Biochimica et Biophysica Acta, 664 (1981) 108–120
Elsevier/North-Holland Biomedical Press

BBA 79256

MEDIATED TRANSPORT OF ANIONS IN BAND 3-PHOSPHOLIPID VESICLES

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(Received November 25th, 1980)

Key words: Reconstitution; Anion transfer; Band 3 protein; Phospholipid; Mediated transport; (Human erythrocyte membrane)

Band 3 protein, extracted from human erythrocyte membranes by Triton X-100, was recombined with egg lecithin/cholesterol mixtures to form small unilamellar vesicles at a yield of 15–20%. These systems exhibited sulfate fluxes which were inhibitable by stilbene disulfonates and other inhibitors. Maximal inhibition could only be obtained when inhibitors were present at both membrane surfaces. Inhibitor constants I_{50} were higher than in the native membrane. Quantitatively, transport function was retained at least 60%, as related to the amount of protein involved. Sulfate transport in the recombinates resembled transport in the native membrane with respect to temperature dependence (E_a = 29–32 kcal/mol), pH dependence between pH 6.5 and 7.8, and the relationship between net and exchange fluxes. In contrast to the native cell, concentration dependence was linear up to 80 mM sulfate, which may be indicative of a lowered affinity for the substrate. Lactate transport in these systems, although substantial, was insensitive to stilbene disulfonates as well as to mercurials, indicating that band 3 is not involved in the specific monocarboxylate transfer in the erythrocyte. Anion transport in band 3-lipid recombinates was insensitive to cholesterol between 0 and 27 mol%. Treatment with proteases, while not affecting transport per se, abolished sensitivity to stilbene disulfonate inhibitors. These observations indicate a number of disturbances of band 3 after recombination, in spite of a preservation of the major transport properties.

Introduction

Of the methods used for the identification of membrane transport systems and their functional analysis, the reincorporation of purified transport proteins into artificial lipid membranes can be regarded as potentially one of the best. Considerable technical problems, however, have restricted the application of this method to the analysis of transport processes [1], while enzymatic functions of reincorporated membrane proteins have been studied by numerous investigators [1,2].

Two passive transport systems of the erythrocyte have so far been isolated and inserted into artificial membranes. Lipid vesicles containing the membrane protein fraction of 45 000–55 000 daltons (band 4.5, according to the nomenclature of Steck [3]), which is assumed to contain the hexose carrier system, exhibited stereoselective, inhibitor-sensitive, saturable glucose permeability [4,5]. It has been argued, however, that the transporting protein fraction may not be a constituent of the native membrane but a product of proteolytic cleavage from band 3 (96 000 daltons), the major intrinsic protein fraction of the erythrocyte membrane [6]. This fraction, on the other hand, catalyzes the coupled exchange of anions, particularly Cl^- against HCO_3^-, across the erythrocyte membrane, which is of paramount physiological significance in CO_2 trans-

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Abbreviations: DIDS, 2,2′-diisothiocyano-stilbene-4,4′-disulfonic acid; DNDS, 2,2′-dinitrostilbene-4,4′-disulfonic acid; Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid; PCMBS, p-chloromercuriphenyl sulfonic acid; SDS, sodium dodecyl sulfate; Pipes, piperazine-*N*-*N*′-bis[2-ethane sulfonic acid].

port and acid-base homeostasis [7]. The identity of this system was disclosed by covalent impermeable inhibitors, in particular DIDS, which bind to band 3 with high selectivity [8].

The identity of the anion exchange system was further confirmed by studies in which membrane vesicles, depleted of almost all peptides other than band 3 by 'negative purification', were shown to retain the capacity to transport anions in a pH-dependent, inhibitor-sensitive fashion comparable to that of native erythrocytes [9]. Moreover, band 3 could be isolated, purified and subjected to an analysis of its structure and composition (see Ref. 10 for a review) due to its abundance in the erythrocyte membrane (25% of the total protein content, equivalent to approx. 1.3 mg/ml cells). Incorporation of crude and purified band 3 preparations into phospholipid vesicles induced anion permeability which shared a number of properties with the anion permeability of the native cells [11–14]. The most successful reconstitutions of this type have been reported by Cabantchik et al. [13] and Wolosin [14].

In the following, another successful reconstitution of anion transport in artificial lipid membranes is reported. It was undertaken in the course of attempts to clarify the relationship between the transport of monocarboxylates (e.g. lactate, pyruvate) and band 3 protein. Monocarboxylates have been shown to permeate the erythrocyte membrane by a process differing from the classical anion exchange system in a number of properties, but sharing other features with that system [15,16,17]. In the course of our studies similarities as well as differences between native and reconstituted anion fluxes could be confirmed and substantiated by further observations.

Materials and Methods

Materials

Human blood was obtained from the local blood bank and used on the same day. Cholesterol (No. 24622) was from Merck, Darmstadt, L-α-phosphatidylcholine (egg yolk, Type V-E, No. 5763) from Sigma, München. Trypsin (33 U/mg) and α-chymotrypsin (A4, 90 U/mg) were purchased from Boehringer, Mannheim; Triton X-100 (analytical grade) from Merck, Darmstadt; *p*-chloromercuriphenyl sulfonic acid (PCMBS) from Sigma, München.

2,2′-Dinitrostilbene-4,4′-disulfonate (DNDS) was from K and K Chemicals, tetrathionate from Fluka (Neu-Ulm) and phloretin from Roth, Karlsruhe. 2,2′-Diisothiocyanostilbene-4,4′-disulfonate (DIDS) was a kind gift from Dr. J.O. Wieth, Copenhagen. Standard chemicals used were of reagent grade.

$Na_2{}^{35}SO_4$ and L-[U-^{14}C]lactate were obtained from Amersham Buchler, Braunschweig, *N*-[*ethyl*-2-^{3}H]ethylmaleimide and [1-^{14}C]dipalmitoylphosphatidylcholine from New England Nuclear, Dreieich, F.R.G.

