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Asymmetric and functional reconstitution of band 3 into pre-formed phosphatidylcholine vesicles

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Abstract

Human erythrocyte band 3 protein was purified in 0.1% Triton X-100 and reconstituted into pre-formed phosphatidylcholine vesicles by a Triton X-100-mediated procedure [1]. Band 3 (and its transmembrane domain) could be asymmetrically reconstituted into phosphatidylcholine vesicles with retention of sulfate transport activity which showed behaviour characteristic of red cell anion transport in response to pH, H_2DIDS and temperature. Successful reconstitution was also possible using high mol ratios of band 3/phosphatidylcholine (1:200), which are not achieved by any other method.

Keywords: Band 3; Membrane protein; Reconstitution; Triton X-100; Anion transport

1. Introduction

The erythrocyte membrane anion exchanger, band 3, is a 95 kDa integral membrane glycoprotein (reviews: [2–4]). Band 3 comprises approximately 25% of the erythrocyte membrane protein [5] and may be rapidly purified in milligram quantities in solution in non-ionic detergents such as Triton X-100 [6].

Band 3 has been extensively studied in the native membrane [7,8], in detergent solution [9,10], and in reconstituted proteoliposomes [11–13]. Several methods have been devised for the production of band 3 proteoliposomes from purified band 3 in detergent solution [14–19,12].

Of these methods, only the method of Scheuring et al. [12] produces proteoliposomes with most of the complex characteristics of red cell anion exchange, such as the variation of anion flux with pH with a maximum around pH 6.2, activation energy of ~ 138 kJ mol^{-1} and ~ 100% inhibition by externally added H_2DIDS [8]. This method uses band 3 purified in the low CMC detergent, Triton X-100, which is favourable for band 3 stability [20]. In order to use dialysis to reconstitute the proteoliposomes, the high CMC, non-ionic detergent, octyl-glucoside, is used to solubilise the system after lipid has been added. However, band 3 purified in octyl-glucoside undergoes irreversible aggregation and denaturation [21] and, possibly because of the destabilising effect of this detergent on band 3, the band 3 recovery rates for this reconstitution method are low (10–20%) [12]. In addition, the presence of Triton X-100 in the protein preparation makes reconstitution with large quantities of band 3 relative to lipid (> 1:500 band 3/lipid mol ratio) very difficult because the removal of octyl-glucoside by dialysis leaves insufficient TX-100 to hold the band 3 and lipid in solution, preventing successful reconstitution by this method.

A novel approach to detergent-mediated membrane protein reconstitution has been developed by Rigaud et al. [1]. Membrane proteins such as bacteriorhodopsin [22], Ca^{2+}-ATPase [23] and CF_0F_1 [24] will insert into lipid vesicles which have been partially, but not totally, solubilised by detergents. After incubation, the detergent may then be removed by direct adsorption onto hydrophobic beads (e.g., Biobeads SM-2) leaving reconstituted proteoliposomes.

We have investigated the use of this method for the reconstitution of band 3, into phosphatidylcholine vesicles, mediated by the detergent Triton X-100. Hen egg phosphatidylcholine is a readily available lipid in which band 3 has been shown to be fully active [12] and Triton X-100 is the

Abbreviations: CMC, critical micellar concentration; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; H_2DIDS, dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonate; PA, phosphatidic acid; PC, phosphatidylcholine from hen egg; TMD, transmembrane domain of band 3; TX-100, Triton X-100.

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detergent in which band 3 has been shown to be most thermodynamically stable [20]. The use of Triton X-100 for membrane protein reconstitution has previously been investigated for bacteriorhodopsin [22] and we here report the successful adaptation of this method for the production of band 3/PC proteoliposomes.

2. Materials and methods

2.1. Materials

Recently outdated packed human red cells were obtained from the Regional Transfusion Service, Birmingham, UK; Hen egg phosphatidylcholine was obtained from Lipid Products, Nuffield Ridge, Surrey, UK; Triton X-100 specially purified for membrane research was obtained from Boehringer-Mannheim, Germany; ^{35}S-labelled Na_2SO_4 was obtained from ICN, Thame, Oxon.

