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Sodium ions protect a membrane transport protein from proteolysis

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Na^+-dependent alanine transport activity in vesicles prepared from pigeon erythrocyte membranes was examined after exposure of the vesicles to some proteinases under various conditions. The presence of sodium ions during proteolysis affords considerable protection of alanine transport activity from the inhibitory action of the proteinases. The concentration of sodium ions required for half-maximum protection is greater than that needed for half-maximum activation of alanine uptake. The site of protective action could be at either or both surfaces of the membrane because the vesicles are very permeable to sodium ions. Neither measurement of residual protein content nor analysis by polyacrylamide gel electrophoresis revealed any differences in the extent of protein degradation occurring in the presence and absence of sodium ions, suggesting that the transporter constitutes only a minor membrane component. We conclude that sodium ions probably induce a conformation change in the transporter.

Introduction

Partial hydrolysis of proteins by limited incubation with proteinases is a technique frequently used either to gain information about specific sites on enzymes, receptors, etc., or to remove unwanted proteins during purification. This approach can be especially useful for the study of an intrinsic membrane protein because much of it is embedded in the lipid bilayer and is unlikely to be accessible to proteinase attack. A particularly valuable finding from such a study was the differential sensitivity of the membrane $(Na^+ + K^+)$-ATPase to digestion with trypsin, according to whether the enzyme was suspended in NaCl or KCl during proteolysis [1]. That observation provided the most convincing evidence for the notion that the ATPase can adopt two clearly distinct conformations, one stabilised by Na^+ and the other by K^+ ions. Also, it was the basis for many subsequent studies of both the structure of the enzyme and its molecular mode of action [2]. Here we describe a somewhat similar finding for the action of proteases on the membrane protein responsible for Na^+-dependent alanine uptake through pigeon erythrocyte membranes, an example of the widespread 'ASC' transport system [3]. A preliminary note of the initial observation has already been reported [4].

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Methods

Preparation of vesicles from pigeon erythrocyte membranes

Preparation of nucleated ghosts. Blood was obtained by decapitation of pigeons that had been anaesthetised with ether. It was collected in a

solution containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4 at 4°C), 0.01 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mg of heparin per ml. White cells were removed by passing the suspension through a column (2.5 cm × 2.0 cm) of α-cellulose that had been equilibrated with the same solution. All subsequent procedures were carried out at 4°C. The erythrocytes were washed twice by centrifugation and resuspension in the isotonic solution without heparin and then haemolysed by slow addition to at least 10 volumes of haemolysis buffer (2 mM $MgSO_4$, 3 mM NaCl, 9 mM Tris-HCl, pH 7.8–8.0 at 4°C). The nucleated ghosts thus obtained were washed repeatedly by centrifugation (20000 × *g* for 2 min) and resuspension in the haemolysis buffer until they were pale pink and the supernatant remained colourless.

Enucleation of the ghosts. One volume of packed ghosts was resuspended by addition of one volume of haemolysis buffer and drawn into a 20 ml syringe. The plasma membranes were then disrupted by forcing the suspension through a No. 27 gauge needle bent into a z shape [5]. (This process was facilitated by the use of a specially designed clamp that enabled a steady force to be applied to the syringe plunger.) The resulting mixture was immediately centrifuged at about 3000 × *g* for 15 min, to yield a dense white pellet of nuclei, a variable-sized middle layer of nucleated ghosts, and an upper layer containing fragmented plasma membranes. This upper layer was collected carefully with a pipette. If a substantial middle layer was present, it was also collected and again subjected to the enucleation process. The nuclear pellets were discarded with care to avoid contamination of the upper layers.

Formation of membrane vesicles. The fragmented membranes were sedimented by centrifugation at 40000 × *g* for 15 min and then resuspended in distilled water to a volume equal to 0.27 of the volume of the original packed erythrocytes. One volume of this suspension was slowly added to 70 volumes of a solution containing 0.2 mM EDTA (pH 11 at 4°C) and the pH readjusted to 11 by addition of NaOH solution. The mixture was stirred gently on ice for 30 min, after which the membranes were sedimented by centrifugation at 75000 × *g* for 30 min. The pellet was resuspended too a volume equal to 0.2–0.3 of the volume of original packed erythrocytes in a solution containing 300 mM mannitol, 2 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.6 at 20°C), 1 mM [^{3}H]sucrose (20 μCi/ml) and either 5 or 10 mM L-alanine. The mixture was homogenised by 20 strokes in a glass/glass homogeniser and the final vesicle suspension was stored on ice until required.

