non for operating our Beckman 890C sequencer, and Robert Huber for a preprint of the paper by Remington et al. (1982). H.W.D. thanks Richard Perham, in whose laboratory this work was begun.

Registry No. NADH, 58-68-4; citrate synthase, 9027-96-7.

References

Alam, T., Finkelstein, D., & Srere, P. A. (1982) *J. Biol. Chem. 257*, 11181-11185.
Atassi, M. Z., & Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 546-553.
Bayer, E., Bauer, B., & Eggerer, H. (1981) *FEBS Lett. 123*, 258-260.
Bloxham, D. P., Ericsson, L. H., Titani, K., Walsh, K. A., & Neurath, H. (1980) *Biochemistry 19*, 3979-3985.
Bloxham, D. P., Parmelee, D. C., Kumar, S., Wade, R. D., Ericsson, L. H., Neurath, H., Walsh, K. A., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5381-5385.
Bloxham, D. P., Parmelee, D. C., Kumar, S., Walsh, K. A., & Titani, K. (1982) *Biochemistry 21*, 2028-2036.
Duckworth, H. W., & Tong, E. K. (1976) *Biochemistry 15*, 108-114.
Duckworth, H. W., & Bell, A. W. (1982) *Can. J. Biochem. 60*, 1143-1147.
Harmey, M. A., & Neupert, W. (1979) *FEBS Lett. 108*, 385-389.
Higa, A. I., & Cazzulo, J. J. (1976) *Experientia 32*, 1373-1374.
Higa, A. I., Massarini, E., & Cazzulo, J. J. (1978) *Can. J. Microbiol. 24*, 215-221.
Juan, S. M., Cazzulo, J. J., & Segura, E. L. (1977) *J. Parasitol. 63*, 921-922.
Koeller, W., & Kindl, H. (1977) *Arch. Biochem. Biophys. 181*, 236-248.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Mendez, E., & Lai, S. Y. (1975) *Anal. Biochem. 68*, 47-53.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88-118.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819-831.
Morse, D., & Duckworth, H. W. (1980) *Can. J. Biochem. 58*, 696-706.
Porter, J. S., & Wright, B. E. (1977) *Arch. Biochem. Biophys. 181*, 155-163.
Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111-152.
Schroeder, W. A. (1972) *Methods Enzymol. 25*, 203-221.
Skiados, D. (1981) *High Speed Analysis of PTH Amino Acids*, Perkin-Elmer, Norwalk, CN.
Somack, R. (1980) *Anal. Biochem. 104*, 464-468.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622-627.
Tong, E. K., & Duckworth, H. W. (1975) *Biochemistry 14*, 235-241.
Weitzman, P. D. J. (1966) *Biochim. Biophys. Acta 128*, 213.
Weitzman, P. D. J., & Danson, M. J. (1976) *Curr. Top. Cell. Regul. 10*, 161-204.
Weng, L., Russell, J., & Heinrikson, R. L. (1978) *J. Biol. Chem. 253*, 8093-8101.
Wiegand, G., Kukla, D., Schotze, H., Jones, T. A., & Huber, R. (1979) *Eur. J. Biochem. 93*, 41-50.

Topological Localization of Proteolytic Sites of Sodium and Potassium Ion Stimulated Adenosinetriphosphatase†

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ABSTRACT: The (Na^+ and K^+)-stimulated adenosinetriphosphatase [(Na^+,K^+)-ATPase] consists of two different polypeptides, α and β, both of which are embedded in the plasma membrane. The α chain from dog kidney (Na^+,K^+)-ATPase can be hydrolyzed at specific sites by trypsin and chymotrypsin [Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221-2228]. In order to position these sites with respect to the lipid bilayer, we have treated sealed, inside out vesicles from human red cells and unsealed kidney enzyme membranes with trypsin and chymotrypsin and have used ouabain-stimulated phosphorylation to identify the (Na^+,K^+)-ATPase and its fragments. All of the proteolytic sites observed in the kidney membranes are accessible in the inside out vesicles. The ouabain-inhibitable uptake of $^{86}Rb^+$ in human red blood cells is resistant to externally added chymotrypsin. These results indicate that the proteolytic sites of the (Na^+,K^+)-ATPase are exposed on the cytoplasmic side of the membrane.

The (Na^+,K^+)-ATPase[1] is an intrinsic membrane protein that couples the hydrolysis of ATP to active transport of Na^+ and K^+ across plasma membranes (Cantley, 1981). In all active preparations of the enzyme, two polypeptides are found in equimolar quantities, a catalytic subunit, α, of approximate M_r 100 000, and a glycoprotein, β, of approximate M_r 60 000. The α chain spans the lipid bilayer; it contains the active site for ATP hydrolysis on the cytoplasmic side, and it can be labeled with photoaffinity derivatives of ouabain (Forbush et al., 1978), a specific inhibitor which binds to the extracellular surface of red blood cells (Perrone & Blostein, 1973). The existence of a carbohydrate moiety on the β chain implies that part of the polypeptide lies on the extracellular side of the membrane. The catalytic subunit can be phosphorylated either

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[1] Abbreviations: (Na^+,K^+)-ATPase, sodium and potassium ion stimulated adenosinetriphosphatase; P_i, inorganic orthophosphate; NaDodSO$_4$, sodium dodecyl sulfate; $K_{0.5}$, concentration of ligand that yields a half-maximal amount of product; PAS, periodic acid-Schiff.

