Asymmetric Reconstitution of the Erythrocyte Anion Transport System in Vesicles of Different Curvature: Implications for the Shape of the Band 3 Protein

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Z. Naturforsch. **45c,** 1021–1026 (1990); received July 18/August 24, 1990

Band 3 Protein, Anion Transport System, Asymmetric Reconstitution, Protein Shape, Erythrocyte Membrane

The anion transport protein of the human erythrocyte membrane, band 3, was solubilized and purified in solutions of the non-ionic detergent nonaethylene glycol lauryl ether and then reconstituted in spherical egg phosphatidylcholine bilayers as described earlier (U. Scheuring, K. Kollewe, W. Haase, and D. Schubert, J. Membrane Biol. **90,** 123–135 (1986)). The resulting paucilamellar proteoliposomes of average diameter 70 nm were transformed into smaller vesicles by French press treatment and fractionated according to size by gel filtration. The smallest protein-containing liposomes obtained had diameters around 32 nm; still smaller vesicles were free of protein. All proteoliposome samples studied showed a rapid sulfate efflux which was sensitive to specific inhibitors of band 3-mediated anion exchange. In addition, the orientation of the transport protein in the vesicle membranes was found to be "right-side-out" in all samples. This suggests that the orientation of the protein in the vesicle membranes is dictated by the shape of the protein's intramembrane domain and that this domain has the form of a truncated cone or pyramid.

Introduction

Numerous attempts have been made to reconstitute the anion transport system of the human erythrocyte membrane in lipid vesicles (for a review, see [1]). However, it was only recently that a fully satisfactory method for the reconstitution of this system was described [2, 3].

The most striking as well as unexpected feature of the successful reconstitution method consists of the virtually complete reconstitution of the right-side-out orientation of the transport protein, band 3, in the vesicle membranes [2, 3]. The unidirectional incorporation of the protein into the membranes, which occurs spontaneously, is most clearly demonstrated by the effect of the combined action of two specific inhibitors of anion transport: inhibition by a mixture of H_2DIDS (which does not penetrate lipid bilayer membranes [4]) and flufenamate (which rapidly penetrates membranes [5]) does not exceed the inhibition produced by H_2DIDS alone [3]. Thus, the H_2DIDS-binding site of virtually all band 3 molecules contributing to the inhibitor-sensitive sulfate efflux is accessible from the vesicle's exterior, in analogy to the situation in the erythrocyte membrane [4].

The reconstitution method discussed above is essentially a detergent dialysis method and uses a combination of two non-ionic detergents: Triton X-100, which is applied for the solubilization and purification of the protein, and octylglucopyranoside. The latter detergent is added to the protein/Triton X-100 mixture only after the addition of the lipid; its removal during analysis is what initiates vesicle formation. In the experiments described below, we have substituted Triton X-100 by nonaethylene glycol lauryl ether ($C_{12}E_9$), a related non-ionic detergent of the same low critical micelle concentration as Triton X-100. The proteoliposomes obtained again contain a reconstituted anion transport system; however, they are much smaller than those formed in Triton X-100 (average diameter 70 nm, as compared to 130 nm [3]). Nevertheless, these proteoliposomes, as well as smaller vesicles of diameters down to approx. 30 nm derived from them by treatment in a French press, again

Abbreviations: $C_{12}E_9$, nonaethylene glycol lauryl ether; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate.

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0341–0382/90/0900–1021 $ 01.30/0

show a virtually complete right-side-out orientation of the band 3 protein. This strongly suggests that, in the liposomes, the right-side-out arrangement is dictated by the structure of the band 3 protein.

Materials and Methods

$C_{12}E_9$ ("Polydocanol") was obtained from Sigma (München, F.R.G.). Before use, the amount needed for the experiment (in ethanol) was run through a column (17 × 1 cm) of aluminium oxide (Serva, Heidelberg, F.R.G.). Sephacryl S-500 was from Pharmacia (Freiburg, F.R.G.). For the sources of all other materials used see [2, 6].

