Incorporation of D-Glucose-, L-Alanine- and Phosphate-Transport Systems from Rat Renal Brush-Border Membranes into Liposomes

By ROLF KINNE

Max-Planck-Institut für Biophysik, Frankfurt/Main, German Federal Republic

and ROBERT G. FAUST*

Department of Physiology, University of North Carolina School of Medicine, Chapel Hill, NC 27514, U.S.A.

(Received 19 August 1977)

An extract of soluble proteins was prepared from rat kidney brush-border membranes by Triton X-100 solubilization followed by centrifugation for 1 h at 100000g. Its protein composition was markedly different from that of the brush-border membranes. Proteoliposomes were formed by co-sonication of the Triton X-100-free extract with a naturally occurring mixture of phospholipids extracted from rat kidney. These proteoliposomes were shown to contain Na⁺-stimulated D-glucose-, L-alanine- and phosphate-transport systems.

During recent years knowledge of the biophysical and cellular events involved in the transport of sugars, amino acids and phosphate by the renal proximal tubule has been advanced considerably by studies on the transport properties of isolated luminal and contraluminal plasma-membrane vesicles (Kinne, 1976). Although the ultimate goal, namely the understanding of these transport processes in molecular terms, has not so far been reached, methods have become available to test the transport characteristics of soluble membrane proteins by reincorporating them into artificial liposomes. By using the techniques developed by Racker (1973), Crane *et al.* (1976) demonstrated that a low-speed supernatant (30000g for 30 min) of a Triton X-100 extract from rabbit kidney brush-border membranes, when recombined with crude soya-bean phospholipids, could exhibit Na⁺-dependent phlorrhizin-inhibitable stereospecific transport of D-glucose. In these studies, however, several questions remained open. The first concerns the membrane extract used for the recombination. Since low centrifugal forces were used, it is possible that small membrane vesicles remained in the supernatant which were responsible for the transport properties of the liposomes (Semenza *et al.*, 1977). Also no further characterization of other transport systems or of the protein extract with respect to its protein composition was performed. In addition, the composition of the lipid used was not investigated.

In the present studies these objections were overcome by using a protein extract that contained only a few soluble brush-border membrane proteins and by using phospholipids of known composition extracted from rat kidney. The mixture of phospholipids was used to enhance the incorporation into liposomes of various transport systems with possible different lipid requirements.

* To whom reprint requests should be addressed.

Materials and Methods

Brush-border membranes were isolated from 6 g of rat kidney cortex by a calcium precipitation method described by Booth & Kenny (1974). After isolation, the membranes (approx. 20 mg of protein) were suspended in 2 ml of KHT buffer (150 mM-KCl, 5 mM-Hepes†/Tris, 10 mM-MgSO₄, pH 7.5), and 2 ml of KHT buffer containing 1 % Triton X-100 was added. After incubation for 10 min at 4°C the suspension was centrifuged for 15 min at 30000g and 4°C and this low-speed supernatant was centrifuged for 1 h at 100000g and 4°C.

The high-speed supernatant was then passed through six Bio-Bead columns to remove the Triton X-100. Each column was equilibrated and eluted with the KHT buffer. The final volume of the Triton-free brush-border membrane extract was adjusted with KHT buffer to 10 ml (approx. 0.8 mg of protein/ml) and it was used immediately in the formation of proteoliposomes.

Phospholipids were prepared from whole rat kidneys by extraction with chloroform/methanol (1:1, v/v) and purified as described by Kates (1972). The final phospholipid preparation contained 17 % sphingomyelin, 34 % phosphatidylcholine, 31 % phosphatidylethanolamine, 9 % phosphatidylinositol and 9 % phosphatidylserine and essentially no cholesterol and cholesterol esters as determined by quantitative t.l.c. described in detail elsewhere (Bode *et al.*, 1976). A portion (260–280 mg) of this phospholipid mixture dissolved in chloroform was transferred to an evaporation flask and the chloroform was initially evaporated under a stream of N₂ and then under vacuum for 30 min. KHT buffer (9 ml) was added and

† Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

the phospholipids were suspended at room temperature (approx. 25°C) in the buffer by gentle shaking for 10 min in an N₂ atmosphere.

The phospholipid mixture (2 ml) and the protein extract (2 ml) were combined in a 5 ml centrifugation tube and sonicated under a stream of N₂ at room temperature for 5 min at 150 W (20 kHz) with a Labsonic 1510 (Braun, Melsungen, W. Germany) sonicator. The proteoliposome suspension thus formed was centrifuged for 15 min at 37 000g and 15°C to sediment large, probably multi-layered, structures. The supernatant was removed and recentrifuged for 30 min at 150 000g and 15°C. The resulting pellets were suspended in 0.4 ml of KHT buffer by mixing on a Vortex shaker, and the suspension (containing, on average, 2 mg of protein and 46 µM-phospholipid phosphorus/ml) was kept at room temperature until the transport studies were performed.