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with ATP in the presence of Mg^{2+} and Na^+ or with P_i in the presence of Mg^{2+} and ouabain to generate similar covalent enzyme–phosphate intermediates (Sen et al., 1969).

Cleavage of the α chain with trypsin and chymotrypsin produces distinct fragments that have been ordered within the linear map of the polypeptide (Giotta, 1975; Jorgensen, 1975; Castro & Farley, 1979). These workers have also shown that the specific sites of hydrolysis depend on the conformational state of the enzyme. The ligands present during proteolysis determine which sets of protease-sensitive bonds are exposed. Because the experiments utilized purified, unsealed membranes of dog kidney (Na^+,K^+)-ATPase, one could not orient the proteolytic sites with respect to the lipid bilayer. Knauf et al. (1974) established that the activity of the (Na^+,K^+)-ATPase in ghosts and its mobility on $NaDodSO_4$ gels were unaffected if intact red cells were first exposed to Pronase. They also observed that Pronase treatment of unsealed ghosts modified both parameters. Giotta (1975) performed similar experiments on red cells and ghosts with trypsin and obtained comparable results. Both Knauf et al. (1974) and Giotta (1975) suggested that the protease-sensitive regions of the enzyme are located on the cytoplasmic side of the membrane. An implicit assumption was that no protease-sensitive bonds became accessible on the extracellular side as a result of cell lysis and ghost preparation. Furthermore, limited cleavage at specific sites was not demonstrated; all of the parameters that were measured were entirely abolished by proteolysis, or totally insensitive. Karlish & Pick (1981) used the kinetics of tryptic inactivation of cholate-reconstituted (Na^+,K^+)-ATPase to suggest that the aforementioned sites are cytoplasmic. Without data on the molecular weights of the fragments, however, it is not clear if the peptide bonds cleaved in their system are identical with those seen in the native, unsolubilized enzyme.

In this work, we examine hydrolysis of the (Na^+,K^+)-ATPase at the tryptic and chymotryptic sites in a system of restricted sidedness that has never been treated with detergent. We use specific labeling of the α chain by phosphorylation with $^{32}P_i$ in the presence of ouabain to characterize the limited proteolysis of the (Na^+,K^+)-ATPase from human red blood cells and from dog kidneys by trypsin and chymotrypsin. Although the (Na^+,K^+)-ATPase of dog kidneys can be purified to homogeneity, the enzyme of human red cells represents less than 0.05% of the total membrane protein (Dunham & Hoffman, 1970). The specificity and sensitivity of ouabain-stimulated phosphorylation is utilized for structural studies in the red cell. We directly demonstrate the location of proteolytic sites by showing that the α chain is specifically cleaved in a membrane preparation in which the cytoplasmic side is exposed and the extracellular side is protected.

Materials and Methods

Materials. Human red blood cells were obtained from the Massachusetts Red Cross, and dog kidneys were generously provided by the Cardiovascular Research Department at the Massachusetts General Hospital. 1-Hexyldecylpyridinium chloride was from MCB. α-Chymotrypsin, 3× crystallized, and trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone to inhibit contaminating chymotryptic activity] were purchased from Worthington. Both proteases were from bovine pancreas.

General Procedures. (Na^+,K^+)-ATPase was purified from dog kidneys by the method of Jorgensen (1974) to a specific activity of 20–30 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C. Protein concentrations were measured by the procedure of Lowry et al. (1951) in the presence of 1% $NaDodSO_4$ with bovine serum albumin as standard. Acetylcholinesterase assays were as described by Steck & Kant (1974). $NaDodSO_4$–polyacrylamide gels were run according to Laemmli (1970) and stained with Coomassie blue or stained with PAS following Fairbanks et al. (1971). Polyacrylamide gel electrophoresis at acid pH using the cationic detergent 1-hexyldecylpyridinium chloride and subsequent autoradiography were carried out following Resh (1982). In agreement with previous results (Amory et al., 1980), molecular weights could not be estimated from the relative mobilities of soluble and membrane-bound marker proteins.