Band 3 protein from human erythrocyte membranes was isolated in solutions of 1% $C_{12}E_9$ as described earlier [7]. Reconstitution of the anion transport system in egg yolk phosphatidylcholine liposomes was performed according to [2], with the modifications described in [3]. However, Triton X-100 was substituted by $C_{12}E_9$. The performance of the flux measurements and the evaluation of the data again followed [2], as did the electron microscopy of negatively stained samples.

For the preparation of smaller proteoliposomes of uniform size, 260 mg of phospholipid and 4–6 mg of band 3 were subjected to the reconstitution procedure, and the lipoprotein material obtained after sucrose gradient centrifugation [2] was pelleted by ultracentrifugation (3 h at 60,000 rpm, rotor Beckman 60 Ti). The pellet was resuspended in 7 ml buffer A (10 mM Hepes (pH 7.2), 10 mM Na_2SO_4, 0.5 mM EDTA, 15 mM β-mercaptoethanol) and passed 4 times through a French press (Aminco; 40 ml capacity, pressure 20,000 psi, flow rate 5 ml/min. Temperature: approx. 4 °C) [8, 9]. Then, protein adsorbed to the outer surface of the proteoliposomes was degraded by trypsin treatment [6], and the sample was again centrifuged to remove aggregated material (1 h at 50,000 rpm, rotor 50 Ti). Afterwards, the proteoliposomes were separated according to size by gel filtration on Sephacryl S-500 (column dimensions: 81 × 2.6 cm) [10, 11]. Fraction size was 4 ml. Selected peak fractions were pooled, concentrated 10–50-fold by ultrafiltration (Amicon cell, volume 50 ml; filters YM 100), and then filtered through a 0.2 μm Nucleopore polycarbonate filter [12]. The absorbance at 320 nm (due to light scattering) of the final samples was 0.2–0.3 (d = 1 cm). The size distribution of the vesicles in these samples was determined from measurements of quasi-elastic light scattering [12].

Results

Primary products of the reconstitution procedure

The reconstitution procedure described above yields band 3-containing liposomes which are heterogeneous in size, as judged from electron micrographs of negatively stained samples (Fig. 1). Average liposome diameter is approx. 70 nm. Only about one third of the liposomes are unilamellar (in contrast to those obtained by the use of Triton X-100 [2]); most of them consist of 2–3 lipid bilayers (Fig. 1). Similar to the lipoprotein vesicles obtained by the use of Triton X-100/octylglucopyranoside mixtures [2], the liposomes contain around 60% of the lipid and 30% of the protein originally present.

Sulfate efflux from the proteoliposomes and from protein-free liposomes prepared analogously is shown in Fig. 2. As can be seen from the figure, sulfate efflux is much more rapid from the band 3-containing vesicles than from the protein-free vesicles. The former can be effectively inhibited by micromolar concentrations of H_2DIDS and flufenamate which, in this concentration range, effec-

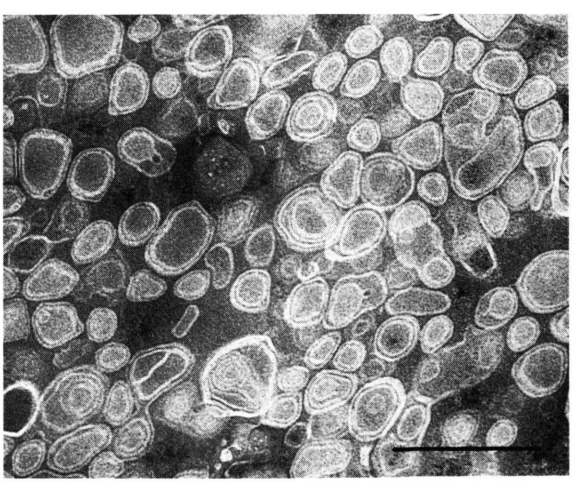

Fig. 1. Morphology of the products of the reconstitution procedure, as revealed by electron microscopy. The bar indicates 200 nm.

