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FORMATION AND PROPERTIES OF TETRAMERS OF BAND 3 PROTEIN FROM HUMAN ERYTHROCYTE MEMBRANES IN PLANAR LIPID BILAYERS

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Lipid bilayer experiments were performed in the presence of solubilized band 3 protein from human red cell membranes. Band 3 protein increased the conductance of the lipid membranes by several orders of magnitude. Membrane conductance was found to be dependent on the fourth power of protein concentration. This shows that four band 3 subunits form an ion permeable pathway in the lipid bilayer membranes. It also shows that, in the membranes, the protein molecules undergo an association equilibrium which involves at least the monomer and the tetramer of the protein, relaxation towards equilibrium being rapid on the time scale of the experiment. The increase in bilayer conductance induced by the band 3 tetramer could be inhibited by pretreatment of the protein with several SH-reagents (pCMB, pCMBS, DTNB) which also inhibit water transport across the human red cell membrane. Other SH-reagents which do not influence water transport (iodoacetamide, *N*-ethylmaleimide) did not show any influence on the band 3 induced conductance increase. A band 3-mediated exchange of anions comparable to that in the erythrocyte membrane did not occur in the system studied by us. Our results suggest that, in the human erythrocyte membrane, a pore formed by the band 3 tetramer could be the pathway responsible for the protein-mediated part of water transport.

Introduction

Work in many laboratories over the past decade has established that the rapid anion exchange across the human red cell membrane is mediated by a 97 kDa polypeptide present in the erythrocyte membrane (for reviews, see Refs. 1 and 2). This polypeptide is the major component of the red blood cell membrane and has been identified in dodecylsulfate gel electrophoresis as 'band 3' [3]. It has also been suggested that this anion exchange system is involved in net anion transport [4–7] and that it may play some role in the transport of non-electrolytes, and, especially, in water flow across the membrane [8–11]. In addition, some authors have claimed that a subpopulation of the band 3 protein transports glucose [12,13]; however, this possibility seems to be definitely ruled out by recent findings [14].

For several years band 3 protein has been regarded as being exclusively a stable noncovalent dimer both in the erythrocyte membrane and after

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2-disulfonic acid; pCMB, *p*-chloromercuribenzoic acid; pCMBS, *p*-chloromercuribenzene sulfonic acid.

solubilization by nonionic detergents [15] (despite an early claim that it is a stable tetramer [16]). It has often been assumed that this dimer is the functional unit of anion as well as of water transport and that transport occurs along the interface between the subunits of the dimer [11,17,18]. However, more recent data suggest that the functional unit of anion transport may be the protometer of band 3 [19,20], transport occurring via a 'gate' located between the two integral subdomains of the polypeptide chain [21]. On the other hand, recent electronmicroscopic studies have suggested that, in the erythrocyte membrane, band 3 may partly or even predominantly exist in the form of tetramers [22–24]. Furthermore, it was demonstrated that band 3 protein isolated either in acetic acid or in nonionic detergents is in a monomer/dimer/tetramer association equilibrium (the stable dimers of the protein observed in earlier studies being artifacts), and it was suggested that band 3 would show the same association behaviour in the intact erythrocyte membrane [25–27]. The band 3 tetramer has thus become an object of obvious interest. Detailed studies on its properties have, however, not yet been performed.

As shown in earlier studies, mixtures of band 3 protein and the sialoglycoproteins of the erythrocyte membrane, which were isolated in acetic acid, induce large changes in the electrical conductance of planar lipid bilayers [28–30]. It was also found that, under some conditions, membrane conductance increases with the fourth power of the concentration of added protein, and it was suggested that this effect is due to the (reversible) formation of band 3 tetramers from monomers of the protein and that the tetramers act as a conducting unit [29,30]. In the present paper these findings have been confirmed and extended by similar experiments on purified band 3 protein. As the main aspect, however, the protein-induced increase in membrane conductance has been used as a probe for the formation and some properties of the band 3 tetramer.

Materials and Methods

Protein preparation. Band 3 protein was isolated from human erythrocyte membranes by zonal electrophoresis in acetic acid/water/sucrose mixtures [31]. The removal of the organic solvent was done as described in Ref. 25. The protein was stored in 1 mM HCl/0.1 mM NaCl/0.1 mM ethylenediaminetetraacetic acid, at a concentration of approx. 200 μg/ml. Before being injected into the measuring cell or being diluted with buffer of pH 7, it was titrated to pH 10.0.

