THE PHOSPHORYLATION AND ISOLATION OF TWO ERYTHROCYTE MEMBRANE PROTEINS IN VITRO*

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SUMMARY

Two proteins of bovine erythrocyte ghost membrane have been phosphorylated with γ-^{32}P-ATP and isolated by SDS polyacrylamide gel electrophoresis. One of the two proteins (MW 98,000) has been identified here as the phosphorylated intermediate of the $Na^+ + K^+$ activated ATPase. The other phosphorylated protein (MW 220,000) is apparently unrelated to the Na-K ATPase, but may be involved in other energy requiring membrane processes.

Sodium and potassium transport across membranes is mediated by a membrane bound, $Na^+ + K^+$ activated, ATPase (Na-K ATPase) (E.C. 3.6.1.3) (1, 2). Erythrocyte ghost membranes have been shown to incorporate ^{32}P from γ-^{32}P-ATP and some of this incorporation is apparently related to the Na-K activated ATPase (3, 4). However, a significant portion of the membrane phosphorylation appears not to be related to the Na-K ATPase enzyme (5) and, as yet, none of the phosphorylated components has been identified or isolated from erythrocyte membranes. This report describes the identification and characterization of two membrane proteins from bovine red cell ghosts that are readily phosphorylated with γ-^{32}P-ATP. The smaller of these two phosphorylated proteins is identified here as the phosphorylated intermediate of the Na-K ATPase enzyme while

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the larger phosphorylated protein is apparently related to membrane structural proteins identified previously (6, 7).

MATERIALS AND METHODS

Erythrocyte ghost membranes were prepared from fresh bovine blood by a modification of the procedure described by Mazia and Ruby (7). Heparinized whole blood (250 ml) was centrifuged at 15,000 xg for 5 min and the pellet was washed three times in 0.3 M dextrose. The packed red cells were lysed by suspending in 250 ml of distilled water containing 0.1% Triton X-100 (Cal Biochem) and 10 gm of mixed ionic exchange resin (501 AG-X8, Biorad. Labs). The cell suspension at approximately pH 7.4 was stirred 3 min, decanted from the resin, and then titrated to pH 6.7 with HCl. The suspension was centrifuged for 25 min at 37,000 xg. The resulting pellet was washed once in 20 mM ammonium acetate adjusted to pH 6.7 with HCl, and then three times in 20 mM ammonium acetate, pH 7.4. The pellet was then washed three times in 50 mM Hepes buffer (Sigma) adjusted to pH 7.4 with NaOH. The membranes were phosphorylated as described in the results. Prior to disc gel electrophoresis the phosphorylated membrane material was washed twice in 15 mM NaCl, and then dissolved in 3% sodium dodecyl sulfate (SDS) containing 0.01 M sodium phosphate buffer, pH 7.1, and 1% 2-mercaptoethanol. The phosphorylated membrane proteins were identified by polyacrylamide gel electrophoresis by methods similar to those of Martonosi (8) and Shapiro (9).

γ-^{32}P-ATP was prepared from a modified procedure of Glynn and Chappell (10). Protein concentrations were determined by the procedure of Zamenhof using crystalline egg albumin as the standard (11).

RESULTS

As may be seen from Fig. 1 only two of the several proteins stained with Coomassie brilliant blue are labeled with ^{32}P. Fig. 1 is representative of nine such figures obtained from five separate experiments. Assuming the intensity of the stain is proportional to the amount of protein then these labeled moieties are major membrane components.

