Biochimica et Biophysica Acta 856 (1986) 496–504
Elsevier

BBA 73032

Tryptic modification of red-cell sodium pump behaviour

William J. Harvey and Rhoda Blostein *

Departments of Biochemistry and Medicine, McGill University, Montreal, Quebec (Canada)

(Received December 3rd, 1985)

Key words: $(Na^+ + K^+)$-ATPase; Na^+ transport; Tryptic digestion

Inside-out membrane vesicles derived from human red cells were used to probe the effects of controlled tryptic digestion on the sodium pump as it exists in situ. Digestion of the enzyme in its E_1 conformation resulted in several alterations which are generally similar to those reported for the purified kidney enzyme, namely (i) greater loss in overall hydrolytic activity compared to level of phosphoenzyme intermediate and (ii) cleavage of the α-subunit by trypsin as well as chymotrypsin at the cytoplasmic surface to yield a fragment of approx. 78 kDa. Tryptic digestion effected similar rates of inactivation of pump-mediated Na^+-$K^+(Rb^+)$ exchange, (ATP- plus ADP)-dependent Na^+-Na^+ exchange and, in the absence extracellular alkali cation, 'uncoupled' Na^+ flux ($Na^+/0$ flux). Alteration in the $Na^+ : Rb^+(K^+)$ stoichiometry following trypsin cleavage could not be detected. The conformational transitions of phosphoenzyme and dephosphoenzyme are affected similarly by trypsin, as evidenced by similar inactivation rates of reactions through the 'forward' sequence involving the E_1P to E_2P transition as well as through the 'reserve' sequence involving the E_1 to E_2 transition.

Introduction

The relationship between structure and function in terms of the various enzymic reactions and conformational changes of the sodium pump has been examined by specific proteolytic cleavage of purified $(Na^+ + K^+)$-ATPase from kidney [1–10]. In those studies, it was observed that trypsin distinguished two principal conformational states of the enzyme: $Na \cdot E_1$ in the presence of Na^+ or choline and $K \cdot E_2$ in the presence of K^+ [1–3]. Moreover, specific cleavage of $Na \cdot E_1$ effected inhibition of the enzyme's conformational transitions [4–6], with the result that overall hydrolytic activity was affected more than either the steady-state level of phosphoenzyme intermediate or the associated partial reaction, ouabain-sensitive Na^+-dependent ADP-ATP exchange [4]. Studies of $(Na^+ + K^+)$-ATPase reconstituted into liposomes showed that proteolysis of $Na \cdot E_1$ by chymotrypsin effected dissimilar losses of Na^+-Na^+ exchange compared to K^+-K^+ exchange [8]. Although decreased $Na^+ : K^+(Rb^+)$ stoichiometry following trypsin treatment was observed in some experiments [9], in others [10] this was not

* To whom correspondence should be addressed at: The Montreal General Hospital Research Institute, 1650 Cedar Avenue, Montreal, Quebec, Canada, H3G 1A4.

Abbreviations: $(Na^+ + K^+)$-ATP, sodium- plus potassium-activated adenosine triphosphatase; Na^+-ATPase, sodium-activated adenosine triphosphatase; Na_{cyt}, K_{cy} or Rb_{cyt}, are Na^+, K^+ or Rb^+ in the medium exposed to the membrane surface which war originally facing the cytoplasm; Na_{ext}, K_{ext} or Rb_{ext}, are Na^+, K^+ or Rb^+ inside inside-out vesicles, i.e., at the membrane surface which was originally facing the external environment. E, the $(Na^+ + K^+)$-ATPase enzyme; EP, the phosphorylated intermediate of E; K·E, the enzyme with bound K^+; Na·E, the enzyme with bound Na^+. E_1 and E_2 are the two conformational forms of E, E_1P and E_2P are the two conformational forms of EP. E·ATP is the enzyme with bound ATP. $(Na)E_1P$ and $(K)E_2$ are occluded forms of $Na \cdot E_1P$ and $K \cdot E_2$, respectively.

the case. The basis for these inconsistencies remains unclear.

In the present study, we have used controlled proteolytic digestion of human red-cell inside-out vesicles to probe structure-function relationships of $(Na^+ + K^+)$-ATPase as it exists in situ. This paper describes the relative effect of trypsin treatment on different pump modes and enzymic reactions of the sodium pump as well as on the $Na^+ : Rb^+$ exchange stoichiometry. Although with inside-out vesicles resolution of cleavage products is confined to visualization of ^{32}P-labelled fragments bearing the phosphorylated intermediate, specific cytoplasmic tryptic and chymotryptic cleavage sites of red-cell $(Na^+ + K^+)$-ATPase have been shown to be similar to those reported for purified kidney enzyme [11]. Furthermore, this preparation has the advantage that not only has sodium pump behaviour been well-established in the red blood cell (for a recent review, see Kaplan, Ref. 12), but also that it has a relatively large volume and low pump density, features which facilitate the accurate measurement of initial pump rates under a variety of conditions.

The location and sidedness of the effects of Na^+, K^+ or Rb^+ are described in terms of their normal cytoplasmic or extracellular orientation designated by the subscripts 'cyt' and 'ext' [13].

A preliminary report of this work was presented at the Annual Meeting of the Biophysical Society, Baltimore, 1985.