2.2. Preparation of band 3 protein

Band 3 protein and its transmembrane domain were purified and characterised as described by Casey et al. [6]. Protein preparations were $> 95\%$ pure as judged by Coomassie-stained SDS-PAGE.

2.3. Preparation of phosphatidylcholine vesicles

Egg phosphatidylcholine (PC) in chloroform/methanol (3:1) was dried to a film by evaporation under nitrogen. Final traces of chloroform/methanol were removed by evaporation under vacuum overnight. Unilamellar PC vesicles were prepared by the method of Almog et al. [25]. The final concentration of lipid was measured by a phosphorus assay (see below).

2.4. Preparation of reconstituted vesicles

Reconstituted vesicles were prepared essentially as described by Rigaud et al. [1] except that band 3 protein was used instead of bacteriorhodopsin. Purified band 3 in 0.1% TX-100 and PC vesicles were mixed to the molar ratio 1:2000 at room temperature. The Triton X-100 concentration was then adjusted such that ratio of effective Triton X-100 to phosphatidylcholine was 1.3:1 and the mixture was gently stirred at room temperature for 2 h. At this point, 80 mg/ml washed Biobeads SM-2 were added to adsorb the Triton X-100 [26] and after gentle agitation at room temperature for 2 h, another 80 mg/ml were added followed by a further 2 h gentle agitation at room temperature. The reconstituted vesicles were then layered onto a sucrose density gradient 5–30% (w/v) which was centrifuged overnight at $50\,000 \times g$, 4°C. One major band of vesicles was usually observed which was collected and dialysed against several changes of the relevant buffer. The vesicles were then stored at 4°C, in the dark, for up to 5 days.

2.5. Protein assays

Band 3 concentrations in Triton X-100 solution in the absence of lipid were measured by a Coomassie-binding assay (Pierce Chemicals). In order to avoid interference by lipid, band 3 concentrations in reconstituted vesicles were measured by the filtration method of [27].

2.6. Lipid assay

Phosphatidylcholine concentration was measured by phosphorus assay [28].

2.7. Triton X-100 content

Triton X-100 was monitored in the reconstitution using tracer ^{3}H-labeled Triton X-100 (New England Nuclear).

2.8. Band 3 activity in reconstituted vesicles

Activity of reconstituted band 3 and the intravesicular volume of the proteoliposomes were measured by the method of Scheuring et al. [12] by monitoring efflux of radioactively labelled sulfate ($^{35}SO_4^{2-}$) at Donnan equilibrium with 10 mM Na_2SO_4.

2.9. Microscopy

Samples for freeze-fracture were prepared by freeze etching by the method of Sternberg et al. [29]. The samples were examined in a Siemens 80 keV electron microscope. Vesicle sizes were measured from electron micrographs manually.

3. Results

3.1. Interaction of Triton X-100 with PC vesicles

The interaction between phosphatidylcholine vesicles and Triton X-100 (TX-100) was investigated by measuring the scattering of the vesicles at 500 nm wavelength [30]. The scattering intensity was found to be dependent upon the parameter R_{eff}, which is defined as the ratio of effective micellar detergent to lipid [30].

At detergent concentrations below the CMC ($R_{eff} < 0$), most of the detergent is present as monomer. Active detergent is defined as being that in excess of the CMC ($R_{eff} > 0$), which is 0.28 mM for TX-100 [31]. Although this is a simplification, it is a useful parameter to use at the higher detergent concentrations ($R_{eff} \gg 0$) generally present during the critical stages of reconstitution. The point at which the scattering intensity becomes a maximum (Fig.

1) is the point at which the vesicles are saturated with intercalated detergent ($R_{eff} = R_{sat}$). At higher concentrations of detergent, the vesicles solubilise to form mixed micelles of detergent and lipid. This process results in a decrease in scattering intensity until all of the vesicles are solubilised ($R_{eff} = R_{sol}$).

