Chemical Characterization and Pronase Susceptibility of the Na:K Pump-Associated Phosphoprotein of Human Red Blood Cells

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ABSTRACT The phosphoproteins formed by incubation of red cell ghosts with [γ-^{32}P]ATP in the presence of Mg and Na + Mg have been characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The ^{32}P-labeled phosphoprotein was seen as a single peak confined to the region of the diffuse 90,000 dalton polypeptide band; labeling with Na + Mg considerably increased the quantity of ^{32}P-phosphoprotein contained in this band relative to labeling with Mg alone. Treatment of intact cells with Pronase known to partially hydrolyze the glycoproteins and the 90,000 daltons polypeptide did not change either the amount or the position of the ^{32}P-phosphoprotein present in the gels. The molecular weight of the ^{32}P-phosphoprotein is estimated to be 103,000. Pronase treatment of intact cells also did not significantly alter any of the transport parameters of the membrane such as the K pump flux, ouabain binding, or Na,K-ATPase. In contrast, treatment of ghosts with Pronase not only resulted in drastic alteration of the transport parameters but also inhibited the formation of the phosphoprotein under all conditions. Thus, while the Na:K pump is not intrinsically resistant to Pronase, those elements of the pump which are susceptible are not accessible from the outside of the cell. Further, SDS-polyacrylamide gel electrophoresis after Pronase treatment of intact cells results in a substantial increase in the purification of the phosphoprotein relative to that which was previously possible in ghosts.

INTRODUCTION

Working with a variety of microsomal preparations, Post and co-workers (9, 10, 29, 30) and others (1, 31, 32) have described phosphorylated proteins whose characteristics suggest that they serve as intermediates in the Na,K-

ATPase reaction. Blostein (6, 7, 8) has found a similar labile phosphoprotein in red cell ghosts which have been exposed to low concentrations of ATP in the presence of Mg and Na. This protein is present in very small amounts (less than 1 pmol/mg ghost protein) commensurate with the number of ouabain binding sites in human red cells (19). Its response to various ions and inhibitors indicates that it is probably associated with the Na:K pump.

The object of the present study has been to characterize and to partially purify this phosphoprotein primarily by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In order to gain information regarding the identity and location of the phosphoprotein its mobility on acrylamide gels has been measured following Pronase treatment of ghosts or intact cells. In addition other functional parameters of the Na:K pump, such as ouabain-sensitive K influx, Na,K-ATPase activity, and ouabain binding, have been studied in cells which were exposed to externally added Pronase. A brief account of this work has been previously presented (21).

METHODS

Packed Red Cell Preparation

Blood was obtained from normal adults by venipuncture and put into flasks containing heparin (0.15 mg/ml). The blood was centrifuged at 20,000 × *g* and the buffy coat removed. The cells were then washed three times in unbuffered 165 mM NaCl and the packed cells used as indicated below.

Red Cell Ghost Preparation

Hemoglobin-free ghosts were prepared by the method of Hoffman and Ryan (see Parker and Hoffman, 27) from unwashed packed cells (20, 000 × *g*) and were stored in a solution containing 15.3 mM NaCl, 1.7 mM Tris, and 0.1 mM EDTA (pH 7.5) at −20°C until used.

^{32}P-Labeling of Ghosts

In general the procedure outlined by Blostein (7) was followed. Frozen ghosts were thawed and washed three times in 17 mM Tris (pH 7.5). The ghosts were resuspended in 10 mM Tris (pH 7.5) and were added to a medium containing (final concentrations) 2 μM [γ-^{32}P]ATP (approximately 10 Ci/mmol, kindly supplied by Dr. Paul Greengard), 12 μM $MgCl_2$, and either 50 mM sodium chloride or choline chloride (Hoffman-Taff, Inc., West Alexandria, Ohio), and 10 mM KCl, when used, to make a 20% suspension (about 5 mg ghost protein/ml or about 7–8 × 10^{12} ghosts/ml). After 15 s at 0°C the reaction was stopped by the addition of 5% trichloroacetic acid (TCA) containing 1 mM orthophosphate (P_i) and 0.1 mM ATP. The precipitate was washed five times with this solution, solubilized in 2.5% SDS and stored in a refrigerator at 4–6°C.

The ^{32}P-labeled proteins formed when Mg alone is present in the labeling medium and the additional phosphoprotein resulting from Na addition will be referred to as the Mg-phosphoprotein and Na-phosphoprotein, respectively.

Pronase Treatment

Packed red cells were suspended at a 45–50% hematocrit in a medium containing 149 mM NaCl, 5 mM $CaCl_2$, and 17 mM Tris (pH 7.4). Pronase (0.2 mg/ml suspension [13] Calbiochem. B grade, Lot 502117, Calbiochem, San Diego, Calif., 45,000 P.U.K./g) was added and the suspension was incubated at 37°C for 30–40 min. The suspension was then centrifuged at 20,000 × *g* and the cells were washed six times with 5 vol of ice-cold isotonic saline. Control cells were treated according to the same procedure except that Pronase was absent. Ghosts were prepared from the control and Pronase treated cells by the procedure outlined above.