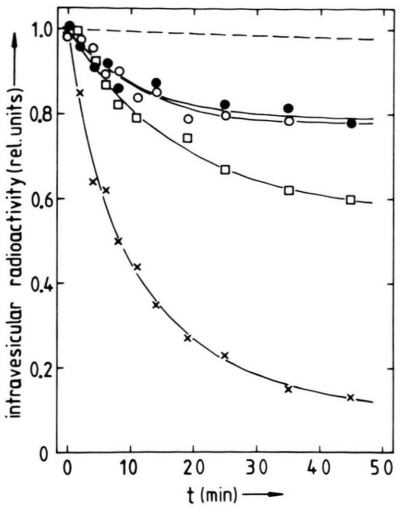

Fig. 2. Sulfate efflux from the band 3-containing liposomes: counting rate n(t) of [^{35}S]sulfate trapped in the liposomes in the absence of inhibitors of anion transport (×) and after addition (at zero time) of 15 μmol/l H_2DIDS (○), 5 μmol/l flufenamate (□) and 15 μmol/l H_2DIDS plus 5 μmol/l flufenamate (●). The protein/lipid weight ratio in the starting mixture was 1:75. Buffer: 10 mM Hepes (pH 7.2), 10 mM Na_2SO_4, 0.5 mM EDTA. θ = 30 °C. The broken line indicates sulfate efflux from vesicles which did not contain band 3 protein but otherwise were prepared in the same way as the proteoliposomes. The solid curves represent the least squares fit to the data according to Eqn. (2) and (3) of [2]. All experimental and fitted data were normalized to the value $n_f(0)$ of the corresponding fitted curve.

tively inhibit anion exchange across the human erythrocyte membrane [4]. This shows that the proteoliposomes contain a reconstituted anion transport system. Another remarkable aspect of Fig. 2 is that the joint application of both inhibitors does not lead to a stronger inhibition of sulfate efflux than application of H_2DIDS alone. As already discussed in the introduction, this means that all band 3 molecules which contribute to the H_2DIDS- or flufenamate-sensitive sulfate efflux are incorporated into the liposomal membranes with their H_2DIDS-binding site pointing outward and hence are in "right-side-out" orientation (at least with respect to the outermost lipid bilayer). The H_2DIDS-insensitive flux component thus represents leak flux [2]. It is also apparent from Fig. 2 that this (protein-induced) leak flux is shown by only a small part of the liposome population (for a detailed discussion see [2]).

Proteoliposomes obtained by French press treatment of the reconstituted material

Passage of the proteoliposomes through a French press yields liposomes which are heterogeneous in size, with diameters between approx. 20 and 70 nm and a maximum of the distribution around 60 nm. Most of the liposomes still consist of two lipid bilayers (Fig. 3). This is in contrast to the effect of French press treatment on protein-free liposomes which can be transformed nearly quantitatively into unilamellar vesicles of diameters between 15 and 30 nm ([9] and own observations on protein-free liposomes prepared analogously to the proteoliposomes). Thus, the incorporated band 3 protein by an unknown mechanism stabilizes the liposomes against the shear-induced rupture and the decrease in diameter.

From the heterogeneous sample of Fig. 3, liposomes of a narrow size distribution were derived by gel filtration on Sephacryl S-500 [10, 11]. The elution profile of the column, together with the position of the fractions pooled and the average diameter of the liposomes in the pooled fractions, are shown in Fig. 4. As indicated, average diameters in the pooled fractions are 63, 51 and 32 nm. According to electron microscopy, the 51 nm-vesicles still predominantly consist of two lipid lamellae, whereas the 32 nm-vesicles are almost exclusively unilamellar. As demonstrated in Fig. 5 for