Methods

Band 3 protein was purified from human erythrocyte membranes and incorporated into lipid vesicles essentially according to procedures described by Wolosin [14] and Cabantchik (personal communication). White ghosts were prepared by hypotonic lysis [18] and stored frozen at −20°C for up to 3 weeks. For preparing band 3 protein, 1 vol. of packed membranes (4 mg protein/ml) was extracted at 4°C with 5 vol. phosphate-buffered saline (20 mM Na_2HPO_4/NaH_2PO_4/147 mM NaCl/0.2 mM NaN_3, pH 8) for 20 min. After centrifugation (10 min, 35 000 × *g*, 4°C) the sediment, depleted of band 6 protein (glyceraldehyde phosphate dehydrogenase) was extracted twice with 6 vols. of 0.05% Triton X-100 in 1 : 5 diluted phosphate-buffered saline (15 min, 4°C) in order to remove glycophorin. The sediment obtained after another centrifugation was washed once in 1 : 5 diluted phosphate-buffered saline, and extracted with 5 vols. of 0.5% Triton X-100. The extract was centrifuged (4°C) for 20 min at 100 000 × *g* in a Beckmann Ultracentrifuge (Rotor SW 56), and the supernatant, containing about 1 mg protein/ml, immediately frozen in liquid N_2.

Incorporation of band 3 into lipid vesicles. L-α-Phosphatidylcholine and cholesterol were dissolved in chloroform, usually at a molar ratio of 3 : 1 (=25 mol% cholesterol) and spread as a thin film in a round-bottom flask by evaporation. The lipids were then taken up in a medium comprising 100 mM D-mannitol/15 mM Na_2SO_4/1 mM EDTA/0.5 mM NaN_3/0.1 mM dithioerythritol/0.5% Triton X-100.

After addition of band 3 protein (0.9 mg, dissolved in 4 ml 1 : 5 diluted phosphate-buffered saline per 20 mg lipid dispersed in 8 ml medium) and the raising of the dithioerythritol concentration to 6.5 mM, Triton X-100 was removed from the suspension by three consecutive 8 h treatments (at 4°C) with Biobeads SM 2 (Bio-Rad, München) pretreated according to methods described in Ref. 19. The total amount of Biobeads was usually 3.6 g wet weight per 12 ml suspension.

The lipid-protein vesicles formed by this procedure were freed from dense lipid-protein aggregates by low speed centrifugation (1750 × *g*, 4°C, 20 min), and concentrated by high speed centrifugation (200 000 × *g*, 4°C, 90 min) (Rotor SW 56). After careful removal of the supernatant, the vesicle pellet was resuspended in a medium comprising 100 mM D-mannitol/10 mM Na_2HPO_4/NaH_2PO_4/10 mM Na_2SO_4/0.2 mM NaN_3, pH 6.8 (medium F).

Preparation of double-labelled protein-lipid vesicles. In order to determine the protein-lipid ration of the vesicles used for flux measurements ^{3}H-labelled band 3 was reconstituted with ^{14}C-labelled phospholipid. Briefly, isolated membranes (40 mg protein in 10 ml solution) were incubated with 3 μmol *N*-[*ethyl*-2-^{3}H]ethylmaleimide (specific activity 25 mCi/mmol) at 37°C, pH 8.0, for 30 min. The membranes were pelleted by centrifugation and resuspended in phosphate-buffered saline. Subsequently, band 3 was purified as described above and mixed for vesicle formation with [^{14}C]phosphatidylcholine (specific activity 1.4 μCi/mmol P_i) and cholesterol (3 : 1, mol/mol). The double-labelled vesicles were passed over a Biogel A 15 column (70 × 1 cm) by elution with a buffer comprising 10 mM Hepes/25 mM Na_2SO_4/0.5 mM NaN_3. Fractions of 800 μl were collected and analyzed for ^{14}C- and ^{3}H-content, using an appropriate evaluation procedure for double-labelled samples.

Flux measurements. The transport properties of the band 3-lipid vesicles were routinely studied by measuring tracer efflux from preloaded vesicles under self-exchange conditions. 400 μl vesicle suspension containing about 2 μmol phospholipid were mixed with 50 μCi $Na_2{}^{35}SO_4$ in 50 μl 10 mM Na_2SO_4 and incubated for 24 h at 4°C. Subsequently, extravesicular radioactivity was removed by gel filtration (6 min) through a Biogel P 4 column (15 × 1 cm) at 4°C using medium F as elution fluid unless otherwise indicated. The fraction of the eluate containing the vesicles was mixed with 22 vols. medium F in order to initiate tracer efflux. 900 μl aliquots of this vesicle suspension were removed after various time intervals and the extracellular medium separated by filtration through cellulose acetate filters (pore size 0.2 μm, diameter 2.5 cm, Sartorius, Göttingen). 500 μl of the filtrate were used for determination of radioactivity by liquid scintillation counting.

For measurements of L-lactate fluxes, vesicles were suspended in media containing 5 mM Na-L-lactate instead of sulfate, and loaded with 10 μCi L-[^{14}C]-lactate. Tracer efflux were measured at 0°C, pH 7.0.

By appropriate evaluation of the time-dependent increase in radioactivity, apparent first-order rate coefficients of tracer efflux (min^{-1}) were obtained. In some instances, fluxes were calculated by multiplying rate coefficients by the sulfate concentration in the vesicles.

In experiments with inhibitors, efflux was initiated as usual. After taking the first sample, the suspension was divided into two parts. Inhibitor was added to one part, the other one served as control. By this procedure statistical errors could be reduced.

Analytical routine procedures. Protein contents were determined by the Lowry procedure as modified by Bensadoun [20], using bovine serum albumin as a standard. Lipid phosphorus contents in vesicle suspensions (Hepes replaced phosphate buffer in such experiments) was quantified according to methods described in Ref. 21. SDS gel electrophoretic patterns of proteins were obtained as described previously [22].