Methods and Materials

Inside-out vesicles were prepared from fresh human erythrocytes without further separation of sealed membranes from porous membrane fragments, as previously described [13,14]. They were stored at 0–4°C for up to 6 days in 20 mM Tris-glycylglycine (pH 7.4)/0.2 mM $MgSO_4$ at a concentration of 2–4 mg protein/ml. For all experiments, vesicles were first equilibrated for 30 min at 37°C with 50 mM chloride salt solutions of either NaCl or choline chloride plus 20 mM Tris-glycylglycine (pH 7.4) and 0.5–2.0 mM $MgSO_4$ with KCl or RbCl added as indicated. Na^+-activated ATP hydrolysis (Na^+-ATPase) and the phosphoenzyme intermediate (EP) were assayed as described previously [15,16]. Assays of ATP-dependent Na^+ and Rb^+ transport ($^{22}Na^+$ influx and $^{86}Rb^+$ efflux) were carried out by the filtration assay described elsewhere [16,17] using Sartorius-type SM filters (1.2 μm pore size). In some experiments, K^+ was equilibrated across the membrane by adding 2.2 μM valinomycin (0.005 vol. of 0.45 mM valinomycin dissolved in ethanol). $^{22}Na^+$ and $^{86}Rb^+$ were used at a specific activity of approx. 1.0 mCi/mmol. Values given for ATP-dependent fluxes represent the differences between mean values of measurements made with and without ATP or ATP plus ADP. Strophanthidin-sensitive *p*-nitrophenylphosphatase activity was assayed as described elsewhere [20,21].

Proteolysis of inside-out vesicles was carried out as follows. Vesicles, at a protein concentration of 3—5 mg/ml, were equilibrated 30 min at 0°C with 50 mM choline chloride salt in 20 mM Tris-glycylglycine (pH 7.4) and 0.2 mM $MgSO_4$ and then incubated for various periods of time, as indicated, with 1.8 μg/ml TPCK-trypsin or 4.5 μg/ml TLCK-chymotrypsin at 37°C. The reaction was stopped by addition of a 10-fold (w/w) excess of soybean trypsin inhibitor (18 μg/ml) or 0.01 mM chymostatin, respectively. Control and trypsinized inside-out vesicles were then phosphorylated in the presence of Na^+ or K^+ as described above.

Peptides bearing the (γ-^{32}P)-labelled phosphoenzyme were visualized following polyacrylamide gel electrophoresis in hexadecylpyridinium chloride at 2°C as described by Amory et al. [18] as modified by Resh [19]. For these experiments, the phosphorylation reaction was stopped by addition of 9 vol. 5% trichlororoacetic acid containing 5 mM Na-ATP and 2.5 mM NaH_2PO_4 [16]. Following centrifugation at 3000 rpm for 20 min, the precipitates were washed twice with 1 mM H_3PO_4 and then solubilized in 35 mM hexadecylpyridinium chloride containing 100 mM KH_2PO_4 (pH 3.0) 1.56% 2-mercaptoethanol, 0.15 M sucrose and 10 μg/ml Malachite green. Following electrophoresis for 5 h at 1–2°C, the gels were stained briefly (20 min) in 50% methanol, 10% acetic acid and 0.03% Coomassie brilliant blue and then destained in 10% acetic acid containing 2% glycerol. The gels were then dried and exposed to Kodak XR-OMAT film at −20°C.

Reagents were obtained from Sigma except for ATP (vanadate-free Na^+ form converted to the Tris-form), which was obtained from Boehringer-Mannheim. $^{22}NaCl$ and $^{86}RbCl$ (carrier-free) were obtained from Amersham or New England Nuclear. [γ-^{32}P]ATP (10–25 mCi/mmol) was synthesized from $H_3{}^{32}PO_4$ [22,23] obtained from Amersham, except for experiments aimed to detect the ^{32}P-labelled phosphoenzyme by autoradiography of polyacrylamide gel electropherograms; in these cases, [γ-^{32}P]ATP (3000 mCi/mmol) was obtained from New England Nuclear.

Results

Kinetics of tryptic inactivation

A controlled and relatively slow rate of tryptic inactivation of sodium pump activity (ATP-dependent $^{22}Na^+$ uptake into inside-out vesicles containing K^+) was effected by adjusting the ratio of trypsin to total membrane protein. As shown in Fig. 1, the time-course of trypsin inactivation of ATP-dependent sodium influx after proteolysis in Na^+ medium (or choline medium, not shown) is clearly different from that obtained in K^+ medium. Moreover, the curve can be described as a single exponential only when K^+ is present during trypsinolysis. This result is generally similar to that obtained with the reconstituted kidney enzyme [1,2]. Similar results were obtained when ($Na^+ + K^+$)-ATPase was measured with either inside-out vesicles or the purified pig kidney enzyme (not shown).