By plotting detergent concentration at maximum scattering intensity ($R_{eff} = R_{sat}$) against lipid concentration, R_{sat} for the interaction of Triton X-100 with PC vesicles can be measured from the gradient to be 0.65 ($\pm$0.2). The CMC of Triton X-100 can also be estimated, by extrapolation of the plot to zero PC concentration, to be 0.3 mM ($\pm$0.1 mM) which compares with a literature value of 0.28 mM [31]. R_{sol} was estimated to be 2.7 ($\pm$0.5).

These values of R_{sat} and R_{sol} for the interaction between Triton X-100 and phosphatidylcholine are almost identical to those obtained by Paternostre et al. [30] for the interaction between Triton X-100 and phosphatidylcholine/phosphatidic acid vesicles ($R_{sat} = 0.64$, $R_{sol} = 2.5$). We therefore concluded that PC vesicles behave similarly to PC/PA vesicles during solubilisation with TX-100 and that, since reconstitution is largely governed by detergent-lipid interactions, the R_{eff} used, during the initial incubation, for reconstituting band 3 into PC vesicles would be similar to the value of 1.3 used for reconstituting BR into PC/PA vesicles [1].

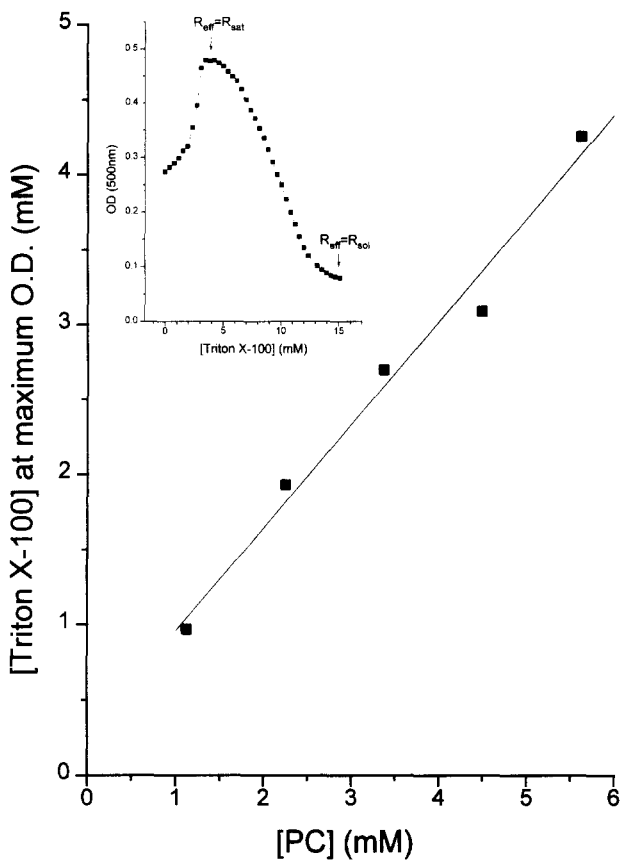

Fig. 1. Plot showing the variation of Triton X-100 concentration at saturation point with concentration of phosphatidylcholine vesicles. The slope gives $R_{sat} = 0.64$ and the intercept gives an estimate of the CMC of 0.3 mM. The inset shows the change in scattering at 500 nm wavelength with addition of Triton to 6 mM PC vesicles. Saturation and solubilisation points are indicated.