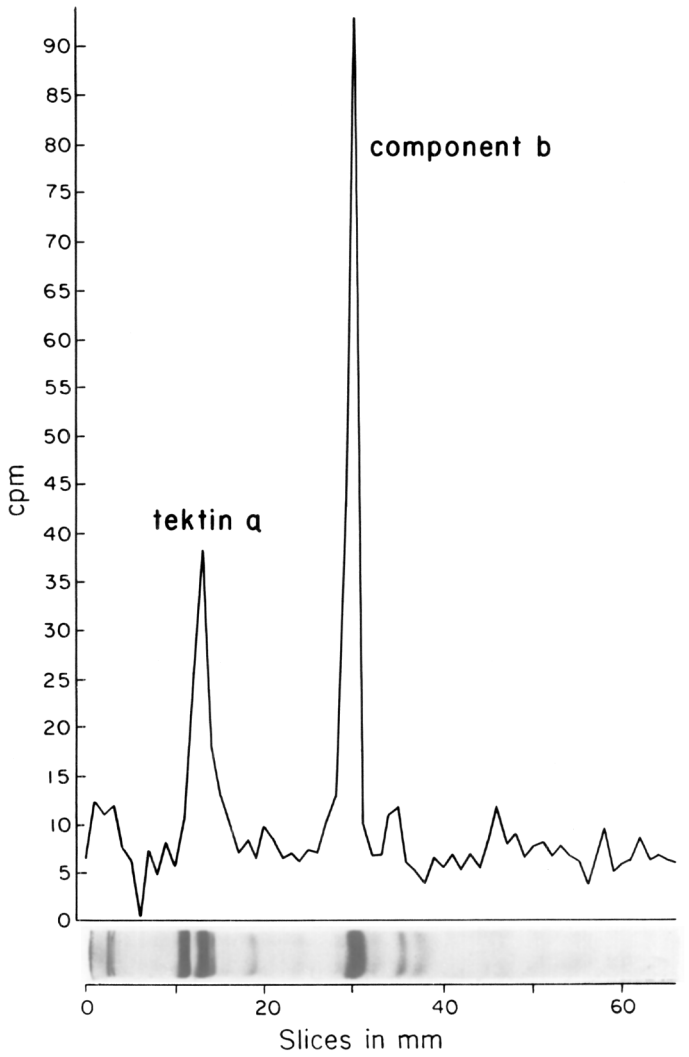

Fig. 1. The suspension of washed erythrocyte ghosts (see Materials and Methods), containing 1 mg of protein, was frozen and thawed twice and then incubated 1 hour at 37°C in a volume of 0.25 ml with the following (final concentrations): 50 mM Hepes-Tris buffer pH 7.4; 2.5 mM $MgCl_2$; 62.5 μM ATP containing 10^6 cpm γ-^{32}P-ATP; and 2.5 mM NaCl. The reaction was stopped by washing twice with 50 mM NaCl previously made to pH 7.4 with Tris. The washed ghost preparation was then dissolved by adding the following (final concentrations): 3% SDS; 0.01 M sodium phosphate buffer, pH 7.1; 1% 2-mercaptoethanol; and 10% glycerol. The preparation was incubated 15 min at 37°C in this solution prior to electrophoresis. Then 20 μg of the dissolved phosphorylated material was layered on 7 cm columns of polyacrylamide gel containing 5% acrylamide monomer, 10% glycerol, 0.1% SDS, 0.1 M sodium phosphate buffer at pH 7.1. The preparations, in duplicate, were electrophoresed for 2.5 hours at 10 ma per tube. The duplicate gels were removed; one was stained in 1% Coomassie blue and the other was frozen and sliced at 1 mm intervals. The gel slices were then treated with NCS solubilizer (Amersham-Searle) and counted. The counts per min were corrected for a background of 10 cpm. The gel was electrophoresed from left to right. The stained and sliced gels were corrected for shrinkage and expansion.

The phosphorylated membrane protein, labeled component b in Fig. 1, is a protein that is phosphorylated by γ-^{32}P-ATP in a manner characteristic of the Na-K activated ATPase intermediate. Table I shows the requirements for phosphorylation of the membrane proteins. It may be seen that the phosphorylation of component b is activated by Na^+, and is inhibited when both K^+ and Na^+ are present. Ca^{2+} cannot substitute for Mg^{2+} in this labeling reaction. Additionally, when the labeling reaction is followed by a reaction with un-

TABLE I

Conditions	cpm in tektin α	cpm in component b
1. Complete	33	102
2. $-Na^+$	30	22
3. $+K^+$	35	36
4. Unlabeled ATP "chase"	30	36
5. $-Mg^{2+}$	0	0
6. $+Ca^{2+}$, $(-Mg^{2+})$	0	0
7. $+^{32}P_i$, $(-^{32}$P-ATP)	0	0
8. Heated to 65°C, 15 min	0	0

All of the above variables were tested twice and in each experiment all six variables were tested simultaneously. The cpm values listed are averages from two experiments and were taken from the gel slices containing only tektin α or component b. The complete condition as listed above represents samples that were run under the same conditions as those used in Fig. 1. Samples were added to or omitted from the same incubation medium that was used in Fig. 1. Calcium was added at the same concentration as was the magnesium that was used in the complete condition. Similarly $^{32}P_i$ (10^6 cpm) was added at the same ATP concentration used in the complete medium. Under the condition where both Na^+ and K^+ were present, their concentrations were 33 mM each. The unlabeled ATP was added at the end of the initial incubation at a final concentration of 2 mM and then reincubated for 15 min. In each of the above experiments the samples were electrophoresed and counted as indicated in the legend to Fig. 1. Heat lability of the labeling reaction was tested by heating the membranes prior to incubation in the labeling reaction.

labeled ATP the amount of labeled intermediate is diminished. No incorporation is observed when γ-^{32}P-ATP is replaced with $^{32}P_i$. The effect of the cardiac glycoside, ouabain, on the labeling of the phosphorylated intermediate is to stimulate phosphorylation in certain preparations, and to inhibit it in others. Consequently, its effect on the labeling reaction remains unclear.

Of the two large molecular weight proteins seen at the left (top) of the gel in Fig. 1 only one is phosphorylated by γ-^{32}P-ATP. These two large molecular weight polypeptides have been characterized elsewhere and have been called tektin α and α' (6, 7). It is apparent from Table I that the phosphorylation of tektin α protein is not influenced by the conditions that characterize the labeling of the Na-K ATPase intermediate. The uniform labeling of tektin α protein may thus be considered a control against which the variations in the labeling of component b can be compared.