Some of the control ghosts were treated with Pronase as follows: The frozen ghosts were thawed and suspended in a medium (subsequently called "Solution A") which contained 15.3 mM NaCl, 1.7 mM Tris, and 0.1 mM EDTA (pH 7.5). After centrifugation at 27,000 × *g*, the packed ghosts were resuspended in Solution A (to which 5 mM $CaCl_2$ had been added) at 37°C. Pronase (0.2 mg/ml) was added and the suspension was incubated at 37°C for 30 min, centrifuged at 27,000 × *g*, and the ghosts were washed twice with 15 vol of Solution A. The ghosts were then labeled with ^{32}P as described above. Controls were treated in an identical manner except without Pronase.

Hydroxylamine Treatment

In accordance with a procedure of Blostein (7) the ^{32}P-labeled ghosts, washed once with 0.01 N HCl, were suspended (final concentration 0.5 mg protein/ml) in 0.08 N acetate (pH 5.3) containing 0.6 N freshly dissolved hydroxylamine hydrochloride obtained from Matheson, Coleman, and Bell, Norwood, Ohio). For the control hydroxylamine was replaced by 0.6 N NaCl. Ghosts were exposed to hydroxylamine for 10 min at 23°C after which 5% TCA was added and the suspension was chilled and centrifuged at 10,000 × *g*. The supernatant was saved for counting. The precipitate was washed three times with 5% TCA containing 1 mM P_i and 0.1 mM ATP and was then solubilized in 2.5% SDS.

SDS-Gel Electrophoresis

The ghost protein was solubilized in 1% SDS according to the method of Fairbanks, et al. (17) except that reduction with dithiothreitol was carried out at pH 7.4. Aliquots containing 50–150 μg protein were applied to 5.6% acrylamide gels and electrophoresis was carried out for 3.5 h at 4 mA/gel. Water at 10–15°C was circulated through the electrophoresis apparatus (Buchler Instruments Div. Nuclear-Chicago Corp., Fort Lee, N.J.).

Some of the gels were stained for protein with Coomassie Blue (17) while others were sliced transversely. The slices were put into scintillation vials containing 1 ml of 0.05% SDS. Slices longer than 0.2 mm were crushed into small pieces with a spatula. The vials were capped and incubated at 37°C for 20–48 h after which 10 ml of triton toluene scintillation fluid (1 liter Triton X-100, 2 liter toluene, 16.9 g Omnifluor, [New England Nuclear, Boston, Mass.]) were added to each. Under these conditions all of the ^{32}P was leached from the slices since removing the slices from the vials did not affect the count rate. The efficiency, measured by adding a known amount of ^{32}P

to each vial, was high and constant even when slices as long as 2 cm were counted: with 0.2-cm gel the efficiency was 85.1 ± 0.1 % (N = 53) while with 2-cm gel it was 84.3 ± 0.5 % (N = 12). Longitudinal slices of other gels were laid on wet Whatman No. 50 filter paper (Reeve Angel Co., Clifton, N. J.) and dried under vacuum (16, 25). These slices were then autoradiographed on either Kodak SB-54 or RP-54 X-ray film (Eastman Kodak Co., Rochester, N. Y.). After development, the autoradiograms were scanned with a Joyce-Loebl calibrated densitometer (Joyce, Loebl & Co., Inc., Burlington, Mass.).

For the determination of molecular weight, gels were calibrated (15, 34) in each experiment using as standards rabbit muscle phosphorylase A (Nutritional Biochemicals Corp., Cleveland, Ohio), ovalbumin (2 × crystallized, Worthington Biochemical Corp., Freehold, N. J.), β-galactosidase, bovine serum albumin, carbonic anhydrase, rabbit muscle creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.), and γ-globulin (fraction II-Lederle Labs. Division, American Cyanamid Co., Pearl River, N. Y.).

Peptic Hydrolysis and Paper Electrophoresis

Approximately 2 mg of the ^{32}P-labeled protein was washed with 0.01 N HCl. The supernatant was removed and 0.2 ml of a 1-mg/ml suspension of pepsin (Worthington-2630 μm/mg) in 0.01 N HCl was added to the precipitate. The reaction was allowed to proceed for 30 min at 23°C after which the mixture was centrifuged (1,000 *g*) and the supernatant was removed and kept frozen until used (3).

Paper electrophoresis of the peptic digest was carried out on Whatman 3 MM filter paper (16.5 cm × 56 cm) with 7 % formic acid. Carrier P_i (~0.1 μmol) was added to each sample. Electrophoresis was run at 15–20°C for 4 h at 1,000 V and 40 mA. The dried electrophoretograms were autoradiographed on Kodak RP-54 X-ray film.

Potassium Influx

Uptake of potassium was measured by adding a trace amount of ^{42}K (0.05–0.1 μCi/ml) to a 3 % suspension of washed red cells at 37°C in a medium containing 8 mM KCl, 140 mM NaCl, 180 mg % glucose, 20 mg % albumin, buffered with 17 mM glycylglycine at pH 7.4. Aliquots of the suspension were removed at 30 and 90 min, centrifuged, and washed twice with ice-cold isotonic $MgCl_2$. The cells were hemolyzed and the hemolysates were assayed for hemoglobin and ^{43}K radioactivity. Influx was calculated as described by Sachs and Welt (33).

ATPase

ATP hydrolysis was determined by incubating ghosts at 37°C in a medium containing 40 mM NaCl, 20 mM KCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM Tris (pH 7.5), and 0.1 mM [γ-^{32}P]ATP. The reaction was stopped by adding an equal volume of ice-cold 6 % perchloric acid. Appearance of inorganic ^{32}P was measured by removing the ATP with charcoal and counting the resulting supernatant for ^{32}P (14). Protein content of the suspension was determined by the method of Lowry et al. (24) and the results are expressed as nanomoles ATP hydrolyzed per milligram protein per hour.

