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PHOSPHOLIPID DEPENDENCE OF THE ANION TRANSPORT SYSTEM OF THE HUMAN ERYTHROCYTE MEMBRANE

STUDIES ON RECONSTITUTED BAND 3/LIPID VESICLES

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Band 3 protein extracted from human erythrocyte membranes by Triton X-100 was recombined with the major classes of phospholipid occurring in the erythrocyte membrane. The resulting vesicle systems were characterized with respect to recoveries, phospholipid composition, protein content and vesicle size as well as capacity and activation energy of sulfate transport. Transport was classified into band-3-specific fluxes and unspecific permeability by inhibitors. Transport numbers (sulfate ions per band 3 per minute) served as a measure of functional recovery after reconstitution. The transport properties of band 3 proved to be insensitive to replacement of phosphatidylcholine by phosphatidylethanolamine, while sphingomyelin and phosphatidylserine gradually inactivated band-3-specific anion transport when present at mole fractions exceeding 30 mol%. The activation energy of transport remained unaltered in spite of the decrease in transport numbers. The results, which are discussed in terms of requirements of band 3 protein function with respect to the fluidity and surface charge of its lipid environment, provide a new piece of evidence that the transport function of band 3 protein depends on the properties of its lipid environment just as the catalytic properties of some other membrane enzymes. The well-established species differences in anion transport (Gruber, W. and Deuticke, B. (1973) J. Membrane Biol. 13, 19–36) may to some extent reflect this lipid dependence.

Introduction

Intrinsic, membrane-spanning proteins are a major element of the fluid-mosaic membrane model [1], presently regarded as an adequate description of the physical reality of biomembranes. Functional, i.e., cognitive and catalytic, properties of membranes are to a major extent based on such proteins which are surrounded by membrane lipids. These lipids can either act as an inert solvent for the protein, or interact with the peptide segments by specific interactions [2] which sometimes may even lead to a formation of a particular lipid anulus surrounding the protein [3–6]. Unspecific solvent properties (fluidity, polarity) as well as specific interactions may therefore be responsible for the functional alterations imposed on membrane receptors [7], membrane enzymes [8] and membrane transport systems [8–14] by alterations and modulation of the membrane lipid.

Due to the intricate heterogeneity of their lipid composition, it has not been possible in many cases to draw unequivocal conclusions on causal molecular mechanisms from experiments on mod-

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Abbreviations: DNDS, 2,2'-dinitrostilbene-4,4'-disulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ified biomembranes. The technique of reconstituting membrane proteins, isolated and delipidated in a functional or reversibly inactivated state, into artificial membrane systems of defined lipids, has to some extent improved the strategic basis for analyzing details of lipid-protein interactions [15,16].

A considerable amount of information has thus become available for receptors [7] and membrane enzymes [5,8]. As concerns transport proteins, the vectorial component in their catalytic function has hampered progress to some extent. The available information on lipid requirements of 'carriers' is largely restricted to actively pumping systems [17–19]. In these systems, the evaluation of lipid dependences of transport capacity requires reliable information on parallel membrane leaks, which are usually not easily quantified [19,20]. For the few passive carrier systems reconstituted as yet in a functional state [17] lipid dependences have not hitherto been investigated.

The anion exchange system of the erythrocyte membrane was successfully reconstituted by a number of groups [21–25]. Due to the abundant availability of the transport protein, band 3 peptide [26], which constitutes about 20% (approx. 1 mg/ml cells) of the total membrane peptide mass of the erythrocyte, this system would seem to be an attractive model for studying the lipid dependency of a passive transport system. Indications for profound effects of membrane lipids on the activity of band 3 protein in its native environment are already available [12–14,27]. The present study describes the influence of the four major phospholipids of the erythrocyte membrane on the reconstituted anion transport system. A preliminary communication has been presented elsewhere [28]. The role of cholesterol has already been treated in a previous report of this series [25].

Materials

Freshly drawn human blood was obtained from the local blood bank and used on the same day. L-α-Phosphatidylcholine (egg yolk, No. 5763), L-α-phosphatidylethanolamine (egg yolk, No. 6386), L-α-phosphatidylserine (bovine brain, No. 8518) were from Sigma, Munich, sphingomyelin (bovine brain, No. 5067-00) from Koch Light Laboratories, Colnbrook, Berks., U.K., and cholesterol (No. 24622) from Merck, Darmstadt. The purity of the phospholipids was checked by two-dimensional thin-layer chromatography. No impurity could be detected. $Na_2^{35}SO_4$ and [2-^{14}C]EDTA were purchased from Amersham Buchler, Braunschweig, [phenyl-^{3}H(n)]Triton X-100 from New England Nuclear, Dreieich, F.R.G. Bio-Beads SM-2 and Bio-Gel P-4 (100–200 mesh) were from Bio-Rad, Munich. 2,2-Dinitrostilbene-4,4′-disulfonate (DNDS) was from K & K Chemicals, New York. All other chemicals were of the highest purity available.