Measurement of Na^+-dependent alanine exchange

The standard incubation medium contained 150 mM NaCl or KCl, 2 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.6 at 20°C), 0.4–1.0 μCi [^{14}C]alanine and, except in a few experiments, 2 mM NaN_3. When required, 10 mM L-serine was also added as a specific inhibitor of the transport system. For some experiments the NaCl or KCl was replaced by other monovalent chlorides, as indicated in the text. Incubations were started by mixing one volume of vesicle suspension with about 44 volumes of incubation medium at 37°C (thus giving a final external alanine concentration of 0.11–0.22 mM) and the mixture was incubated at 37°C. After the required time, usually 10 min, a sample of the mixture containing vesicles with a total internal volume of 0.05–0.10 μl was removed, rapidly filtered on a membrane filter (Millipore, HAWP 025) under reduced pressure, and the vesicles trapped on the filter were washed twice with 4 ml of KCl medium. The filters were dried, dissolved overnight in a scintillation cocktail containing methylcellosolve and toluene (1 : 4, v/v) and then the amounts of trapped [^{3}H]sucrose and [^{14}C]alanine were measured by liquid scintillation counting. The total internal volume of the trapped vesicles was calculated from the [^{3}H]sucrose present and the apparent concentration of alanine in the vesicles was calculated with the use of the specific radioactivity of alanine in the initial incubation mixture.

Other assays and materials

Protein concentrations were determined by the method of Peterson [6]. Polyacrylamide gel electrophoresis [7] and membrane phospholipid assays [8] were carried out as described previously. Details of all materials used are also as described before [9,10].

Results

Membrane vesicles

The method used to enucleate the erythrocyte ghosts was based on that described by Granger et al. [5] for chicken erythrocytes and differed from that used previously [10] in two ways. First, the concentration of Mg^{2+} ions was kept relatively high (2 mM) and, second, the shearing force used was increased. Lower concentrations of Mg^{2+} (0.2 μM or less) render the plasma membrane more fragile and easier to break, but the same applies to the nuclear membrane so that contamination of the plasma membranes with DNA and histones was often a problem with the previous method. If the contamination was high no active vesicles could be formed from the membranes [10]. DNA assays [11] on four of the membrane preparations used here gave a value of 18 ± 5 ng DNA/mg vesicle protein (mean $\pm$ S.E.), whereas the previous method gave values in the range 10–50 μg DNA/mg vesicle protein [10]. As is indicated below, no differences were detected in the transport properties of the vesicles formed by the two methods.

Sodium-dependent exchange of alanine by vesicles

In the presence of Na^+ ions labelled alanine enters the vesicles rapidly, reaching a peak around 10 min, after which the concentration declines more slowly (Fig. 1). Previous work showed that this pattern results from a rapid Na^+-dependent exchange of labelled alanine from the medium with the unlabelled alanine inside the vesicles, whilst there is also a net efflux until equilibrium with the external medium is attained [9]. Addition of 10 mM serine to the medium abolishes the rapid exchange (Fig. 1), and replacement of external Na^+ ions by K^+ ions has the same effect (Table I). Thus serine-inhibited uptake of alanine is equivalent to Na^+-dependent uptake. Table I also shows that the basic amino acid lysine, which is not transported by this system [3,9] has no significant effect on alanine influx, thus confirming the specificity of the transport system. In many of the experiments described below, complete removal of Na^+ ions from the vesicle suspension was difficult to achieve. We have therefore used serine as a specific inhibitor instead of replacing Na^+ ions with K^+ ions and, from the results in Table I, define Na^+-dependent alanine exchange as the difference in uptake measured in the presence and absence of serine in the Na^+-medium.

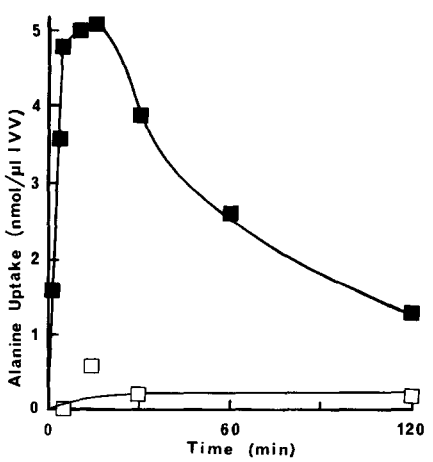

Fig. 1. Uptake of alanine by membrane vesicles. The uptake of ^{14}C]alanine by membrane vesicles containing 10 mM unabelled alanine was measured during incubation in standard Na^+-medium, with (□) and without (■) 10 mM serine, as described in the text.