Ouabain Binding

According to the method of Hoffman (19) ghosts were exposed to approximately 1×10^{-7} M [^{3}H]-ouabain for 30 min at 37°C in a medium containing 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM Tris (pH 7.5), and 1.5 mM ATP. Subsequently the ouabain-sensitive ATPase activity was determined as the difference between the original activity and that in the presence of 10^{-4} M ouabain. The amount of [^{3}H]-ouabain bound was determined by liquid scintillation counting as previously described (19).

Reproducibility of the Results

It should be understood that while the results of single experiments are sometimes presented similar findings were obtained in at least two and in most cases several similar experiments except for the data given in Tables II and III, where only single experiments were performed but in which the results presented are the average of duplicate determinations.

RESULTS

The first series of experiments was directed toward separating the phosphoproteins formed in the presence of Mg or Mg + Na from other red cell membrane proteins by the method of SDS-polyacrylamide gel electrophoresis. The photograph at the top of Fig. 1 shows a gel containing ghost proteins stained with Coomassie Blue. It exhibits the band pattern previously observed in red cell ghosts (17), with approximately 30% of the Coomassie Blue staining material forming a dense diffuse band (region III) whose leading edge corresponds to a molecular weight of about 90,000.

The upper graph in Fig. 1 presents the density trace of an autoradiogram of a gel to which have been added well-washed solubilized ghosts labeled with [γ-^{32}P]ATP in the presence of Mg. The density profile has a single peak which falls within the diffuse band of the 90,000 dalton polypeptide. When 50 mM Na is added as shown in the middle graph no additional peaks of ^{32}P-labeled material are seen but the amount of the phosphorylated protein is increased as indicated by an increase in the area under the peak. When 10 mM K is added together with 50 mM Na as shown in the bottom graph the amount of phosphorylated protein decreases to the same level as in the absence of Na. Quantitation of the number of counts in these peaks by counting sliced gels confirms the results seen in these autoradiograms (Table I); sodium stimulates the phosphorylation and potassium abolishes the stimulation.

It is important to emphasize that the results in Fig. 1 were obtained with ghosts which had been very extensively washed (>6 times at 4°C) in 17 mM Tris at pH 7.5. Ghosts washed only three times in Tris showed variable amounts of an additional phosphorylated protein of higher molecular weight (about 150,000) as shown in Fig. 3. Other experiments described in the

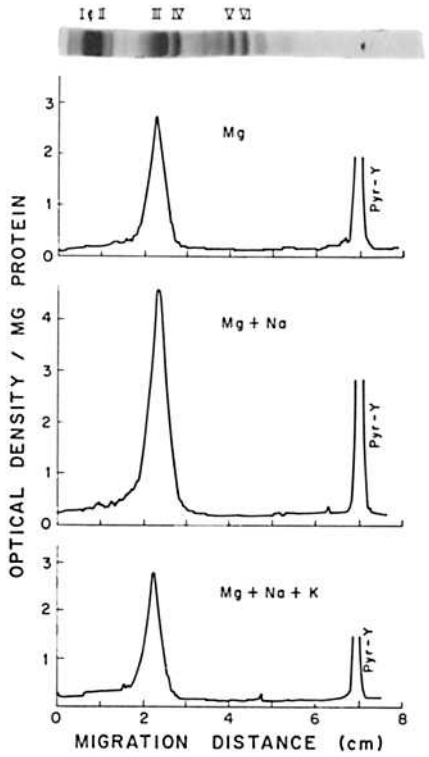

FIGURE 1. SDS-acrylamide gel electrophoresis of phosphorylated ghosts. The photograph at the top shows a gel on which solubilized ghost proteins have been electrophoresed and stained with Coomassie Blue. Bands are labeled as described by Fairbanks et al. (17). The graphs are densitometer traces of autoradiograms of gels on which phosphorylated ghosts have been electrophoresed. The ordinate represents the density of the autoradiogram at a distance from the top of the gel (given by the abscissa) per milligram of ghost protein applied to the gel. The ordinate is proportional to the number of counts of ^{32}P per unit length of gel per milligram ghost protein applied to the gel. From top to bottom the three graphs depict ghosts phosphorylated in the presence of Mg, Mg + Na, and Mg + Na + K. The sharp peak to the right of each trace indicates the position of the Pyronin-Y marker dye. Although the density traces are roughly aligned with the photograph according to the position of the Pyronin-Y, accurate comparison of bands is difficult because of possible stretching of the gels during preparation for autoradiography.

following paper (22) indicate that this second peak can be prevented by more thorough washing and can be restored and greatly increased by adding Ca to the reaction mixture. The calcium dependence suggests that this may reflect the activity of a Ca-ATPase which is separate from the Na,K-ATPase (22).

When the reaction with [γ-^{32}P]ATP was stopped by means other than addition of 5% TCA such as by adding 6% perchloric acid or 5% SDS to the reaction mixture the peak was found in the same region of the gel, indicating

that the phosphorylated protein formed is relatively independent of the means used to terminate the reaction. To see whether the phosphorylation was affected by trace impurities in the [γ-^{32}P]ATP, phosphorylation was measured with ATP at a 50-fold lower specific activity. As shown in Table II the same results were obtained regardless of the specific activity of the ATP.