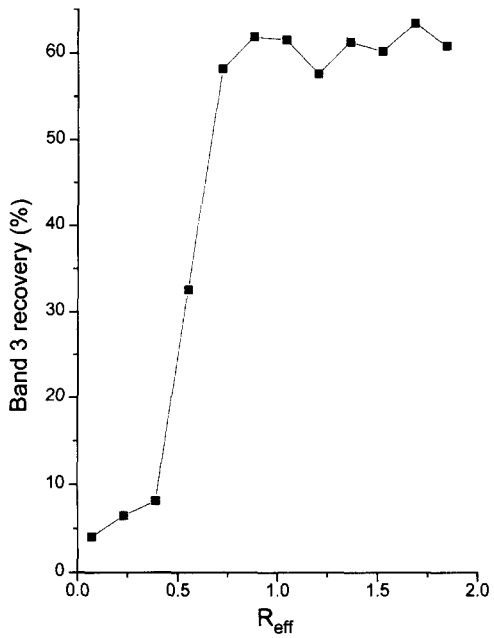

Fig. 2. The effect of R_{eff} upon the amount of band 3 incorporated into PC vesicles.

3.2. Reconstitution of band 3/phosphatidylcholine vesicles

Fig. 2 shows the variation of R_{eff} during the reconstitution upon the amount of band 3 incorporation. At $R_{eff} < R_{sat}$ ($\sim$ 0.6), there is no solubilization of the lipid vesicles and consequently, very little or no band 3 incorporation. At R_{eff} values between 0.6 and 1.3, high levels of incorporation were observed and the vesicles sedimented as more than one band in the sucrose density gradient, with a large population of vesicles at low sucrose density corresponding to protein-free lipid vesicles and a smaller band further down the gradient corresponding to reconstituted proteoliposomes. At R_{eff} values of 1.3 and above, the reconstituted vesicles sedimented as a single band. Therefore, an $R_{eff} = 1.3$ was taken to be the optimal for band 3/PC reconstitution, the same as previously reported for bacteriorhodopsin/PC/PA reconstitution [1].

Band 3 recovery rates of 40–60% were observed for initial band 3/PC ratios of 1:2000 using reconstitution with $R_{eff} = 1.3$ for the stages up to and including the sucrose density gradient centrifugation. The final band 3/PC mol ratio was 1:3000 $\pm$ 200. Dialysis to remove the sucrose resulted in some loss of vesicles giving a final yield of 20–30% based on the initial amount of protein used in the reconstitution and freeze-fracture electron micrographs show that this protein is incorporated into the

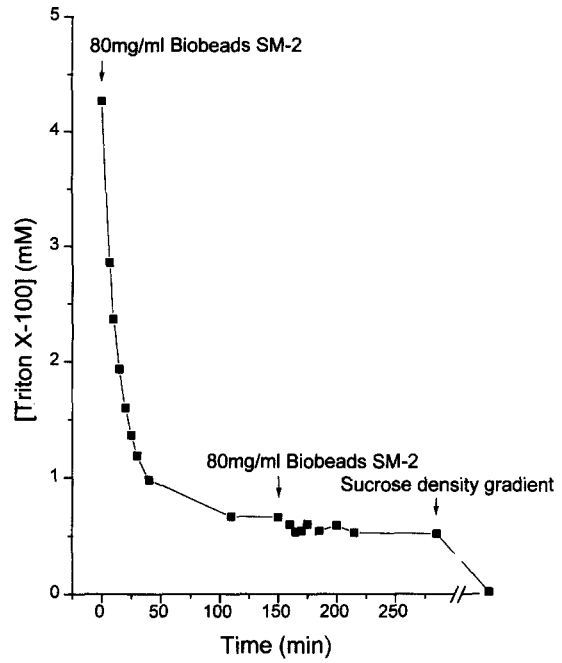

Fig. 3. The removal of Triton X-100 from reconstituted vesicles. The additions of 80 mg/ml batches of Biobeads SM-2 are indicated as is the sucrose density gradient step. Final Triton X-100 levels were equivalent to 0.6% of PC.

reconstituted membrane (Fig. 4). This yield represents an improvement upon previously published methods [17,12] which give recovery rates of 10–20%.