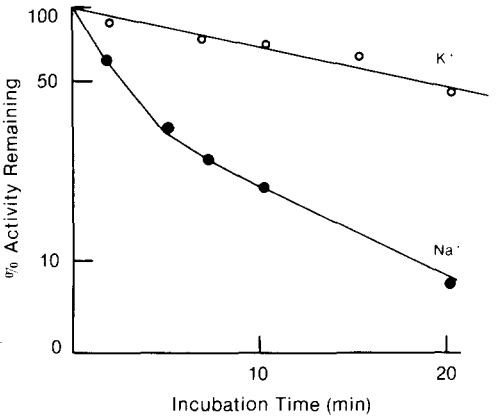

Fig. 1. Semilorgarithmic plot of the time-course of tryptic inactivation of ATP-dependent $^{22}Na^+$ uptake in inside-out vesicles. 1.5 ml of inside-out vesicles (4.5 mg protein/ml) in 25 mM NaCl and 25 mM choline chloride (●), or 10 mM KCl and 40 mM choline chloride (○), in 0.2 mM $MgSO_4$ and 20 mM Tris-glycylglycine (pH 7.4) were incubated with 1.5 μg TPCK-trypsin at 37°C. All inside-out vesicles contained 2.2 μM valinomycin to equilibrate K^+. 20 μl aliquots were removed at intervals as indicated and added to 80 μl soybean trypsin inhibitor (0.2 mg/ml in either 2.5 mM KCl, 47.5 mM choline chloride and 0.2 mM $MgSO_4$ for choline choride-loaded inside-out vesicles or 6.25 mM NaCl and 43.75 choline chloride for inside-out vesicles originally in KCl plus choline chloride). 25 μl of inside-out vesicles were then mixed with 225 μl of prewarmed (37°C) reaction medium containing 10 mM $^{22}NaCl$, 38 mM choline chloride, 0.2 mM $MgSO_4$, 2 mM KCl and 20 mM Tris-glycylglycine (pH 7.4), with or without 0.45 mM Tris-ATP. Incubation was carried out for 5 min at 37°C and the uptake of $^{22}Na^+$ was measured as described in Methods and Materials. The experiment shown is representative of three similar experiments. Each point shown is the average of duplicate determinations.

Phosphorylated tryptic fragments

Following trypsin treatment in choline chloride, inside-out vesicles were phosphorylated with [γ-^{32}P]ATP for 15 s at 37°C in the presence of of 50 mM NaCl to label the phosphorylated intermediate of the α-subunit of the ($Na^+ + K^+$)-ATPase. As shown in Fig. 2, trypsin has relatively little effect on the steady-state level of Na^+-dependent phosphoenzyme (EP) compared to the marked decrease in Na^+-ATPase. In the experiment shown in Fig. 3, the ^{32}P-labelled trypsinized inside-out vesicles were solubilized and subjected to polyacrylamide gel electrophoresis using cationic detergent at pH 2.0 as described in Methods and Materials. Na^+-dependent phosphorylation of the ($Na^+ + K^+$)-ATPase was apparent as a ^{32}P-labelled 100 kDa band in the control and trypsinized samples. In addition, ^{32}P-labelled bands, presumably tryptic fragments, are observed at a position close to the 100 kDa α-subunit, presumably the 78 kDa fragment, and as a weakly visible diffuse band at a molecular weight of about 45 kDa. Another visible band at approx. 15 kDa is apparent in some but not all electropherograms, e.g., Fig. 3, but only after prolonged (30 min) trypsinolysis. This pattern is generally similar to that reported for purified kidney ($Na^+ + K^+$)-ATPase [3]. It is relevant to note also that, like the kidney enzyme [3,8], the red-cell ($Na^+ + K^+$)-ATPase is cleaved by chymotrypsin to yield only

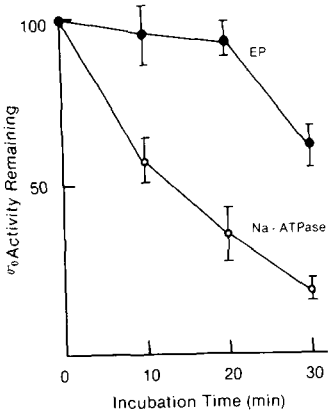

Fig. 2. Effect of trypsin on Na^+-ATPase activity and steady-state level of phosphoenzyme intermediate. Inside-out vesicles (4 mg proten/ml) were treated for 10, 20 and 30 min at 37°C with 1.8 μg/ml TPCK-trypsin in 50 mM choline chloride and the reaction was stopped with 20 μg/ml soybean trypsin inhibitor. Inside-out vesicles (30 μl) were then incubated for 15 s at 37°C in a final volume of 200 μl containing (final concentrations) 50 mM NaCl or 50 mM KCl containing 0.2 mM $MgSO_4$ and 0.5 μM [γ-^{32} P]ATP. ○, Na^+-ATPase activity; ●, EP. Each point represents the mean ± S.E. of the differences of two sets of quadruplicate samples, one with NaCl and the other with KCl (K^+ baseline).