Results

Recombination of band 3 protein with lipids. Band 3 protein, the purported anion exchange system of the erythrocyte membrane, can be purified and solubilized by consecutive treatments of isolated membranes with increasing concentrations of the nonionic detergent, Triton X-100 [13,14,23]. Using this procedure we were able to obtain band 3 preparations containing at least 80–85% of the protein, as substantiated by gel electrophoretic analyses. A

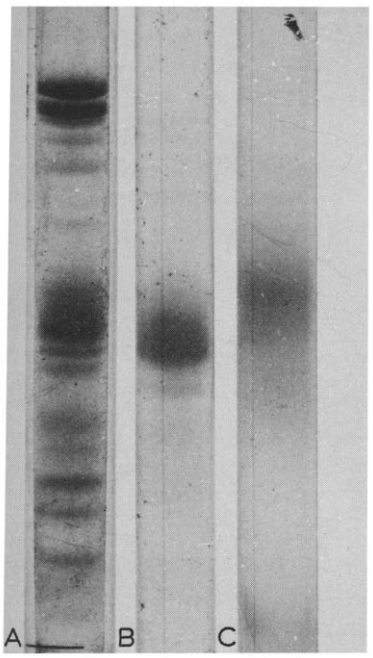

Fig. 1. SDS-gel electrophoresis of (A) native erythrocyte membranes prepared according to Ref. 18 and solubilized by 2% SDS, (B) protein material (in 2% SDS) obtained by extracting membranes with 0.5% Triton X-100 after preceeding treatment with 0.05% Triton X-100 in 1 : 5 diluted PBS buffer, (C) protein material in recombined band 3-lipid vesicles after solubilization by 2% SDS. Gels (5% acrylamide, 0.25% bisacrylamide, 1% SDS) stained with Coomassie Blue.

representative example is given in Fig. 1. Contaminating peptide fractions were mainly observed in the regions of band 4 and sometimes band 7. Periodic acid-Schiff reagent positive glycoproteins were usually absent from the preparations, except for traces of fraction PAS II (nomenclature as in Ref. 3).

Purified band 3 protein was recombined with a mixture of pure egg yolk phosphatidylcholine and cholesterol (3 : 1, mol/mol) at an initial ratio of 1 mg protein per 22 mg lipids as described in the Methods. Since the protein/lipid ratio of the original recombination does not necessarily reflect the protein/lipid ratio in the vesicles finally used for transport studies, relative losses of lipid and protein during vesicle preparations were followed using band 3 protein labelled by *N*-[^{3}H]ethylmaleimide (3.8 mol per mol of Band 3) and ^{14}C-labelled phospholipid. Band 3 protein could be prepared from *N*-[^{3}H]-ethylmaleimide-labelled erythrocyte membranes with approximately the same yield as from unmodified membranes.

As becomes evident from Table I, the preparation of vesicles involves a loss of about 75–85% of the lipid and the protein material originally suspended. This loss affects phospholipid and protein to almost the same extent.

The pellet collected by the ultracentrifugation step consists of phospholipid (and presumably cholesterol) as well as protein, firmly associated with each other, as indicated by our finding (Fig. 2) that lipid and protein elute with the same pattern from Biogel A 15 columns. Peaks containing only one of the two components were not detectable. The protein migrates to the position of band 3 on SDS gels (Fig. 1C), although the band has somewhat more diffuse edges than normal. The presence of unusually high amounts of lipid may be responsible

TABLE I

LOSS OF MATERIAL DURING PREPARATION OF BAND 3 LIPID VESICLES

Vesicles were formed from band 3 labelled with *N*-[*ethyl*-2-^{3}H]ethylmaleimide and [^{14}C]phosphatidylcholine as described in the Methods. Samples were taken for radioactivity measurements at the stages indicated in the Table.

	Content of			
	^{3}H-Band 3		^{14}C-Phospholipid	
	cpm × 10^{-5}	%	cpm × 10^{-5}	%
Initial	2.42	100	0.52	100
Supernatant after treatment with Bio-Beads	1.91	79	0.36	70
Sediment after centrifugation at $2 \cdot 10^5 \times g$	0.59	24	0.08	16

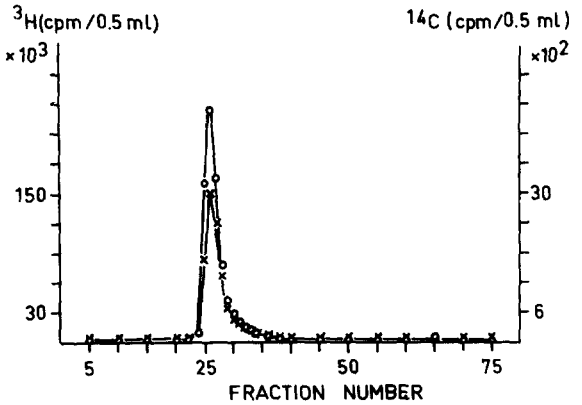

Fig. 2. Co-elution of band 3 labelled by *N*-[^{3}H]ethylmaleimide and ^{14}C-labelled phospholipid. Protein and lipid, labelled and recombined as described in the Methods, were passed over a Bio Gel A 15 column (70 × 1 cm) by elution with 10 mM Hepes/25 mM Na_2SO_4/0.5 mM NaN_3. 800 µl fractions were collected and analyzed.

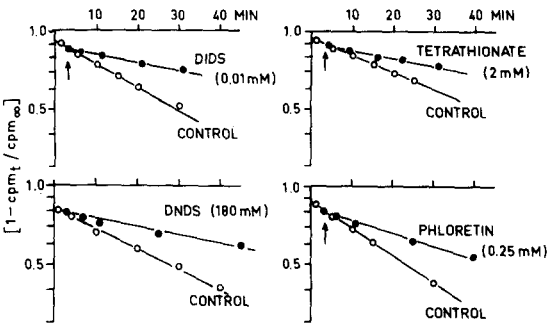

Fig. 4. Inhibitor sensitivity of sulfate fluxes in band 3-lipid vesicles. Vesicles loaded with unlabelled and labelled sulfate were separated from the loading medium by passage over a Bio-Gel P 4 column. Tracer efflux (10°C, pH 6.8) was initiated by suspending an aliquot of the loaded vesicles in medium F (see Methods). Inhibitors were added to one part of the vesicle suspension at the time marked by the arrow. Aliquots of extravesicular medium were sampled for counting by filtration (see Methods).

for this effect. Proteolytic degradation products of band 3 could not be detected. From these observations it appears that band 3 can be recombined with lipids at an approximate yield of 15–20%. The recombinate consists mainly of single-shelled vesicles with diameters ranging from 0.04–0.15 µm as demonstrated by electron microscopy (Fig. 3).