Preparation of Cells. Red blood cells were washed twice with a modified Ringer's buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM Na_2HPO_4 (pH 7.4), 1 mM $MgSO_4$, and 10 mM glucose and then washed once and suspended to 50% hematocrit in a buffer containing 0.25 M sucrose, 25 mM tris(hydroxymethyl)aminomethane (pH 7.4), and 5 mM $MgCl_2$. The cells were incubated for 30 min at 23 °C either with the addition of 0.1 mM strophanthidin (a stock solution of 30 mM in ethanol) or with the addition of the same volume of ethanol. This was followed by an incubation for 5 min at 37 °C in the presence or absence of 0.3 mg/mL chymotrypsin. All samples were then treated with phenylmethanesulfonyl fluoride. The cells were centrifuged, resuspended in modified Ringer's buffer, and incubated for 60 min at 23 °C. This washing procedure was repeated 3 more times. Trypsin treatment of intact red cells was carried out in the same sucrose buffer for 5 min at 37 °C in the presence of 0.06 mg/mL trypsin. Samples were quenched with soybean trypsin inhibitor and washed as above.

Ghosts and Inside Out Vesicles. Red cell ghosts and inside out vesicles were prepared following the procedure of Steck & Kant (1974), except that 10 μM ouabain was included in the vesiculation medium, 0.5 mM Na_2HPO_4 (pH 8.0), to prepare vesicles with internally trapped ouabain. After centrifugation on a dextran cushion, the vesicles were gently resuspended in a buffer containing 25 mM imidazole (pH 7.4) and 5 mM $MgCl_2$ and then washed several times with the same buffer. They were suspended to a final protein concentration of about 1 mg/mL.

$^{86}Rb^+$ Transport. Washed cells were suspended to 50% hematocrit in the modified Ringer's buffer in the absence or presence of 60 μM ouabain and warmed to 37 °C. Transport assays were initiated by the addition of 2 μCi of tracer $^{86}Rb^+$ to each mL of cell suspension. Aliquots of 0.1 mL were removed at timed intervals and added to 0.9 mL of ice-cold buffer. The samples were centrifuged, the supernatant was removed, and 0.9 mL of 10% trichloroacetic acid was added to the cell pellet. After thorough mixing and centrifugation, 0.5 mL of the clarified supernatant was added to 3.5 mL of Aquasol for scintillation counting.

Phosphorylation of the (Na^+,K^+)-ATPase. All phosphorylation reactions were performed in 25 mM imidazole (pH 7.4) and 5 mM $MgCl_2$. Aliquots (50 μL) of ghosts or vesicles containing about 50 μg of membrane protein were preincubated for 30 min at 23 °C in the presence of 10 μM KH_2PO_4 (pH 7.4) to permit binding of externally added or intravesicular ouabain. Labeling was initiated by the addition of 10 μL of [^{32}P]orthophosphoric acid (2 mCi/mL in imidazole/$MgCl_2$) and quenched after 2 min with 0.9 mL of ice-cold 5% trichloroacetic acid and 50 μL of 0.6 M KH_2PO_4 (pH 2.0). The samples were centrifuged, and the pellets were vigorously suspended and washed twice with the same volumes of ice-cold trichloroacetic acid and KH_2PO_4. After a final, gentle wash with 0.2 mL of ice-cold 0.15 M KH_2PO_4 (pH 2.0), the pellets were solubilized in 50 μL of acid gel sample buffer containing

Table I: $^{86}Rb^+$ Uptake by Red Blood Cells[a]

pretreatment	$^{86}Rb^+$ uptake (cpm × 10^{-2})[b] −ouabain	+ouabain	ouabain inhibitable
none	16.5	4.5	12
chymotrypsin	15	4.5	10.5
strophanthidin	16	4	12
chymotrypsin + strophanthidin	16	4.5	11.5

[a] Pretreatment of cells and assays of $^{86}Rb^+$ transport were performed as described under Materials and Methods. In all cases rates of uptake were linear for at least 60 min, and the amount of isotope trapped at 60 min was less than 10% of total isotope added. [b] Values from two experiments were normalized and averaged to represent rates of $^{86}Rb^+$ uptake assayed in 1.0 mL of a 50% cell suspension. These values of $^{86}Rb^+$ uptake are time points taken at $t = 10$ min.

0.25 M sucrose, 0.1 M KH_2PO_4 (pH 4.0), 70 mM hexyldecylpyridinium chloride, 10 μg/mL pyronin Y, and 5% (v/v) 2-mercaptoethanol.

Purified kidney enzyme (0.5 μg) was phosphorylated similarly except that only 1–2 μL of the $^{32}P_i$ solution was used, and bovine serum albumin (50 μg) was added as carrier after quenching with trichloroacetic acid.

Proteolysis. The (Na^+,K^+)-ATPase was phosphorylated with ouabain bound. However, 2 min after the addition of $^{32}P_i$, proteases were added to the reaction mixture, and the digestion was stopped with inhibitors before addition of trichloroacetic acid. Except as noted, α-chymotrypsin digestions were performed for 10 min at 37 °C in the presence of soybean trypsin inhibitor at a weight ratio of chymotrypsin/trypsin inhibitor of 10/1 and were quenched with phenylmethanesulfonyl fluoride. All trypsin digestions were performed with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin for 5 min at 37 °C and were stopped with soybean trypsin inhibitor. Other conditions of proteolysis are described in the figures.