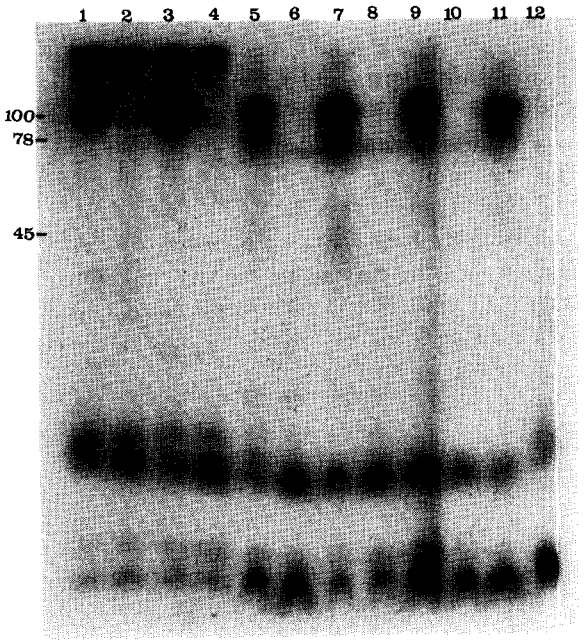

Fig. 3. Identification ^{32}P-phosphorylated tryptic and chymotryptic fragments of red-cell ($Na^+ + K^+$)-ATPase. Inside-out vesicles (5.4 mg protein/ml) were treated with TPCK-trypsin or TLCK-chymotrypsin in 50 mM choline chloride, 0.2 mM $MgSO_4$ and 20 mM Tris-glycylglycine (pH 7.4), as described in Methods and Materials. Phosphorylation was carried out for 15 s at 37°C in medium containing 0.2 mM $MgSO_4$, 0.5 [γ-^{32} P]ATP (2900 mCi/mmol), 20 mM Tris-glycylglycine (pH 7.4) and either 25 mM NaCl or 25 mM KCl. Aliquots (25 μg protein) were applied to each slot and subjected to electrophoresis for 5 h at 4°C using 40 mA current per gel slab as described in Methods and Materials. Molecular masses are designated in kilodaltons. Lanes 1, 3, 5, 7, 9, and 11. Phosphorylation in the presence of 25 mM NaCl: lanes 1, 5 and 7, trypsin treatment for 0, 10 and 25 min, respectively; lanes 3, 9 and 11, chymotrypsin treatment for 0, 30 and 60 min, respectively. Lanes, 2, 4, 6, 8, 10, and 12. Phosphorylation in the presence of 25 mM KCl: lanes 2, 6 and 8, trypsin treatment for 0, 10 and 25 min, respectively; lanes 4, 10 and 12, chymotrypsin treatment for 30 and 60 min, respectively.

the 78 kDa fragment close to the α-subunit (Fig. 3).

Effect of trypsin on vesicle permeability

($Na^+ + K^+$)-ATPase was measured in control and trypsinized vesicle preparations using the hydrophilic, impermeable cardiac glycoside, ouabain (0.2 mM), to inhibit the activity of membrane fragments or porous vesicles; the hydrophobic glycoside, strophanthidin (0.02 mM), was used to inhibit total ($Na^+ + K^+$)-ATPase. The difference between the two activities, the ouabain-insensitive ($Na^+ + K^+$)-ATPase, reflects, presumably, the intact inside-out vesicles. Typically, trypsin effected a time-dependent inactivation of strophanthidin-sensitive and ouabain-sensitive activities. In a representative experiment (not shown), one-third of the original activity remained after 30 min of trypsin treatment. In contrast, the percentage inside-out vesicles, based on the ratio of ouabain-insensitive ($Na^+ + K^+$)-ATPase to total ($Na^+ + K^+$)-ATPase, was decreased only slightly by trypsin, i.e., from an initial value of 71% to 62% after 30 min with trypsin; these estimates of percentage inside-out vesicles are similar to those obtained from measurements of the effects of trypsin on detergent-stimulated acetylcholinesterase activity (not shown). Under these conditions of trypsinolysis, overall Na^+ permeability evidenced as $^{22}Na^+$ uptake into inside-out vesicles appears to increase and then decrease somewhat with time of exposure to trypsin (Fig. 4). However, quantitative assessment of the effects of trypsin is not com-

promised by these permeability changes. Thus, as shown in Fig. 4, when membrane vesicles were treated with trypsin and subsequently equilibrated without or with 50 mM NaCl and then incubated in a medium containing 5 mM ^{22}NaCl, $^{22}Na^+$ influx was similar in both Na^+-free (choline-loaded) and Na^+-loaded vesicles; ATP-dependent Na^+ uptake into both is also affected similarly by trypsin, i.e., the percentage activity remaining was 40%, 22% and 16% for choline loaded and 38%, 24% and 16% for Na^+-loaded inside-out vesicles after 3, 6 and 10 min of trypsin treatment, respectively.

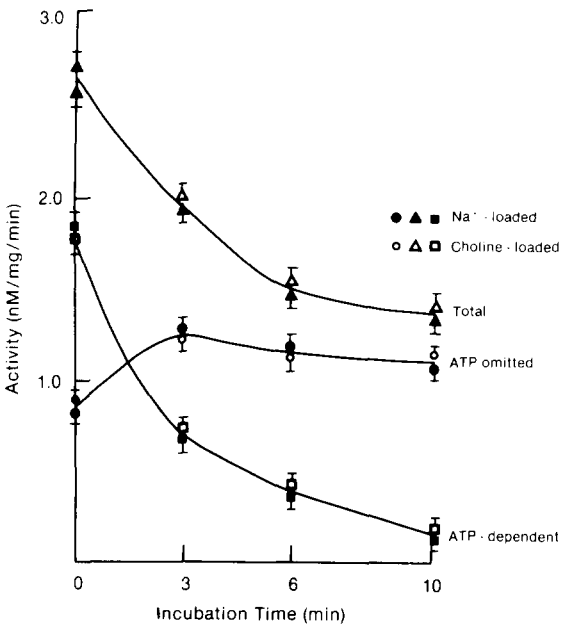

Fig. 4. Effect of intravesicular Na^+ on the influx of $^{22}Na^+$ into control and trypsin-treated inside-out vesicles in the absence or presence of ATP. Inside-out vesicles (4 mg protein/ml) in 50 mM choline chloride, 20 mM Tris-glycylglycine (pH 7.4) and 0.2 mM $MgSO_4$ were treated with 7 μg/ml TPCK-trypsin at 37°C. The reaction was stopped with 20 μg/ml soybean trypsin inhibitor. Following treatment, the vesicles were divided and equilibrated at 37°C for 10 min with either 5 mM RbCl and 45 mM choline chloride or 5 mM RbCl, 40 mM NaCl and 5 mM choline chloride in the presence of 2.2 μM valinomycin. 25 μl of inside-out vesicles were then mixed with 225 μl prewarmed (37°C) medium containing (final concentrations) 4 mM ^{22}NaCl, 0.5 mM RbCl, 45.5 mM choline, 1 mM $MgSO_4$ and 20 mM Tris-glycylglycine (pH 7.4) in the presence and absence of 0.2 mM Tris-ATP. After 4 min, 225 μl samples were removed for the assay of $^{22}Na^+$ uptake. Na^+-equilibrated inside-out vesicles: ●, minus ATP and, ▲, plus ATP; choline-equilibrated inside-out vesicles: ○, minus ATP and, △, plus ATP. Each point represents the mean ± S.E. of quadruplicate samples.

