*IDENTIFICATION OF COMPONENTS OF ($NA^+ + K^+$)-ADENOSINE TRIPHOSPHATASE BY DOUBLE ISOTOPIC LABELING AND ELECTROPHORESIS**

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Abstract.—A microsomal adenosine triphosphatase (ATPase) that requires both sodium and potassium ions is thought to be identical with, or an integral part of, the active cation transport system located in cell membranes. Attempts to isolate and purify ($Na^+ + K^+$)-ATPase have met with limited success because solubilization of microsomal protein causes partial, if not complete, loss of enzymatic activity. We now report the isolation from rat kidney microsomes of proteins which, though enzymatically inactive, could still be identified as components of the ($Na^+ + K^+$)-ATPase system.

Phosphoproteins known to be intermediates in the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase were prepared by incubating rat kidney microsomes with γ-labeled ATP^{33} in the presence of sodium or with P^{32}-orthophosphate in the presence of ouabain. After the P^{32}- and P^{33}-labeled microsomes had been dissolved in phenol-acetic acid-urea, the resultant solutions were mixed and subjected to polyacrylamide gel electrophoresis. The radioactivity from both phosphorus isotopes was found almost exclusively in one of the resultant 21 protein bands. In contrast, the radioactive protein from DFP^{32}-labeled microsomes moved slightly faster than the radioactive protein from microsomes labeled with P^{33}-orthophosphate in the presence of ouabain. DFP inhibits ($Na^+ + K^+$)-ATPase by reacting with a nucleophilic site at or near the active site. These results suggest that while a single protein component of ($Na^+ + K^+$)-ATPase accepts the terminal phosphate from ATP, the final splitting of this phosphoprotein intermediate may be catalyzed by nucleophilic sites on a second protein.

Cell membranes contain an energy-dependent pump which is responsible for the active transport of sodium and potassium ions. When cellular organization is destroyed by homogenization, it is thought that this cation transport system manifests itself as a Mg^{++}-dependent microsomal ATPase[1] which is stimulated by the simultaneous presence of sodium and potassium ions.[2-4] It is to be hoped that elucidation of the mechanism of ($Na^+ + K^+$)-ATPase will shed light on the molecular events taking place during active cation transport.

Attempts to isolate and purify ($Na^+ + K^+$)-ATPase have thus far met with only limited success. Treatment of microsomes with strong salt solutions[5] or low concentrations of detergents[6, 7] considerably enhances ($Na^+ + K^+$)-ATPase activity, provided that the enzyme is still associated with an insoluble microsomal particle. However, as soon as microsomal protein is solubilized, e.g., by detergents, much of the ($Na^+ + K^+$)-ATPase activity is lost. This suggests that the forces which are important for retaining ($Na^+ + K^+$)-ATPase

in the insoluble microsomal particle may also be responsible for holding the enzyme in a catalytically active conformation. Alternatively, ($Na^+ + K^+$)-ATPase may consist of several protein subunits which must act cooperatively in order to display enzyme activity. Thus, solubilization of microsomal protein may cause a loss in conformational stability on the one hand, or dissociation of subunits on the other. In either case, the result would be a loss of enzymatic activity.

We now report the isolation from rat kidney microsomes of proteins which, though enzymatically inactive, could still be identified as components of ($Na^+ + K^+$)-ATPase. Phosphoproteins, known to take part in the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase, were prepared by incubating rat kidney microsomes with ATP^{33}, P_i^{33}, P_i^{32}, or DFP^{32}. The P^{32} or P^{33}-labeled microsomes were dissolved in phenol-acetic acid-urea and then subjected to polyacrylamide disc gel electrophoresis.[8] Results show that only one out of the 21 proteins was labeled by ATP^{33}, P_i^{32}, or P_i^{33} while DFP^{32} also labeled a protein with a slightly higher electrophoretic mobility. This suggests that while a single protein component of ($Na^+ + K^+$)-ATPase accepts the terminal phosphate from ATP, the final splitting of this phosphoprotein intermediate may be catalyzed by nucleophilic sites on a second protein.

Materials and Methods.—Rat kidney microsomes were prepared by the method of Skou.[9] Terminally labeled ATP^{33} (sp. act. 5–10 mC/μmole) was prepared by previously reported methods.[10] DFP^{32} (sp. act. 300 μC/mg) was purchased from Amersham Searle. P_i^{33} and P_i^{32} were obtained from Tracerlab.

Labeling of microsomes: P^{33} was incorporated into the microsomes by incubation with ATP^{33} in the presence of Na^+ as previously described.[11] In some experiments oligomycin (0.1 mg/ml) was included in the incubation. The microsomes were washed successively with ice-cold 10% TCA, 5% TCA (containing carrier ATP and H_3PO_4), and finally with 0.01 N HCl.