Band 3 was also reconstituted into PC vesicles at mol ratios of 1:200 using $R_{eff} = 1.3$. Recovery rates for band 3 were 30–40% and final reconstituted band 3/PC mol ratios, after sucrose density gradient centrifugation, were 1:350 (±50). This is approximately equivalent to the band 3:lipid ratio in the native erythrocyte membrane [17]. The transmembrane domain of band 3 (TMD), purified by proteolytic removal of the cytoplasmic domain [6], was also successfully reconstituted, using $R_{eff} = 1.3$, at TMD/PC ratios of 1:2000 and 1:200 with similar results.

3.3. Removal of Triton X-100

The removal of Triton X-100 was monitored during reconstitution using $R_{eff} = 1.3$ using tritiated Triton X-100. Counts in 100 μl were converted to Triton X-100 concentration and plotted against time in Fig. 3. Contrary to Levy et al. [22], Biobeads SM-2 do not seem to be able to remove all the Triton X-100 from the reconstituted vesicles. A residual amount remains, at a concentration of approximately the CMC, and this can only be removed finally by sucrose density gradient centrifugation.

3.4. Characterisation of reconstituted band 3 / PC vesicles

Fig. 4 shows freeze-fracture electron micrographs of band 3 reconstituted using $R_{eff} = 1.3$ at final band 3/PC

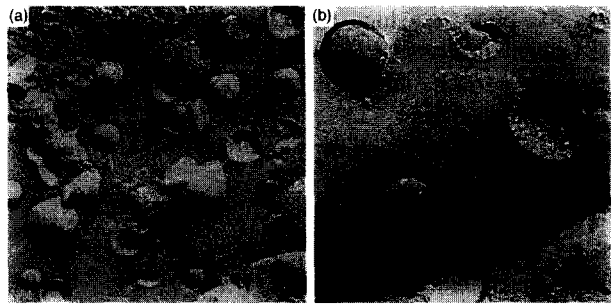

Fig. 4. Freeze-fracture electron micrographs of band 3-phosphatidylcholine reconstituted vesicles at final band 3/PC mol ratios of (a) 1:3000 and (b) 1:350. Scale bar represents 100 nm.

ratios of 1:3000 and 1:350. In both cases, it can be seen that protein is incorporated into the reconstituted membrane. Reconstituted vesicle sizes averaged 120 nm (±20 nm) for 1:3000 and 200 nm (±30 nm) for 1:350 band 3/PC final ratios, respectively.

The anion exchange function of the reconstituted band 3 was examined and compared with band 3 in the native membrane [2,8,32]. Because of the very high sulfate efflux rate in high protein/PC (~ 1:350) vesicles, even at reduced temperatures, it was only possible to perform functional studies on low band 3/PC (~ 1:3000) vesicles. These vesicles showed a sulfate transport number (T_N) of 17 SO_4^{2-}/min/band 3 monomer which compares with a T_N of 20 SO_4^{2-}/min/B3 for band 3 in erythrocytes [8,12].

Fig. 5 shows the sulfate transport in reconstituted vesicles compared with the same vesicles inhibited with satu-

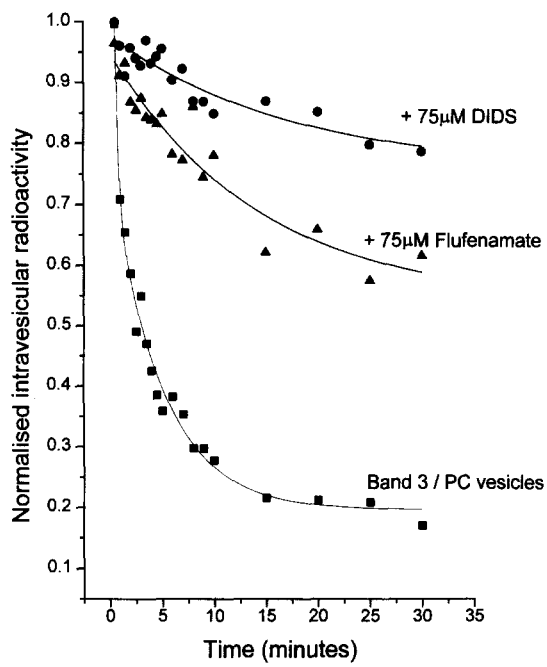

Fig. 5. Efflux of sulfate from band 3-PC vesicles and its inhibition by saturating concentrations (75 μM) of membrane impermeable (DIDS) and membrane permeable (flufenamate) inhibitors. Temperature = 37°C, pH 7.2.