Other materials. Oxidized cholesterol was prepared from cholesterol (Eastman, Rochester, NY, U.S.A.) according to Ref. 32. Egg phosphatidylcholine was either prepared according to Ref. 33 or purchased from Avanti Biochemicals (Birmingham, AL, U.S.A.). Phosphatidylethanolamine was obtained from Supelco (Bellafonte, PA, U.S.A.), phosphatidylserine from Avanti Biochemicals and glycerolmonooleate from Sigma (St. Louis, MO, U.S.A.). *n*-Decane-was from Fluka (Buchs, Switzerland ('purum')). *n*-Pentane and squalene were obtained from Eastman and pCMBS and pCMB from Sigma. Ammonyx-LO was from Onyx Chemical Co. (Jersey City, NY, U.S.A.), and ^{36}Cl from New England Nuclear (Boston, MA, U.S.A.). H_2DIDS was a kind gift of Professor H. Passow. All other reagents were from Merck (Darmstadt, F.R.G.) and were of analytical grade.

Formation of artificial bilayer membranes. Artificial lipid bilayer membranes were obtained by two different methods. Solvent containing membranes were formed from 1% (w/v) solutions of oxidized cholesterol or of egg phosphatidylcholine in *n*-decane. Solvent-free membranes ('folded' bilayers) were formed from monolayers spread on the air-water interface from solutions of different lipids in *n*-pentane using the technique first described by Montal and Mueller [34]. The aperture in the Teflon partition separating the two aqueous compartments was pretreated with a 2% solution of squalene in *n*-pentane. The membrane cells were made from Teflon. The circular hole between the two aqueous compartments had either an area of 1.5 mm^2 (in the case of solvent-containing membranes) or was varied from 10^{-2} to 2 mm^2 (for the folded membranes used for single-channel and flux measurements).

Electrical measurements. For the electrical measurements, Ag/AgCl or calomel electrodes with salt bridges were inserted into the aqueous compartments on both sides of the membrane. The conductance and zero-current potential measure-

ments were performed with a Keithley 610 C electrometer. The current in the single-channel experiments was amplified using a current amplifier and was monitored in a storage oscilloscope and recorded in a stripchart or a tape recorder. In both the conductance and the single-channel experiments the protein was either added to one or both sides of preformed bilayers, or the membranes were formed in the presence of the protein. Zero-current membrane potentials were measured by a slight modification of an earlier described method [35]: Solvent-containing bilayer membranes were formed in a 10^{-2} M salt solution. After the membranes had turned completely black, the band 3 protein was added in a final concentration around 5 μg/ml while stirring. The membrane conductance reached a quasi-stationary level within about 20 min. Then the salt concentration on one side of the membrane was raised by the addition of small amounts of concentrated salt solutions. After about 10 min the zero-current membrane potential reached a steady-state value.

Chloride fluxes. The unidirectional flux of chloride was measured with ^{36}Cl essentially as described previously [36]. In brief, solvent-free bilayers were formed, and after bilayer formation, one of the chambers was perfused with 5 ml of the initial solution and the perfusate used to determine background. After this, an aliquot of the radioactive solution was added to one chamber (*cis*) either before or after interaction with band 3 and a small portion (50 μl) of the *cis* solution was removed to determine the specific activity present in this chamber (the volume of the chambers was either 1.5 or 3.0 ml). Samples from the *trans* compartment were then obtained at different time intervals by perfusion of this compartment. At the end of the washout, no radioactivity could be detected in the *trans* chamber. The samples thus collected were then counted in a liquid scintillation counter (Packard Tri-Carb, Packard Instrument Co., Downers Grove, IL, U.S.A.) after addition of liquid scintillation cocktail.

Other methods. Analytical ultracentrifugation was performed as described earlier [25,26], as was the procedure used in the lipid monolayer experiments [37,38].