Methods

Purification and reconstitution of band 3 protein

The procedure of purification and reconstitution of band 3 protein has been described earlier [23]. Briefly, erythrocyte membranes ('ghosts') are solubilized by the non-ionic detergent Triton X-100 and band 3 protein is purified by differential extraction with increasing concentrations of Triton X-100. After addition of defined lipids, solubilized in Triton X-100 too, and removal of the detergent by absorption to Bio-Beads SM-2, bilayer membranes of lipid and protein form spontaneously. Small unilamellar vesicles produced under these conditions are most interesting with regard to transport studies, and can be isolated by differential centrifugation.

Characterization of band 3 / lipid vesicles

Yields of vesicles. Yields of vesicles prepared from different phospholipids were quantified by phosphate analysis (29) after reconstitution and purification of the protein/lipid-recombinates via a Biogel P 4 column in Hepes buffer (10 mM Hepes/100 mM D-mannitol/10 mM Na_2SO_4/0.1 mM NaN_3 (pH 8)).

Lipid composition. The lipid composition of these vesicles was determined by thin-layer chromatography [30] after extraction of the lipids [31]. Phospholipid fractions on the silica plates were quantified by phosphate analyses [29].

Protein contents. Protein contents in the vesicles were quantified by amino acid analysis. For this purpose, an aliquot of the vesicle sediment after reconstitution and ultracentrifugation was resus-

pended in Hepes buffer, and hydrolysed at 105°C in 6 M HCl. Amino acids were separated in a Beckman Autoanalyzer (Column length: 40 cm, column material: Beckman M 81). In order to calculate the amino acid composition of the sample, standards of known concentrations were used. The band 3 content of the samples was derived from the concentrations of five representative amino acids (asparagine + aspartate, glutamine + glutamate, alanine, valine, leucine), using for calibration the quantitative amino acid composition of band 3 protein given in Ref. 24. The protein content was related to the lipid-phosphorus content of the sample determined in parallel.

Specific vesicle volumes. Volumes were derived from the trap of [^{14}C]EDTA during the reconstitution. For this purpose, 5 μCi [^{14}C]EDTA (spec. act. 10 mCi/mmol) were added to a reconstitution suspension (containing 10 mM Na_2SO_4/20 mM NaCl/3.6 mM EDTA-Na_2/2.6 mM phosphate buffer (pH 8)) before extraction of the detergent. After vesicle formation and centrifugation, extravesicular radioactivity was removed by gel filtration through a column containing Bio-Gel P-4 at 4°C in a buffer containing 100 mM mannitol/10 mM Na_2SO_4/10 mM Hepes (pH 6.9). The specific volume, i.e., the volume (μl H_2O) entrapped per μmol vesicle phospholipid, was calculated from the entrapped radioactivity (cpm/μmol lipid P) and the radioactivity in the supernatant (cpm/μl H_2O).

Residual Triton X-100 concentrations. Concentrations of Triton X-100 in the vesicles after reconstitution were measured by carrying ^{3}H-labelled Triton X-100 through the procedure. Concentrations were related to the phospholipid content of the vesicle preparation.

Flux measurements

The transport activity of band 3 protein in vesicles of different lipid composition was determined by measuring [^{35}S]sulfate efflux under equilibrium exchange conditions as described previously [25]. 'Band-3-specific transport' was defined as the fraction of sulfate efflux susceptible to high doses (180 μM = 150 × ID_{50}) of the impermeable stilbene disulfonate inhibitor DNDS. In all vesicle preparations the inhibition by these concentrations of DNDS was the maximal one obtainable by the inhibitor, since it could not be enhanced by raising the inhibitor concentration to 0.5 mM. Transport numbers (TN) (defined as sulfate molecules transported per band 3 protein molecule per minute at a standard sulfate concentration) were calculated by the following equation [21]:

$$TN = \frac{k_{spec} \cdot C_{SO_4} \cdot \bar{V}}{\bar{C}_{Prot}}$$

where k_{spec} is the rate coefficient of the fraction of sulfate efflux sensitive to inhibition by external DNDS, C_{SO_4} the standardized sulfate concentration in the vesicles (10 mM), $\bar{V}$ the specific vesicle volume (μl H_2O enclosed per μmol vesicle phospholipid) and $\bar{C}_{prot}$ the mean number of protein molecules per μmol phospholipid. Activation energies of band-3-specific transport were calculated from rate coefficients of sulfate exchange at 10°C, 20°C and 25°C.

Results

The anion exchange system of the human erythrocyte membrane can be solubilized by the non-ionic detergent Triton X-100 and purified to about 85% [22,23]. Functional reconstitution of the purified protein into vesicle systems of different phospholipid composition is demonstrated in this study. All preparations contained 27 mol% cholesterol in the lipid dispersion used for reconstitution. In order to be able to compare transport activities of band 3 protein in the different kinds of vesicle, the vesicle volumes and their protein contents were first determined. As a further characterization of the vesicle system, the phospholipid composition and the residual amount of detergent in the vesicle membrane after the reconstitution procedure were analyzed.