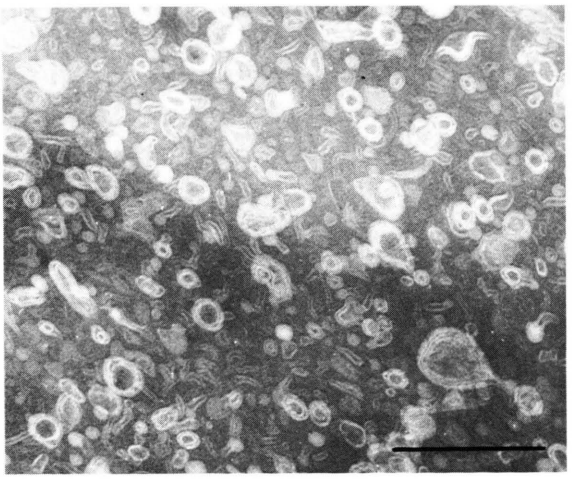

Fig. 3. An electron micrograph of the proteoliposomes of Fig. 1 after treatment of the material in a French press. Bar: 200 nm.

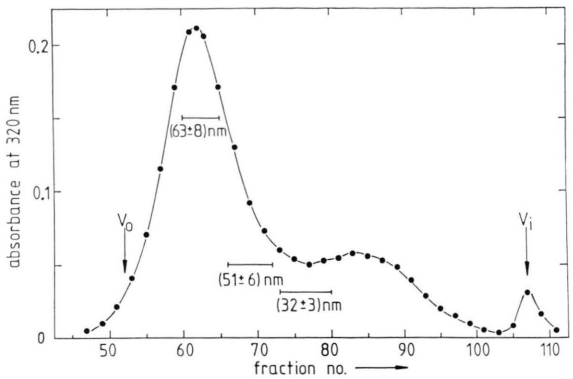

Fig. 4. Fractionation of the proteoliposomes, after treatment in a French press, by gel filtration on Sephacryl S-500. V_o and V_i are the elution positions of the void and total volume, respectively; they were determined as in [3]. The fractions indicated were pooled, concentrated, and then used for the dynamic light scattering and sulfate efflux measurements. The figures given are the average mass-weighted diameter and the standard deviation of the diameter of the vesicles in the pooled fractions.

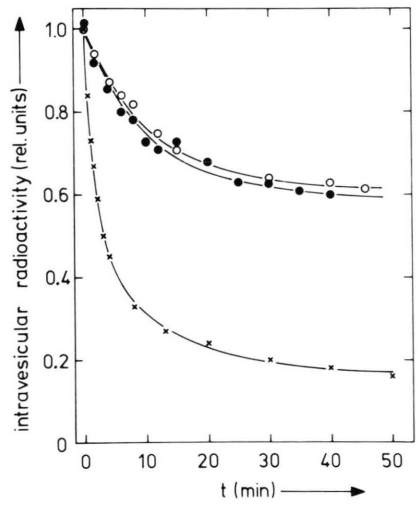

Fig. 5. Sulfate efflux from the proteoliposomes of diameters (51 ± 6) nm (see Fig. 4), measured in the absence of inhibitors (×) and in the presence of 15 μM H_2DIDS (○) and 15 μM H_2DIDS plus 5 μM flufenamate (●). θ = 37 °C. For further details see legend to Fig. 2.

the 51 nm-vesicles, the sulfate efflux curves for the different samples, in the absence and presence of the two inhibitors applied, are completely analogous to that shown in Fig. 2 and thus again demonstrate both the presence of a reconstituted anion transport system and the right-side-out orientation of the transport protein in the vesicle membranes. On the other hand, the vesicles of diameters smaller than approx. 28 nm contained little or no protein and did not show a rapid sulfate efflux.

Our primary aim in investigating the products of French press treatment was to establish a system which would allow us to study whether or not monomeric band 3 protein is able to transport anions. The 32 nm-vesicles turned out to represent such a system ([13] and S. Lindenthal and D. Schubert, manuscript in preparation)*. They may be useful also for analogous studies on other membrane proteins.

Discussion

The different proteoliposome samples prepared and studied in this paper apparently represent a reconstitution of the most conspicuous transport properties of the erythrocyte anion transport system: they show a rapid anion efflux (under exchange conditions) which is inhibitable by micromolar concentrations of H_2DIDS or flufenamate. In addition, they share a basic structural property with the native system, namely the unidirectional, right-side-out orientation of the transport protein.