Results

Band 3-induced conductance changes and the structure of the conducting unit

Fig. 1 shows that addition of band 3 protein promotes the increase of the bilayer conductance by several orders of magnitude. This effect is qualitatively that described earlier, using impure preparations of band 3 (approx. 80% band 3 as compared to > 95% in this paper) [29,30]. In the present study, however, it was found that the conductance values obtained at a fixed band 3 concentration were larger than in the earlier studies. In order to try to resolve the origin of these quantitative differences, we compared the conductance induced by impure and purified band 3 when the organic solvent in all cases was removed according to Ref. 25 (see Materials and Methods). In this case we found that the conductance increase induced by the different band 3 preparations, at the same band 3 concentration, was virtually identical in all cases (not shown). Thus, we conclude that the differences in the results stem from differences in the methods used to remove

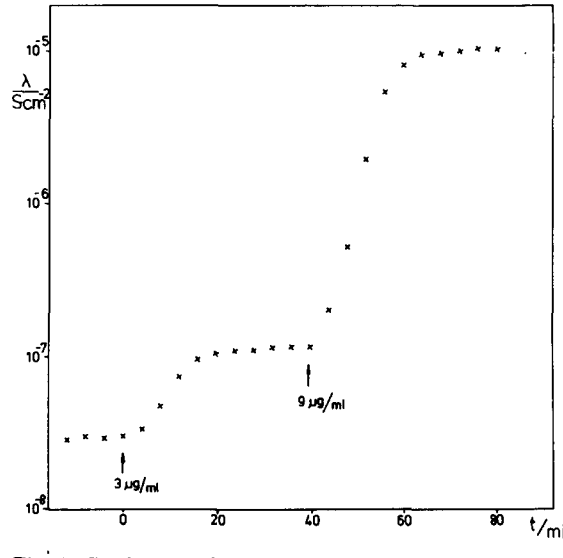

Fig. 1. Conductance increase of a membrane from egg phosphatidylcholine/*n*-decane in the presence of band 3 protein. The membrane was formed in 0.1 M NaCl/5 mM sodium phosphate (pH 7.0). Band 3 protein was added in a concentration of 3 μg/ml 10 min after blackening of the membrane (arrow). 40 min after the first addition, the total concentration of band 3 protein was increased to 9 μg/ml (arrow). $T = 25\,^{\circ}C$; applied voltage $V_m = 10$ mV.

the organic solvent during protein isolation.

Fig. 1. suggests that the band 3-induced increase in membrane conductance is a high power function of the protein concentration in the aqueous phase, as shown earlier for impure band 3 preparations [29,30]. This is confirmed in Fig. 2 which shows a double logarithmic plot of the conductance versus protein concentration curves for two different membrane systems. In this figure, as well as in nearly all other corresponding measurements, for conductance values above those obtained in the absence of protein the data can be fitted by a straight line of slope 4.0. This suggests that the conducting unit assembled by the protein consists of four building blocks, and that these building blocks and the tetramer formed by them are in an association equilibrium the relaxation of which towards equilibrium is rapid on the time scale of the experiment. Two lines of evidence indicate that the building blocks are monomers of band 3 protein:

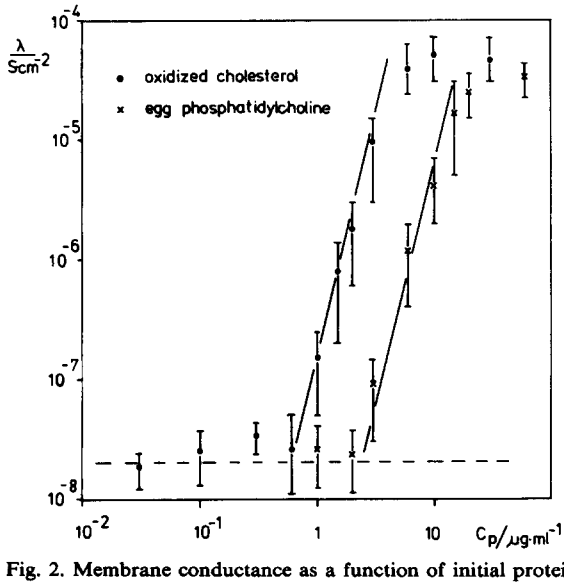

Fig. 2. Membrane conductance as a function of initial protein concentration in the aqueous phase for membranes formed from egg phosphatidylcholine/*n*-decane or from oxidized cholesterol/*n*-decane. The aqueous phase contained 0.1 M NaCl/5 mM sodium phosphate (pH 7.0). The points represent the mean ± S.D. of conductance values from at least three membranes measured about 30 min after blackening of the membranes (if the protein was present prior to membrane formation) or after addition of the protein. The broken line corresponds to the average value of membrane conductance in the absence of protein. $T = 25\,^{\circ}C$, $V_m = 10$ mV.