In Table I different vesicle systems are compared (1) with respect to the recovery of phospholipids and proteins, relative to the amount originally introduced for reconstitution, and (2) with respect to their fractional contents of these constituents. Vesicle systems with high fractional contents of phosphatidylcholine and phosphatidylserine could be reconstituted at high yields,

TABLE I

CHARACTERISTICS OF RECONSTITUTED BAND 3/LIPID VESICLES

Characteristics of band 3/lipid vesicles reconstituted from different phospholipid dispersions, following the general procedure described in Ref. 25. For details of the analytical techniques see Methods. Mean values (±S.D.) from n experiments. n.d. = not determined; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Lipids used for reconstitution (mol% in addition to 27 mol% cholesterol)	Phospholipid recovery (% of initial amount) (n = 2–9)	Protein recovery (% of initial amount) (n = 2)	Relative protein contents (nmol/μmol phospholipid) (n = 2)
1 PC (73)	28.1 ± 4.1	20	0.33
2 PC/SM (55/18)	17.0 ± 4.4	n.d.	n.d.
3 PC/SM (37/36)	16.7 ± 3.6	19	0.87
4 SM (73)	7.4 ± 3.7	30	1.97
5 PC/PE (37/36)	39.8 ± 1.3	n.d.	n.d.
6 PE (73)	7.5 ± 5.1	3	0.44
7 PE/PS (48/25)	34.8 ± 15.6	n.d.	n.d.
8 PE/PS (29/44)	38.1 ± 8.5	29	0.35
9 PS (73)	41.2 ± 11.6	52	0.68

while systems with high mole fractions of phosphatidylethanolamine and sphingomyelin suffered a remarkable loss of lipid material during the reconstitution procedure. The recovery of proteins in the vesicles, as estimated by amino acid analyses, varied in parallel with the phospholipid yield in most of the systems, suggesting that protein and lipid were incorporated into the artifical membrane to almost the same extent regardless of the absolute yield. In two vesicle systems the relative protein content was greater than in phosphatidylcholine-containing vesicles: in those containing predominantly phosphatidylserine by a factor of about 2, in sphingomyelin vesicles even by factors of 3–5, depending on the sphingomyelin content. Residual detergent concentrations in the vesicles amounted to 0.8–1.5 molecules of Triton X-100 per 100 lipid molecules (data not shown) in agreement with earlier data [32], but below the results of recent other measurements [33,34]. These numbers are below the range of Triton concentrations producing inhibition of anion transport and acceleration of unspecific ion permeabilities in the native erythrocyte membrane (Deuticke, unpublished data) and do not enhance ion permeability in vesicle systems [35].

In Table II the phospholipid patterns of the reconstituted lipid/protein vesicles are compiled. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine are incorporated into the vesicles essentially at the amounts introduced into the reconstitution procedure. Sphingomyelin is incorporated only to a lower extent. The presence of phosphatidylethanolamine in vesicles nominally free of phosphatidylethanolamine results from contamination of the band 3 preparations with erythrocyte membrane lipids. It has been shown that phosphatidylethanolamine is enriched relative to the other membrane phospholipids in band 3/Triton X-100 micelles [36,37]. Vesicles reconstituted from sphingomyelin-containing phospholipid dispersions considerably differ in their fractional phospholipid composition from the initial phospholipid dispersion. This difference is most likely due to poor solubilization of sphingomyelin by Triton X-100. In systems composed of sphingomyelin and an additional phospholipid (e.g., phosphatidylcholine) this poor solubilization leads to a relative enrichment of the additional phospholipid, in pure sphingomyelin systems it results in a markedly reduced yield of vesicles (see Table I).

Mean relative vesicle volumes, measured by trapping of the impermeable marker EDTA, generally ranged from 0.8 to 1.2 μl H_2O/μmol phospholipid. As an exception, enclosed volumes of 2.5

TABLE II

LIPID COMPOSITION OF BAND 3/LIPID VESICLES USED FOR FLUX MEASUREMENTS

Mean values from three experiments. Values as mol% of phospholipids used for reconstitution. Abbreviations as in Table I.

Initial fractional content (mol% of the phospholipids used for reconstitution)				Final fractional phospholipid content after reconstitution			
PC	SM	PE	PS	PC	SM	PE	PS
100				94.9 ± 2.8	1.3 ± 2.3		
75	25			76.5 ± 12.4	18.1 ± 10.0	5.5 ± 2.3	
50	50			67.7 ± 6.4	26.9 ± 6.2	7.2 ± 5.1	
	100			7.5	81.1	9.7	1.8
50		50		48.6		51.4	
		100		9.1 ± 12.9		90.8 ± 12.9	
		66	34	1		71.5 ± 6.3	25.7
		27	73			28.7	71.3
			100			7.8	92.2

μl H_2O/μl phospholipid were found in phosphatidylcholine/ phosphatidylethanolamine vesicles. The mean volume obtained for vesicles of a certain lipid composition, however, varied to some extent (up to a factor of 2) from one reconstitution to another.

Sulfate transport in the different band 3/lipid vesicles was determined under self-exchange conditions. The studies routinely included transport measurements with and without added DNDS in two parallel samples from one vesicle population [25]. In this way statistical errors of the fractional inhibitions by DNDS could be reduced.