Only about 35–45% (Method A, Table I) of the total counts in the TCA

TABLE I

AMOUNT OF ^{32}P-LABELED PROTEIN RECOVERED FROM ACRYLAMIDE GELS UNDER DIFFERENT CONDITIONS

Electrophoresis was carried out in expt A as described in Methods: current was 4 mA/gel and water at 10–15°C with circulated through the electrophoresis apparatus. In B, the current was also 4 mA/gel but there was no water cooling. In C, the current was 8 mg/gel without cooling. Recovery is expressed as the percentage of the total counts added to the gel which were recovered in the region between 1 and 3-cm migration distance containing the phosphoprotein peaks.

Labeling conditions	Amount ^{32}P in peak			Recovery		
	A	B	C	A	B	C
	(*% relative to Mg*)			(%)		
Mg	100	100	100	42.9	25.6	17.5
Mg+Na	175	188	166	42.8	25.6	15.1
Mg+Na+K	99	96	80	39.5	24.8	12.5

TABLE II

EFFECT OF ATP-SPECIFIC ACTIVITY ON PHOSPHATE BINDING TO GHOSTS

In A, ghosts were phosphorylated with ATP at high specific activity (>10 Ci/mmol) as described in Methods. In B, the specific activity was 50-fold less.

Labeling condition	Phosphate binding	
	(A) High specific activity	(B) Low specific activity
	(*picomol/mg protein*)	
Mg	0.81	0.79
Mg+Na	1.36	1.38

precipitate before solubilization are found in the peak. The rest of the ^{32}P radioactivity is found in a diffuse band which runs ahead of the Pyronin-Y marker dye out of the gel (and therefore is not shown in Fig. 1). Since ATP and orthophosphate (P_i) run in the same region and since this region contains no detectable protein (17), this band probably represents $^{32}P_i$ released from the protein during the solubilization and electrophoresis procedures, along with a small contribution from [γ-^{32}P]ATP loosely bound to the TCA precipitate which fails to exchange with the ATP in the 5% TCA washes.

The comparatively low recovery of protein-bound ^{32}P in the gels is an expected result in view of the lability of the phosphoprotein (7). In order to

examine the source of this breakdown, however, with a view towards improving the recovery, the effect of variations in the solubilization time at 37 °C on the recovery was studied (Table III). In this case electrophoresis was carried out for only 50 min so that none of the $^{32}P_i$ left the gels. The percent recovery was only slightly dependent on the time of incubation. It was, however, somewhat greater than the recovery from gels which had been run for the full 3.5 h particularly when phosphoprotein was formed in the presence of Mg + Na. This together with the fact that electrophoresis at 8 instead of 4 mA/gel (which noticeably warms the gels) results in very low recoveries (10–20%) suggest that much of the breakdown of ^{32}P-labeled protein occurs during electrophoresis. This conclusion is further supported by the consistent observation of a higher background of ^{32}P counts in gel slices following the

TABLE III

EFFECT OF INCUBATION AT 37°C ON RECOVERY

Samples were added to a solubilizing solution containing dithiothreitol and 1% SDS as described in Methods. After incubation at 37°C for the indicated times, the samples were chilled in a water bath at 10–15°C. Samples were electrophoresed for 50 min at 4 mg/gel in an electrophoresis apparatus cooled with water at 10–15°C. Gels were then sliced transversely and counted. Counts in the first 1-cm slice (including the phosphoprotein peaks) are expressed as a percentage of the total counts in the gel.

	% counts in first slice	
Minutes incubation at 37°C	Mg	Mg + Na
0	49.9	57.0
5.0	49.6	55.2
15.3	43.7	52.9

peak than in slices preceding it. (This is not shown in the autoradiograms since the technique used in their preparation leaches most if not all of the P_i from the gel.) Unfortunately the temperature of electrophoresis could not be reduced below 10–15°C without precipitating the SDS, so this breakdown of phosphoprotein could not be avoided.

As may be seen from Table I, however, large variations in the recovery (due to different electrophoresis conditions) have little effect on the relative amounts of ^{32}P-labeled protein formed in the presence of various ions. This means that the labilities of the ^{32}P-labeled proteins formed under different ionic conditions are quite similar and further suggests that the ^{32}P-protein remaining after gel electrophoresis is a representative sample of that which was present before solubilization and electrophoresis.

By comparison with standards the molecular weight of the phosphoprotein was found to be about 103,000 ± 4,000. (This standard error was calculated from 10 determinations with five separate phosphoprotein preparations and

includes errors due to gel stretching during preparation for autoradiography. The true error is somewhat greater because of errors in determining the mobilities and molecular weights of the standards.) The same molecular weight was found for the phosphoprotein formed either in the presence or absence of Na.

This observation suggests that the phosphoproteins formed in the presence of Mg or Mg + Na may be identical. In order to investigate this question, as well as to shed further light on the chemical nature of the protein-phosphate bond, we treated the phosphorylated ghost protein with hydroxylamine, a procedure known to hydrolyze acyl phosphate bonds (23). Previous results of Blostein (7) had suggested that the Mg phosphoprotein might be more resistant to hydroxylamine than the Na-stimulated component.

The results are shown in Table IV. In this experiment ghosts were phosphorylated for 5 s at 37°C as well as under the usual conditions, 15 s at 0°C. In neither case was the Mg-phosphoprotein significantly less sensitive than the Na-phosphoprotein to either hydroxylamine or to incubation at pH 5.3. The small residual phosphorylation which survives hydroxylamine treatment may well represent bound ATP and is certainly not related to the 103,000 dalton phosphoprotein since not even a vestigial peak could be detected when the hydroxylamine-treated material was run on acrylamide gels.