(1) The same behaviour is shown by a subfraction of impure band 3 preparations which is characterized by an $s_{20,w}$ value of 4 S [30]. This s value corresponds to that of monomeric band 3 [25,26].

(2) In aqueous solutions, the band 3 samples used in the present study are in a monomer/dimer/tetramer association equilibrium [25]. At the low protein concentrations present in the aqueous solutions in the cell, only monomers of the protein will be present, provided that the time interval τ between dilution of the protein and membrane formation is sufficiently large to ensure complete equilibration [25]. We have verified that the conductance values obtained are indeed independent of τ up to τ values of several hours (data not shown).

The dependency of membrane conductance on the fourth power of protein concentration, indicative of a monomer/(dimer)/tetramer association

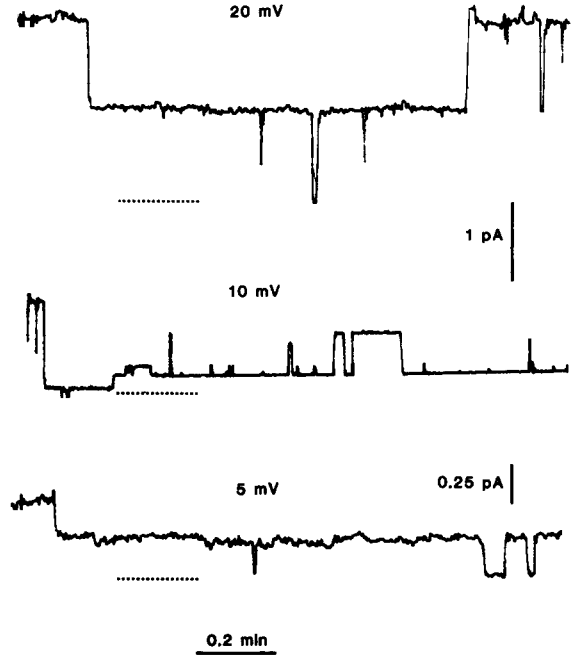

Fig. 3. Current fluctuations of a bilayer exposed to 10 ng/ml band 3 polypeptides on the *cis* side. Bilayer composition: phosphatidylethanolamine/phosphatidylserine (1:1) (*trans*); phosphatidylcholine/cholesterol (2:1) (*cis*). Aqueous phase: 160 mM NaCl/5 mM EDTA/10 mM Tris-HCl (pH 7.3). The upper current scale corresponds to the curves labelled 20 mV and 10 mV. The interrupted lines show the current level for the bare bilayer. Membrane conductance: 10^{-9} S/cm^2.

equilibrium with rapid relaxation towards equilibrium, was observed also when the protein batch used was one of the rare batches which, in aqueous solution, show slow relaxation towards association equilibrium [25] (data not shown). Thus, incorporation into a lipid environment shifts relaxation towards association equilibrium from 'slow' to 'rapid'.

The ion specificity of the conductance pathway induced by the band 3 tetramer was studied by zero-current potential measurements. The results indicate that the pathway is almost nonselective (ratio of the permeability coefficients P_c/P_a of cation and anion 1.1). Thus, the conductive units represent relatively large water-filled pores through which anions and cations can cross the membrane.

We have also found that upon addition of low concentrations of protein (10–30 ng/ml) to 'folded' bilayers, it is possible to observe current fluctuations at fixed potentials suggestive of channel formation (Fig. 3). The conductance per channel was found to be 50 pS and voltage independent. These channels were ill defined or absent in decane-containing bilayers.

The influence of membrane composition.

The dependence of membrane conductance on the fourth power of the concentration of added band 3 protein described above was observed with a variety of different lipids and lipid mixtures (e.g., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, oxidized cholesterol and various mixtures of phospholipids or phospholipids plus cholesterol). In addition, it was observed regardless of the type of bilayer (i.e., solvent-poor or solvent-containing membranes). Differences were observed, however, with respect to the magnitude of the conductance increase. Thus, at fixed protein concentration the conductance increase shown by membranes from oxidized cholesterol/*n*-decane was considerably larger than for membranes made from phospholipids (which, on the other hand, showed a quite uniform response regardless of the phospholipid species used) (Fig. 2). Similarly, at the same concentration of added protein, solvent-free membranes showed a conductance which was 2 to 3 times higher than the conductance of solvent-containing membranes made from the same phospholipids.