P^{32} from DFP^{32} was incorporated[12] into microsomal protein (1 mg) by incubation for 2 hr at 37° with 1 ml of a solution containing $MgCl_2$ (25 mM), Tris-HCl, pH 7.4 (100 mM), DFP^{32} (5 mM), Tris-HCl, pH 7.4 (100 mM), and ATP (25 mM) where indicated. An additional 25 μmoles of ATP was added after 1 hr of incubation.

Orthophosphate was incorporated into the microsomes by a ouabain-dependent reaction.[13] Kidney microsomes (1 mg) were incubated for 20 min at room temperature with 1 ml of a solution containing $MgCl_2$ (5 mM), Tris-HCl, pH 7.4 (100 mM), and ouabain (1 mM) where indicated. P_i^{32} or P_i^{33} (0.1 mM) was then added and the incubation was continued for another 10 min and then stopped by the addition of an equal volume of cold 10% TCA. The precipitate was washed with cold 10% TCA, 5% TCA, 0.01 N HCl, and finally resuspended in water.

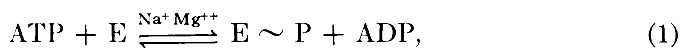
Solubilization of labeled protein: Labeled protein was suspended in water, and solid urea added to a final concentration of 8 M. To 1 vol of this suspension were added 3 vol of phenol-acetic acid (2:1 w/v), with shaking. The solution was complete within 1 min. Aliquots (30–50 μl) of the resultant solution, containing 0.10–0.15 mg protein, were then subjected to disc electrophoresis as described below.

Disc gel electrophoresis: Acrylamide gels (7.5%, 45 mm long) were polymerized by the method of Cotman and Mahler[8] and then soaked for at least 3 days in a phenol-acetic acid-water mixture (2:1:1, w/v/v) containing 2 M urea. Samples for electrophoresis were placed on the gel and acetic acid (75%) was layered on top. Electrophoresis was carried out for 2 to 4 hr at 4° using either 2.5 mA or 5.0 mA per tube. Because the fixing and staining procedures resulted in a loss of radioactivity from the microsomal proteins, it became necessary to cut the gels longitudinally[14] so that one half could be stained while

the other half was used to measure the radioactivity. The separated proteins were fixed and stained by immersion overnight in 7% acetic acid containing 1% Amido Schwartz. Excess stain was removed by shaking the gels for 48 hr with successive changes of 7% acetic acid. The gel halves for counting were sectioned transversely[15] and the slices placed in scintillation vials containing 0.5 ml hydrogen peroxide (30%). After the vials had been incubated at 50° overnight, 2 ml of hyamine hydroxide (Packard Instrument Company) and 10 ml of Bray's solution[16] were added and the radioactivity was determined in a Packard liquid scintillation counter. With appropriate settings of the discriminator, P^{32} and P^{33} could be counted separately in the same sample.

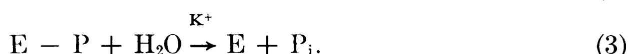
Results: Rat-kidney microsomal protein solubilized by treatment with phenol-acetic acid-urea was separated by disc gel electrophoresis into 21 distinguishable bands (Fig. 1). Because the protein in bands 3, 9, and 14 stained darker than the rest, they were used as reference points for the numbering and identification of the other bands (Fig. 2).

The protein bands associated with $(Na^+ + K^+)$-ATPase were detected by their radioactive phosphorus label, which was incorporated into the microsomes prior to solubilization and electrophoresis. The choice of the labeling techniques was based on the following biochemical events[4] which are known to be associated with the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase:



$$ATP + E \underset{}{\overset{Na^+ \; Mg^{++}}{\rightleftharpoons}} E \sim P + ADP, \quad (1)$$

$$E \sim P \overset{Mg^{++}}{\rightarrow} E - P, \quad (2)$$



$$E - P + H_2O \overset{K^+}{\rightarrow} E + P_i. \quad (3)$$

The first step is a reaction between ATP and $(Na^+ + K^+)$-ATPase to form a high-energy phosphoprotein ($E \sim P$). This reaction permits the introduction of P^{33} from γ-labeled ATP^{33} into a protein which must be a component of $(Na^+ + K^+)$-ATPase. The reversible formation of this high-energy phosphoprotein ($E \sim P$) can only be demonstrated when step 2 is inhibited by lowering the concentration of magnesium ions[17] or by adding *N*-ethylmaleimide or oligomycin.[18–20] In step 2 the high-energy phosphoprotein ($E \sim P$) rearranges to a lower energy form ($E - P$) which is rapidly dephosphorylated (step 3) in the presence of potassium ions. Radioactivity from orthophosphate is not normally incorporated into microsomal protein but recent evidence shows that $P_i{}^{32}$ is incorporated in the presence of the cardiac glycoside ouabain.[13, 22] It is possible that ouabain holds the microsomal protein in a conformation such that the over-all ATPase