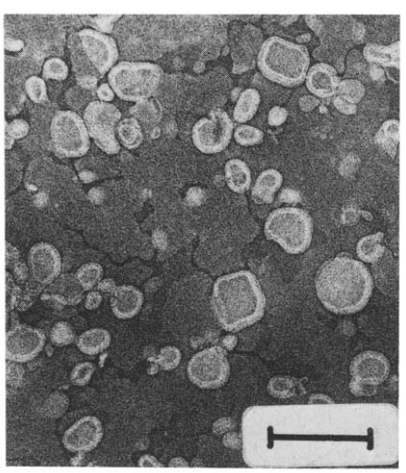

Fig. 3. Morphology of band 3-lipid vesicles. Vesicles prepared as described in the Methods were diluted 1 : 10 in medium F, spread on Teflon grids, and prepared for electron microscopy by negative staining with aurothioglucose (2%, pH 5.0). Grids were inspected in a Siemens Electronmicroscope EM 101 at 100 kV. The bar indicates 0.2 µm.

Transport properties of band 3-lipid recombinates. For measuring the transport properties of band 3 incorporated into lipid vesicles we used efflux techniques. Vesicles were loaded with labelled sulfate after vesicle formation in order to reduce the contribution, in the calculation of rate coefficients, of vesicles containing but not releasing labelled sulfate. When loaded vesicles were resuspended in tracer-free but sulfate-containing medium, the appearance of radioactivity in the filtrate followed first order kinetics (Fig. 4) during the egress of the first 40% of the trapped radioactivity. At 10°C, a mean value (±S.D.) of 37 ± 9 min was obtained for the half-time of sulfate efflux from 22 experiments.

In order to demonstrate the involvement of the anion exchange protein in this efflux, effects of characteristic and to some extent specific inhibitors of anion exchange were studied. Sulfate efflux could be inhibited by about 55–65% by stilbene disulfonates (DIDS, DNDS), by tetrathionate and by phloretin applied externally during the efflux period (Fig. 4). The concentrations used in these experiments are sufficent to produce maximal inhibition of anion exchange when added to suspensions of intact erythrocytes containing the same concentration of band 3 per vol. of solvent ($3 \cdot 10^{13}$ copies

per ml, equivalent to 3 μl packed cells per ml suspension).

Inhibition by the impermeable stilbene disulfonates could be increased to a maximum of about 80% when both the outside and the inside of the vesicle membrane were exposed to inhibitor. To this end, high concentrations of DNDS (150 μM) were introduced into the vesicles during their preparation, and DNDS or DIDS added from the outside during efflux. Thus, at least 80% of the sulfate leaving the vesicles may be assumed to penetrate via band 3 protein.

The failure to obtain maximal inhibition by externally applied stilbene disulfonates, which was also observed by Cabantchik et al. [13], requires comment, since in native erythrocytes maximal inhibition can be achieved from the outside. DNDS present on the cytoplasmic membrane surface has no inhibitory effect up to millimolar concentrations [24]. This pattern indicates an asymmetric arrangement of the DNDS binding site in the erythrocyte membrane. Our findings suggest a more random arrangement of band 3 protein in the vesicle membrane. Assuming equal affinity constants of the DNDS binding sites on both faces of the vesicle membrane and identical transport properties of band 3 independent of its orientation in the vesicles, the inhibition observed in our experiments suggests that 75% of the binding sites are oriented outwards, while 25% face inwards.

The DNDS affinity of the outward facing binding sites is somewhat different from the affinity of DNDS binding sites in the native cell under equivalent conditions, i.e., at low substrate concentration and zero chloride. This can be inferred from the dose

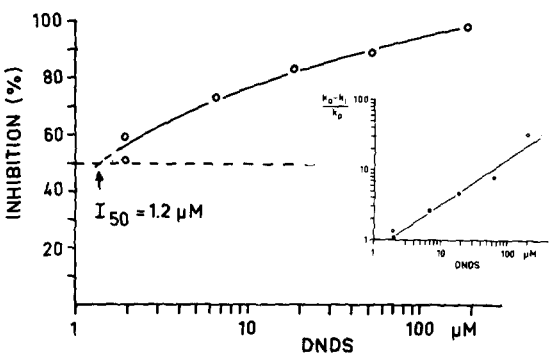

Fig. 5. Dose response curve for the inhibition of sulfate fluxes in band 3 lipid vesicles by a reversibly binding stilbene disulfonate. Fluxes measured as described in Fig. 4 and in the Methods. Inhibition related to the maximum of inhibition (63% of the total flux) obtained by external DNDS. Insert: double logarithmic plot of the dose response curve, rearranged according to methods described in Ref. 43. i = fractional inhibition. In this Hill type plot a slope of 1.0 indicates a 1 : 1 interaction of the inhibitor with the transport system.

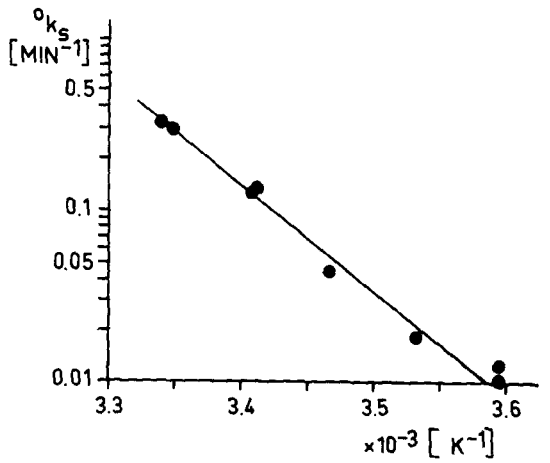

Fig. 6. Temperature dependence of sulfate fluxes in band 3-lipid vesicles, presented as an Arrhenius diagram. From the slope a constant activation energy of 29 kcal/mol is obtained for this vesicle preparation.