The results described show that reversible formation of band 3 tetramers from monomers in the lipid membranes occurs in any of the different lipid environments studied. This is in agreement with the recent finding that it also occurs in the lipid-like environment provided by micelles of nonionic detergents [26,27]. The higher values of membrane conductance observed with membranes from oxidized cholesterol could be explained by a strong and specific band 3-cholesterol interaction [37] or by unspecific protein-sterol interactions [39], which both could facilitate insertion of the protein into the membranes. Similarly, the higher conductance values found with solvent-free membranes could be due to facilitated insertion of the protein caused by the reduction of membrane thickness.

The effect of H_2DIDS and SH-reagents on band 3-induced membrane conductance

H_2DIDS is a potent inhibitor of the anion transport system of the erythrocyte membrane [1,2] (but probably not of water transport [40]). In the intact membrane, it binds to band 3 in a biphasic manner: first rapidly and reversibly, then within 30–60 min covalently and irreversibly. Both types of binding inhibit anion exchange and net anion transport [1,2]. We have studied the effect of the compound on the increase in membrane conductance induced by the band 3 tetramers (PC/*n*-decane membranes). The results obtained depend on the experimental conditions used. Addition of H_2DIDS (final concentration 10 μM) to membranes containing band 3 protein did not lead to a significant decrease in membrane conductance, though the slow phase of the conductance increase (2–3-fold between 20 and 40 min after protein addition) following the rapid increase (for $t < 20$ min) seemed to be reduced. On the other hand, protein preincubated in a H_2DIDS solution for $\geqq 30$ min was no longer able to increase membrane conductance (Fig. 4). This suggests that, in our experiments, H_2DIDS is able to act via covalent but not via non-covalent binding. It should, however, be considered that H_2DIDS, during interaction with solubilized band 3 protein, may react not only with the two lysine residues to which it binds in intact red cells [1,2,21] but also with other amino acid side chains, including that

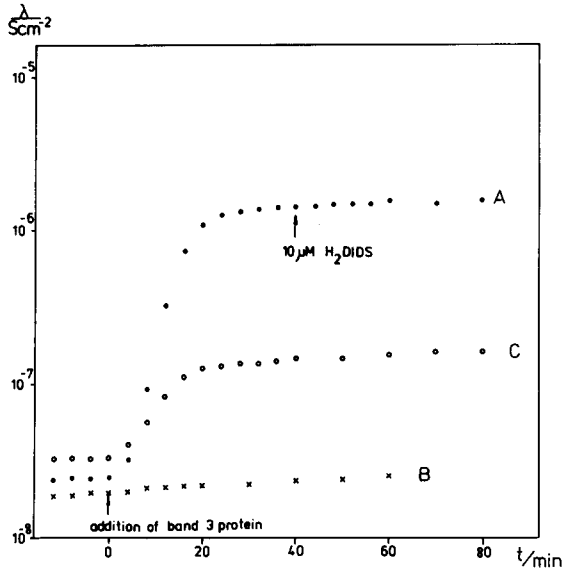

Fig. 4. Influence of H_2DIDS on the band 3-induced conductance pathway in lipid bilayer membranes. The membrane were formed from egg phosphatidylcholine/*n*-decane. The aqueous phase contained 0.1 M NaCl/5 mM sodium phosphate (pH 7.0). $T = 25\,°C$, $V_m = 10$ mV. (A) Band 3 protein was added in a final concentration of 6 μg/ml to a black membrane (arrow). 40 min after addition of the protein H_2DIDS was added in a concentration of 10 μM (arrow). (B) Band 3 protein was preincubated, at pH 7.0 and 25 °C, for 30 min in 10 μM H_2DIDS and then added to a black membrane in a final concentration of 6 μg/ml (arrow). Identical results were obtained when preincubation was performed at pH 10.0, $T = 0\,°C$. (C) Same as in curve B, except that preincubation of the protein in H_2DIDS was for 10 min.