Previously published methods for the reconstitution of transport systems in liposomes have, with a few exceptions, led to an approximately symmetrical orientation of the transport proteins with respect to the membrane's midplane [1, 15]. This is true also for the erythrocyte anion transporter [1]. On the other hand, the reconstitution method for the latter system described by our group [2, 3] and modified and varied in the present paper leads to a virtually complete right-side-out incorporation of the transport protein into the vesicle membranes. The asymmetric orientation certainly cannot result from an insertion of the protein into a preformed vesicle membrane, as discussed for other membrane proteins with which it has been achieved [15, 16]: this mechanism requires that the protein is inserted with its more

* This application of the 32 nm-vesicles also dictated the choice of $C_{12}E_9$ instead of Triton X-100 [2, 3] as the primary detergent: The density of $C_{12}E_9$ is very near to that of egg phosphatidylcholine [14], which allows the determination of the protein content of the vesicles by analytical ultracentrifugation, by simultaneously matching the densities both of the lipid and of residual detergent possibly retained in the samples [13, 14].

hydrophobic moiety first, whereas, with band 3 protein, it would be just the highly charged cytoplasmic domain (M_r = 43,000) [4] which would have to cross the lipid bilayer. The unidirectional orientation of the protein is thus spontaneously generated during the process of vesicle formation from mixed protein/phospholipid/detergent micelles, regardless of whether Triton X-100 [2, 3] or $C_{12}E_9$ is used as the primary detergent and regardless of the protein content of the vesicles [2]; it is also found with sphingomyelin vesicles [6]. As shown in this paper, it is, in addition, preserved during disruption of the vesicles by French press treatment and subsequent formation of vesicles of much smaller diameter. Thus, there must be an intrinsic structural property of the band 3 protein which, during the original insertion of the protein into a nascent spherical lipid bilayer as well as during vesicle formation from disrupted bilayer sheets, strongly favours the right-side-out configuration.

What structural property of the anion transport protein could be responsible for its asymmetric orientation in the vesicle membranes? It has to be considered that the diameter of the vesicles originally formed in the reconstitution process is only around 50–60 nm (larger vesicles are the result of fusion) [17] and that most of the vesicles formed by French press treatment have an even smaller diameter. Thus, the vesicle membranes are strongly curved. If, in this situation, the intramembrane domain of the protein would taper towards one of the membrane surfaces, the presence of the protein in the nascent lipid bilayer membrane or in a bilayer sheet generated in the French press would be most likely to dictate the direction of vesicle closure: it could strongly favour that direction in which the thicker end of the protein's intramembrane domain would be placed in the outer monolayer of the membrane (Fig. 6). This view is in agreement with results from theoretical considerations [18]. The right-side-out arrangement of the band 3 protein in the vesicle membranes observed in all our experiments therefore suggests that the intramembrane domain of the band 3 protein has the form of a truncated cone or pyramid the base of which is directed towards the outside of the cell.

Two aspects have to be added to the considerations described. (1) The structural properties of the band 3 protein do not necessarily lead, during reconstitution, to a right-side-out configuration but must be supported by suitable experimental conditions. This is shown by the lack of a distinct asymmetry of protein orientation in the reconstitution experiments of other authors [1]. (2) The right-side-out orientation of the band 3 protein in the vesicle membranes was observed with vesicles of a wide range of diameters. Nevertheless, it seems probable that the conformation of the protein may be affected by the curvature of the membranes and that the transport function may be impaired in the highly curved membranes. In fact we have found that, in the 32 nm-vesicles, the turnover number of band 3 for sulfate transport is smaller than in the 130 nm-vesicles [19] by a factor of approx. 15 (S. Lindenthal and D. Schubert, manuscript in preparation). It should be recalled that the vesicles of diameter <28 nm do not contain detectable amounts of protein, which suggests that the very strong curvature of these vesicles is incompatible with the presence of band 3.