To test further the similarity of the Mg- and Na-phosphoproteins, the preparation was treated with pepsin and the resulting peptides were electrophoresed on paper in 7% formic acid. The electrophoretograms were autoradiographed to reveal the ^{32}P-labeled peptides, yielding the result shown in Fig. 2. The patterns for Mg alone and for Mg + Na are virtually identical

TABLE IV

EFFECT OF HYDROXYLAMINE TREATMENT ON ^{32}P-LABELED PROTEIN

Phosphorylation and treatment with hydroxylamine or buffer were as described in Methods, except that labeling was carried out for 5 s at 37°C (two right-hand columns) as well as under the usual conditions (two left-hand columns). Amount of phosphorylated protein is expressed as picomoles per milligram ghost protein or as percent of the ^{32}P-labeled protein formed when ghosts from the same donor were labeled under the same conditions but were not further treated.

^{32}P-labeling	Incubation	[γ-^{32}P]ATP at 0° for 15 s	%	[γ-^{32}P]ATP at 37° for 5 s	%
		pmol P/mg protein		*pmol P/mg protein*	
Mg	None	0.625	100	0.797	100
Mg	NH_2OH	0.095	15	0.095	12
Mg	Buffer	0.44	70	0.355	45
Na+Mg	None	0.935	100	1.325	100
Na+Mg	NH_2OH	0.08	9	0.10	8
Na+Mg	Buffer	0.605	65	0.74	56

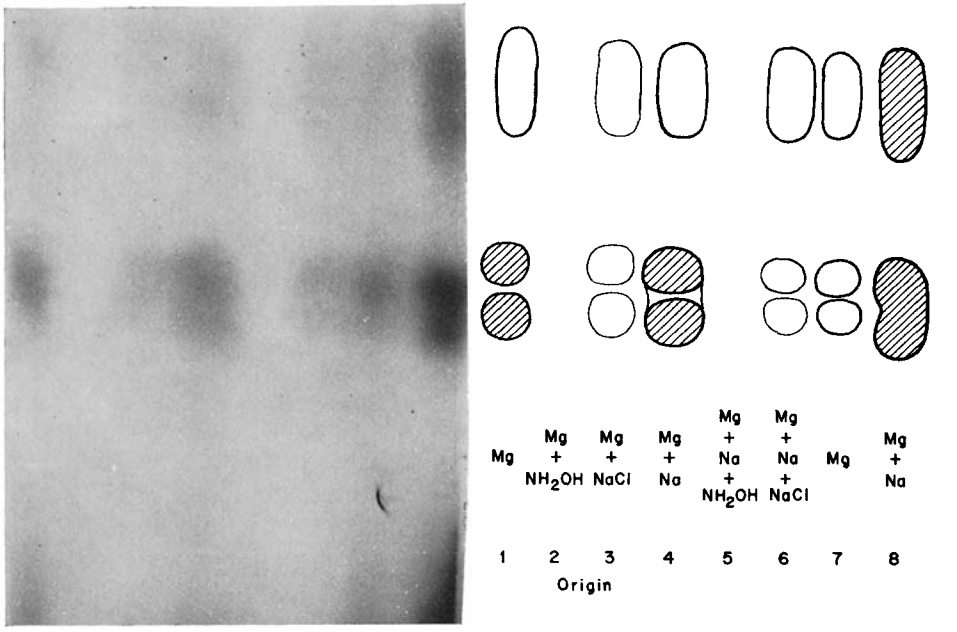

FIGURE 2. Paper electrophoresis of ^{32}P-labeled peptides. Phosphorylation, hydroxylamine treatment, peptic digestion, paper electrophoresis, and autoradiography were performed as described in Methods. Hydroxylamine treatment preceded peptic digestion. Autoradiogram exposure was for 1 mo on Kodak RP54 X-ray film. The origin is at the bottom of the photograph and the cathode is at the top. The drawing at the right represents the principal features of the autoradiogram, as well as the order in which the following samples were applied to the origin:

Sample	Amount*	Phosphorylation conditions	Treatment after phosphorylation
1	1	Mg	None
2	1	Mg	NH_2OH
3	1	Mg	NaCl
4	1	Mg+Na	None
5	1	Mg+Na	NH_2OH
6	1	Mg+Na	NaCl
7	2	Mg	None
8	2	Mg+Na	None

* Each unit represents roughly the same amount of protein added to the paper.

and almost all of the material is sensitive to hydroxylamine, as indicated by the absence of ^{32}P radioactivity in the peptides from the hydroxylamine-treated material. These results strongly indicate that the amino acid sequences near the phosphate in the two proteins are very similar if not identical (see Discussion).

The results so far described indicate that the phosphoprotein runs in a

region of the gel containing one of the major polypeptide components of the red cell ghost protein. Therefore acrylamide gel electrophoresis provides only a small (perhaps a threefold) purification of the phosphopeptide. Treatment of intact red cells with Pronase (a collection of proteolytic enzymes from *Streptomyces griseus*) has been shown to partially hydrolyze the 90,000 dalton polypeptide (5) to yield a fragment of lower molecular weight. This effect of Pronase on membrane proteins is shown in the lower gel photograph in Fig. 3. It is known that Pronase also hydrolyzes the major red cell glycoproteins (which cannot be seen with Coomassie Blue), one of which runs near the 90,000 dalton polypeptide. To determine whether the protein which is phosphorylated is 90,000 dalton polypeptide or another protein migrating in the same region of the gel, intact red cells were treated with Pronase as described in Methods.