TABLE I

CRITERIA FOR ASSAY OF Na^+-DEPENDENT TRANSPORT OF ALANINE

The uptake of [^{14}C]alanine by membrane vesicles containing 5 mM unlabelled alanine was measured, as described in the text, during a 10 min incubation in either Na^+-medium or K^+-medium (without NaN_3) in the presence and absence of 10 mM serine or lysine, as indicated. Transport activity is expressed as nmol alanine/μl IVV per 10 min (IVV, intra-vesicle volume).

Sample	Alanine uptake			
	nmol/μl IVV			% of control
	(*A*) Na^+-medium	(*B*) K^+-medium	(*A* − *B*)	
Control	2.15	0.27	1.88	100
+10 mM serine	0.35	0.27	0.08	4
+10 mM lysine	1.95	0.34	1.61	86

Protective effect of Na^+ ions during tryptic digestion

Preliminary experiments showed that exposure

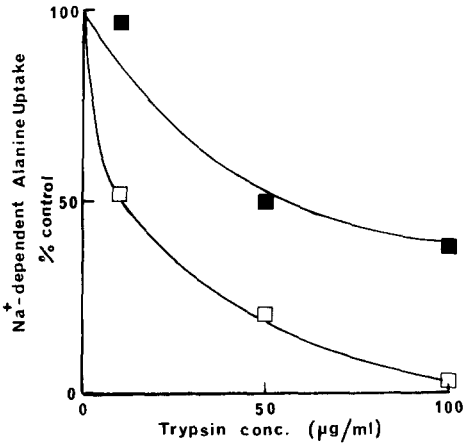

Fig. 2. Differential inhibition of alanine transport activity after exposure of vesicles to trypsin in the presence of NaCl or KCl. Test samples of membrane vesicles (final protein concentration 1.2 mg/ml) were incubated with the indicated concentrations of trypsin for 30 min at 37 °C in a solution containing 5 mM alanine, 260 mM mannitol, 2 mM $MgSO_4$, 10 mM Tris-HCl (pH 7.6 at 20 °C) and either 20 mM NaCl (■) or 20 mM KCl (□). Control samples were incubated without the trypsin. Then a chilled solution of trypsin inhibitor and PMSF was added to all samples to give final concentrations of 0.5 mg/ml and 0.05 mM, respectively, followed by enough trypsin to give 0.1 mg/ml in each tube. The vesicles were then sedimented by centrifugation and resuspended in mannitol medium. [^{14}C]alanine uptake by the vesicles was measured, as described in the text, during 10 min incubation in standard Na^+-medium in the presence and absence of 10 mM serine. The results are mean values of duplicate measurements from a single experiment.

TABLE II

THE PRESENCE OF Na^+ IONS DURING EXPOSURE OF VESICLES TO TRYPSIN HAS A PROTECTIVE EFFECT ON THE TRANSPORT ACTIVITY

The methods were exactly as described in the legend to Fig. 2, except that the solutions used for the preliminary incubation of the vesicles (with or without trypsin) contained the indicated salts at 100 mM, plus only 100 mM mannitol. The combined results from two experiments are given. IVV, intra-vesicle volume.

After preliminary incubation in	Na^+-dependent uptake of alanine (nmol/μl IVV)		100 (*B*/*A*)
	(*A*) − trypsin	(*B*) + trypsin	
NaCl	2.86	2.36	83
KCl	2.20	0	0
LiCl	1.81	0.36	20
RbCl	2.00	0.19	10
Choline chloride	1.90	0.18	9

of the vesicles to trypsin inhibited subsequently measured alanine transport activity. However, incubation with trypsin in the presence of 20 mM NaCl caused less inhibition than did incubation with the proteinase in the presence of 20 mM KCl (Fig. 2). This differential effect was even more pronounced if the digestions were carried out in the presence of 100 mM NaCl or KCl, and tests with other salts revealed that they all mimicked the effect of KCl (Table II). Hence Na^+ ions must have a protective action on the alanine transporter during tryptic digestion of the vesicles.