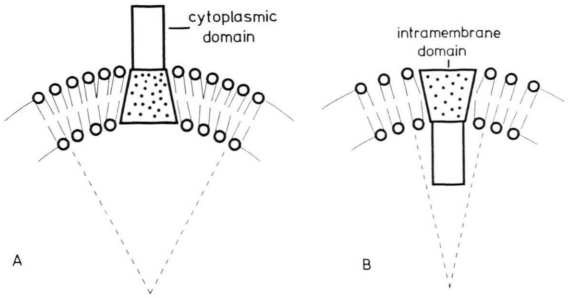

Fig. 6. A model attributing the observed right-side-out orientation of the band 3 protein in the vesicle membranes to a tapered shape of the intramembrane domain. (A) "Inside-out" incorporation of the protein into the spherical vesicle membrane leads to an energetically unfavourable, strong local distortion of the lipid arrangement in a large area surrounding the protein. (B) A much smaller lipid area is distorted if the protein is incorporated into the vesicle membrane "right-side-out". Assumed vesicle dimensions: outer diameter 50 nm, inner diameter 45 nm.

Acknowledgements

We are grateful to Professor H. Passow for stimulating discussions, to Mrs. G. Grieshaber for performing some preliminary experiments, to Mrs. D. Gahl for skilful technical assistance, and to the Deutsche Forschungsgemeinschaft for financial support (SFB 169).

[1] Z. I. Cabantchik and A. Darmon, in: Structure and Properties of Cell Membranes, **Vol. III** (G. Benga, ed.), pp. 123–165, CRC Press, Boca Raton, Fl. 1985.
[2] U. Scheuring, K. Kollewe, W. Haase, and D. Schubert, J. Membrane Biol. **90,** 123–135 (1986).
[3] U. Scheuring, G. Grieshaber, K. Kollewe, Z. Kojro, H. Ruf, E. Grell, W. Haase, and D. Schubert, Biomed. Biochim. Acta **46,** S46–S50 (1987).
[4] H. Passow, Rev. Physiol. Biochem. Pharmacol. **103,** 61–203 (1986).
[5] J. L. Cousin and R. Motais, Biochim. Biophys. Acta **687,** 147–155 (1982).
[6] U. Scheuring, W. Haase, and D. Schubert, FEBS Lett. **231,** 232–236 (1988).
[7] G. Pappert and D. Schubert, Biochim. Biophys. Acta **730,** 32–40 (1983).
[8] Y. Barenholz, S. Amselem, and D. Lichtenberg, FEBS Lett. **99,** 210–214 (1979).
[9] R. L. Hamilton, J. Goerke, L. S. S. Guo, M. C. Williams, and R. J. Havel, J. Lipid Res. **21,** 981–992 (1980).
[10] J. A. Reynolds, Y. Nozaki, and C. Tanford, Anal. Biochem. **130,** 471–474 (1983).
[11] P. Schurtenberger and H. Hauser, Biochim. Biophys. Acta **778,** 470–480 (1984).
[12] H. Ruf, Y. Georgalis, and E. Grell, Methods Enzymol. **172,** 364–390 (1989).
[13] S. Lindenthal and D. Schubert, 19th FEBS Meeting, Rome, abstract TU 363 (1989).
[14] C. Tanford and J. A. Reynolds, Biochim. Biophys. Acta **457,** 133–170 (1976).
[15] G. D. Eytan, Biochim. Biophys. Acta **694,** 185–202 (1982).
[16] M. K. Jain and D. Zakim, Biochim. Biophys. Acta **906,** 33–68 (1987).
[17] M. Ueno, C. Tanford, and J. A. Reynolds, Biochemistry **23,** 3070–3076 (1984).
[18] H. Gruler, Z. Naturforsch. **30c,** 608–614 (1975).
[19] U. Scheuring, S. Lindenthal, G. Grieshaber, W. Haase, and D. Schubert, FEBS Lett. **227,** 32–34 (1988).