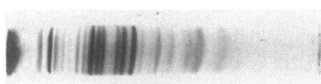


FIG. 1.—Polyacrylamide disc gel electrophoresis of rat-kidney microsomes solubilized in phenol-acetic acid-urea. Electrophoresis was for 2 hr at 5 mA.

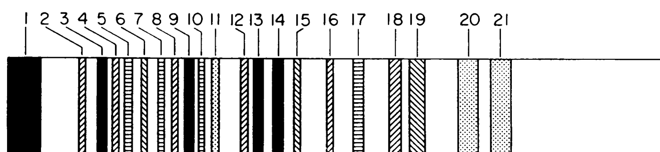


FIG. 2.—Diagrammatic representation of the protein bands obtained by disc gel electrophoresis of rat-kidney microsomes.

reaction is inhibited while at the same time exchange with orthophosphate is facilitated.[13] Still a third means for labeling $(Na^+ + K^+)$-ATPase has been provided by Hokin and Yoda,[12] who have shown that DFP inhibits beef kidney $(Na^+ + K^+)$-ATPase. The incorporation of DFP^{32} into beef kidney microsomes was prevented by ATP, suggesting that DFP may react with a nucleophilic site at or near the active center.

Radioactive phosphorus incorporated into microsomes from ATP or orthophosphate was lost from the polyacrylamide gel bands during the several days' exposure to acetic acid that was required for the fixing and staining process. It was therefore necessary to cut the gels longitudinally and to compare the distribution of radioactivity in transverse segments from one half with the distribution of stained protein bands in the other half. This technique introduced an uncertainty of the order of 1 mm into the location of the radioactive bands. When the radioactivity from P^{32}- and P^{33}-labeled microsomes appeared in a region (Figs. 3 and 4) where the protein bands were close together, it was decided to use double-labeling techniques. This made it possible to determine unequivocally whether the same proteins were being labeled even though there was some uncertainty in the assignment of radioactivity to specific bands in the gel pattern.

Microsomes that had been labeled with ATP^{33} in the presence of sodium ions were mixed with ouabain-treated microsomes labeled with P_i^{32} and the mixture was separated by electrophoresis on the same gel. Because the P^{32} and P^{33} appeared almost exclusively in band 3 (Fig. 3), it was concluded that the isotopes had labeled the same protein. The inclusion of oligomycin (0.1 mg/ml) in the incubation medium with ATP^{33} did not affect the position of the radioactive band. When DFP^{32}-labeled microsomes were mixed with ouabain-treated microsomes labeled by P_i^{33}, the main peaks of radioactivity were also found in band 3 (Fig. 4). However, the DFP^{32}-labeled microsomes always displayed a leading edge in which there were no P^{33} counts (Fig. 4). Apparently a second faster-moving protein (possibly represented by band 4) was also labeled.

Discussion.—The certainty with which protein bands may be identified as components of $(Na^+ + K^+)$-ATPase must ultimately depend on the specificity of the reactions used to label them. There is now abundant evidence[4] that in the presence of sodium ions, the terminal phosphate group of ATP is incorporated into a phosphoprotein component of microsomal $(Na^+ + K^+)$-ATPase. The

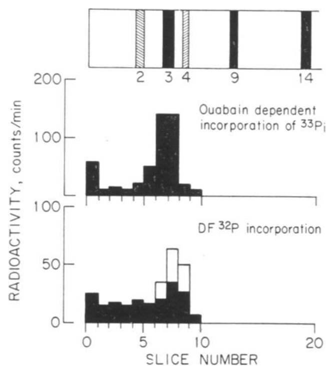


FIG. 3.—Separation of labeled components of rat-kidney microsomes by disc gel electrophoresis. Labeled microsomes were mixed after solubilization with phenol-acetic acid-urea and run on the same gel. In control experiments run separately, no radioactivity entered the gel when microsomes were incubated with P_i^{32} in the absence of ouabain or with ATP^{33} in the absence of sodium ions. Electrophoresis was for 4 hr at 2.5 mA.