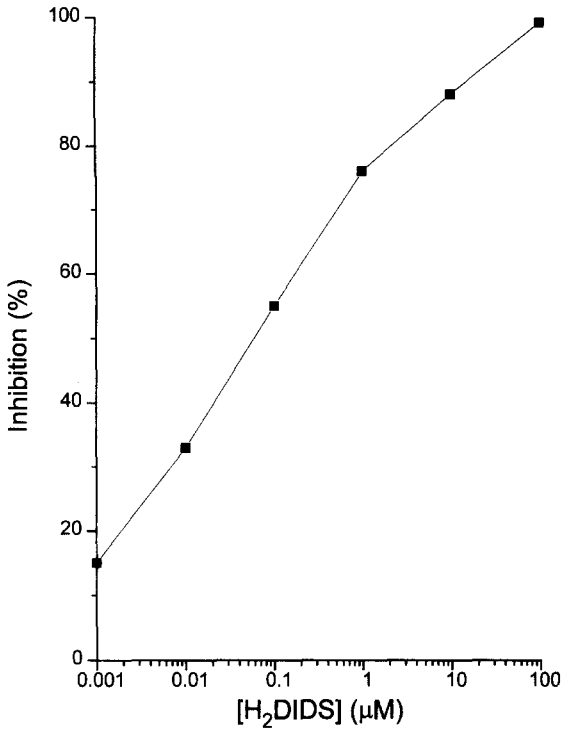

Fig. 6. Dose–response curve for inhibition of reconstituted band 3 by H_2DIDS. IC_{50} = 0.1 μM and the Hill coefficient = 0.4. Temperature = 37°C, pH 6.5.

rating concentrations of the non-membrane permeable stilbenedisulfonate, DIDS, and the membrane permeable flufenamate. DIDS inhibition was > 85% (which compares with an expected value of ~ 50% for a symmetric reconstitution) and was at least as effective as flufenamate, indicating a predominantly right-side-out asymmetrically oriented reconstitution. A similar result was reported by Scheuring et al. [12] for band 3-PC vesicles reconstituted by octyl-glucoside-Triton X-100 dialysis. Inhibition by DIDS at the concentration of 75 μM was not considered to be diagnostic of band 3-mediated anion exchange because of the possibility of non-specific effects [33]. We therefore titrated the inhibition of reconstituted band 3 with the related non-membrane permeable inhibitor H_2DIDS.

Inhibition by H_2DIDS was investigated at pH 6.5 where binding of H_2DIDS by band 3 is almost entirely non-covalent and reversible [32]. As with erythrocyte membranes, half-maximal inhibition was attained in band 3/PC vesicles (1:3000 mol ratio) using H_2DIDS concentrations of around 0.1 μM (Fig. 6). A Hill plot of the inhibition data gives a slope of 0.4 which compares to a value in native membranes of 1.0. This implies a less than stoichiometric interaction of H_2DIDS with band 3 in the reconstituted vesicles. However comparable values have been observed in previous reconstitutions [12,34].