In Fig. 1, the time course of sulfate efflux from band 3/phosphatidylcholine vesicles is demonstrated as a characteristic example before and after addition of high concentrations of DNDS. In contrast to anion transport in the native membrane, sulfate efflux is not completely inhibited by DNDS. The extent of inhibition depends on temperature. Sulfate efflux from band 3/lipid recombinates is thus composed of two fractions, one susceptible to externally added DNDS, defined in this study as 'band-3-specific', and another, termed 'unspecific'.

In Table III rate coefficients are compiled for sulfate efflux, at 10°C, from vesicle systems of different lipid compositions. In membranes composed, in addition to 27 mol% cholesterol, only of phosphatidylcholine or phosphatidylethanolamine, the band-3-specific transport amounts to about 60% of the total sulfate efflux. Cholesterol per se has no effect on band-3-specific efflux in combination with phosphatidylcholine [25].

The following effects of changes of the vesicle phospholipid composition on the rate coefficients of sulfate efflux become evident from Table III.

(1) Band-3-specific transport and unspecific efflux are inhibited by high vesicle contents of sphingomyelin. At 73 mol% the band-3-specific transport decreases to zero, the unspecific flux is lowered considerably.

(2) Phosphatidylethanolamine can replace phosphatidylcholine in vesicles without significant changes in the specific or the unspecific transport rates. The somewhat lower rate coefficients for phosphatidylcholine/phosphatidylethanolamine

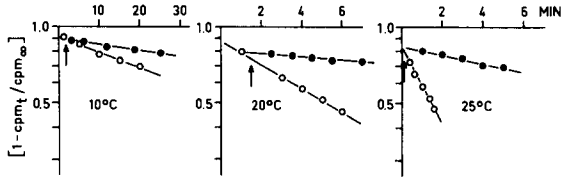

Fig. 1. Time course of [^{35}S]sulfate efflux from band 3/phosphatidylcholine/cholesterol-vesicles under self-exchange conditions at three temperatures in the absence (○) or presence (●) of 180 μM externally added DNDS. 10 mM sulfate (pH 6.9). For further details see Methods and Ref. 25. Inhibitor was added to one part of the vesicle suspension at the times indicated by arrows.

TABLE III

RATE COEFFICIENTS OF SULFATE EQUILIBRIUM EXCHANGE

Rate coefficients of sulfate equilibrium exchange (pH 6.9, 10°C, 10 mM sulfate) in band 3/lipid vesicles of different phospholipid composition. DNDS was added externally at a concentration of 180 μM. Mean values from 3–5 experiments, when errors are given.

Lipids used for reconstitution (mol% in addition to 27 mol% cholesterol)	k_{total} (min^{-1})($\times 10^2$)	Maximal inhibition by DNDS (%)	$k_{specific}$ (min^{-1})($\times 10^2$)	$k_{unspecific}$ (min^{-1})($\times 10^2$)
PC (73)	1.94 ± 0.39	63 ± 4	1.16 ± 0.2	0.79 ± 0.21
PC/SM (55/18)	2.59 ± 0.56	51.7 ± 3.8	1.35 ± 0.35	1.24 ± 0.25
PC/SM (37/36)	2.19 ± 0.97	48.0 ± 7.0	1.09 ± 0.61	1.09 ± 0.38
SM (73)	0.51	0	0	0.51
PC/PE (37/36)	1.43 ± 0.07	60.6 ± 2.6	0.87 ± 0.07	0.56 ± 0.03
PE (73)	1.93 ± 0.30	58.3 ± 5.2	1.18 ± 0.26	0.75 ± 0.05
PE/PS (48/25)	1.76 ± 0.32	54.9 ± 6.4	0.97 ± 0.27	0.78 ± 0.11
PE/PS (29/44)	1.11	20.7	0.23	0.88
PS (73)	1.17 ± 0.25	0	0.06 ± 0.08	1.11 ± 0.18

vesicles as compared to phosphatidylcholine vesicles may be related to the larger volume of the former vesicles.

(3) Phosphatidylserine, used in this study in combination with phosphatidylethanolamine, does not affect band-3-specific transport up to mole fractions (of the phospholipids) of 0.33. Upon a further rise of the phosphatidylserine content, however, band-3-specific sulfate transport is reduced, while unspecific fluxes are not affected. At phosphatidylserine concentrations exceeding 70 mol% the specific transport is reduced to zero. The volumes of these vesicles do not differ from phosphatidylcholine containing vesicles.