The protective action is a saturable function of the concentration of Na^+ ions, half-maximum protection being given by about 20 mM NaCl (Fig. 3).

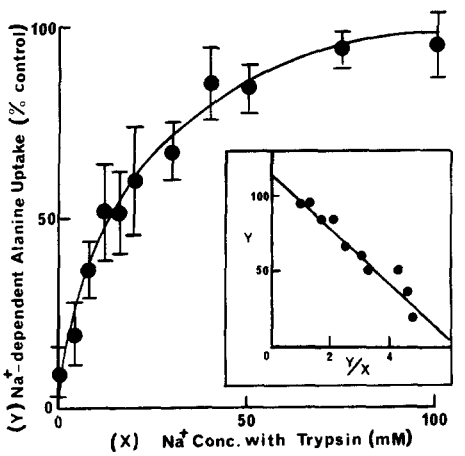

Fig. 3. Variation of inhibitory action of trypsin with concentration of Na^+ ions during digestion. Test samples of vesicles (containing 5 mM alanine) were incubated for 30 min at 37 °C with 0.1 mg/ml trypsin in solutions containing 5 mM alanine, 100 mM mannitol, 2 mM $MgSO_4$, 10 mM Tris-HCl (pH 7.6 at 20 °C) and 0 to 100 mM NaCl. Iso-osmolarity was maintained with either KCl or choline chloride in place of NaCl. Control samples were incubated without trypsin. Then a chilled solution of trypsin inhibitor and PMSF was added to all samples to give final concentrations of 0.5 mg/ml and 0.05 mM, respectively. The vesicles were sedimented by centrifugation, resuspended in mannitol medium containing 5 mM alanine, and then assayed for alanine transport activity as described in the text. Na^+-dependent uptake of alanine by the test vesicles is expressed as a percentage of the values given by the control samples. Mean values (±S.E.) from three experiments are given. The insert shows a linear transformation of the results, indicating that half maximum protection required about 20 mM NaCl.

TABLE III

SIMILAR EFFECTS OF SEVERAL DIFFERENT PROTEINASES

Test samples of membrane vesicles (containing 5 mM alanine) were incubated at 37 °C for 30 min in a solution containing 100 mM mannitol, 2 mM $MgSO_4$, 10 mM Tris-HCl (pH 7.6 at 20 °C), 5 mM alanine, either 100 mM NaCl or 100 mM KCl, and 0.1 mg/ml of the indicated proteinase. Control samples were incubated without the proteinases. The suspensions were then diluted with 10 volumes of chilled mannitol medium, the vesicles sedimented by centrifugation (15 min at $100000 \times g$) and then resuspended to their initial volumes in mannitol medium containing 5 mM alanine. Uptake of [^{14}C]alanine by the vesicles was subsequently measured during 10 min incubation in standard Na^+-medium in the presence and absence of 5 mM serine, as described in the text. Na^+-dependent transport of alanine by the test vesicles is expressed as a percentage of that by the corresponding control samples. Mean values (± S.E. where possible) from the indicated number of experiments (N) are given.

After preliminary incubation with	Na^+-dependent uptake of alanine (% of control)		N
	in NaCl	in KCl	
Trypsin	83±6	23±7	9
Papain	78±6	16±4	3
α-Chymotrypsin	66	10	2
Thermolysin	77	15	2

Similar effects of other proteinases

Three other proteinases, papain, thermolysin and α-chymotrypsin, were tested with the vesicles under the same conditions as were used routinely for trypsin digestion. The effect of each one on alanine transport was similar to that of trypsin (Table III).

Lack of effect of alanine during tryptic digestion

During the proteolytic digestions described above the vesicles initially contained 5 mM alanine and the digestion media contained 1–5 mM alanine. Hence it was possible that the protective effect described could have resulted not simply from an action of Na^+ ions alone, but from a synergic action of Na^+ ions with alanine. To test this possibility vesicles were prepared in the absence of alanine, incubated with trypsin in the absence of alanine, and then allowed to equilibrate with 5 mM alanine during incubation at 4 °C for 24 h before being assayed for alanine

TABLE IV

PROTECTION BY Na^+ IONS IN THE ABSENCE OF ALANINE

Vesicles prepared in the absence of alanine were incubated for 30 min at 37 °C with or without trypsin (0.1 mg/ml) in either NaCl or KCl. Trypsin inhibitor and PMSF were then added to all samples to give final concentrations of 1 mg/ml and 0.05 mM, respectively, followed by trypsin to the controls. Alanine was added to each sample to a final concentration of 5 mM and the suspensions were incubated at 4 °C for 24 h. Alanine exchange activity was then assayed in the usual way (10 min uptake) except that the external alanine concentration was 0.43 mM (Expt. 1) or 0.3 mM (Expt. 2). IVV, intra-vesicle volume.

