PARTIAL SEPARATION OF A SODIUM-DEPENDENT TRANSPORT SYSTEM FOR AMINO ACIDS IN AVIAN ERYTHROCYTE MEMBRANES

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1. Introduction

The mediated movement of molecules through cell membranes has been well characterized kinetically but, with the notable exception of the systems for transporting some inorganic ions, the molecular structures involved remain largely unidentified. The recent progress that has been made in this area has been confined mostly to the facilitated diffusion systems for D-glucose, anions and water in the human erythrocyte membrane [1–3]. However, the most widespread transport systems of considerable physiological importance are probably the Na-dependent ones that bring about the active movements of amino acids and sugars into the epithelial cells of the mammalian small intestine and kidney proximal tubules [4,5]. Amino acids are also accumulated in a Na-dependent fashion by other mammalian tissues, by Ehrlich Ascites tumour cells and by the nucleated pigeon erythrocyte. The energy required for these active transport processes is generally believed to be furnished, at least in part, by independently-generated concentration gradients of Na^+ across the cell membranes [5]. The clearest way to further our understanding of these complex systems seems to be to isolate and to reconstitute them [5,6]. This would not only reveal the molecular complexes involved but also would enable the importance of the cation gradients to be assessed when the metabolic and morphological integrity of the intact cells has been destroyed.

Here we describe the separation of a functioning Na-dependent transport system for amino acids in vesicles derived from the plasma membrane of the pigeon erythrocyte [7] after removal of most major membrane proteins [8]. Polyacrylamide gel electrophoresis shows that these vesicles are characterized by only three main protein bands, these being in the band 2 (spectrin) and band 3 regions of the profile according to the nomenclature in [9] for the human erythrocyte membrane.

2. Materials and methods

Pigeon erythrocyte plasma membranes were purified as in [7] in the form of whole cell 'ghosts', not membrane fragments, and suspended in a solution containing 0.2 mM Mg^{2+}, 3 mM NaCl and 8.5 mM Tris/HCl (pH 7.8) [7]. One volume of the suspension was mixed with 70 vol. 0.2 mM EDTA pre-adjusted to pH 11 and kept at 4°C for 20 min. The mixture was then centrifuged at 60 000 × *g* for 45 min and the sedimented pellets were resuspended by gentle homogenization in a solution containing 140 mM choline chloride, 10 mM sucrose, 2 mM $MgCl_2$, 0.1 mM NaN_3 and 20 mM Tris/HCl (pH 7.5). Where appropriate, 5 mM L-alanine was also present. Membrane vesicles were formed by sonicating the mixture at 4°C for 20 min in a bath-type sonicator [2], followed by incubation at 36°C for 2 h. Incubation media for transport activity measurements consisted of the choline chloride medium described above, or similar solutions in which the choline chloride had been replaced with either NaCl or KCl, plus [^{14}C]alanine. The assays were started by the addition of 10 μl vesicle suspension to 0.4 ml incubation medium, so that the final concentration of alanine was 0.12 mM, with spec. act. 1 μCi/μmol. When alanine was not

included in the solutions used to form the vesicles, sufficient unlabelled alanine was added to the final incubation media to give the same final concentrations and specific activity. Incubation was continued at 36°C for varying time intervals and finally stopped by the addition of 5 ml ice-cold choline chloride medium, followed by rapid filtration through cellulose acetate filters (Sartorius SM 11106 pore size 0.45 μm) under reduced pressure. The vesicles were washed once on the filter with an additional 5 ml quenching buffer. The filters and adhering vesicles were dried in air at 60°C and assayed for radioactivity by scintillation spectroscopy. Non-specific binding of alanine to the filters was measured by dilution of the radioactive incubation medium with the quenching solution before addition of the vesicles. The possibility that microbial contamination contributed to the measured uptake of alanine was excluded by pre-incubating the various solutions for 10 min at 36°C, then carrying out the procedure for assay of non-specific binding. Measurement of lipid phosphorus has been detailed in [7].

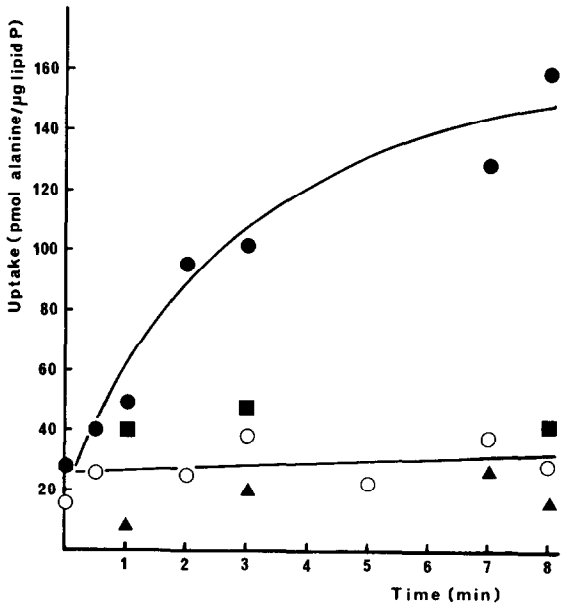

Fig.1. Uptake of L-alanine by membrane vesicles prepared in the absence (○) or presence (●, ▲, ■) of L-alanine (5 mM) and incubated in media containing Na^+ (○, ●), choline ions (▲) or K^+ (■). Data from 4 separate membrane preparations are included in the figure. The displacement along the ordinate at time zero represents the non-specific binding of L-alanine to the filters. For details see section 2.