The molecular weights of the labeled proteins were measured by electro-

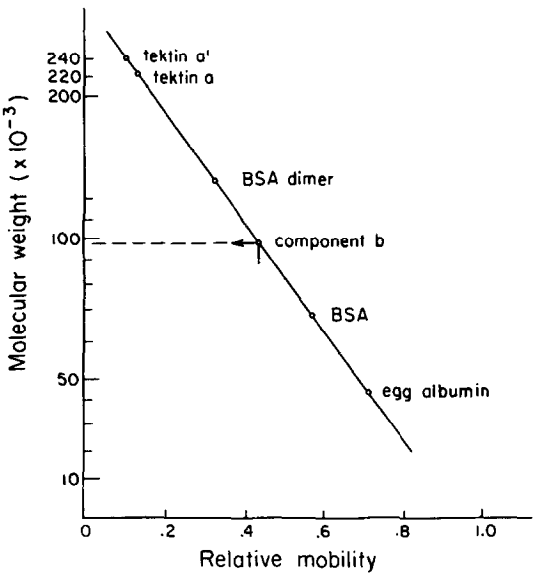

Fig. 2. The molecular weights of the phosphorylated proteins, component b and tektin α, were measured using bovine serum albumin (BSA) (Sigma), its dimer and egg albumin (Sigma) as molecular weight standards. The protein mobilities were measured relative to the tracking dye.

phoresis in SDS polyacrylamide gels using egg albumin and bovine serum albumin and its dimer as molecular weight standards. It may be seen in Fig. 2 that the molecular weight of the phosphorylated Na-K ATPase intermediate, component b, is approximately 98,000 and the molecular weight of the other phosphorylated protein, tektin α, is approximately 220,000. There was little or no variation in molecular weight measurements of the labeled proteins when they were electrophoresed in SDS gels of varying gel concentration.

DISCUSSION

Several investigators have attempted to identify a phosphorylated intermediate of the Na-K ATPase in red cell membranes (3, 4, 5). The method employed in these cases has been to phosphorylate the red cell membrane with ^{32}P from γ-^{32}P-ATP under appropriate conditions, and then to count the labeled phosphorylated membrane residue after extensive washes. These attempts to label the Na-K ATPase intermediate were obscured by the relatively large amount of stable ^{32}P material formed during incubation of ghosts with ^{32}P-ATP. Both Heinz (5) and Blostein (3) report difficulty in correlating the amount of phosphorylation of red cell ghosts with the observed ATPase activity. In the most recent report by Blostein (4) the Na-K ATPase intermediate has been identified, but only a portion of the total labeling is influenced by sodium and potassium. The evidence presented in this investigation suggests an explanation for the previous problems in identifying the Na-K ATPase. From Fig. 1 and Table I it may be seen that in addition to a residual portion of component b being labeled in a manner that did not react like the Na-K ATPase intermediate, a significant portion of the total label is incorporated into another protein.

Some recent evidence suggests that component b chromatographed in the gel system used in this investigation is heterogeneous. The work of Bretscher (12, 13) and others (14-16) using similar 5% acrylamide gel systems have shown that the protein identified in the present investigation as component b

consists of two proteins, one of which is a glycoprotein that is possibly located on the outside of the erythrocyte ghost (12). The molecular weight of the glycoprotein is uncertain, but is generally thought to be considerably less than 100,000 (17). It has been identified by staining with Schiff's reagent, and it is known not to migrate according to molecular weight in SDS polyacrylamide gels. The other protein is not a glycoprotein and therefore migrates on SDS gels according to its molecular weight (13). The Na-K ATPase enzyme intermediate identified in this investigation as component b is probably not related to the glycoprotein because the labeled protein does migrate according to its molecular weight. Additionally, the Na-K ATPase enzyme in relatively pure form contains less than 3% carbohydrate (18).

It has been suggested by Bretscher (13) that the protein which does not contain carbohydrate spans the thickness of the red cell membrane. It is possible that the protein identified in the present investigation as the Na-K ATPase intermediate may be this protein. It is indeed possible that the enzyme, responsible for Na^+ and K^+ transport across the membranes, may itself span the red cell membrane.

The molecular weight of the Na-K ATPase phosphorylated intermediate has been measured in intracellular membrane preparations from several sources such as brain microsomes (19) and renal tissue (20). The molecular weight of a purified enzyme preparation from bovine brain microsomes was reported to be 94,000 (18), which agrees well with the molecular weight of 98,000 identified in this investigation.

The observation that tektin α protein is phosphorylated is interesting from the standpoint of trying to speculate a function for the tektin proteins. The fact that one of the two tektin polypeptides is enzymatically labeled with ^{32}P from γ-^{32}P-ATP suggests that possibly the protein is operating in some energy related biochemical process: perhaps erythrocyte shape maintenance.

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