In Fig. 2 the temperature dependences for sulfate efflux from vesicles composed of the different phospholipids used in this study are demonstrated as Arrhenius diagrams, which allow calculations of the activation energies of band-3-specific transport and unspecific fluxes. In the case of phosphatidylcholine- or phosphatidylethanolamine-containing vesicles, distinct and differing activation energies can be obtained for the band-3-specific and the unspecific sulfate flux. In vesicle systems containing only sphingomyelin or phosphatidylserine, however, no band-3-specific transport could be measured. The temperature dependence of total sulfate efflux represents the activation energy for the unspecific permeability. The

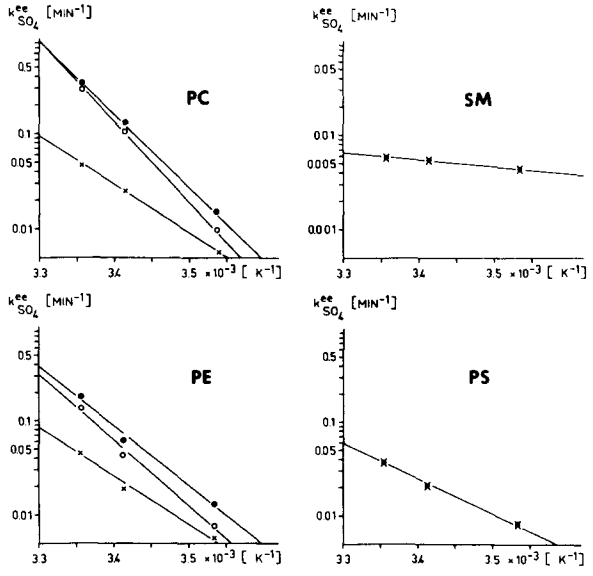

Fig. 2. Temperature dependence of sulfate equilibrium exchange (pH 6.9, 10 mM sulfate) in band 3/lipid-vesicles from different phospholipids, presented as Arrhenius diagrams. The vesicles were prepared using lipid dispersions containing 73 mol% of the phospholipid indicated in each panel (in addition to 27 mol% cholesterol). The final lipid composition of the vesicles was slightly different from that of the initial dispersion, as demonstrated in Table II. ●, total flux; ○, DNDS-sensitive (= band-3-specific) flux; ×, DNDS-insensitive (= unspecific) flux. DNDS added externally at 180 μM.

activation energies for all types of vesicle used in this study are compared in Table IV. In vesicles exhibiting band-3-specific transport, the activation energies of the specific pathway consistently range from 33–36 kcal/mol, in good agreement with the activation energy of sulfate transport in the native membrane [38]. Obviously, the activation energy of band-3-specific transport is not influenced by the lipid environment. The activation energy of the unspecific sulfate permeability is considerably lower, ranging from 17 to 20 kcal/mol. As an exception, the unspecific permeability in vesicles containing only sphingomyelin has an activation energy as low as 3 kcal/mol.

For further quantification of the activity of the transport protein in different lipid environments, we calculated transport numbers per band 3 protein molecule using the equation given in the Methods. The results are compiled in Fig. 3, which summarizes the influence of phospholipids on band-3-specific transport. For native human erythrocyte membranes one can calculate transport numbers of eight sulfate ions per band 3 per min at 25°C from data given by Schnell [39]. Transport numbers for phosphatidylcholine or phosphatidylethanolamine vesicles amount to about 80% of this value. In vesicles containing 37 to 44 mol% of sphingomyelin or phosphatidylserine, transport numbers are markedly lower and decrease to zero in vesicles consisting only of one of these two phospholipids.

TABLE IV

ACTIVATION ENERGIES OF SULFATE EQUILIBRIUM EXCHANGE

Activation energies of sulfate equilibrium exchange (pH 6.9, 10 mM sulfate) in band 3/lipid vesicles of different phospholipid composition. Mean values (±S.D.) from three different reconstitutions (flux measurements at 10°C, 20°C and 25°C). Abbreviations as in Table I.

Lipids used for reconstitution (mol% in addition to 27 mol% cholesterol)	Activation energy (kcal/mol) for:		
	k_{total}	$k_{specific}$	$k_{unspecific}$
PC (73)	31±2	35±3	19±4
PC/SM (55/18)	29±2	34±1	18±6
PC/SM (37/36)	28±3	34±6	17±3
SM (73)	3		
PC/PE (37/36)	32±2	36±1	21±1
PE (73)	29±1	34±4	22±1
PE/PS (48/25)	31±2	35±3	22±2
PE/PS (29/44)	24	36	17
PS (73)	16±2		16±2