After Pronase treatment the cells were washed six times with 5 vol of 165 mM NaCl to remove all traces of Pronase and ghosts were prepared and phosphorylated in the standard manner. The result of Pronase pretreatment of intact cells is shown in the middle graph of Fig. 3. Despite drastic changes in the protein pattern in the region containing the phosphoprotein the mobility of the ^{32}P-labeled protein is not detectably altered in ghosts from Pronase-treated cells. This is most clearly shown in the lower graph which was obtained by electrophoresing a 50:50 mixture of solubilized protein from control and Pronase-treated cells. Here the two different samples have been run on the same gel yet only one peak is seen in the 103,000 dalton region. The maximum change in molecular weight of the phosphopeptide which might have occurred upon Pronase treatment is less than 10%. In addition Pronase had no effect on the number of counts in the peak, nor on the total amount of TCA-precipitable phosphoprotein formed in the reaction with ATP (Table V). Except for the size of the peaks, the same results were obtained when Mg alone was present in the phosphorylating medium. These results indicate that Pronase does not have access to the phosphorylating mechanism or the phosphoprotein in the intact cell.

The effect of Pronase on ghosts is shown in Fig. 4. The upper graph shows control ghosts incubated for 30 min at 37 °C without Pronase and then washed as described in Methods. The faster-moving peak is similar to the control; the slower-moving band is absent because these ghosts like those in Fig. 1 have been extensively washed. Ghosts treated with Pronase (lower graph) exhibit considerable protein hydrolysis and very little phosphorylated protein is formed. This provides evidence that the phosphorylating mechanism is not intrinsically resistant to Pronase. Evidently the failure of Pronase to affect phosphorylation in the intact cell indicates that the vital parts of the phosphorylating mechanism are inaccessible to the externally added enzyme.

In addition to phosphorylation the effect of Pronase on other parameters

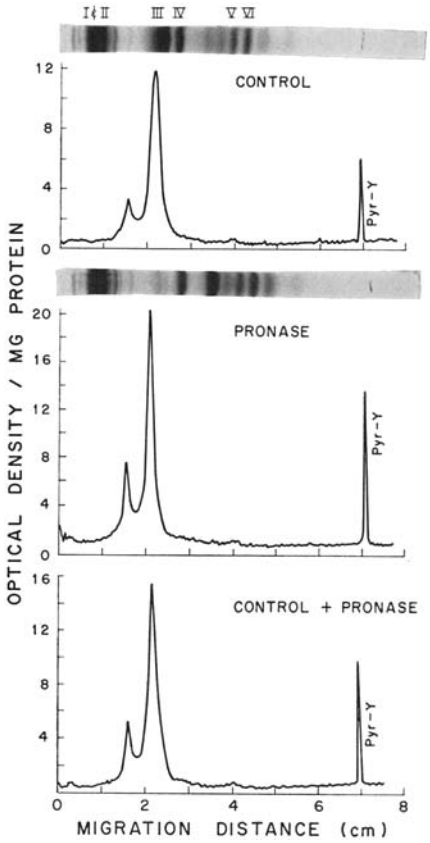

FIGURE 3

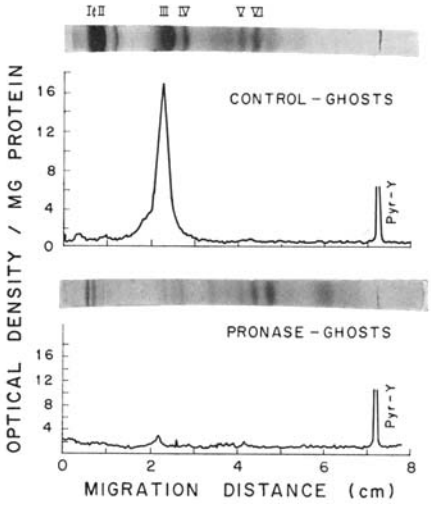

FIGURE 4

FIGURE 3. Gel electrophoresis of ghosts from control and Pronase-treated cells. The upper and lower photographs show gels on which solubilized proteins from ghosts of control and Pronase-treated cells have been electrophoresed and stained with Coomassie Blue. Note the shift in position of band III (90,000 dalton polypeptide) to lower molecular weight (5). The graphs are densitometer scans of autoradiograms of gels on which phosphorylated ghosts have been electrophoresed. Ordinate and abscissa are as described in Fig. 1. The densities are higher because the specific activity of the [γ-^{32}P]ATP was higher in this case. From top to bottom the graphs represent ghosts from control cells, Pronase-treated cells, and a 50:50 mixture of ghosts from Pronase-treated and control cells, all phosphorylated in the presence of Mg + Na.

FIGURE 4. Gel electrophoresis of control and Pronase-treated ghosts. The upper and lower photographs show Coomassie Blue stained gels on which phosphorylated control and Pronase-treated ghosts have been electrophoresed. Note the extensive hydrolysis in the lower photograph resulting from Pronase treatment of ghosts. The upper and lower graphs are densitometer traces of autoradiograms of similar gels. Phosphorylation was performed in the presence of Mg + Na following Pronase treatment. Ordinate and abscissa are as described in Fig. 1. Procedures for Pronase treatment, phosphorylation, solubilization, electrophoresis, and autoradiography were as described in Methods.

related to the Na:K pump was also investigated. Table VI shows the effect of Pronase on potassium influx in the presence and absence of ouabain. Although the ouabain-insensitive potassium influx is very slightly increased in Pronase-treated intact cells in agreement with Passow's observations on net K flux (28), the pump flux represented by the ouabain-sensitive component is not significantly altered.