	After incubation in	Na^+-dependent uptake of alanine		
		nmol/μl IVV		% of control
		(A) without trypsin	(B) with trypsin	100 (B/A)
1	NaCl (105 mM)	2.38	2.33	98
	KCl (105 mM)	2.24	0	0
2	NaCl (112 mM)	3.68	3.38	92
	KCl (112 mM)	2.40	0.22	9

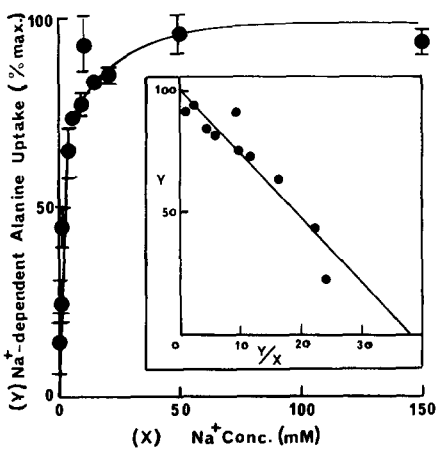

Fig. 4. Effect of Na^+ ion concentration on uptake of alanine by membrane vesicles. Uptake of [^{14}C]alanine by membrane vesicles (containing 5 mM unlabelled alanine) was measured, as described in the text, during incubation for 10 min at 37 °C in standard media containing the indicated concentrations of NaCl. Iso-osmolarity was maintained with KCl and the external concentration of alanine was 0.1 mM. In each experiment Na^+-dependent uptake of alanine (as defined in the text) under each condition was expressed as a percentage of the maximum observed, and the mean values (± S.E.) from three experiments are given. The insert shows the linear transformation of the results used to estimate the concentration of Na^+ ions needed for half-maximum uptake: 2.7 mM.

exchange activity. The results show clearly that alanine is not necessary for the protective action of Na^+ ions (Table IV).

Effects of Na^+ ion concentration on alanine uptake

Na^+-dependent uptake of alanine by the vesicles is a saturable function of the Na^+ ion concentration and half-maximum uptake requires only about 3 mM Na^+ ions (Fig. 4).

Permeability of vesicles to Na^+ ions

The efflux of Na^+ ions from vesicles was rapid under all conditions tested, the half-time for equilibration with the different incubation media being about 3.5 min (Fig. 5). Hence the vesicles are very permeable to Na^+ ions and, in all experiments, the internal concentration of Na^+ ions must have approached the external Na^+ ion concentration.

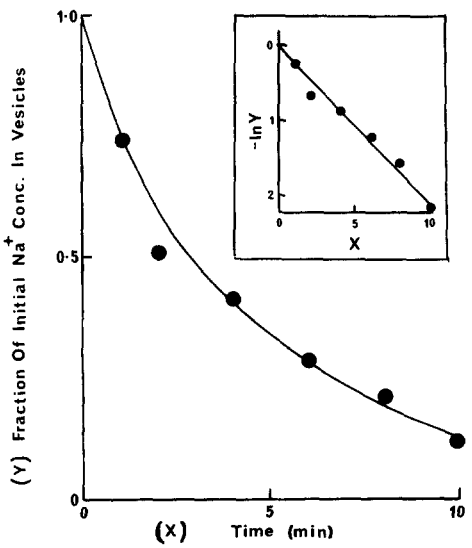

Fig. 5. Permeability of membrane vesicles to Na^+ ions. Membrane vesicles containing 20 mM [^{22}Na]NaCl (0.7 Ci/mol), 5 mM [^{3}H]sucrose (1.8 Ci/mol) and 5 mM alanine (unlabelled) were prepared as described in the text and samples incubated at 37 °C in 40 volumes of standard medium containing either NaCl, KCl or choline chloride as the main salt. The suspensions were samples at the indicated times and the vesicles were quickly separated by filtration and assayed for ^{22}Na and ^{3}H, as described in the text. The values obtained after 30 min incubation provided estimates of non-specific binding of the ^{22}Na to the membranes and filteres, and were used to correct the other values. The values shown are the means obtained from the three different media in a single experiment, there being no significant differences among the results in the different media. The insert shows that the efflux of Na^+ ions followed simple first-order kinetics: $t_{1/2}$ about 3.5 min.