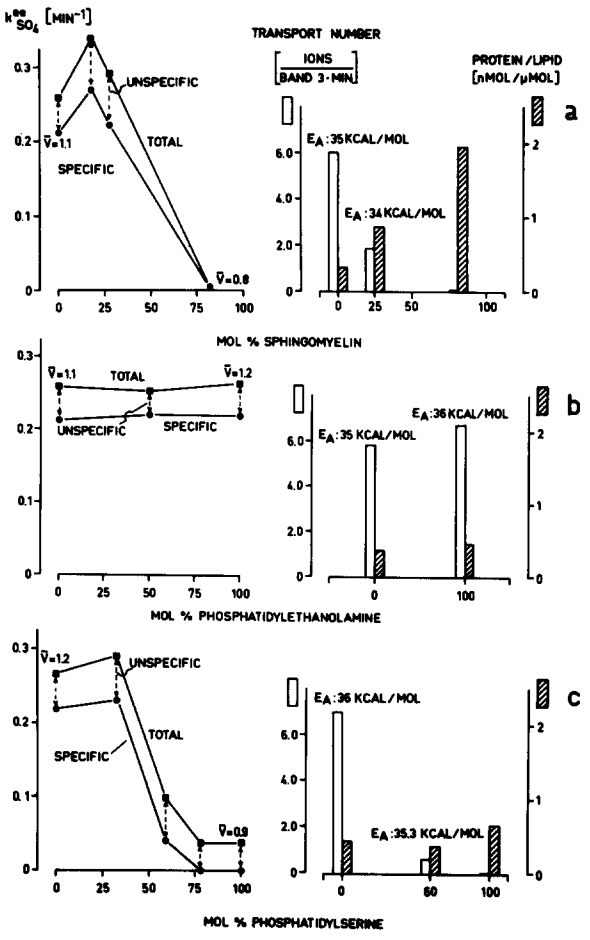

Fig. 3. Transport properties (at 25°C, pH 6.9, 10 mM sulfate) of band 3/lipid vesicles containing phospholipids in varying proportions. The vesicles were prepared from cholesterol and phospholipid at a molar ratio of 1:2.7. The mole fractions of phospholipids given on the abscissa refer to phospholipid analyses (cf. Table II) of the vesicles used for flux measurements. Definitions of total, specific and unspecific fluxes as given in Fig. 2. $\bar{V}$ = specific volume of the vesicles (μl H_2O/μmol phospholipid), transport numbers and protein/lipid ratios determined as described in the Methods. Activation energies for the specific flux (i.e., for the transport numbers) are taken from Table IV. (a) Mixtures of sphingomyelin and phosphatidylcholine; (b) mixtures of phosphatidylethanolamine and phosphatidylcholine; (c) mixtures of phosphatidylserine and phosphatidylethanolamine.

Discussion

Different activities of the anion transport system in erythrocytes from various mammalian species have been interpreted by the hypothesis that the lipid composition of the membranes might affect the transport properties of band 3 protein [27]. This concept was further supported experimentally by variation of the membrane cholesterol content [12] and limited modification of the lipid domain by phospholipases [13,14]. Reconstitution of transport proteins into artificial membranes provides the possibility of varying the lipid environment of transport proteins almost without limitation and to study the consecutive changes in transport. This strategy should therefore be suitable for obtaining further insight into the possible rôle of lipids in the anion transport capacity of band 3 protein.

The general features of our preparation have been outlined elsewhere [25]. In the studies described here, we have used techniques and adduced procedural information previously not available.

(1) The protein content of the vesicle preparation was derived from amino acid analyses, an approach avoiding the shortcomings of the Lowry procedure when used for the determination of proteins in presence of a large excess of lipids.

(2) The residual amounts of the detergent used for solubilization, Triton X-100, were quantified and found to be one order of magnitude below the level inhibiting anion exchange in the native membrane. The lowest membrane concentration affecting band-3-mediated anion exchange in erythrocytes is about 1 μmol/ml cells (Deuticke, B., unpublished data), i.e., 15 molecules of Triton per 100 phospholipid molecules as opposed to 0.8–1.5 per 100 phospholipid molecules in our vesicles.

(3) The quality of the reconstitution was characterized by a 'transport number' derived in a straightforward manner from the flux and the protein content of the vesicles. These numbers revealed a surprisingly high functional survival of band 3 protein during the whole procedure of isolation and reconstitution, as compared to other transport systems, e.g., the glucose transporter of the erythrocyte membrane [40], the adenine nucleotide carrier [41] of the mitochondrial membrane or the acetylcholine receptor from *Torpedo marmorata* [42].

(4) In order to characterize further the transport activity in vesicles of different lipid composition, activation energies for anion fluxes were also calculated from measurements at three temperatures. Unlike rate coefficients, activation energies of transport should not be influenced by uncertainties related to estimations of vesicle volume and amount of protein incorporated (see below).

For the comparative evaluation of the rate coefficients of transport measured in different types of vesicle, additional information was required concerning the geometry and the protein incorporation of the vesicles. Problems concerning the interpretation of these data will be discussed in the following. Specific vesicle volumes, calculated from the trap of the impermeable anion EDTA were of the order of 1 μl H_2O/μmol phospholipid and generally not affected by the lipid composition of the vesicles. Strictly speaking, these numbers describe a ratio of volume to surface area and can be related to a mean vesicle diameter using the approach of Fraley et al. [43]. A mean value of 40 nm obtained for our preparation agrees well with data reported by other authors [44] *.