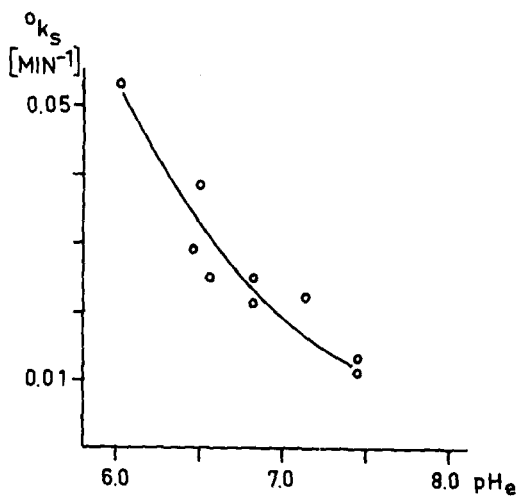

Fig. 7. pH dependence of sulfate fluxes in band 3-lipid vesicles. Vesicles were prepared, as described in the Methods, at pH 6.8. Efflux was measured into media composed as medium F, but having different pH values. 10°C, values from three different experiments.

response curve in Fig. 5. Our I_{50} value of 1.2 μM, obtained at 10°C, has to be compared with the I_{50} value of 0.45 μM obtained at 25°C in native cells (Fig. 8 of Ref. 25) for low sulfate concentrations. The slope of a Hill plot for the inhibition, given in the insert, is only 0.5, while for native cells a value of 1.0 has been reported [25].

Characteristics of anion transport via band 3 in vesicles. Among the characteristic features of sulfate exchange in human erythrocytes, a high activation energy, a bell-shaped pH-dependence curve with a maximum between pH 6.0 and 6.5 and saturation kinetics with self-inhibition at substrate concentrations above 80 mM have attracted particular interest (see Ref. 26 for a review).

Sulfate fluxes via band 3 incorporated into vesicles are also highly temperature dependent (Fig. 6). From all our data, an apparent activation energy of 28–30 kcal/mol can be derived. This value is higher than values reported previously for reconstituted band 3 systems [13,14,27], but closely agrees with the activation energies observed in intact cells (32.8, pH 6.75 [28]). pH dependence (Fig. 7) also closely resembles the pH dependence of sulfate exchange in

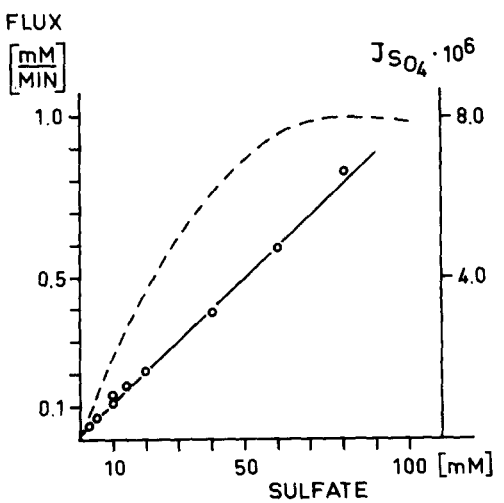

Fig. 8. Concentration dependence of sulfate fluxes in band 3-lipid vesicles. Vesicles were formed in a medium of the usual composition (see Methods) but containing sulfate at the required concentrations. The efflux medium contained the required sulfate concentrations in addition to the other components. 10°C, pH 6.8. Fluxes calculated as described in the Methods. Dotted line: data for ghosts studied under comparable conditions, taken from Fig. 3b of Ref. 29.

resealed ghosts [29]. The flux rates increase by a factor of 3.5 in both systems when pH is lowered from 7.5 to 6.5, although the pH was varied on both sides of the membrane in the case of ghosts, while our vesicles were prepared at a uniform internal pH of 6.8 and exposed to solutions of varying external pH during the efflux measurements.

The concentration dependence of sulfate movements in the band 3-lipid recombinates is quite different from that observed in the native membrane. Sulfate fluxes (Fig. 8) increase linearly with sulfate concentration up to 80 mM while in ghosts the flux/concentration relationship already markedly deviates from linearity in this range of concentration [29]. The linear concentration dependence agrees with results by Cabantchik et al. [13].

The anion transport system of the erythrocyte is specialized on anion exchange. Net movements of anions (accompanied by cations) are slower by orders of magnitude in the case of chloride [30]. In the case of sulfate, net movements have been estimated to proceed at a rate of about 1/10 to 1/5 of the rate of coupled self-exchange [31,32]. In a number of experiments we tried to study the extent of coupling of sulfate transport in band 3-lipid vesicles.

As shown in Fig. 9, sulfate efflux into a medium free of permeable anions * occurs at a rate not significantly different from the rate at sulfate equilibrium. Addition of Cl^- to this anion-free medium does not accelerate sulfate efflux. The net efflux of sulfate is sensitive to DIDS to the same extent as efflux at sulfate equilibrium.

Lactate movements in band 3-lipid vesicles. Lactate and other monocarboxylates permeate the erythrocyte membrane mainly by a transport system differing in its kinetic properties from the inorganic anion exchange system [15–17]. It was, therefore, a major object of our study to investigate whether lactate migrates via band 3 incorporated into lipid vesicles. At pH 7.0 and 0°C and a concentration of L-lactate of 5 mM, a rate coefficient was obtained

* Hepes behaves like a highly impermeable anion in a number of indirect assays (Deuticke, B., unpublished results). Measurements of tracer permeability for this zwitterion are not available.

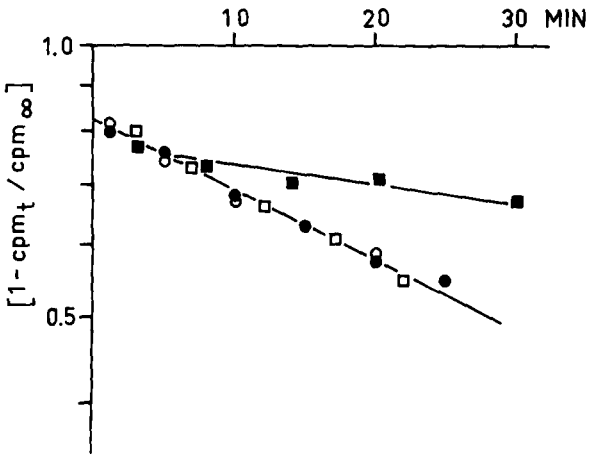

Fig. 9. Comparison of net and exchange fluxes in band 3 lipid recombinates. Vesicles were prepared as usual and separated from the anion-containing loading medium by a passage over a Bio Gel P4 column, equilibrated with an anion-free medium (100 mM mannitol/10 mM Hepes-Na/ 1 mM NaN_3). This medium was also used for net efflux measurements. ○, sulfate exchange; •, sulfate net flux; ▪, sulfate net flux + DIDS 10 μM; □, sulfate efflux into medium containing 10 mM Cl.