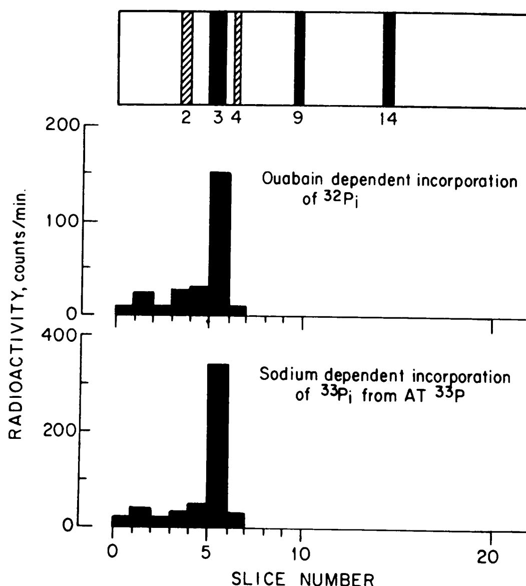


FIG. 4.—Separation of labeled components of rat-kidney microsomes by disc gel electrophoresis. Microsomes labeled with P_i^{33} in the presence of ouabain were mixed with those labeled by DFP^{32} before solubilization and electrophoresis. The unshaded bars in the DFP^{32} graph represent that part of the labeling which was prevented when the DFP^{32} labeling was carried out in the presence of ATP. Electrophoresis was for 4 hr at 2.5 mA.

Mg^{++} concentration present during the labeling of the kidney microsomes by ATP^{33} was probably sufficient to catalyze step 2. Thus, it was the low-energy form of the phosphoprotein (E — P) which was found in band 3 (Fig. 3). However, when step 2 was inhibited by oligomycin, the labeling pattern was unchanged. Thus, the terminal phosphate of ATP must remain attached to the protein found in band 3 during the entire enzymatic process. The conversion of the phosphoprotein intermediate from a high-energy (E $\sim$ P) to a low-energy (E — P) form may therefore represent a change in conformation. Jardetsky has proposed[21] that such protein conformational changes may be responsible for the transfer of sodium and potassium ions across membranes.

Oubain stimulates the incorporation of P_i^{32} into microsomes from eel electroplax and calf brain.[13, 22] Albers and co-workers have suggested[13] that the introduction of P_i is facilitated because ouabain stabilizes a conformation in which there is little difference between the free and phosphorylated forms of (Na^+ + K^+)-ATPase. Our results show that the radioactivity from P_i^{32} appears in the same protein that is labeled by ATP^{33} (Fig. 3). This again suggests that the protein found in band 3 (Fig. 3) is a component of (Na^+ + K^+)-ATPase.

The inhibition of beef kidney (Na^+ + K^+)-ATPase by DFP is dependent on magnesium ions, enhanced by potassium ions, and antagonized by ATP.[12] Although Hokin and Yoda found[12] that DFP^{32} was incorporated into serine, they suggested that this amino acid was unlikely to be the site of phosphorylation by ATP. Nevertheless, the 50 per cent reduction in DFP^{32} labeling[12] (cf. Fig. 4) observed in the presence of ATP did suggest that a serine group was near the active site. Serine itself is not normally sufficiently nucleophilic to react with DFP.[23] Thus, the reactivity of the serine residues in (Na^+ + K^+)-ATPase which were labeled by DFP^{32} may have been modified by interaction with neighboring amino acids at the active site.

Although there was some overlap with band 3, rat-kidney protein labeled by DFP^{32} invariably moved ahead of that labeled by P_i^{33}. These results suggest that while a single protein may accept the terminal phosphate from ATP, a second protein may also be involved in the enzyme mechanism. Such a protein might carry a nucleophilic group to catalyze the release of phosphate from the phosphoprotein intermediate.

The identification of at least one protein as a component of $(Na^+ + K^+)$-ATPase should greatly facilitate its isolation and chemical characterization. Disc gel electrophoresis can be used, for example, to locate this component in protein fractions obtained from microsomes by less destructive methods of separation. However, when the protein associated with band 3 is isolated, it is not anticipated that it will have enzymatic activity. The DFP^{32}-labeling experiments have already suggested that another protein may be involved in the $(Na^+ + K^+)$-ATPase mechanism. Albers has also pointed out[13] that the intrinsic properties of the enzyme suggest that $(Na^+ + K^+)$-ATPase is a multicomponent system. It might be anticipated that other proteins, possibly involved in the binding of sodium and potassium ions, may have to be assembled together with the phosphate carrier identified in this communication before enzyme activity is observed.

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[1] The following abbreviations are used: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; P_i, orthophosphate; DFP, diisopropylfluorophosphate; TCA, trichloroacetic acid.

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