In contrast to vesicle size, protein incorporation into the vesicle membrane is to some extent dependent on phospholipid composition (Table I). Vesicles prepared from lipid mixtures containing sphingomyelin or phosphatidylserine incorporate more protein than vesicles composed of phosphatidylcholine or phosphatidylethanolamine. As compared to phosphatidylcholine vesicles, containing one molecule of band 3 per 3000 phospholipid molecules, sphingomyelin vesicles contain one molecule of band 3 per 500 phospholipids. This value is still below the band 3/phospholipid ratio of the native erythrocyte (1 : 300) by a factor of

* In principle, determination of the trapped volume of small vesicles by a charged anion such as EDTA might be affected by Donnan exclusion of the anion from the vesicle interior whenever headgroups bearing a net charge are involved. At the ionic strength of the reconstitution medium used (approx. 0.07) (see Methods) such Donnan exclusion, leading to an underestimation of vesicle volume, cannot be discarded for phosphatidylserine (and phosphatidylethanolamine) vesicles, while vesicles prepared from neutral phospholipid should be free of such artifacts.

about 2. The different protein/lipid ratios may, on the one hand, be due to poor phospholipid solubilization, as, for example, in case of sphingomyelin [45], providing only a low amount of lipid for recombination with protein. On the other hand, the different phospholipids might be bound to the Bio-Beads during removal of the detergent to a differing extent. Alternatively, however, differences in the affinity of phospholipids for band 3 might be involved. Such differences in the binding affinity of band 3 protein to different lipids are suggested by studies [46] in which the changes in the surface pressure of monolayers prepared from various lipids were studied after addition of band 3 to the subphase. For glycophorin, another major erythrocyte membrane protein, preferential affinity for anionic phospholipids such as phosphatidylserine has been reported [47].

As becomes evident from our results, band-3-mediated transport is impaired in vesicles containing high concentrations of sphingomyelin or phosphatidylserine (Fig. 3). Possible explanations for these inhibitory effects shall now be discussed. A decreased incorporation of transport protein into the vesicles can probably be discarded in view of our measurements of protein/lipid ratios. Changes in the orientation of the protein in the membranes, however, must be considered. Band 3 protein has been shown to be oriented randomly in reconstituted systems [22,24,25], in contrast to the native membrane, where all stilbene disulfonate binding sites of band 3 are exposed to the external membrane surface [48]. The randomization of binding sites in the vesicles causes a decrease in the fraction of transport sites sensitive to external DNDS. A further change in the orientation of band 3, shifting even more of the DNDS binding sites to the inside of the vesicles, could simulate an inhibition of band-3-specific transport. Although this interpretation cannot completely be discarded, it seems unlikely in view of the fact that in the phosphatidylserine or sphingomyelin vesicles having a lower rate of band-3-specific transport, as defined above, the total rate of transport is decreased by the same amount as the band-3-specific transport. A mere change in the orientation of band 3 should increase the 'unspecific' transport by the amount lost in band-3-specific transport but leave the total transport more or less unaltered.

It seems justified, therefore, to presume a real inactivation of the transport protein in vesicles containing high fractions of sphingomyelin or phosphatidylserine. Several mechanisms can be discussed to explain this inhibition.

Membranes containing high amounts of sphingomyelin are known to be rather rigid due to their long, saturated alkyl chains and a marked tendency to form hydrogen bonds in the interfacial region [49,50]. A rigid structure is probably also characteristic for the polar headgroup region of phosphatidylserine domains [51]. On the other hand, it seems rather unlikely that crystalline domains, which in many cases do not incorporate proteins in artificial lipid membranes (Refs. 52, 53 (also for further references)), are present in our reconstitution systems, since the presence of cholesterol in the vesicles will suppress the formation of crystalline gel phases [54,55]. Moreover, the relative protein contents of the different vesicle preparations was never lower than that in the phosphatidylcholine vesicles used in our earlier [25] studies (cf. Table I). It should also be mentioned that the phosphatidylserine used for our reconstitution is essentially not different in its fatty acid pattern from the phosphatidylethanolamine. Thus, the effect of phosphatidylserine cannot be related to its hydrophobic moiety.

Generalizing, it might be deduced from the inhibitory action of phospholipids with rigid domains that the normal function of the anion transporter band 3 protein requires a certain extent of structural flexibility in its lipid environment. This concept is also supported by the observation [12] that elevation of the cholesterol content in the erythrocyte membrane, which increases rigidity [56] inhibits anion exchange via band 3, while cholesterol depletion, which fluidizes the membrane [56], has the opposite effect. The absence of such effects of cholesterol in reconstituted vesicles, described in an earlier report [25,37], is probably due to the low concentrations of cholesterol (0–27 mol%) used in that study.

Intuitively, one would tend to attribute fluidity-dependent changes in carrier transport function to rate-controlling processes, i.e., to the rate of conformational changes responsible for the reorientation of the substrate-binding site. On the

other hand, fluidity could also affect the native conformation of the protein and thus alter the affinity of its binding site by an 'allosteric' mechanism. Dunker et al. [57] have recently demonstrated an α-helix $\rightleftharpoons$ β-sheet transition in a phage coat protein as a function of membrane lipid composition, which demonstrates the principal possibility of fluidity-dependent conformational transitions. Lipid-dependent changes in antibody-binding affinity of human red cell blood group receptors [58,59] can also be taken as evidence for such mechanisms. The answer to the question whether K_T or V_{max} – or both – of anion transport are affected by lipid fluidity will have to await detailed studies on the transport kinetics of the reconstituted system, which have hitherto met limited success at best [23,25].