ATP-dependent Rb^+ efflux

As shown earlier [17] addition of ATP to $^{86}Rb^+$-loaded vesicles effects a rapid loss of $^{86}Rb^+$ from a fraction of the vesicles. The remaining $^{86}Rb^+$ is lost slowly, with a rate constant similar to that observed before ATP addition, and represents, presumably, vesicles without pumps. Fig. 5 shows that the trypsinized inside-out vesicles passively accumulate $^{86}Rb^+$ to the same extent and have a rate of passive $^{86}Rb^+$ efflux similar to that of the control vesicles. In contrast, trypsin effects a marked decrease in the apparent rate constant for ATP-dependent $^{86}Rb^+$ efflux (0.28 min^{-1} compared to 1.40 min^{-1} for control inside-out vesicles), as well as a decrease in the fraction of vesicles with pumps.

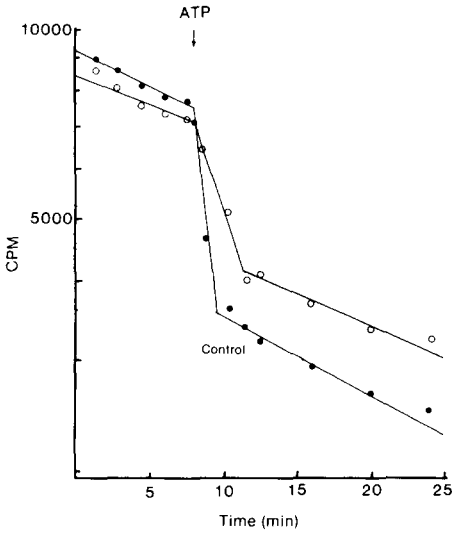

Fig. 5. Effect of trypsin on ATP-dependent $^{86}Rb^+$ efflux from inside-out vesicles. Control (●) and trypsin-treated inside-out vesicles (○) at a concentration of 3.1 mg protein/ml (15 min trypsin treatment in 50 mM choline chloride as in Fig. 2) were equilibrated with 0.4 mM ^{86}RbCl for 30 min at 37°C after which 350 μl were added to prewarmed (30 °C) medium (3.15 ml) containing 2 mM NaCl, 1 mM $MgSO_4$ 0.2 mM EGTA and 20 mM Tris-glycylglycine (pH 7.4). At intervals, aliquots (250 μl) were removed for the measurement of intravesicular $^{86}Rb^+$ as described in Methods and Materials except that 0.1 mM nonradioactive RbCl was included in the wash medium. Tris-ATP (8 μl) was added to the reaction mixture at the indicated time (final concentration, 0.2 mM).

$Na^+ : Rb^+$ stoichiometry

ATP-dependent $^{22}Na^+$ uptake and $^{86}Rb^+$ efflux were measured concurrently in control and trypsinized inside-out vesicles (Table I). Preliminary experiments were carried out to establish conditions under which the amount of ATP-mediated $^{86}Rb^+$ loss would not limit intravesicular $^{86}Rb^+$. Thus, ATP-dependent $^{2}Na^+$ uptake continuing after $^{86}Rb^+$ depletion, presumably the 'uncoupled' Na^+ efflux described earlier by Glynn and Karlish [24] would occur to a greater extent in more active unmodified vesicles than trypsinized vesicles, resulting in an apparently higher $Na^+ : Rb^+$ stoichiometry in the latter. In the experiment shown in Table I, the conditions chosen (0.25 mM ATP and 1 min incubation at 30°C) obviated this problem; the ATP-dependent decrease in $^{86}Rb^+$ in control vesicles was 33% and decreased to 22% and 16% after 10 and 40 min, respectively, of trypsin treatment. As shown, a significant difference in ATP-dependent uptake of $^{22}Na^+$ relative to loss of $^{86}Rb^+$ could not be detected following trypsin treatment.

TABLE I

COMPARISON OF ATP-DEPENDENT ^{22}Na UPTAKE AND ^{86}Rb EFFLUX IN CONTROL AND TRYPSIN-TREATED INSIDE-OUT VESICLES

Inside-out vesicles (2.5 mg/ml in 50 mM choline chloride) were incubated with 1.8 μg/ml TPCK-trypsin at 37°C. Samples (800 μl) were removed at 0, 10 and 40 min and added to 800 μl 40 μg/ml soybean trypsin inhibitor, after which aliquots (300 μl) were equilibrated with either 0.5 mM RbCl and 49.5 mM choline chloride for the measurement of $^{86}Rb^+$ efflux. The reaction was initiated by adding inside-out vesicles (20 μl) to 80 μl prewarmed medium containing 2.5 mM $^{22}NaCl$ or NaCl, 0.5 mM $MgSO_4$ and 47.5 mM choline chloride with and without 0.2 mM ATP. After 1 min at 30°C, 80 μl of the mixture were removed for the measurement of intravesicular $^{22}Na^+$ (ATP-dependent $^{22}Na^+$ uptake) or $^{86}Rb^+$ (ATP-dependent $^{86}Rb^+$ efflux) as described in Methods and Materials. Each point represents the mean ± S.D. of quadruplicate samples.