As judged by electron-microscopical investigation of thin sections of the proteoliposomes, their diameters ranged from 0.1 to 0.4 µm and their walls consisted of a bilayer of phospholipids.

The transport studies were initiated by the addition of 20 µl of the proteoliposome suspension to 170 µl of incubation medium. After different time periods samples of 50 µl, or for longer incubation periods 20 µl, were withdrawn, added to 1 ml of ice-cold stopping solution and rapidly filtered through a Millipore filter (0.22 µm pore size). The incubation medium adhering to the proteoliposomes was re-

moved by washing the filter with 5 ml of ice-cold stopping solution. All experiments were performed in duplicate. The exact composition of the incubation medium and the stopping solutions is given in the legends to the Figures. Each incubation medium contained 25 µCi of radioactively labelled substrate. The radioactivity remaining on the filter was counted in a Packard Tri-Carb 3380 liquid-scintillation counter with Rothscint (Roth, Karlsruhe, W. Germany) as scintillation fluid. The labelled compounds were obtained from New England Nuclear, Boston, MA, U.S.A. All other chemicals were purchased from Merck A.G., Darmstadt, W. Germany, in analytical-grade purity.

For polyacrylamide-gel electrophoresis in SDS, the samples (approx. 50 µg of protein) were delipidated with 1 ml of chloroform/methanol (1:1, v/v) and the aggregated proteins were resuspended by incubation at 37°C for 14 h in 1% SDS, 0.04 M-dithiothreitol, 0.001 M-EDTA and 0.04 M-Tris/acetate, pH 6.1. Electrophoresis was then performed in 7.5% (w/v) polyacrylamide gels purchased from Bio-Rad, Richmond, CA, U.S.A. The gels were stained with Coomassie Blue and destained with 10% (v/v) propan-2-ol and acetic acid. Protein was determined in the presence of 1% SDS by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

At first it was essential to demonstrate that under the conditions chosen to investigate the incorporation of Na⁺ co-transport systems into the proteoliposomes, namely substrate transport in the presence of NaSCN compared with KCl in the incubation medium, the volume of the proteoliposomes is not altered during the transport experiment. [³H]Mannitol, for which apparently no transport system exists in the brush-border membrane, was used as a reference substance. Fig. 1 shows that the uptake of mannitol, even in the presence of D-glucose for which a transport system is present in the proteoliposomes (see below), is not influenced by the replacement of NaSCN by KCl. Consequently, the intraliposomal space remained constant under the experimental conditions, rendering our experimental protocol feasible for further studies.

Fig. 2 shows that NaSCN, the salt maximally effective in creating an electrochemical potential difference for Na⁺ in intact vesicles (Kinne *et al.*, 1975), stimulated D-glucose uptake by proteoliposomes. This uptake is decreased by 35% at maximum when KCl is substituted for NaSCN. The difference is statistically significant (*P* < 0.05) after 10, 20 and 40 min of incubation. The same decrease is observed when NaSCN is replaced by KSCN (results not shown). Furthermore this uptake is inhibited by 0.2 mM-phlorrhizin to the same extent as by the

Fig. 1. Uptake of mannitol by proteoliposomes formed from the co-sonication of a mixture of rat kidney phospholipids and a Triton X-100-free extract of rat kidney brush-border membranes

The incubation medium before the proteoliposomes were added contained 0.25 mM-[³H]mannitol, 0.25 mM-D-glucose, 5 mM-Hepes/Tris, pH 7.5, 10 mM-MgSO₄ and 150 mM-NaSCN or -KCl. The medium inside the proteoliposomes used in this study and in all subsequent studies to be described contained 150 mM-KCl, 5 mM-Hepes/Tris and 10 mM-MgSO₄, pH 7.5. Uptake was performed at 25°C. The stopping solution was 190 mM-NaCl, 5 mM-Hepes/Tris, pH 7.5, and 0.5 mM-phlorrhizin. ●, +NaSCN; ○, +KCl.

Fig. 2. Uptake of D-glucose by proteoliposomes. The incubation medium before the proteoliposomes were added contained 150 mM-NaSCN or -KCl, 10 mM-MgSO₄, 5 mM-Hepes/Tris buffer, pH 7.5, and 0.25 mM-D-[³H]glucose. To terminate the D-glucose uptake a stopping solution was used which contained 190 mM-NaCl, 5 mM-Tris/Hepes, pH 7.5, and 0.5 mM-phlorrhizin. Values are means $\pm$ S.E.M. of at least five experiments; for the phlorrhizin experiments the average of two experiments is given. $\bullet$, +NaSCN; $\circ$, +KCl; $\blacktriangle$, +NaSCN + 0.2 mM-phlorrhizin.

replacement of Na⁺ by K⁺, and thus the same properties of the glucose/Na⁺ co-transport system as found in isolated brush-border-membrane vesicles are observed (Kinne *et al.*, 1975).