Extent of degradation of membrane proteins

To examine the overall degradation of membrane proteins during proteolysis of the vesicles, samples were washed and then assayed for both protein and phospholipid contents. Values of the ratio: mg protein/μmol phospholipid were then used to compare the effects of the proteases under the different conditions used. Table V shows that the average result, for all the proteinases, was a loss of about 30% of vesicle protein during digestion in either NaCl or KCl. In several experiments with trypsin there did appear to be a greater loss of protein during digestion in KCl than in NaCl; but consideration of all the results shows that there was no statistically significant difference.

Analysis of residual proteins after proteolysis

TABLE V

EFFECT OF PROTEOLYSIS ON THE CONCENTRATION OF PROTEIN IN VESICLE MEMBRANES

Test and control samples of vesicles were incubated with and without, respectively, the indicated proteases exactly as described in Table III. Then the suspensions were diluted with chilled mannitol medium containing trypsin inhibitor (final concentration 2 mg/ml) and PMSF (final concentration 0.05 mM) and the vesicles were sedimented by centrifugation at 100000 × g for 10 min. The vesicles were washed three times by resuspension (in mannitol medium) and sedimentation and then their contents of protein and phospholipid assayed as described in the text. Ratios of (mg protein/μmol phospholipid) in the test vesicles were calculated and expressed as percentages of the values in the corresponding controls. Mean values (±S.E. where possible) are given for the indicated number of experiments (N). None of the values obtained after treatment in NaCl is significantly different from the corresponding value obtained after treatment in KCl.

After digestion with	Residual (protein/phospholipid) (% of control)			N
	(A) in NaCl	(B) in KCl	($A-B$)	
Trypsin	75±6	56±6	−18±7	5
α-Chymotrypsin	62	74	+12	2
Papain	77	71	−6	2
Thermolysin	70	70	0	2
Mean values	71±3	68±4	−3±6	4

by polyacrylamide gel electrophoresis similarly failed to reveal any obvious differences between Na^+ and K^+ conditions (Fig. 6). It is clear that the large proteins constituting Bands 1–3 (see Ref. 12 for nomenclature) were subject to considerable degradation; but the appearance of several smaller degradation products renders further interpretation difficult and unreliable. In addition, these analyses revealed the presence of endogenous proteolytic activity, as is shown by comparison of lane A with lanes D1 and D2 in Fig. 6. We do not know the origin of this activity, but it varies somewhat in its intensity from one preparation to another. It seems most likely to account for the observation that vesicles incubated as controls in the absence of Na^+ ions sometimes showed lower transport activity than controls incubated with Na^+ ions. Compare, for example, the various control values in Tables II and IV.