The effects of Pronase on the Na,K-ATPase are shown in Table VII. Neither the ouabain-insensitive ATPase nor the ouabain-sensitive component

TABLE V

PHOSPHORYLATION OF MEMBRANE PROTEIN IN GHOSTS FROM CONTROL AND PRONASE-TREATED CELLS

Conditions of Pronase treatment and phosphorylation were as described in Methods. Amount of phosphorylation is expressed as picomoles per milligram ghost protein. Each value is the average of duplicate determinations on the same ghost preparation.

	Mg	Mg + Na	ΔNa
Control cells	0.91	1.91	1.00
Pronase cells	0.84	1.83	0.99

TABLE VI

POTASSIUM INFLUX

Potassium influx in the presence or absence of 10^{-4} M ouabain was determined using ^{42}K as described in Methods. Values represent the mean of four determinations on cells from a single donor ± standard error of the mean.

	+ouabain	−ouabain	ouab sensitive
	(mmol/liter cells/h)		
Control	0.238±0.002	1.805±0.019	1.567±0.019
Pronase	0.260±0.005	1.767±0.042	1.507±0.042

TABLE VII *a*

ATP HYDROLYSIS

In all cases ATPase activity was determined on ghosts in the presence or absence of 1×10^{-4} M ouabain as described in Methods. Values represent the average of two determinations in a single experiment. The label "Cells" or "Ghosts" refers to the state of the cells or membranes at the time of incubation at 37°C with or without Pronase as described in Methods.

	−ouabain	+ouabain	ouab sensitive
	(mμmol/mg protein/h)		
Control-Cells	235	100	135
Pronase-Cells	229	89	140
Control-Ghosts	160	66	94
Pronase-Ghosts	27	18	9

are significantly affected by Pronase treatment of intact cells. In contrast, ghosts pretreated with Pronase show a pronounced inhibition of both ATPase activities in comparison with ghosts treated in the same fashion but without Pronase.

As shown in Table VIII, Pronase treatment of intact cells also did not significantly affect ouabain binding. Pronase-treated ghosts on the other hand

TABLE VII *b*

SUMMARY OF EXPERIMENTS WITH PRONASE-TREATED CELLS

In all cases ATPase activity was determined on ghosts in the presence or absence of 1×10^{-4} M ouabain as described in Methods. Values are the average of at least four separate experiments ± standard deviation. *P* is the probability that the means of the Pronase-treated and control cells are equal, as evaluated by Student's *t*-test.

ATPase activity	Pronase/Control	±SD	*P*
Ouabain-sensitive	0.949	0.081	>0.2
Ouabain-insensitive	0.997	0.046	>0.8

TABLE VIII

EFFECT OF PRONASE TREATMENT ON OUABAIN BINDING

Pronase treatment, ouabain-binding determination, and ATPase activity measurement were performed as described in Methods. Values are the average of at least two determinations. The right-hand column represents the percent inhibition of Na,K-ATPase activity as compared to a paired control treated in an identical manner, except without 1×10^{-7} M ouabain.

Pretreatment	Min exposure to 10^{-7} M ouabain	ouabain bound	inhibition of ATPase activity
		pmol/mg protein	%
Control	10–15	1.56	99
	30	1.60	91
Cells Pronase-treated	10–15	1.77	93
	30	2.07	97
Ghosts Pronase-treated	10–15	2.64	~0
	30	3.71	~0

exhibited enhanced ouabain binding. This binding appears to be nonspecific and unrelated to the Na:K pump since it was not associated with inhibition of the Na,K-ATPase.[1]

[1] It should be noted that the number of molecules of ouabain bound per ghost, as presented in Table VIII, is between 2–4 times larger than values we have previously reported (19) but similar to estimates recently proposed by Gardner and Conlon (18). As pointed out before (19, p. 347s) estimates of the number of ouabain-binding sites as determined using ghosts are considerably less accurate than those determined using intact cells. Thus the values reported in Table VIII are only of value in the context presented. (The basis for the discrepancy in the numbers of ouabain-binding sites per intact cell obtained by us (19) compared to those reported by Gardner and Conlon (18) will be considered in a separate paper.)

DISCUSSION

The principal finding reported in this paper is that a phosphorylated protein associated with the Na,K-ATPase can be purified on SDS-polyacrylamide gels and that its molecular weight was determined to be about 103,000. These observations are supported by those recently published by Avruch and Fairbanks (2). Although their conditions of phosphorylation were somewhat different they found a Na-stimulated phosphoprotein in human red cell ghosts with a molecular weight of 105,000 which is almost certainly identical to that which we observed. The fact that they obtained similar results at pH 2.4 (which should inhibit the hydrolysis of the phosphoprotein (7)) provides further evidence against the existence of other undetected highly labile phosphoproteins.

Avruch and Fairbanks' findings differ from ours in that they observed two additional peaks, one of which appeared to be bound ATP and the other of which ran near the Pyronin-Y marker. In our system no ATP was seen, since the TCA precipitate was thoroughly washed and since at pH 7.4 ATP runs ahead of the Pyronin-Y marker dye and therefore off the gel in most cases. When ghosts were phosphorylated for 20 min at 0°C a small peak was seen near the Pyronin-Y but this was never observed under the usual phosphorylating conditions.