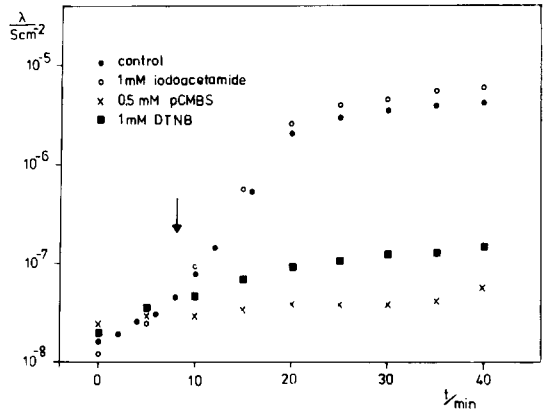

Fig. 5. Influence of SH-reagents on the band 3-induced conductance pathway in lipid bilayer membranes. The membranes were formed, at zero time, from egg phosphatidylcholine/*n*-decane. The aqueous phase contained 0.1 M NaCl, 5 mM sodium phosphate (pH 7.0) and either no SH reagent (control), 1 mM iodoacetamide, 0.5 mM pCMBS or 1 mM DTNB. The protein was added to these solutions 30 min before membrane formation, at a final concentration of 7 μg/ml. The arrow indicates the time when the membranes had become completely black. $T = 25\,°C$, $V_m = 10$ mV. The curves obtained with 2 mM *N*-ethylmaleimide were virtually identical to the control (not shown).

of cysteine [41] (see below).

In an effort to characterize further the properties of the conducting unit formed by the band 3 tetramer, we have studied the effect of incubation of solubilized band 3 protein in solutions of pCMBS and pCMB (before addition of the protein to the membrane cell) on the protein-induced increase in membrane conductance. These SH-reagents are inhibitors of both the osmotic and diffusional water transport across the erythrocyte membrane [10,11,42]. The results are shown in Fig. 5. It is apparent from the figure that preincubation of band 3 protein with pCMBS, under conditions which lead to an inhibition of water transport across the erythrocyte membrane [10,11,42], virtually completely blocks the band 3-induced conductance increase. pCMB showed the same effect as pCMBS. Preincubation of the protein with DTNB, which has also been reported to inhibit osmotic water transfer across the erythrocyte membrane [10,42,43] (but see also Ref. 44), led to a similar though somewhat smaller inhibition of the conductance increase (Fig. 5). On the other hand, pretreatment of the protein with iodoacetamide or *N*-ethylmaleimide, which do not inhibit water transport in red cells [10,42], does not block the conductance increase induced by the band 3 tetramer (Fig. 5).

Addition of pCMBS (final concentration 1 mM) to membranes into which band 3 had already been incorporated only led to a slow and relatively small decrease in membrane conductance, with a half-time $\tau_{1/2} = 30$–60 min. This value of $\tau_{1/2}$ is similar to that observed in erythrocyte membranes for the inhibition of water transport [45].

Studies using analytical ultracentrifugation showed that incubation of band 3 with H_2DIDS or pCMBS does not influence the self-association of the protein. In addition, we have found that it does not influence the interaction of the protein with phospholipid monolayers at the air/water interface. This suggests that the effect of pretreatment of band 3 with H_2DIDS or pCMBS on the

band 3-induced changes in membrane conductance is not due to a blockage of the formation of band 3 tetramers or of incorporation of the protein into the lipid membrane, but must be due to the blockage of a transport pathway within the tetramers.

Flux measurements

In order to get an insight into the functional state of the band 3 molecules incorporated into the lipid membranes, we have performed measurements of chloride flux across bilayers exposed to the protein, using a variety of membranes of different lipid composition. We were unable to detect anion fluxes significantly different from those of the control bilayers after the anion-exchange protein had interacted with the membranes to produce a substantial increase in the membrane conductance. This could be due to a lack of sensitivity of our measurements or to a failure of the incorporated band 3 polypeptides to enhance the flux of anions or to a combination of both. However, we feel that with our present sensitivity we would have detected a change in the anion fluxes had it existed, since we can measure fluxes as low as 10^{-16} mol/s. This flux would be the one present across a red cell containing 1000 band 3 monomers (given a chloride flux across red cells of $2 \cdot 10^{-8}$ mol/cm^2 per s [46] and a band 3 density of $3 \cdot 10^{11}$ molecules/cm^2). This flux would also be the one across a bilayer with a conductance of (50 pS/band 3 molecules) $\times$ (1000 molecules band 3)/$(10^{-2}$ cm$^2) = 5 \cdot 10^{-6}$ S/cm^2, assuming that there is one molecule per channel (minimum number), and that all of the molecules can engage in anion-exchange. Thus, we have to conclude that the protein-lipid system used by us does not represent a reconstitution of the anion transport system of the erythrocyte membrane.

