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NEW APPROACHES FOR THE RECONSTITUTION AND FUNCTIONAL ASSAY OF MEMBRANE TRANSPORT PROTEINS

APPLICATION TO THE ANION TRANSPORTER OF HUMAN ERYTHROCYTES

A. DARMON, M. ZANGVILL and Z.I. CABANTCHIK

Department of Biological Chemistry, The Hebrew University of Jerusalem, Institute of Life Sciences, 91904 Jerusalem (Israel)

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The human red blood cell anion transport protein, band 3, was isolated and reconstituted into lipid vesicles. The main feature of the new reconstitution is the replacement of native lipids and of solubilizing detergent by externally added lipids, while band 3 protein is immobilized on a gel matrix. The vesicles formed upon detergent removal and sonication are unilamellar and sealed, and band 3 protein is the major polypeptide detectable in them. The method consists of: (a) solubilization of alkali-treated red blood cell membranes by Triton X-100; (b) binding of glycophorin and band 3 protein to diethylaminoethyl (DEAE)-cellulose in Triton X-100 solution, followed by high ionic strength elution; (c) band 3 protein complexation to organomercurial Sepharose; (d) exchange of the Triton X-100 with the dialyzable detergent octylglucopyranoside, while band 3 protein is complexed to the column; (e) elution of band 3 by cysteine (5 mM) in the presence of octylglucopyranoside; (f) addition of lipids (asolectin or egg phosphatidylcholine) to the protein-detergent suspension; and (g) dialysis of the mixture against 1% bovine serum albumin to remove the detergent completely. The vesicles were assayed for anion transport capacity by a novel procedure which is based on the fluorescent substrate *N*-(2-aminoethylsulfonate)7-nitrobenz-2-oxa-1,3-diazole (NBD-taurine) and on anti-NBD-antibodies as quenchers of extravesicular NBD-taurine fluorescence. Efflux of NBD-taurine from vesicles was monitored in a continuous mode as a decrease in intravesicular fluorescence. The band 3-mediated flux was approx. 50% inhibitable by externally added disulfonic stilbenes, indicating the random distribution of band 3 protein in reconstituted vesicles. Both the specific transfer rate (i.e., nmol substrate / mg protein per min) of band 3 and its energy of activation (E_a) in the artificial lipid milieu were similar to those obtained with the native system. Glycophorin incorporation into this milieu had no significant effect on the associated anion transport properties.

Introduction

The red blood cell is endowed with a specialized system for the exchange of anions, which subserves the physiological requirement of CO_2-removal from tissues to lungs (see Refs. 1–3 for reviews). The assignment of the transport function

Abbreviations: NBD-taurine, *N*-(2-aminoethylsulfonate)-7-nitrobenz-2-oxa-1,3-diazole; CMTF, continuous monitoring of anion transport by fluorescence; pHMB, *p*-hydroxymercuribenzoate; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonic acid; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; PMSF, phenylmethylsulfonylfluoride; TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone.

to the predominant membrane polypeptide of M_r 95 000 (band 3, according to sodium dodecyl sulfate polyacrylamide gel electrophoresis [4], relied primarily on the specific labeling of the anion transporter with affinity and photoaffinity binding probes [2,5–7] and on the functional isolation of the protein in its native lipid milieu by negative purification [8,9].

Several attempts have been made to detergent solubilize the protein, partially purify it [10–16] and either reconstitute it into lipid vesicles [9,11–17] or implant it into cells lacking the anion exchange capacity [9,14,15,18]. However, functional studies with the reconstituted band 3 protein have been hampered both by lack of reliable tools for quantitative estimation of anion transport capacity in systems of high surface area : volume ratio such as vesicles and by an uncontrolled variability in the transport capacity of the system in the reconstituted state. A critical assessment of factors affecting the functional properties of reconstituted band 3 protein has been given elsewhere [9,14]. A major factor invoked was the inefficient and irreproducible removal of the hitherto most efficient detergent for this system, Triton X-100, and its replacement by appropriate lipids by means which are conservative of the function.

In this work we introduce a procedure of band 3 protein isolation which comprises steps used in previous works [8,10,13] and a novel approach to reconstitution which facilitates substitution of Triton X-100 by dialyzable detergents and incorporation of phospholipids. The substitution of detergent is carried out while the band 3 protein is immobilized on a pHMB-Sepharose affinity column. In this work we also assess the functional status of the reconstituted band 3 protein by a novel technique for continuous monitoring of anion transport by fluorescence (CMTF), which is based on the fluorescent anion substrate NBD-taurine [19] in conjunction with membrane-impermeant anti-NBD antibodies which serve as quenchers of extravesicular fluorescence [20]. This technique circumvents the need for vesicle separation from medium and allows precise determinations of transport profiles.

Our quantitative studies of anion transport in the reconstituted preparations indicate that the novel procedure for band 3 protein isolation and reconstitution into either soya-bean phospholipids or egg phosphatidylcholine-containing vesicles preserved the basic features of anion transport such as anion transport capacity, temperature dependence and susceptibility to specific inhibitors of anion exchange.

Preliminary reports of this work were presented at the 13th FEBS Meeting, Jerusalem (1980) S1-P36 and at the Israel Biochemical Society Meeting, Rehovot, (1982) [39].