Ghosts phosphorylated for 20 min at 0°C also exhibited a peak at high molecular weight (>200,000). Faint labeling of this peak was occasionally seen under our usual phosphorylating conditions and this labeling was abolished by 0.5 mM Ca. This peak may be associated with the tektin A protein observed in bovine (11, 35) and human (12) erythrocyte ghosts. We also noted higher molecular weight phosphoproteins in an experiment where the ghosts were phosphorylated for 5 s at 37°C. These ghosts exhibited less Mg-stimulated labeling in the 103,000 dalton region than ghosts phosphorylated at 0°C. The three minor peaks of higher molecular weight were not affected by Na addition. Further characterization was not attempted.

A second important finding reported in this paper is that all of the pump parameters tested (the mobility of the Na-phosphoprotein, the amount of Na-stimulated phosphorylation, the ouabain-sensitive potassium influx, the ouabain-sensitive ATPase activity, and the ouabain binding) remained the same after Pronase treatment of intact cells. Pronase treatment of ghosts on the other hand resulted in marked changes in Mg- and Na,K-ATPase activities, ouabain binding, and phosphorylation, indicating that these aspects of the pump complex are not intrinsically resistant to Pronase. The resistance of these elements to externally added Pronase therefore provides evidence that they are not accessible to Pronase and therefore are not located at the

outer surface of the membrane near the glycoproteins and the 90,000 dalton polypeptide.

Summed together the Mg- and Na-phosphoproteins represent less than 0.02% of the total ghost protein (assuming one phosphate atom per molecule). This alone makes it unlikely that any of the major ghost proteins might be the phosphoprotein, unless only a very small fraction of the molecules are phosphorylated. Williams (35) has suggested the possibility that in bovine erythrocytes a Na-stimulated phosphoprotein (mol wt 98,000) might be associated with the dense protein band of similar molecular weight. Our results with Pronase-treated cells in which the 90,000 dalton polypeptide can be partially hydrolyzed without affecting the phosphoprotein rule out this possibility in human red cells.

When ghosts from Pronase-treated cells are electrophoresed, very little protein is found in the region containing the phosphoprotein. This technique therefore provides a very substantial (about 100-fold as judged from the intensity of the Coomassie Blue stain) purification of the phosphoprotein, which compares favorably with the highest purification previously attained (about 10-fold) by Dunham and Hoffman (14). The phosphate appears to be associated with one of the very faint bands remaining after Pronase treatment but it could be bound to a protein which is not detectable with Coomassie Blue since each gel contains only about 15–20 ng of phosphoprotein. Although the quantities of protein which may be obtained by this method are probably insufficient for further chemical characterization, the separation of the Mg- and Na-phosphoproteins from other phosphorylated proteins (e.g. Ca-stimulated phosphoproteins) provides an unprecedented opportunity to study them separately (see 22).

Since the Mg- and Na-phosphoproteins have the same molecular weight (103,000), this could mean that there are two different proteins with coincidentally similar molecular weights or else the same phosphoprotein might form part of two distinct enzyme complexes in which other subunits determine the specificity for Mg or Mg + Na. These two different enzyme complexes might phosphorylate the protein at the same or different sites.

Both phosphoproteins are sensitive to hydroxylamine (Table IV) suggesting that they are both acyl phosphates. Paper electrophoresis of the peptic fragments revealed the same patterns for both phosphopeptides (Fig. 2) and virtually all of the material was hydroxylamine sensitive. Bader et al. (4) reported that erythrocyte ghosts phosphorylated at 40 μM ATP exhibited a different electrophoretic pattern from that of all other preparations tested and that a large portion of the material was resistant to hydroxylamine. The pattern seen in our preparation (at 2 μM ATP) is very similar to that which they observed for a variety of microsomal preparations from such tissues as guinea pig kidney, octopus gill, frog brain, and the electric organ of the

electric eel. The erythrocyte phosphopeptide formed at 2 μM ATP (in contrast to that formed at higher ATP concentrations) is evidently very similar to the phosphopeptides formed by most other ATPase preparations. If so then the size of the peptides found in our preparation are estimated from the similarity of the results of other (3, 29) to be about a decapeptide or larger.

The similarlity of the Mg- and Na-phosphoproteins might be explained if most of the Mg-phosphoprotein were actually Na-phosphoprotein resulting from Na already present in the ghost preparation. This is unlikely since the Na concentration in the washed ghosts is very low (<0.02 mM) and since the addition of K (which dephosphorylates the Na-stimulated component) does not reduce the labeling much below that seen when Mg alone is present.

All of our data suggest that phosphorylation occurs at the same site on the same protein whether Mg or Mg + Na are present. On the contrary, evidence for different enzyme complexes is provided by recent experiments of Proverbio and Hoffman (in preparation) in which they have succeeded in separating the Mg- and Na-phosphoproteins by centrifugation on sucrose gradients (14). Under these conditions large protein aggregates are present rather than individual polypeptide chains as is probably the case with SDS gel electrophoresis. These conflicting results may be reconciled if the same phosphoprotein forms a part of two different enzyme complexes. The higher molecular weight found for the Na,K-ATPase by the sucrose gradient technique (400,000 [4]) and by other methods (250,000 [20]; 500,000 [26]), in contrast to the comparatively low molecular weight of the phosphopeptide (103,000) tends to support this interpretation.

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