Fig. 3(a) gives an example of the effect of NaSCN and KCl on the uptake of L-alanine by the proteoliposomes. A lower uptake is observed in the presence of KCl; NaSCN increases L-alanine uptake by approx. 48% at maximum. Thus an Na⁺-stimulated transport system for L-alanine also seems to have been incorporated into the proteoliposomes.

In Fig. 3(b) the uptake of P_i into the proteoliposomes is depicted. The highest uptake rate is observed in the presence of NaSCN. In the presence of KCl the uptake is inhibited by almost 50% at maximum ($P < 0.02$ at 10, 20 and 40 min). An inhibition is also observed if 10 mM-arsenate is added to the incubation medium containing NaSCN. In the last case, as expected for a competitive inhibitor (Hoffmann *et al.*, 1976), the degree of inhibition decreases with time.

One feature common to all three transport systems found in the proteoliposomes is the lack of 'overshooting' uptake which can be observed with intact brush-border vesicles in the presence of an Na⁺ gradient across the membrane (Kinne, 1976). Since the 'overshoot' is due to the relative magnitude of Na⁺-dependent to -independent influx and the duration of the Na⁺ gradient (Evers *et al.*, 1976), it could be argued that the lack of 'overshoot' in the proteoliposomes is because the substrate/Na⁺ co-transport influx is too small and the Na⁺-independent substrate influx is relatively too large and/or the duration of the

Fig. 3. Uptake of L-alanine (a) and P_i (b) by proteoliposomes. The incubation medium before the proteoliposomes were added contained 0.25 mM-L-[³H]alanine or 0.86 mM-[³²P]P_i. For termination of the alanine uptake the same stopping solution as detailed in the legend of Fig. 2 was used; for phosphate uptake the solution contained 10 mM-sodium arsenate instead of phlorrhizin. The results represent the average of two experiments. $\bullet$, +NaSCN; $\circ$, +KCl; $\blacktriangle$, +NaSCN + 10 mM-arsenate.

Fig. 4. Protein pattern of the proteoliposomes, the Triton X-100-free membrane extract and the brush-border membranes as revealed by electrophoresis in 7.5% polyacrylamide gels in the presence of 0.205 M-Tris/acetate buffer, 0.1 (w/v) SDS at pH 6.1

The standards (BDH, Poole, Dorset, U.K.) represent a mixture of the monomer and oligomers of peptides with mol.wts. 14 300 and 53 000 respectively.

Na⁺ gradient is too short. We think, however, that an 'overshoot' in the proteoliposomes would occur if there were many more Na⁺/substrate co-transport systems per membrane area.

In Fig. 4 the protein gel pattern of the proteoliposomes, the brush-border-membrane extract and the brush-border membranes are compared with proteins of known molecular weights. The proteoliposomes contain only a few bands, two prominent ones, with apparent mol. wts. approx. 100 000 and approx. 130 000, and several very faint bands, with mol.wts.

in the region 40 000–45 000, 60 000 and 90 000. The high-molecular-weight bands can also be observed in the membrane extract and the brush-border membranes, although more protein bands are seen in the latter. Several proteins with apparent mol.wts. approx. 45 000 and 35 000, which are found in both the brush-border and the membrane extract, do not seem to be present in the proteoliposomes. This could indicate that only a few proteins, perhaps the most hydrophobic ones, are incorporated into the proteoliposomes. Among these are the transport systems for D-glucose, L-alanine and phosphate.

We thank Professor K. J. Ullrich and Dr. H. Murer for their helpful discussions. Thanks are also due to Ms. G. Zimmerschied and Mr. A. Nagl for their excellent technical assistance and to Dr. W. Haase for electron microscopy. R. G. F. was a recipient of a Senior International Fellowship from the Fogarty International Center for Advanced Studies, National Institutes of Health, Bethesda, MD, U.S.A., while on sabbatical leave (1976–1977) at the Max-Planck-Institut für Biophysik.

References

- Bode, F., Baumann, K. & Kinne, R. (1976) *Biochim. Biophys. Acta* **433**, 294–310
- Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581
- Crane, R. K., Malathi, P. & Preiser, H. (1976) *FEBS Lett.* **67**, 214–216
- Evers, J., Murer, H. & Kinne, R. (1976) *Biochim. Biophys. Acta* **426**, 598–615
- Hoffmann, N., Thees, M. & Kinne, R. (1976) *Pflügers Arch.* **362**, 147–156
- Kates, M. (1972) *Techniques of Lipidology*, pp. 394–395, North-Holland, Amsterdam
- Kinne, R. (1976) *Int. Rev. Physiol.* **11**, 170–210
- Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M. & Sachs, G. (1975) *J. Membr. Biol.* **21**, 375–395
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Racker, E. (1973) *Biochem. Biophys. Res. Commun.* **55**, 224–230
- Semenza, G., Tannenbaum, C., Kessler, M., Toggengruber, G. & Wahlgren, L. (1977) *FEBS Symp.* **42**, 269–279