Time with trypsin (min)	ATP-dependent $^{22}Na^+$ uptake		ATP-dependent $^{86}Rb^+$ efflux	
	(nmol/mg per min)	%	(nmol/mg per min)	%
0	0.39 ± 0.05	100 ± 12	0.32 ± 0.03	100 ± 8
15	0.19 ± 0.01	49 ± 6	0.15 ± 0.01	47 ± 7
40	0.13 ± 0.01	33 ± 9	0.09 ± 0.01	28 ± 6

Effect of trypsin on sodium pump modes

Under normal conditions, the sodium pump effects ATP-dependent Na^+-K^+(Rb^+) exchange. ATP-dependent $^{22}Na^+$ transport is observed also in the absence of extracellular cations. This so-called 'uncoupled' Na^+ efflux appears to occur

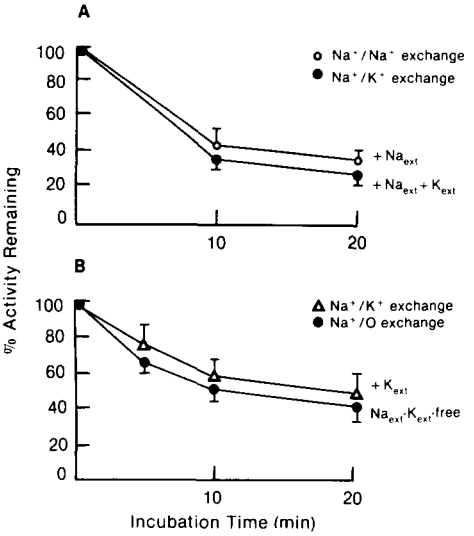

Fig. 6. (A) Effect of trypsin treatment on Na^+-K^+ exchange and Na^+-Na^+ exchange. 2 ml inside-out vesicles (3.4 mg protein/ml) were equilibrated with 50 mM NaCl, 0.2 mM $MgSO_4$, 20 mM Tris-glycylglycine (pH 7.4) and then incubated with 1.8 μg/ml trypsin at 37°C for 0, 15 or 30 min. The vesicles were the divided and one half was equilibrated with 0.1 vol. 5.0 mM KCl, 49,5 mM chloride with 2 μM valinomycin for Na^+-K^+ exchange (●), which was measured in the absence and presence of 0.1 mM Tris-ATP; the remainder was equilibrated with 0.1 vol. 50 mM choline chloride for Na^+-Na^+ exchange (○), which was measured in the absence or presence of 0.1 mM ATP plus 0.5 mM ADP. Each point represents the mean ± S.E. of quadruplicate samples. (B) Comparison of Na^+-K^+ exchange and anion-coupled Na^+ flux in control and trypsin-treated inside-out vesicles. 2 ml inside-out vesicles (4.8 mg protein/ml) were treated with trypsin as in Table I. Each sample was then divided: one-half was equilibrated with 0.3 mM KCl, 49,7 mM choline chloride and 2.2 μM valinomycin for 30 min at 37°C for Na^+-K^+ exchange (△) and the other portion was equilibrated with 50 mM choline chloride to measure Na^+ influx (●). ATP-dependent $^{22}Na^+$ uptake was measured as described in Fig. 1 under the following conditions: inside-out vesicles (25 μl) were added to 200 μl prewarmed (37°C) medium containing 2 mM NaCl, 0.5 mM $MgSO_4$ and 20 μM Tris glycylglycine (pH 7.4). 0.3 mM KCl was included in the medium used for measuring Na^+-K^+ exchange. Assays were carried out for 5 min in the presence or absence of 0.2 mM Tris-ATP. Each point represents the mean ± S.E. of quadruplicate samples.

with the co-transport of anions [25]. These two pump modes reflect, presumably, the entire reaction sequence. In addition, the pump can effect other modes of ion translocation, including Na^+-Na^+ exchange which, in the 'classical mode', is associated with the partial reaction characterized by Na^+-dependent ADP-ATP exchange [26,27]. Comparison of the relative effects of trypsin treatment of inside-out vesicles on these pump modes is shown in Fig. 6. Fig. 6A shows Na^+-K^+ exchange compared to ATP- plus ADP-dependent Na^+-Na^+ exchange and Fig. 6B, Na^+-K^+ exchange compared to the 'uncoupled' Na^+ flux ($Na^+/0$ flux, presumably anion-coupled, cf. Ref. 24). As shown, no significant difference in relative losses due to trypsin treatment can be detected.

Effect of trypsin on p-nitrophenylphosphatase

K^+-activated ouabain- or strophanthidin-sensitive *p*-nitrophenylphosphatase activity reflects, presumably, a component of the K^+-dependent dephosphorylation reaction of the $(Na^+ + K^+)$-ATPase. This activity can be stimulated either by K_{cyt} or by K_{ext} provided Na_{cyt} and micromolar amounts of ATP are present [20]. Thus, it has been suggested that *p*-nitrophenylphosphatase is catalyzed by the $K \cdot E_2$ form of the $(Na^+ + K^+)$-ATPase, reflecting either the partial reaction $E_1 + K_{cyt} \rightarrow K \cdot E_1 \leftrightarrow K \cdot E_2$ (K_{cyt} present) or the sequence $E_1 + Na^+ + ATP \rightarrow Na \cdot E_1P \rightarrow E_2P + Na_{ext}$; $E_2P + K_{ext} \rightarrow K \cdot E_2 + P_i$ (Na_{cyt}, K_{ext} and ATP present [20,28]). When *p*-nitrophenylphosphatase was measured in inside-out vesicles by both pathways after trypsin treatment, no difference in relative losses of activity could be detected; no difference in relative losses of K^+-dependent *p*-nitrophenylphosphatase compared to overall $(Na^+ + K^+)$-ATPase activity could be detected, either (experiments not shown).