Results and Discussion

Castro & Farley (1979) have shown that chymotrypsin digestion of dog kidney (Na^+,K^+)-ATPase in the absence or presence of ligands produces a fragment of M_r 77 000[2] from the α chain. A second chymotryptic site is exposed after ouabain binds, and cleavage of the M_r 77 000 fragment at this site yields fragments of M_r 40 000 and 35 000. Human red blood cells were treated with chymotrypsin in the absence and presence of strophanthidin[3] in order to hydrolyze the α chain at these sites. Extensive washing removed the inhibitor, and the cells were then assayed for $^{86}Rb^+$ uptake. The results presented in Table I show that the ouabain-inhibitable Rb^+ transport is resistant to extracellular chymotrypsin. Since cleavage at the ouabain-independent site abolishes ATPase activity (Castro & Farley, 1979), one can infer that this site is located on the cytoplasmic side. The effect of cleavage at the ouabain-dependent site on ATPase activity is unknown, and therefore no suggestion about the topological position of this site can be made. The resistance of red cell (Na^+,K^+)-ATPase activity to external chymotrypsin is similar to that observed with Pronase and trypsin (Knauf et al., 1974; Giotta, 1975).

[2] We refer to proteolytic fragments by their apparent mass in 10% $NaDodSO_4$–polyacrylamide gels.

[3] Strophanthidin is similar to ouabain in its specific inhibition of (Na^+,K^+)-ATPase but has a higher rate of dissociation from the enzyme (Yoda & Hokin, 1970).

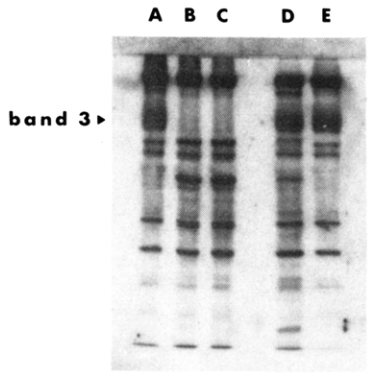

FIGURE 1: Extracellular proteolysis of red cell membrane proteins. Intact red cells were treated with proteases, and ghost membranes were prepared, analyzed on a 10% $NaDodSO_4$ gel, and stained with Coomassie blue as described under Materials and Methods. Each lane contained 12.5 μL of a 2 mg/mL sample. (A and E) Controls, in the absence of protease. (B) Chymotrypsin. (C) Chymotrypsin in the presence of strophanthidin. (D) Trypsin.

In control experiments, membranes from similarly treated cell samples were analyzed by $NaDodSO_4$ gel electrophoresis as shown in Figure 1. Comparison of lanes B and C with lane A demonstrates that band 3 has been quantitatively hydrolyzed at its extracellular chymotryptic site (Steck et al., 1976). Also presented in Figure 1, lanes D and E, is the resistance of the Coomassie blue stained proteins of the red cell membrane to digestion by extracellular trypsin (Steck et al., 1976). In agreement with previous results (Steck et al., 1971), we also observed in parallel gels that extracellular trypsin virtually eliminated PAS staining of PAS-1 and PAS-2 and that extracellular chymotrypsin generated a new PAS species migrating between PAS-1 and PAS-2. Thus, under these experimental conditions, both trypsin and chymotrypsin are capable of cleaving peptide bonds.

The membrane of the human red blood cell has already been established as a well-defined system that can be manipulated to produce sealed, inside out vesicles (Steck & Kant, 1974). The ouabain-stimulated phosphorylation of the (Na^+,K^+)-ATPase by $^{32}P_i$ (Sen et al., 1969) was examined as a specific marker for the α chain. Preliminary experiments with ghosts indicated that exchange of label into the phosphorylated enzyme reached saturation within 2 min with an approximate $K_{0.5}$ = 0.1 μM for ouabain under the reaction conditions. Portions of the same membrane samples displayed in Figure 1 were phosphorylated in the presence (10 μM) or absence of ouabain. The results are shown in Figure 2.[4] The intact ATPase is identified as the major labeled band and also as the only band whose labeling is stimulated by ouabain. Furthermore, since equal quantities of protein were loaded in

[4] We tentatively explain the ouabain-independent labeling in Figures 2–4 as follows. The label trapped at the top of the stacking gel probably represents free phosphate, which is not removed by the brief wash prior to drying the gel. Coomassie blue staining of acid gels reveals no protein at the top of the stacking gel and variable amounts at the top of the separating gel. We believe incomplete solubilization and aggregation produce material that fails to enter the running gel. In $NaDodSO_4$ gels we have observed identical behavior, that solubilization at 23 °C is sometimes incomplete, that boiling (which was avoided in this system to preserve the covalent intermediate) reduces the amount of large aggregates, and that proteolysis also diminishes the amount of protein that remains at the interface. The identities of the low molecular weight bands, which migrate with the tracking dye, seen in the red cell membrane sample lanes are unknown. Most of the ouabain-independent bands seen in Figure 2 are no longer present in Figure 3, after the red cell ghosts have been stripped of some peripheral proteins during vesiculation.