A characteristic feature of band 3-mediated sulfate transport is the strong dependence upon pH with a maximal activity at about pH 6.2 and a sharp decrease in activity at lower pH [8]. A qualitatively similar behaviour

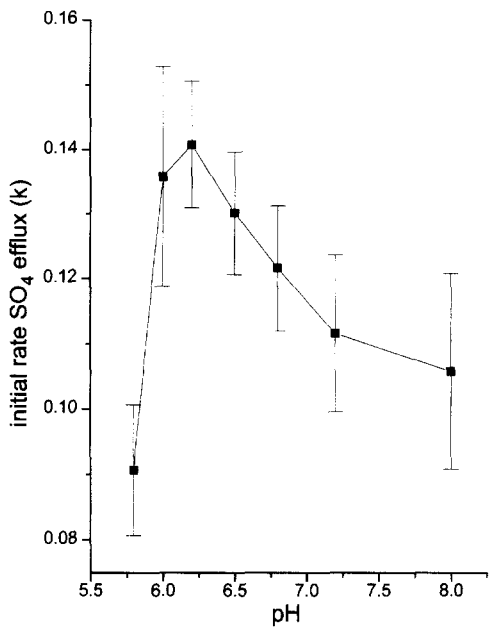

Fig. 7. Effect of pH upon the rate constant for sulfate efflux from band 3-PC reconstituted vesicles showing a characteristic 'bell-shaped' response. Temperature = 37°C.

is observed in the reconstituted vesicles prepared here (Fig. 7).

Fig. 8 shows the variation of the flux constant k with (temperature)$^{-1}$ to give an estimate of the activation energy (E_a) for sulfate transport of 132 (±10) kJ mol^{-1}, which compares with a value for native membranes of 138 (±10) kJ mol^{-1} [2].

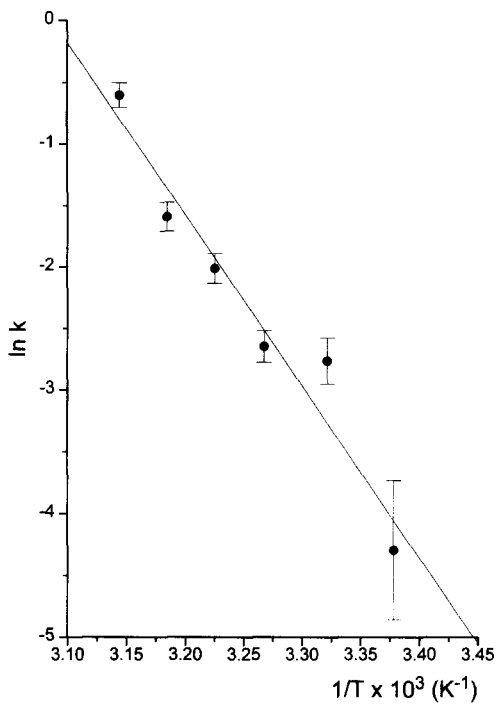

Fig. 8. Arrhenius plot showing the effect of temperature upon the sulfate transport activity of band 3-PC reconstituted vesicles. The slope gives the activation energy for reconstituted transport of 132 kJ/mol. pH 7.2.

4. Discussion

We have applied the reconstitution method of Rigaud et al. [1], developed for bacteriorhodopsin, to the reconstitution of band 3 into phosphatidylcholine vesicles mediated by Triton X-100. This reconstitution behaves similarly to the reconstitution of bacteriorhodopsin into PC/PA vesicles described by Rigaud et al. [1] implying, firstly, that PC vesicles interact with Triton X-100 in an essentially identical way to PC/PA vesicles, and secondly, that the controlling factor in the reconstitution is the interaction of the detergent and lipid. This is confirmed by the use of the method to reconstitute proteins as diverse as bacteriorhodopsin [22], Ca^{2+} ATPase [23] and CF_1F_0 [24]. It thus seems that this method may be generally applicable to reconstitution of detergent-solubilized membrane proteins into lipid vesicles.

The removal of Triton X-100 from the reconstituted vesicles was not complete using Biobeads SM-2 alone. Since virtually complete removal of TX-100 was observed by Levy et al. [22], it is unlikely that this is because of non-adsorption of non-micellar TX-100. It therefore seems most likely that there is a relatively strong interaction between TX-100 and band 3 which prevents complete removal of the detergent by Biobeads SM-2. This interaction may account for the high thermodynamic stability of band 3 in Triton X-100 compared with other detergents [20].