Discussion

Trypsin treatment of human erythrocyte inside-out membrane vesicles results in changes and losses of activity and proteolytic cleavage patterns essentially similar to those reported by others using purified kidney $(Na^+ + K^+)$-ATPase. Taken together, these observations indicate that the sites accessible to tryptic cleavage are similar whether digestion is carried out with red cell membranes or detergent-purified kidney enzyme; whether the relative accessiblity of the sites is the same remains to be determined (see below).

Trypsin treatment, at least at the relatively low concentrations used in these experiments, effects only a small increase in the proportion of membrane vesicles which are permeable to ouabain. Moreover, it is unlikely that trypsin alterations of ATP-dependent $^{22}Na^+$ efflux are secondary to permeability changes. Thus, an increased outward leak of $^{22}Na^+$ taken up was not observed, since the $^{22}Na^+$ uptake was not altered by preloading the vesicles with non-radioactive NaCl (see (Fig. 4); more importantly, the percentage decrease in the ATP-stimulated component of Na^+ uptake effected by trypsin is the same in Na^+-loaded and choline-loaded vesicles. It should be noted also that the $^{86}Rb^+$ contents of control and trypsinized vesicles are similar (Fig. 5).

With the assumption that pump molecules are inserted independently into the membrane and are thus distributed in a Poisson fashion and that the diameter distribution of vesicles is Gaussian, Appel and Marcus [29] have derived a statistical model for ATP-dependent ^{86}Rb efflux applicable to data from red-cell inside-out vesicles obtained earlier from our laboratory [17]. The model predicts that a change in only the fraction of vesicles with active pumps reflects a change in pump density, whereas a change in only the half-time for ATP-driven $^{86}Rb^+$ efflux reflects a change in pump turnover. Although our data are consistent with both types of change (see Fig. 5), it is difficult to reconcile them with the quantitatively similar effects of trypsin on the various pump modes. Thus, according to the following minimal sequence

$$E_1 \xrightarrow{Na^+} Na \cdot E_1P \rightarrow Na \cdot E_2P \begin{array}{l} \xrightarrow{K^+} K \cdot E_2P \rightarrow (K)E_2 \\ \searrow Na^+ \end{array}$$

$$\rightarrow K \cdot E_2 \rightarrow K \cdot E_1 \begin{array}{l} \longrightarrow E_1 \\ \searrow K^+ \end{array}$$

one would expect Na^+-K^+ exchange and Na^+-Na^+ exchange to be affected more than uncoupled Na^+ efflux, which is limited, presumably, by the rate of dephosphorylation of E_2P. This not being the case, it may be concluded that the transport rates measured following trypsin treatment reflect

only the activity of 'undamaged' pumps. Thus, it is unlikely that the change in apparent rate constant for $^{86}Rb^+$ efflux observed in Fig. 5 reflects a change in turnover. In fact, as pointed out by Appel (personal commmunication), the rather high turnover of the sodium pump taken together with the efflux data indicate that the enzyme is probably clustered; a change in the size of clusters is a feature which could not be incorporated into their model.

A significant change in $Na^+ : Rb^+$ stoichiometry could not be detected after trypsin treatment of inside-out vesicles. Although Jorgensen and Anner [9] reported a decreased stoichiometry in trypsinized kidney $(Na^+ + K^+)$-ATPase reconstituted in liposomes, Anner et al. [30] have suggested recently that the change in stoichiometry reflects, in some way, an effect of the large amount of soybean trypsin inhibitor used in their experiments. In the present study, trypsinization of erythrocyte inside-out vesicles was inhibited with an amount of soybean trypsin inhibitor equivalent to 0.4% of total protein, whereas with kidney $(Na^+ + K^+)$-ATPase the amount of trypsin inhibitor added represented about 30% of total protein.

Unlike the aforementioned pump modes, K^+-K^+ exchange has been difficult to detect and quantitate in red-cell inside-out vesicles (experiments not shown). Alternatively, we have utilized K_{cyt}-dependent *p*-nitrophenylphosphatase as a measure of the partial reaction $E_1 + K \leftrightarrow K \cdot E_1 \leftrightarrow K \cdot E_2$, whereby dephosphorylation of *p*-nitrophenylphosphatase takes place when the enzyme is presumably in the $K \cdot E_2$ form [20]. Thus, as mentioned above, comparison of trypsin treatment on K_{cyt}-activated *p*-nitrophenylphosphatase on the one hand, with either $(Na_{cyt} + K_{ext} + ATP)$-activated *p*-nitrophenylphosphatase or $(Na_{cyt} + K_{ext} + ATP)$-activated ATPase on the sequence

$$E_1 \rightarrow E_1P \rightarrow E_2P \xrightarrow{K^+} K \cdot E_2,$$

shows similar losses in activities. This result supports the notion that the conformational transitions of dephosphoenzyme ($E_1 \leftrightarrow E_2$) or phosphoenzyme ($E_1P \leftrightarrow E_2P$) are affected similarly. The present findings confirm those of others using the purified kidney enzyme [1,5] that trypsin-modified $(Na^+ + K^+)$-ATPase retains the initial phosphorylation reaction step and Na^+-dependent ADP-ATP exchange. Inhibition of the conformational transitions $E_1 \leftrightarrow E_2$ and $E_1P \leftrightarrow E_2P$ as mentioned above is probably the mechanistic basis for slowing of overall hydrolysis and *p*-nitrophenylphosphatase activities.