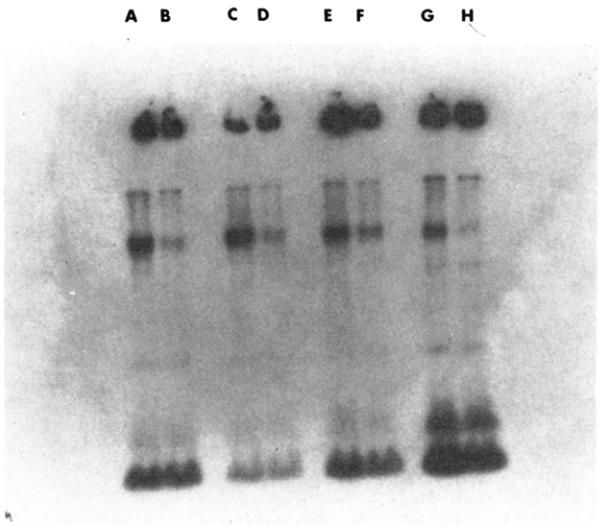

FIGURE 2: Phosphorylation of the red cell (Na^+,K^+)-ATPase. Ghost membranes from the samples shown in Figure 1 were phosphorylated and analyzed by acid gel electrophoresis and autoradiography as described under Materials and Methods. Each lane contained 50 μL of a 1.5 mg/mL sample. The left lane of each pair was phosphorylated in the presence of ouabain and the right lane in the absence of ouabain. (A and B) Controls, in the absence of protease. (C and D) Chymotrypsin. (E and F) Chymotrypsin in the presence of strophanthidin. (G and H) Trypsin.

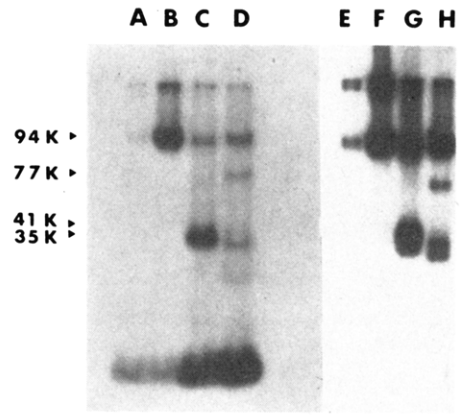

FIGURE 3: Proteolysis of the phosphorylated (Na^+,K^+)-ATPase. Inside out vesicles and purified kidney enzyme were phosphorylated, digested, and analyzed by acid gel electrophoresis and autoradiography as described under Materials and Methods. Lanes A–D and lanes E–H are from two separate gels. (A–D) Inside out vesicles; 50 μL of a 0.65 mg/mL sample was loaded in each lane. (E–H) Kidney enzyme; 20 μL of 10 μg of ATPase/mL of sample was loaded in each lane. (A and E) Phosphorylation in the absence of ouabain. (B–D and F–H) Phosphorylation in the presence of ouabain. (C) Phosphorylated vesicles digested with 20 μg/mL trypsin. (D) Phosphorylated vesicles digested with 0.65 mg/mL chymotrypsin. (G) Phosphorylated ATPase digested with 1 μg/mL trypsin. (H) Phosphorylated ATPase digested with 40 μg/mL chymotrypsin.

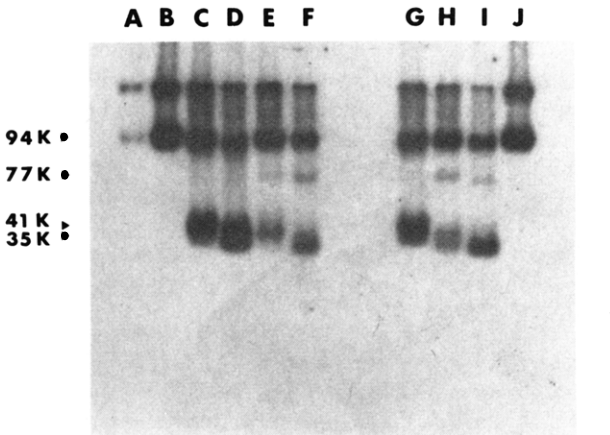

FIGURE 4: Proteolysis of the phosphorylated kidney (Na^+,K^+)-ATPase. Phosphorylation, proteolysis, acid gel electrophoresis, and autoradiography were performed as described under Materials and Methods. Each lane contained 50 μl of 10 μg of ATPase/mL of sample. (A) Phosphorylation in the absence of ouabain. (B–J) Phosphorylation in the presence of ouabain. (C, D, and G) Phosphorylated ATPase digested with 0.5, 2, and 1 μg/mL trypsin, respectively. (E and F) Phosphorylated ATPase digested with 10 and 50 μg/mL chymotrypsin in the absence of soybean trypsin inhibitor. (H) Phosphorylated ATPase digested with 40 μg/mL chymotrypsin in the presence of 3 μg/mL soybean trypsin inhibitor. (I) Phosphorylated ATPase digested for 5 min at 37 °C with 40 μg/mL chymotrypsin, then 1 μg/mL trypsin added, and digestion continued for 5 min at 37 °C.

each lane, the qualitative equivalence in the intensity of labeling suggests that both trypsin and chymotrypsin, when present at the extracellular side, do not alter the molecular weight of the α subunit of the (Na^+,K^+)-ATPase of human red cells.