Band 3 reconstituted into phosphatidylcholine vesicles exhibits many of the features of the native anion exchanger. The transport number (T_N), the number of sulfate anions transported per min per band 3 monomer, is 17 min^{-1} which is close to the values in erythrocyte ghost membranes of 20 min^{-1} [8] and in previous successful reconstitutions of 16 ($\pm$5) min^{-1} [12]. Transport numbers of 400–1000 min^{-1} were reported by Scheuring et al. [35,36] using vesicles of reconstituted band 3/PC at mol ratios of $<$ 1:10000. These measurements may reflect a higher rate of anion transport by the band 3 monomer although [37] measured the monomer T_N at 25–30 min^{-1}.

The 90–100% inhibition by externally administered stilbenedisulfonates DIDS and H_2DIDS (Fig. 6), indicates a predominantly 'right-side-out' orientation. This is similar to the orientation of bacteriorhodopsin reconstituted by the same method [1] and of band 3 reconstituted by dialysis of octyl-glucoside and Triton X-100 [36].

The binding of stilbenedisulfonates has been extensively studied in erythrocyte membranes and the stoichiometry of covalent binding to band 3 measured at 1 inhibitor per band 3 monomer [38,39]. Non-covalent binding of DIDS and H_2DIDS may be studied at pH 6.5 at which covalent binding is negligible. The Hill coefficient for non-covalent binding of H_2DIDS to erythrocyte membranes was $\sim$ 1, implying no cooperativity in binding [32]. The inhibition of band 3 reconstituted into phosphatidylcholine vesicles by H_2DIDS is described by a Hill coefficient of 0.4 ($\pm$0.1) for our proteoliposomes, which compares with a value of 0.5 ($\pm$0.1) for the proteoliposomes of Scheuring et al. [12]. The discrepancy in H_2DIDS binding between band 3 in the native membrane and band 3 reconstituted into artificial proteoliposomes could be caused by a minor change in band 3 conformation during purification and reconstitution as has been suggested before [12,34].

Other characteristics of the reconstituted proteoliposomes are similar to native erythrocyte membranes. The pH dependence of sulfate transport, which decreases sharply below pH $\sim$ 6.2, is caused by the co-transport of a proton with sulfate by band 3, which results in lower transport at high pH, and by allosteric inhibition of transport at low pH [2]. The activation energy of 132 ($\pm$ 10) kJ mol^{-1} is also close to that measured in ghost membranes of 138 ($\pm$ 10) kJ mol^{-1} [8]. We therefore conclude that the band 3/PC proteoliposomes reconstituted by the method of Rigaud et al. [1] contain fully functional band 3 in a right-side-out orientation.

The recovery of band 3 reconstituted into proteoliposomes was 40–60% of the initial band 3/PC mol ratio of 1:2000. Some band 3 was lost during dialysis to remove sucrose after sucrose density gradient centrifugation to give a final recovery of 20–30%. This compares well with recovery rates of 10–20% using the method of Scheuring et al. [12]. This improvement in recovery rate is probably the result of using only Triton X-100 as detergent throughout the reconstitution rather than introducing octyl-glucoside [21].

Because the method outlined above uses direct adsorption onto Biobeads SM-2, rather than dialysis to remove detergent, it is applicable at higher band 3/PC ratios than are possible with the method of Scheuring et al. [12]. Even though the band 3 recovery rates at these high band 3/PC mol ratios are lower than for proteoliposomes prepared with a lower density of band 3, the electron micrographs show that the protein is also successfully reconstituted in these vesicles. The rate of sulfate transport in these proteoliposomes was too rapid to conduct activity assays, but there is no reason why the activity of band 3 should be affected given that these vesicles have a similar protein/lipid ratio to native erythrocyte membranes.

In conclusion, the reconstitution method of Rigaud et al. [1] has been successfully applied to the reconstitution of active band 3 into PC vesicles at both low (1:2000) band 3/PC mol ratios for functional studies, and at high (1:200) band 3/PC ratios suitable for biophysical studies.

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