Besides fluidity, other parameters controlling the interplay of intrinsic proteins and surrounding lipids may be involved in the modulation of protein function by the lipid environment. Thus, differences in the packing properties of the lipids and the tendency to optimize the interactions between membrane-spanning proteins and the surrounding lipids may induce [60] conformational changes of band 3 protein responsible for the inhibitory influence of sphingomyelin or phosphatidylserine.

In the case of phosphatidylserine vesicles, a high density of negative surface changes could also play a role in the suppression of anion transport. These negative charges could prevent the access of anions to the membrane interface, thereby reducing the apparent substrate affinity of the system. The relevance of this parameter has been clearly demonstrated in mitochondrial membrane enzyme systems [61] and may also play a role for the Ca^{2+}-ATPase of the sarcoplasmic reticulum [19].

It might be tempting to draw conclusions on the mechanism of the inactivation of transport by sphingomyelin or phosphatidylserine from the fact that the activation energy of band-3-specific transport remains unaltered as long as any transport can be measured. This could indicate an all-or-none mechanism of inactivation permitting either fully active or totally inactive transport proteins. The reduced transport numbers in systems of intermediate lipid composition would be a composite in this interpretation arising from a mixture of fully active and totally inactive transport proteins. This interpretation implicitly invokes a lateral domain structure in intermediate systems. The extent to which such lateral domains occur is a matter of present debate [55,62]. Alternative interpretations of the constant activation energy, however, must certainly be considered, since it has been shown that alterations of the transport function of band 3, for example by changes in pH [38], cholesterol content (Deuticke, unpublished results) or by inhibitors [63,64] are without effect on the activation energy.

The unspecific component of sulfate fluxes (insensitive to DNDS from the outside) also deserves comment. This fraction contributes between 20 and 50% of the total flux in different vesicle preparations. By definition it comprises mediated transport via band 3 molecules oriented with their binding site to the vesicle interior as well as simple diffusion via undefined leak pathways. The ratio of the contributions of these two components is about 1 : 1 in phosphatidylcholine/cholesterol vesicles [37], but as yet unknown for vesicles of other phospholipids. The activation energy of the unspecific flux reflects the composite nature of this fraction. It is considerably lower than the activation energy of band-3-mediated transport, but higher than values for anion permeability of pure lipid membrane systems [65]. A very low activation energy for the unspecific leak flux in sphingomyelin vesicles suggests a low-resistance (aqueous?) pathway in this system. In the other vesicles, the non-mediated leak pathways may be represented by the contact areas between lipids and proteins. The mere incorporation of peptides has been shown to enhance the permeability of vesicle systems [66,67].

The results of our study clearly suggest that the function of band 3 protein is not compatible with the exclusive presence of either of two phospholipids, phosphatidylserine or sphingomyelin in its environment. The evaluation of this finding in terms of the lipid composition of the erythrocyte membrane has to take into consideration the asymmetric distribution of phospholipids between the two layers of the membrane, phosphatidylethanolamine and phosphatidylserine facing inward, phosphatidylcholine and sphingomyelin facing outward predominantly [68]. Since the total fractional content of phosphatidylserine amounts to

only about 15% in all mammalian erythrocytes studied so far [69,70], the segments of band 3 protein spanning the inner layer of the erythrocyte membrane are located in an environment containing about 30% phosphatidylserine, while the segments in the outer half are not in contact with this phospholipid. It is interesting that a mole fraction of phosphatidylserine of 30% is just the limiting amount of this phospholipid tolerated by the anion transport protein without impairment of its function (see Fig. 3c). In case of sphingomyelin the opposite situation prevails. The segments of band 3 spanning the outer leaflet of the bilayer are in contact with a phase containing between 20 and 100 mol% sphingomyelin, depending on the animal species [68–70]. The transport rate of anions in various mammalian species decreases with increasing sphingomyelin content [27]. This relationship is in qualitative agreement with the findings on the reconstituted vesicles. However, the transport rate in erythrocytes diminishes only by a factor of 6 between 20 mol% (rat, guinea pig) and 100 mol% (sheep) sphingomyelin in the outer lipid layer [27], while a complete blockage occurs in the vesicles. This difference may result from the asymmetric distribution of sphingomyelin in the erythrocyte membrane, i.e., its absence from the inner leaflet. The asymmetry probably guarantees some extent of conformational mobility for the membrane-spanning peptide segments of band 3 protein likely to be involved in anion transport [71–74]. Evidence that remarkable conformational changes are involved in, and are probably required for, the function of band 3 also comes from a number of other investigations, including inhibitor binding studies and physical probing of protein conformation [73–77]. A marked tendency of band 3 protein to undergo conformation changes quite generally also follows from the considerable sensitivity of its function to various covalently and non-covalently bound ligands acting as inhibitors or activators [70,74,78–82]. Taken together, the available evidence thus suggests that the anion exchange system may belong to a class of intrinsic membrane proteins of a high conformational flexibility, which also accounts for its sensitivity to membrane lipids.

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