We report elsewhere that proteolysis of $(Na^+ + K^+)$-ATPase in the E_1 conformation uncouples hydrolytic from pump activity [31]. As a result, trypsinized inside-out vesicles probably comprise not only inactive enzyme, devoid of both functions, but also enzyme with only hydrolytic activity (uncoupled enzyme) as well as residual unmodified enzyme. Presumably pump activity reflects only the latter species. The similar losses in $(Na^+ + K^+)$-ATPase and K_{cyt}-activated, strophanthidin-sensitive *p*-nitrophenylphosphatase described in the present study indicate that the uncoupled enzyme retains the same proportion of phosphatase and hydrolytic activities as the unmodified enzyme. In contrast, trypsinization of detergent-purified kidney enzyme inactivates *p*-nitrophenylphosphatase more rapidly than $(Na^+ + K^+)$-ATPase [4,7] and, unlike red-cell inside-out vesicles, uncoupling of $(Na^+ + K^+)$-ATPase was not apparent [8]. It is plausible that the proteolytic cleavage sites, albeit similar for both enzymes, differ in their relative accessibility and hence rates of cleavage, depending on the source of tissue and/or prior exposure to detergent. Controlled tryptic cleavage of the E_1 form of $(Na^+ + K^+)$-ATPase produces not only the 78 and 45 kDa fragments bearing the phosphorylation site, but releases also 10–20 amino acid residues from the N-terminus, which is relevant to loss of activity but is not readily detected [4]. As a result, the modified enzyme comprises a mixture of altered species, each with distinct functional alterations. Trypsinized red-cell inside-out vesicles and purified kidney $(Na^+ + K^+)$-ATPase probably differ with respect to the relative proportions of modified species. It remains to be determined whether trypsinization of the kidney enzyme after reconstitution into liposomes (cf. Ref. 10) behaves like the (red cell) enzyme as it exists in situ with respect to the kinetics of inactivation of the various pump modes and enzymic reactions.

Acknowledgement

This work was supported by a grant (MT-3876) from the Medical Research Council of Canada.

References

1 Jørgensen, P.L. (1975) Biochim. Biophys. Acta 401, 399–415
2 Koepsell, H.O. (1979) J. Membrane Biol. 48, 67–94
3 Castro, J. and Farley, R.A. (1979) J. Biol. Chem. 254, 2221–2228
4 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97–108
5 Jørgensen, P.L. and Klodos, I. (1978) Biochim. Biophys. Acta 507, 8–16
6 Jørgensen, P.L. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 597, 305–317
7 Robinson, J.D. (1982) J. Bioenerg. Biomembranes 14, 319–333
8 Jørgensen, P.L., Skriver, E., Hebert, H. and Maunsbach, A.B. (1982) Ann. NY Acad. Sci. 402, 207–225
9 Jørgensen, P.L. and Anner, B.M. (1979) Biochim. Biophys. Acta 555, 485–492
10 Karlish, S.J.D. and Pick, U. (1981) J. Physiol. (London) 312, 505–529
11 Chin, G. and Forgac, M. (1983) Biochemistry 22, 3406–3410
12 Kaplan, J.H. (1985) Annu. Rev. Physiol. 47, 534–544
13 Blostein, R. and Chu, L. (1977) J. Biol. Chem. 252, 3035–3043
14 Steck, T.L., Weinstein, R.S., Straus, J.M. and Wallach, D.F.H. (1970) Science 168–255–257
15 Blostein, R. (1968) J. Biol. Chem. 243, 1957–1965
16 Blostein, R. (1979) J. Biol. Chem. 254, 6673–6677
17 Blostein, R. (1983) J. Biol. Chem. 258, 12228–12232
18 Amory, A. Foury, F. and Goffeau, A. (1980) J. Biol. Chem. 255, 9353–9357
19 Resh, M.D. (1982) J. Biol. Chem. 257, 6978–6986
20 Drapeau, P. and Blostein, R. (1980) J. Biol. Chem. 255, 7827–7834
21 Ottolenghi, P. (1975) Biochem. J. 151, 61–66
22 Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147–149
23 Post, R.L. and Sen, A.K. (1967) Methods Enzymol. 10, 773–776
24 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. (London) 256, 465–496
25 Dissing, S. and Hoffman, J.F. (1983) Curr. Top. Membrane Transp. 19, 693–695
26 Glynn, I.M. and Hoffman, J.F. (1971) J. Physiol. (London) 218, 239–256
27 Kaplan, J.H. (1982) J. Gen. Physiol. 80, 915–937
28 Robinson, J.D., Levine, G.M. and Robinson, L.J. (1983) Biochim. Biophys. Acta 731, 406–414
29 Appel, H.J. and Marcus, M.M. (1985) in The Sodium Pump (Glynn I.M. and Ellory, J.C., eds.) The Company of Biologists Limited, Cambridge, in the press
30 Anner, B.M., Ting-Beall, H.P. and Robertson, J.D. (1984) Biochim. Biophys. Acta 773, 262–270
31 Harvey, W.J. and Blostein, R. (1986) J. Biol. Chem. 261, 1724–1729