whittam, R., Biochem. J., 84, 110 (1962).