Forbush (1982) has recently reported a purification of sealed, right side out vesicles consisting of 30% (Na^+,K^+)-ATPase from dog kidneys. He finds that bovine trypsin inactivates the ATPase activity in the absence of detergent; however, the results of Giotta (1975) and those in this paper indicate that extracellular trypsin, applied to intact cells, does not affect the ATPase activity or the ouabain-stimulated phosphorylation of the enzyme. It is possible that this apparent inconsistency is due to differences in the membrane environment of the enzyme in these two sources.

The incorporation of label into inside out vesicles prepared with and without ouabain (10 μM) was strictly dependent on internally trapped ouabain. In control experiments, externally added ouabain produced little or no stimulation of labeling, suggesting that most of the (Na^+,K^+)-ATPase is in sealed vesicles and is oriented with the extracellular side facing inward and inaccessible from the medium. Acetylcholinesterase assays in the absence and presence of Triton X-100 showed that only 10–15% of the total esterase activity (2 μmol min^{-1} mg^{-1} at 23 °C) was exposed to the medium.

Inside out vesicles and purified kidney enzyme membranes were phosphorylated with $^{32}P_i$ in the absence or presence of ouabain and then were digested with trypsin or chymotrypsin. Control experiments showed that no proteins were labeled after phosphorylation and proteolysis in the absence of ouabain; this indicates that all of the labeled peptides represent the α chain of the (Na^+,K^+)-ATPase and its fragments and do not derive from possible proteolytically activated phosphorylation of other proteins. The results of parallel digestion, acid gel electrophoresis, and autoradiography are shown in Figure 3. The α chain of both preparations is the M_r 94 000 labeled band whose phosphorylation is stimulated by ouabain. Limited tryptic digestion of the red cell (lane C) and kidney (lane G) (Na^+,K^+)-ATPases produces bands of similar electrophoretic mobility; these correspond to the doublet of fragments of M_r 41 000 (Castro & Farley, 1979) as described below. Close examination of the patterns in lanes C and G (and seen more easily in comparison of lanes B, C, and D of Figure 4) shows that a small peptide is removed from both α chains by trypsin. We think that this small shift in mobility is significant because it has been reproducibly observed in four separate experiments. This is in agreement with earlier reports of a tryptic site near the NH_2 terminus (Jorgensen, 1977; Castro & Farley, 1979). Chymotryptic digestion of inside out vesicles (lane D) produces partial fragmentation of the α chain to a smaller peptide which is in turn cleaved to a doublet. Hydrolysis of the kidney enzyme (lane H) at one or both of the chymotryptic sites yields