3. Results and discussion

Figure 1 shows that L-alanine accumulated on the filters in a time-dependent and Na-dependent fashion when the incubation media contained the vesicles. No such accumulation occurred when Na^+ were replaced by either K^+ or choline ions. The possibility that the accumulation was merely Na-dependent binding of alanine to the vesicles was rendered very unlikely by the absence of the Na-dependent component when the isotonic quenching and washing solutions were replaced with hypotonic solutions, because such conditions should favour binding but cause lysis of vesicles. Further confirmation that we were measuring uptake of amino acid into an enclosed space was provided by the striking dependency of the uptake on the presence of alanine inside the vesicles. Vesicles prepared in the absence of alanine showed very slow accumulation of labelled amino acid, thus closely reflecting the behaviour of the exchanging transport system in both intact erythrocytes [10] and resealed 'ghosts' containing the cell nucleus [11]. The specificity of the system also appeared to have been conserved because the Na-dependent uptake of alanine by the vesicles was completely inhibited in the presence of 1 mM L-serine, but was unaffected by 1 mM l-lysine. Washes with isotonic buffer solutions at 37°C, instead of at 0°C, did not change the results; nor did prolonged filtration times of up to 3 min, indicating that the vesicles were tightly sealed. When a finer filter (pore size 0.2 μm) was inserted beneath the normal filter (pore size 0.45 μm) the second filter did retain some alanine in a Na-dependent manner, but it amounted to only 9% of that found on the first filter. Hence the efficiency of trapping of active vesicles by the 0.45 μm pore size filters must have been about 90%, unless there were significant numbers of vesicles with diameters < 0.2 μm.

We have shown [7] that the patterns of polypeptides obtained by polyacrylamide gel electrophoresis of membrane proteins from pigeon erythrocytes are similar to those obtained from human erythrocytes. The pattern obtained for the avian cells is shown in fig.2 with that of the membrane vesicles prepared

by the alkaline extraction process described above. The only major bands retained by the vesicles are band 2 and bands 3.1 and 3.2. Band 2 has been well characterized in the human erythrocyte and is known to be attached to the cytoplasmic surface of the membrane [12]. If we assume that it is similarly located in the pigeon erythrocyte, it seems most likely that one or both of the band 3 group of polypeptides are involved in the Na-dependent transport of amino acids. At the moment this must be considered only as a possibility because it is also possible that some minor protein component, not easily detectable on the gels, is responsible. It is nonetheless a very intriguing interpretation since the band 3 polypeptides of human erythrocytes appear to be involved in both anion and water transport through the human erythrocyte membrane [2,3]. Moreover, the major subunit of the $(Na^+ + K^+)$-dependent ATPase, responsible for the active transport of Na^+ and K^+, also migrates to this region of the gels [13]. And some evidence indicates that the protein involved in the facilitated diffusion of glucose through the human red cell membrane is a band 3 polypeptide, although the most recent report suggests that a smaller minor protein is involved [1].

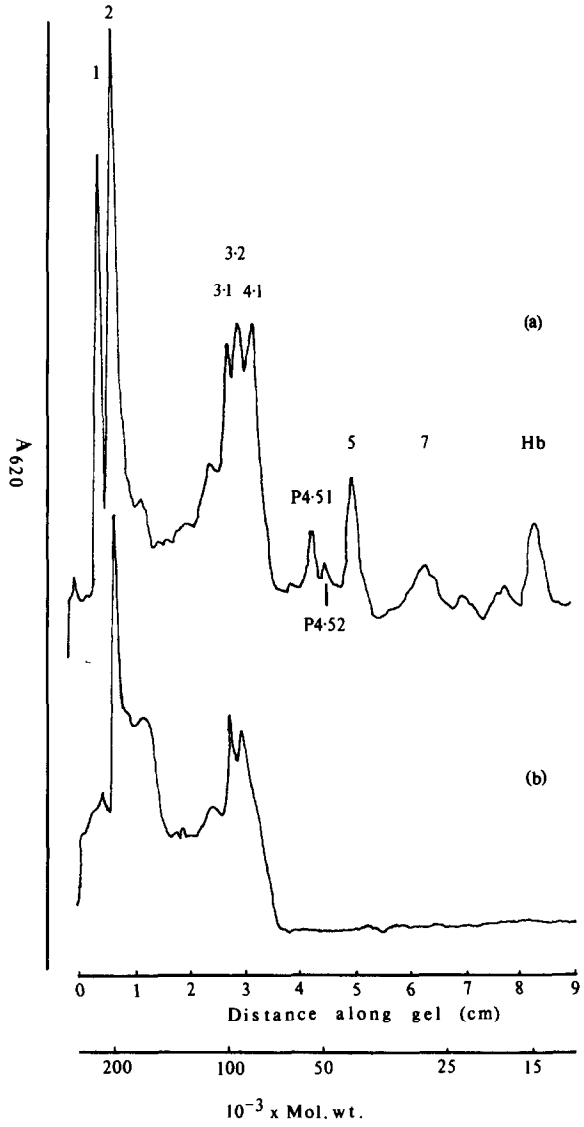

Fig.2. Electrophoretic profiles of (a) purified pigeon erythrocyte plasma membranes and (b) vesicles derived from them by alkaline EDTA treatment. Band nomenclature is based on that suggested in [9] for the human erythrocyte membrane and the electrophoresis procedure was the one described in [17].

Conclusive identification of the protein component responsible for the Na^+-dependent transport of alanine will constitute a further and perhaps more difficult stage in the development of studies of these linked transport systems, which up to now have depended on the use of whole cells [10,14,15] or resealed 'ghosts' [11,16]. We believe that this vesicle preparation of protein-depleted membranes will, in time, provide important insights into both the molecular nature of these cation-dependent transport systems and the way in which cation concentration gradients control their function.

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