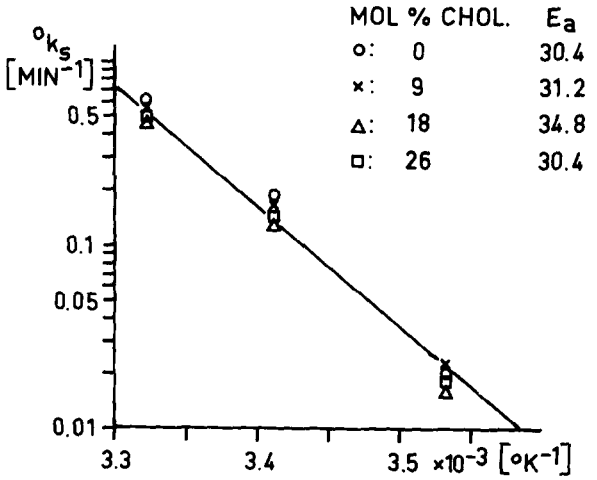

Fig. 10. Lack of influence of cholesterol on the activation energy E_a of sulfate fluxes. Vesicles were formed from phospholipid/cholesterol mixtures of the composition given on the diagram.

of 0.0200 ± 0.0044 min^{-1} (n = 6), to be compared with a value for sulfate at this temperature (extrapolated from Fig. 6) of about 0.0035. This lactate efflux, however, could not be inhibited by DIDS. Moreover, PCMBS, a potent inhibitor of lactate movements via the monocarboxylate transport system, did not inhibit lactate movements.

Influence of lipid composition of vesicles and of enzyme treatment on transport by band 3. Changes in the cholesterol content of the erythrocyte membrane between −50% and +100% of the normal level (cholesterol/phospholipid = 0.7 mol/mol) have been shown to affect anion transport via band 3 [33]. Band 3-lipid recombinates make it possible to study the effects of cholesterol at much lower cholesterol to phospholipid ratios. Band 3 was incorporated into vesicles containing between 0 and 27 mol% cholesterol. Higher mole fractions could not be tested

TABLE II

LOSS OF DIDS-SENSITIVITY OF SULFATE FLUXES IN BAND 3-LIPID VESICLES TREATED WITH PROTEOLYTIC ENZYMES

Vesicles, formed as described in the Methods, were exposed to the enzymes at the indicated concentrations and temperatures for 30 min or 12 h, respectively, after the loading of the vesicles with labelled and unlabelled sulfate, but still in the presence of both. After passage over a Biogel P 4 column, sulfate fluxes in the absence or presence of DIDS were measured as usual in a split sample.

	Rate coefficient of tracer flux (k (min^{-1})(×10^{-2}))					
	No enzyme		Trypsin		Chymotrypsin	
	30 min 10°C	12 h 21°C	180 μg/ml 30 min 10°C	25 μg/ml 12 h 21°C	180 μg/ml 30 min 10°C	25 μg/ml 12 h 21°C
Control	1.39	1.00	1.41	1.04	1.51	1.03
DIDS 10 μM	0.40	0.40	0.40	1.03	0.40	1.03

since vesicles did not reseal to sulfate under such conditions.

The rate coefficient of ^{35}S-sulfate efflux decreased by about 30% when cholesterol was increased from 0 to 27 mol% (Fig. 10). Its temperature dependency, on the other hand, remained constant. The decrease of the rate coefficient is most likely not due to a direct effect of cholesterol on the membrane transport, since it corresponds well to the 30% increase of the internal volume reported for lipid vesicles with increasing cholesterol level [14,34].

In a further attempt to alter the transport properties of the vesicles by biochemical means, band 3-lipid recombinates were treated with proteolytic enzymes. Trypsin and chymotrypsin did not affect anion fluxes (Table II) when the vesicles were exposed to high concentrations for a short time or to low concentrations for an extended time. Under the conditions of our treatment, band 3 in vesicles has been shown to be cleaved by chymotrypsin but not by trypsin [35]. An interesting effect became evident after prolonged exposure of the vesicles to trypsin and chymotrypsin: anion transfer was no longer sensitive to DIDS, while control vesicles incubated for the same time without enzyme were inhibited as normal. Unspecific inactivation of DIDS by binding to residual enzyme protein cannot account for the loss of the effect of DIDS, since in vesicles exposed to much higher concentrations of enzyme for a short time inhibition was unaffected.

Discussion

Re-incorporation of isolated transport proteins into artificial lipid membranes has been shown in a number of cases to produce systems which exhibit properties of the transport system in its native environment [1]. In order to serve as a model of the native system a recombined system should be shown to have retained a maximum of properties of the native system, or the reasons of differences between the recombined and the native system should be clarified. The band 3-lipid recombinate described in our study is characterized by similarities as well as differences in relation to the native anion exchange system of the erythrocyte, which shall be discussed in the following.

Protein/lipid recombination

The protein used for recombination was purified but not free of contaminating peptides. However, since the identification of band 3 as the anion transporter was not the purpose of our investigation, these impurities were thought to be tolerable. By using labelled band 3 protein and labelled phospholipid we could establish the protein to lipid ratio of our vesicles as well as the yield obtainable by the Triton/Biobead technique. The 20% recovery agrees with the data given by others [14,27] for the same or different reconstitution techniques.

A major loss of material occurs because 30–40% of both band 3 protein and lipid cannot be pelleted during the final high speed centrifugation of the recombinate, probably due to the formation of very small vesicles. From the ratio $^{3}H/^{14}C$ in the recombinates a mean molar ratio of protein to phospholipid of 1 : 2000 could be estimated, which corresponds to the ratio in the original mixture of solubilized band 3 and phospholipids, but is only 1/6 of the band 3 to lipid ratio in the native cell. Vesicle sizes correspond to those usually observed under comparable conditions of preparation [14,35]. Only a minor fraction of the vesicles was multilamellar.