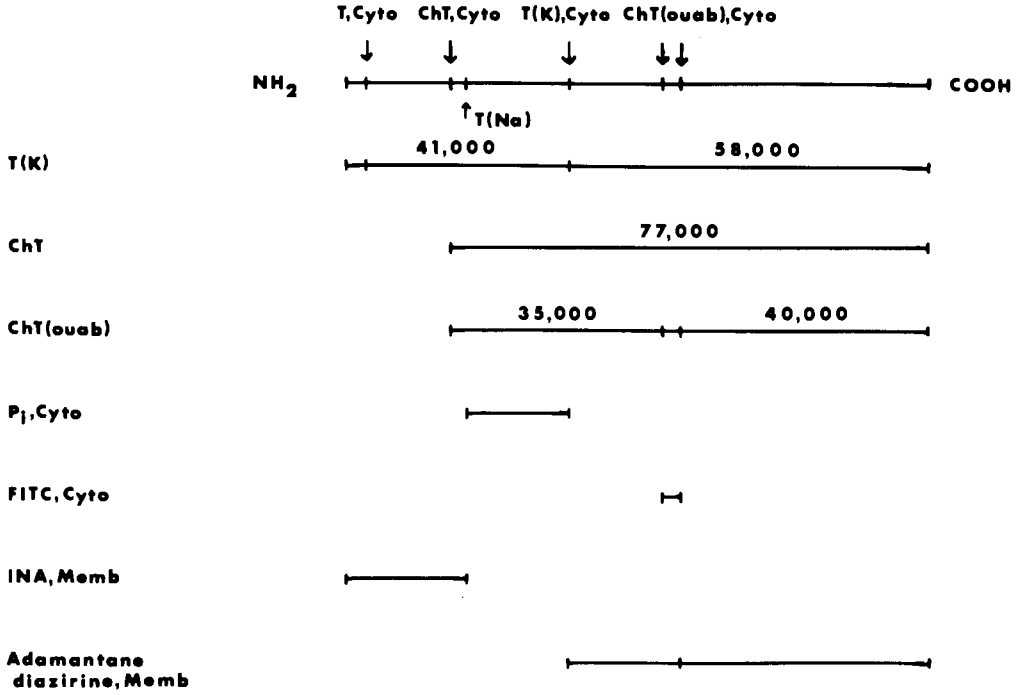

FIGURE 5: Structure of the α chain of the (Na^+,K^+)-ATPase. The linear arrangement of the fragments has been previously determined (Castro & Farley, 1979). The locations of the proteolytic sites with respect to the membrane have been described in the text. The chemically labeled regions of the polypeptide and their suggested positions with respect to the membrane have been identified (Karlish et al., 1977; Farley et al., 1980; Carilli et al., 1982). T, trypsin; ChT, chymotrypsin; ouab, ouabain; Cyto, cytoplasmic; Memb, membrane embedded; FITC, fluorescein 5′-isothiocyanate; INA, 5-iodonaphthyl 1-azide.

phosphorylated peptides of M_r 77 000 and 35 000 as discussed below. The digestions with chymotrypsin were performed at relatively high ratios of protease to membrane protein to improve detection.

The results of digestion of kidney enzyme at different concentrations of protease are presented in Figure 4. Lanes C, D, and G show a conversion of the initial M_r 41 000 doublet to one of slightly smaller molecular weight with increasing amounts of trypsin. A similar modification of the M_r 35 000 fragment with increasing levels of chymotrypsin is seen in lanes E, F, and H. Identical behavior of the red cell (Na^+,K^+)-ATPase can be produced with chymotrypsin but is not observed with trypsin (G. Chin and M. Forgac, unpublished experiments). Finally, digestion of chymotrypsin-treated kidney enzyme with trypsin (lane I) converts both the M_r 77 000 and M_r 35 000 labeled polypeptides to slightly smaller fragments. Examination of digested kidney enzyme by Na-DodSO$_4$ gel electrophoresis (data not shown) revealed that the expected cleavage patterns (Castro & Farley, 1979) had been produced and supported the assignment of molecular weights. These results characterize the (Na^+,K^+)-ATPase peptides in Figure 3 and confirm the identity of the labeled bands in the acid gels.

From the essentially identical proteolysis of the (Na^+,K^+)-ATPases from inside out vesicles and from dog kidneys, we conclude that all of the proteolytic sites mapped within the α chain lie on the cytoplasmic side of the membrane. These include the two major chymotryptic sites that define phosphorylated peptides of M_r 77 000 and 35 000 along with a third site that isolates from the M_r 35 000 peptide a small fragment that may contain the residue modified by fluorescein 5′-isothiocyanate (Carilli et al., 1982). These also include the tryptic site near the NH_2 terminus and the site that specifies a phosphorylated peptide of M_r 41 000 in the presence of K^+ or ouabain. The third major tryptic site, which is exposed in the presence of Na^+ (Giotta, 1975; Jorgensen, 1975; Castro & Farley, 1979), is not easily observed with ouabain bound. Nevertheless, the small shifts in molecular weights of the M_r 77 000 and 35 000 chymotryptic fragments are probably due to hydrolysis at this site, located near the NH_2 termini of these peptides. Another possibility is tryptic cleavage at unrelated sites.

Other workers have used chemical labels in combination with proteolysis to determine either exposed or membrane-embedded regions of the α chain (Karlish et al., 1977; Castro & Farley, 1979; Farley et al., 1980; Carilli et al., 1982). Figure 5 presents a summary of the proteolytic sites, their positions with respect to the membrane and within the polypeptide, and the locations of labeled fragments.

Acknowledgments

We thank Guido Guidotti, in whose laboratory this research was done, for his constant encouragement and advice, Marilyn Resh for her descriptions of phosphorylation and acid gel electrophoresis procedures, and Lewis Cantley for his many helpful suggestions.

Registry No. ATPase, 9000-83-3; proteinase, 9001-92-7; trypsin, 9002-07-7; chymotrypsin, 9004-07-3.

References

Amory, A., Foury, F., & Goffeau, A. (1980) *J. Biol. Chem. 255*, 9353–9357.

Cantley, L. C. (1981) *Curr. Top. Bioenerg. 11*, 201–237.

Carilli, C. T., Farley, R. A., Perlman, D. M., & Cantley, L. C. (1982) *J. Biol. Chem. 257*, 5601–5606.

Castro, J., & Farley, R. A. (1979) *J. Biol. Chem. 254*, 2221–2228.

Dunham, P. B., & Hoffman, J. F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 936–943.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Farley, R. A., Goldman, D. W., & Bayley, H. (1980) *J. Biol. Chem. 255*, 860–864.

Forbush, B. (1982) *J. Biol. Chem. 257*, 12678–12684.