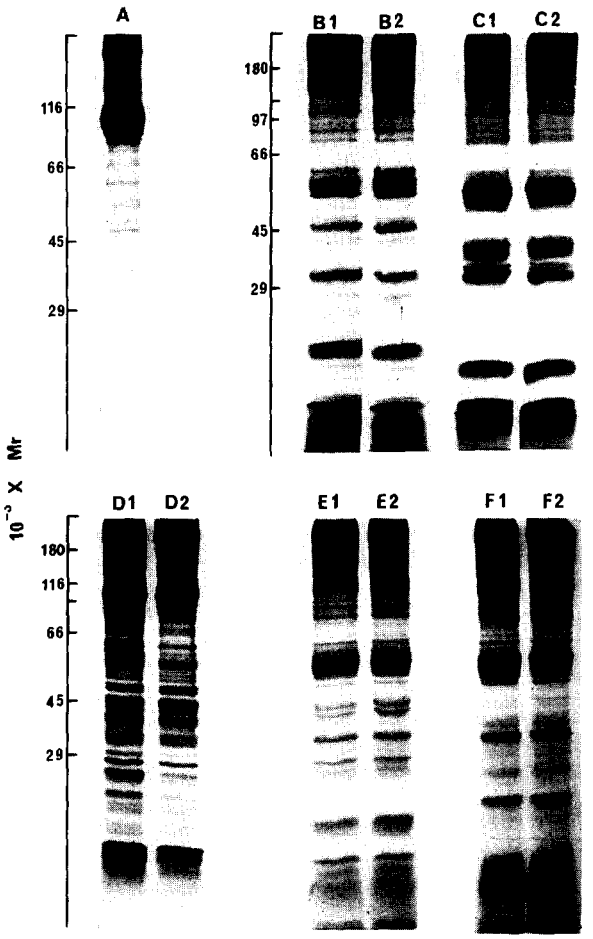

Fig. 6. Residual membrane protein profiles after proteolysis. Vesicles were treated with the proteases as described in Table III except that the suspensions were diluted 25-fold after the incubations. Samples for polyacrylamide gel electrophoresis were prepared with the addition of 2-mercaptoethanol. Lane A, non-incubated control; lane D1, control incubated in NaCl; lane D2, control incubated in KCl. The other lanes show the residual protein profiles after incubation in either NaCl (1) or KCl (2) with: B, trypsin; C, thermolysin; E, α-chymotrypsin and F, papain.

Discussion

Effects of Na^+ ions

The simplest interpretation of the results described above is that Na^+ ions induce a conformation change in the alanine transport protein such that it is much less accessible to attack by proteinases than it is in the absence of Na^+ ions. Since the same effect occurred with proteinases of different specificities (Table III), this finding cannot be explained in terms of accessibility of only one or two specific amino acid residues. One possibility is that part of the protein lies clearly outside the membrane in the absence of Na^+ ions, but moves closely adjacent to the membrane surface, or becomes actually embedded in the lipid bilayer, in the presence of Na^+ ions.

If this concept of a sodium-induced conformation change is accepted, the question arises as to whether it reflects an effect of Na^+ ions that is important for the normal functioning of the protein during amino acid transport. For example, does the variation in alanine transport with Na^+ ion concentration, observed in experiments in vitro, occur because of a Na^+-induced conformation change in the transport protein? One approach to this question seemed to be to compare the effects of Na^+ ion concentration on proteolysis with those on alanine transport. The results in Figs. 3 and 4 reveal almost an order of magnitude difference in the concentrations of Na^+ ions required for half maximum effects on the two processes. However, this comparison may not be valid because (a) the K_m value for Na^+ ion activation of alanine transport appears to be different at the inner and outer membrane surfaces, and (b) the vesicles are permeable to Na^+ ions (Fig. 5). The erythrocyte membrane is almost certainly inside-out in the vesicles, because trypsin had no

detectable effect on intact cells (data not shown), yet caused extensive degradation of protein bands 1 and 2 in the vesicles (Fig. 6), which are known to be interior extrinsic proteins in intact erythrocytes [12]. Hence the low K_m value for Na^+ ion activation of alanine uptake by vesicles (Fig. 4) seems to be characteristic of the normally inner surface of the membrane. This interpretation is supported by the fact that higher K_m values are observed for Na^+ ion activation of alanine uptake by intact cells. (Values ranging from 11 to 40 mM have been recorded, depending on the alanine concentration (unpublished results and Ref. 13).) These differences between the membrane surfaces seem reasonable in view of the normal, physiological, intracellular and extracellular Na^+ ion concentrations of about 20 mM and 150 mM, respectively. However, the high permeability of the vesicles to Na^+ ions makes it impossible to tell if protection against proteolysis arises from interaction of the ions with either or both membrane surfaces. Further, more detailed studies will obviously be required before these intriguing questions can be clearly answered.

Molecular size of the transporter

Earlier work showed that the formation of these vesicles involved the depletion of membrane proteins with M_r values of less than 100000 (that is, smaller than Band 3) and so it was suggested that the transporter might be a component of Band 3 [10]. However, the present work has shown that residual protein bands with M_r values below 100000 can be detected on gtels provided enough vesicle membrane is used: i.e., when the gel is overloaded with respect to Bands 1–3 (Fig. 6, lane A). Also the absence of any clear differences in the residual protein bands after proteolysis in NaCl compared with KCl suggests that the alanine transporter can be only a minor protein. At present therefore it is impossible to make any estimation of its size.

Attempts to solubilise and reconstitute the transporter into liposomes have been unsuccessful. Perhaps protease treatment in the presence of sodium may increase the relative levels of the alanine transporter and facilitate reconstitution and characterisation.

Acknowledgements

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