Sulfate transport

Inhibitor sensitivity. Band 3-lipid vesicles were capable of anion transport sensitive to stilbene disulfonates as in former studies by other investigators [11,13,14]. Stilbene disulfonates belong to the most potent competitive inhibitors of band 3-mediated anion exchange [26]. In addition we could demonstrate inhibition by two agents, phloretin and tetrathionate, supposed to inhibit non-competitively [36].

All these inhibitors suppress anion exchange in native erythrocytes by at least 98%, when present in the external medium. Anion transport in band 3-lipid vesicles, in contrast, could be diminished only by about 40% by external inhibitor, while at least 80% was reached in the presence of inhibitor on both membrane surfaces, indicating the presence of right-side-out (75%) and inside-out (25%) copies of band 3 in the vesicles. A similar conclusion was reached by Cabantchik et al. [13].

The stilbene disulfonate-insensitive flux of sulfate most probably reflects the unspecific permeability

of the vesicles' lipid domain. The lower maximal inhibitor-sensitivity of the recombinates (80% as compared to more than 98% in native cells) does not necessarily indicate that this unspecific permeability is higher in the vesicles. The difference may in part be artifactual since the band 3/lipid ratio in our recombinates was only 1/6 of that in the native cells. It can be calculated that at a physiological band 3/lipid ratio about 95% of the sulfate exchange in our vesicles would be inhibitor-sensitive.

Dose response curves for DNDS inhibition of the right-side-out oriented fraction of transport sites yield an I_{50} value about 3-times higher than that in the intact cell [25]. Taking into account the different temperatures at which measurements in cells (25°C) and vesicles (10°C) were carried out, the I_{50} value is probably as much as 6-times higher in vesicles than in the native cell [36]. The slope of the dose response curve is flatter than would be expected for a simple 1 : 1 interaction of the inhibitor with a uniform set of sites [25]. A population of sites which is non-uniform with respect to its affinity to the inhibitor, however, would give rise to such flat curves. The inhibitor data therefore may indicate subtle changes in the band 3 protein after recombination, leading to a lower and more heterogeneous affinity for the competitive inhibitor.

Turnover numbers

The comparison of the turnover numbers of band 3-lipid recombinates with those of the native anion exchange system has been used previously to estimate the functional preservation of the recombinate relative to the native system. Wolosin [9,14] has proposed a procedure for this purpose, based on a comparison between the ratio of rate coefficients of tracer fluxes measured at sulfate equilibrium under identical conditions in vesicles and intact cells (or ghosts), on the one hand, and the ratio of the surface area to volume ratios for the two systems, on the other. The tacit assumption underlying this type of calculation is that of a homogeneous distribution of the proteins over the available lipid bilayer surface. Different surface densities of band 3, defined by the band 3/lipid ratio (mg/ml), can also be taken into account.

We have applied this procedure using our rate coefficients for vesicles at 25°C (Fig. 6) and comparing them to rate coefficients for ghosts, derived from measurements of Schnell et al. (Ref. 19, Fig. 3). Moreover, we took into consideration the differing band 3/lipid ratios (0.25 for ghosts [37,38] and 0.045 for our vesicles (see Methods and Tables)) and used an area-to-volume ratio of resealed ghosts of $(1.2–1.6) \cdot 10^4$ cm^{-1}. Depending on the choice of the area-to-volume ratio of the spherical vesicles, as calculated from the measured radii (0.02–0.075 μm), we obtained a function preservation greater than or equal to 65%. The results of such calculations, however, depend critically on the choice of parameters which are not easily measurable with high precision and may, therefore, be subject to some error. Nevertheless, this range of values seems reasonable when it is taken into account that (1) contaminating proteins are present in our preparation and (2) functional disturbances of the recombinates are indicated by the changes of affinity to be discussed below.

Temperature dependence

The activation energy of anion transfer has been reported to be lower in reconstituted band 3-lipid vesicles than in the native system [14,27]. The highest values observed for vesicles were about 22 kcal/mol. The difference from the value for the native erythrocytes, 33 kcal/mol [28], has been attributed to alterations of the proteins or, particularly, to the different lipid environment [14]. In our studies we obtained activation energies which were very close to those for intact cells. Essentially normal activation energies can thus be preserved in band 3-lipid recombinates. Moreover, these activation energies are not affected by changes of the cholesterol contents of vesicles (Fig. 10) over a range known to affect markedly the microviscosity of the lipid domain [39]. The conspicuously high activation energy of anion exchange thus does not seem to arise from frictional resistances related to lipid microviscosity.

pH and concentration dependence

The alkaline branch of the pH-dependence curve of sulfate exchange in erythrocytes is preserved in our preparations as in those of others [13,14]. The quantitative agreement with data obtained for intact cells under rather similar conditions (5 mM SO_4^{2-}/ 0 mM Cl^-, 25°C; Ref. 29, Fig. 7b), indicates preser-

vation of the proton binding site responsible for this alkaline branch [40]. A significant alteration of the transfer system seems at first sight to be indicated by the linear concentration-dependence of sulfate efflux under equilibrium conditions observed between 3 and 100 mM sulfate in our preparation, contrary to the saturation (and self-inhibition) kinetics well established for the native system. The same discrepancy was observed by Cabantchik et al. [13], while Wolosin [14] obtained evidence for saturation between 2.5 and 100 mM sulfate.

A true loss of saturability in our preparation seems unlikely since the persistence of a limited number of transfer sites is indicated by the persistent sensitivity of the transfer to competitive inhibitors. As an alternative, a decrease of the affinity of the transfer site for substrate and inhibitors might be envisaged, also indicated by the lower affinity of the recombined system for the competitive inhibitor DNDS (see above). Studies extended to higher concentrations of sulfate will be required to clarify this issue.