Forbush, B., Kaplan, J. H., & Hoffman, J. F. (1978) *Biochemistry 17*, 3667-3676.
Giotta, G. J. (1975) *J. Biol. Chem. 250*, 5159-5164.
Jorgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36-52.
Jorgensen, P. L. (1975) *Biochim. Biophys. Acta 401*, 399-415.
Jorgensen, P. L. (1977) *Biochim. Biophys. Acta 466*, 97-108.
Karlish, S. J. D., & Pick, U. (1981) *J. Physiol. (London) 312*, 505-529.
Karlish, S. J. D., Jorgensen, P. L., & Gitler, C. (1977) *Nature (London) 269*, 715-717.
Knauf, P. A., Proverbio, F., & Hoffman, J. F. (1974) *J. Gen. Physiol. 63*, 305-323.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Perrone, J. R., & Blostein, R. (1973) *Biochim. Biophys. Acta 291*, 680-689.
Resh, M. D. (1982) *J. Biol. Chem. 257*, 6978-6986.
Sen, A. K., Tobin, T., & Post, R. L. (1969) *J. Biol. Chem. 244*, 6596-6604.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172-180.
Steck, T. L., Fairbanks, G., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2617-2624.
Steck, T. L., Ramos, B., & Strapazon, E. (1976) *Biochemistry 15*, 1154-1161.
Yoda, A., & Hokin, L. (1970) *Biochem. Biophys. Res. Commun. 40*, 880-886.

Binding of Phosphorylated Effectors by Active and Inactive Forms of Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: Activation of ribulose-1,5-bisphosphate carboxylase by CO_2 and Mg^{2+} is slow and reversible. At subsaturating concentrations of CO_2 and Mg^{2+}, positive effectors increase and negative effectors decrease the amount of active enzyme at equilibrium. Preequilibrium experiments indicated that both positive and negative effectors inhibit the rates of enzyme activation and deactivation. Greater than 99% inhibition of the activation and deactivation rates was observed at high effector concentrations, indicating that the binding and release of the activators CO_2 and Mg^{2+} occur only with effector-free enzyme. The deactivation rate K_i values for the negative effector ribose 5-phosphate and the positive effectors inorganic phosphate, fructose 1,6-bisphosphate, and 6-phosphogluconate were smaller than the corresponding activation rate K_i values by factors of 2, 6, 25, and 670, respectively. Thus, phosphorylated effectors impede deactivation more than activation. Equilibrium binding studies indicated that the active and inactive enzyme forms have similar affinities for the positive effectors inorganic phosphate (K_D = 650 μM) and fructose bisphosphate (K_D = 11 μM). The positive effector 3-phosphoglycerate was bound with greater affinity by the inactive enzyme (K_D = 25 μM) than by the active enzyme (K_D = 76 μM). Thus, preferential binding of positive effectors to the active enzyme form is not responsible for the enhancement of enzyme activation at equilibrium. The promotion of activation by positive effectors is mediated by altering the relative rates of activation and deactivation to favor active enzyme. Equilibrium binding studies indicated that the inactive enzyme had a much greater affinity for ribose 5-phosphate (K_D = 42 μM) than did the active enzyme (K_D = 480 μM). Preferential binding of this negative effector to inactive enzyme exceeds its stabilizing effect on the active enzyme form and thus causes an overall reduction of activation at equilibrium.

Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) requires the addition of CO_2 and a divalent metal ion in forming an activated enzyme complex capable of catalyzing the carboxylation and oxygenation of RuBP[1] (Lorimer et al., 1976). The kinetics of this process have been well characterized for several RuBPCase enzymes purified from microbial and plant sources (Badger, 1980; Christeller & Laing, 1978; Laing & Christeller, 1976). The rate-limiting step in CO_2/Me^{2+} activation is carbamate formation at a lysine residue (Lys-201) on the 56-kDa catalytic subunit of the enzyme (Lorimer, 1981; Lorimer & Miziorko, 1980), and this is followed by rapid addition of the metal cofactor as shown in eq 1.

$$\underset{\text{inactive}}{E} \overset{\text{slow}}{\rightleftharpoons} E\text{-}CO_2 \overset{\text{fast}}{\rightleftharpoons} \underset{\text{active}}{E\text{-}CO_2\text{-}Me^{2+}} \quad (1)$$

Although the mechanisms of activation and inhibition of RuBPCase by sugar phosphates and other anions have received considerable attention, these processes are not well resolved. Because of their potential regulatory role in vivo, chloroplast metabolites such as 6-P-gluconate, FBP, inorganic phosphate, and NADPH have been of primary interest. Several sugar phosphates enhance the activation state of RuBPCase which is partially activated with respect to CO_2 and Mg^{2+}, while other sugar phosphates lower the activation state of the enzyme

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[1] Abbreviations: RuBP, ribulose 1,5-bisphosphate; RuBPCase, ribulose-1,5-bisphosphate carboxylase; CABP, 2-carboxyarabinitol 1,5-bisphosphate; FBP, fructose 1,6-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; P, phosphate; kDa, kilodalton; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-3410$01.50/0