Discussion

In aqueous solutions, the detergent-free preparations of band 3 protein used in this study are in a monomer/dimer/tetramer association equilibrium [25] and thus show the same association behaviour as band 3 carefully isolated in the non-ionic detergents Ammonyx-LO [26], nonaethylenglycol lauryl ether [26] and Triton X-100 [27]. Among these detergent-solubilized samples of the anion transport protein, at least those prepared in Triton X-100 (see, for example, Refs. 47 and 48) and Ammonyx-LO (Scheuring, U., personal communication) allow a reconstitution of the anion transport system of the erythrocyte membrane in lipid vesicles and therefore seem to represent undenatured band 3 protein. Thus, our detergent-free samples seem to reproduce the association behaviour of native band 3 protein. We therefore expect that those of our results which concern problems of band 3 self-association in artificial lipid bilayers will be applicable to the problem of the self-association of the protein in the erythrocyte membrane. On the other hand, the system used by us does not exchange anions in a way comparable to the erythrocyte membrane, so that we cannot expect that it will yield reliable information on anion transport. The system does, however, respond to covalent binding to the protein of known inhibitors of the water transport system of the erythrocyte membrane, which suggests that our results may have some relevance for this transport pathway.

What can our results tell about band 3 self-association in the human erythrocyte membrane? Firstly, they tell that band 3, in a lipid environment, readily forms tetramers and that these tetramers are linked to monomers of the protein in an association equilibrium (which, most probably, will also include dimers of band 3 as an intermediate of the monomer/tetramer association). Secondly, they tell that the formation of band 3 tetramers and the occurrence of an association equilibrium of band 3 protein is virtually independent of the lipid species surrounding the protein. Thirdly, they show that the formation and disintegration of the band 3 tetramers and thus relaxation towards association equilibrium in the lipid environment is rapid on the time scale of the conductance measurements *. The first and second of these points

* Band 3 protein solubilized by nonionic detergents seems to show, in the detergent solutions, an association equilibrium relaxation of which towards equilibrium is intermediate on the time scale of ultracentrifugation experiments [26] (in these experiments it is, however, difficult to distinguish between intermediate and rapid relaxation [49]). However, association and dissociation of protein molecules embedded into detergent micelles will need rearrangement of the micelles. This may be the rate-limiting step in the reaction [50].

strongly support the suggestion derived from ultracentrifugation experiments on solubilized band 3 protein that, in the lipid bilayer of the erythrocyte membrane, the protein will show a monomer/dimer/tetramer association equilibrium [25,26]. The third point supplements this suggestion.

With respect to the transport properties of the band 3 tetramer reported in this study, it is clear that the increase in membrane conductance induced by the protein aggregate is due to the formation of a relatively large water-filled pore, probably in the centre of the aggregate. The detailed structure of this pore will certainly be different from that of a possible channel between the subunits of the band 3 tetramers in the erythrocyte membrane, since the conductivity of the former is both too high and too unspecific as compared to that which would be expected for the latter one [1,2]. However, this difference may be due to rather subtle displacements of charged amino acid side chains in the artificial system, so that the gross structure of the channel could be very similar in the two systems. We do not know whether the native channel may represent a pathway (possibly one out of several) by which a net transfer of anions across the erythrocyte membrane could occur. Our experiments suggest, however, that the channel formed by the band 3 tetramer could be the structure responsible for the band 3-induced part of the water transport across the erythrocyte membrane: the channel is blocked by three SH-reagents which inhibit water transfer, whereas it is not blocked by two SH-reagents which do not influence water transport. This suggestion would be consistent with recent findings, by freeze fracture electron microscopy, on abnormal erythrocyte membranes of the McLeod type: In these membranes, a reduction in the (average) size of the intramembrane particles (the larger of which seem to represent band 3 tetramers [22–24]) is accompanied by a decrease in water permeability [51].

Acknowledgements

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