Comparison of net and exchange flux

[^{35}S]Sulfate net efflux from band 3-lipid vesicles into media free of permeable anions, while sensitive to stilbene disulfonates, proved to proceed at the same rate as sulfate exchange at equilibrium or sulfate efflux into chloride-containing solutions (Fig. 9). In this respect our recombined system differs from band 3-lipid vesicles prepared by 'negative purification' [9]. In such vesicles net sulfate movements were negligible unless cation permeability was enhanced by ionophores.

DIDS-sensitive anion efflux into anion-free media may be assumed to occur by anion conductance. In native erythrocytes sulfate conductance has been reported to contribute about 20–30% to the total sulfate flux under conditions where anions are present on both sides of the membrane [31,32]. In band 3 vesicles obtained by negative purification, on the other hand, sulfate net flux, in the presence of valinomycin, was of the same order of magnitude as sulfate self-exchange [9].

In our studies, we observed equal rates of net flux, into anion free media, and exchange flux in the absence of any ionophore in vesicles containing sodium sulfate. For reasons of electroneutrality it must be postulated that sodium ions leaked out of the vesicles together with sulfate under net flux conditions. It could not be decided unequivocally, on the basis of the data available on sulfate conductance, whether the quantitative agreement between net and exchange flux indicates an alteration of band 3 or reflects a normal mode of operation.

In an attempt to clarify this problem, the experimental conditions of our net flux experiments on band 3-lipid vesicles were applied to native cells by replacing all intracellular chloride by sulfate and then measuring [^{35}S]sulfate efflux into media containing only mannitol and an impermeable buffer (Pipes/Na salt). According to our results, sulfate leaves the cells under these conditions by a fully DIDS-inhibitable process at a rate not different from that of sulfate self-exchange measured under the same conditions. This sulfate net flux was paralleled by cation leakage in spite of the absence of an ionophore, was not affected by addition of chloride, and could only be slightly (2-fold) stimulated by ionophores. The reason for the increase of cation permeability is obscure as yet. Equally high rates of net and exchange flux of sulfate can thus be observed in native cells. The quantitative agreement, however, is probably fortuitous and results from the actual value of cation permeability. This follows from our observation that after a further increase of cation permeability by an ionophore the rate of sulfate net efflux exceeds that of equilibrium exchange. Our findings on native cells, which are corroborated by observations on sulfate fluxes in valinomycin-treated ghosts (Ref. 41 and Gerhardt, S. and Schnell, K.F., personal communication) and on vesicles prepared by negative purification [9], thus demonstrate that under suitable conditions (external medium free of permeable anions, elevated cation permeability) band 3 in its native environment mediates sulfate net fluxes as high as exchange fluxes. The lack of trans-effects in recombined vesicles therefore does not necessarily indicate an alteration of band 3.

Influence of proteolytic enzymes

In spite of the fact that many properties of the transport system in band 3 are retained after recombination, the protein is certainly no longer in its native orientation with respect to the hydrophobic

barrier of the membrane. This clearly emerges from our finding that trypsin and chymotrypsin abolish the sensitivity of transport to disulfonate inhibitors while not affecting transport per se. This pattern of reaction poses problems for interpretation, since the stilbene disulfonates are considered to act competitively. One possible explanation is that proteolysis, while abolishing band 3-mediated transport, produces unspecific DIDS-insensitive pathways for sulfate in the vesicles. The similarity of the fluxes under control conditions in proteolyzed and nonproteolyzed vesicles would then result from a fortuitous compensation of the two processes. Data suggesting the possibility of such a mechanism have been obtained in native cells extensively treated with pronase [42]. Studies of the temperature-dependence of sulfate flux in proteolyzed vesicles will be required to check this explanation. Alternatively, the findings could be rationalized by the assumption that enzyme treatment, while not disturbing the substrate binding site, perturbs the arrangement of adjacent sites required for the binding of stilbene disulfonates [43]. These groups are localized on two domains of the native band 3 (17 kdalton and 35 kdalton), which can be cleaved by chymotrypsin (but not trypsin) without changing their position relative to each other [44]. It may be speculated that either this close association between the 17 kdalton and the 35 kdalton fragment after proteolysis is lost in the vesicles or that additional sites for enzymatic cleavage are exposed. Both hypotheses are, however, difficult to reconcile with the observation [35] that trypsin produces no detectable cleavage of band 3 in lipid vesicles, while chymotrypsin acts as in intact cells.

In summary, our study thus demonstrates that numerous basic properties of the anion transport system of band 3 endure an isolation of the peptide and its recombination with lipid. Detailed analysis, however, clearly reveals changes in the transport properties indicative of minor alterations of the protein's conformation.

Lactate transport via band 3?

The rate of lactate exchange across the band 3-lipid vesicle membranes, although rather substantial, was insensitive to PCMBS. This finding provides further evidence that a protein component of the erythrocyte membrane other than band 3 is responsible for the saturable, SH-dependent transport of monocarboxylates. DIDS and DNDS did not inhibit lactate movements, although in native cells about 4% of the total flux of lactate occurs via band 3 [17]. This process should be detectable in band 3-lipid vesicles, since the PCMBS-sensitive lactate transfer system, which accounts for about 92% of the transfer in native cells, is no longer present.

DIDS-sensitive lactate transport should also be detectable in view of the following quantitative considerations: in native cells the ratio k_{Lac}/k_{SO_4} for transfer via band 3 amounts to 2 (unpublished results). In the band 3 vesicles we would, therefore, predict, at 0°C, a k value for DIDS-sensitive lactate exchange of 0.0042 min^{-1}, derived from the k_{SO_4} value of 0.0035 and a maximum of 60% inhibition by external stilbene disulfonates. Lactate exchange has a total k value of 0.0200 min^{-1} at 0°C. The maximal expectable decrease to 0.0160, i.e., by 20%, in the presence of stilbene disulfonates, should be observable. Its absence remains to be explained. The total lactate flux occurring in our vesicles must be assumed to represent nonionic diffusion via the lipid phase.

Acknowledgements

We are indebted to Dr. W. Baumeister, Institut für Biophysik und Elektronenmikroskopie, Universität Düsseldorf, for preparing the electron micrograph and to G. Kirschbaum and K. Schwister for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungbereich 160/C3. The invaluable help of Mrs. H. Thomas and Mr. F.-J. Kaiser in preparing the manuscript and the graphs is gratefully acknowledged.

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