Materials and Methods

Chemicals and radiochemicals used in this work were from the following sources: asolectin, egg phosphatidylcholine, from Sigma; Triton X-100 from Serva; octylglucopyranoside and butylated hydroxytoluene from Sigma; Nonidet P-40 from British Drug House; *p*-hydroxymercuribenzoate (pHMB) and 6-mercaptoguanosine from Sigma; 4,4′-dinitro-2,2′-stilbenedisulfonic acid (DNDS) from ICN K&K Chemicals; DEAE-cellulose (DE-52) from Whatman, and Sepharose 4B from Pharmacia. Ultrafiltration membranes PM-30 were purchased from Amicon Corp. and dialysis bags from Fischer Scientific Instrument; Dowex citrate (50–100 mesh) from Bio-Rad; [^{3}H]H_2DIDS from Nuclear Research Center, Negev, Israel; [^{14}C]octylglucopyranoside and [^{3}H]Triton X-100 from New England Nuclear.

The following materials were synthesized according to previously published methods: 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and [^{3}H]H_2DIDS (100 mCi/mmol), according to Ref. 5; NBD-taurine, according to Ref. 19; rabbit anti-NBD antibodies were prepared as previously described [20]; pHMB-Sepharose was prepared as described before [21]. The pHMB coupled to Sepharose was determined by spectrophotometric titration with mercaptoguanosine as described elsewhere [22]. The coupling ratio was 2–4 μmol pHMB/ml wet gel; SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [23]. Gels were fixed and stained with Coomassie blue and subsequently sliced, dissolved with 30% H_2O_2 (16 h at 60°C), and counted in a Packard Prias II scintillation counter.

All protein measurements were carried out

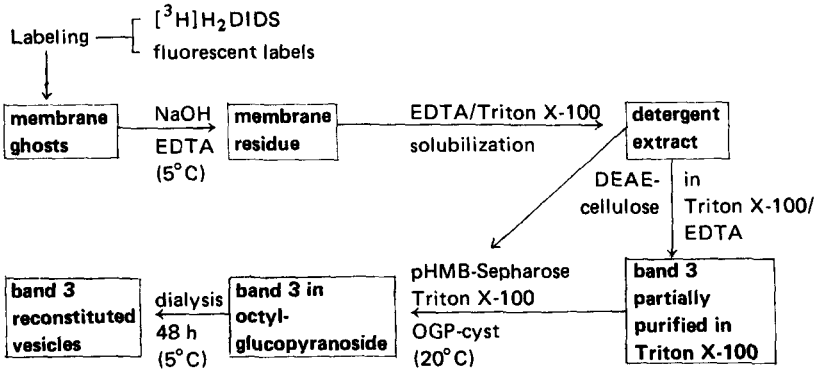

Fig. 1. Schematic representation of band 3 protein isolation and reconstitution. OGP-cyst: octylglucopyranoside in cysteine-containing buffer 2.

according to the SDS method of Lowry et al. [24] and phospholipids according to the method of Stewart [41]. Buffers used throughout this work had the following compositions and final osmolarities (Wescor Instruments): buffer 1, phosphate-buffered saline: 147 mM NaCl/20 mM PO_4 buffer (pH 7.4), unless otherwise specified (310 mosM) or Tris-buffered saline: 140 mM KCl or NaCl/20 mM Tris (pH 7.4) (310 mosM); buffer 2: 36 mM NaH_2PO_4 (pH 7.4) (100 mosM); buffer 3: 32 mM Na_2SO_4/5 mM Tris (pH 7.4) (100 mosM); buffer 4: 100 mM Na_2SO_4/5 mM Tris (pH 7.4) (310 mosM); buffer 5: 5 mM NaH_2PO_4/Na_2HPO_4 (pH 8).

A mixture of anti-protease agents (Sigma) was used at the following final concentrations: PMSF, 20 μM; soybean trypsin inhibitor, 10 μg/ml; pancreatic trypsin inhibitor, 10 μg/ml; TLCK, 10 μg/ml; TPCK, 10 μg/ml; aprotinin, 1 U/ml; benzamidine, 200 μM; and pepstatin A, 10 μg/ml.

Isolation of band 3 polypeptides

A schematic representation of band 3 protein isolation is depicted in Fig. 1. The method includes steps introduced in previous works [8–10,12,13], with appropriate modifications. All steps were carried out at 5°C with solutions gased with N_2 prior to use, unless otherwise specified. In order to follow the concentration and yield of band 3 protein throughout all stages of isolation, reconstitution and anion transport measurements, we used band 3 polypeptides isolated from [^{3}H]H_2DIDS-labeled cells. In this case, cells (10% hematocrit) were labeled with 0.5 μM [^{3}H]H_2DIDS (1 Ci/mmol) for 30 min at 37°C. The labeling yields were usually in the range of $(1–4)\cdot 10^5$ dpm/mg band 3 protein, which represented approximately one H_2DIDS-labeled band 3 per 100 band 3 polypeptides.

Red blood cell ghosts prepared from outdated blood (Hadassah Blood Bank) by hypotonic lysis [25] were washed with buffer 1 (pH 8) to yield a white membrane pellet (membrane ghosts Fig. 1). Alkali extraction of the pellet was carried out for 30 min with 5 vol. of a 2 mM EDTA (pH 9–10) solution. After centrifugation at 40000 × g for 30 min, the procedure was repeated and the final pellet washed with buffer 2 to yield a membrane residue. The latter was extracted at 5°C for 15–18 h with 20 vol. 0.5% (w/v) Triton X-100 in buffer 2, containing 2 mM EDTA and 20 μM butylated hydroxytoluene, under N_2 atmosphere. The supernate obtained after the 30 min 100000 × g spin (detergent extract) was concentrated 5-fold in an Amicon ultrafiltration device, and the resulting suspension was chromatographed on a DEAE-cellulose column (3 ml wet resin/mg detergent-extract protein) at 5°C, and eluted with high ionic strength buffer (150 mM NaCl/150 mM phosphate/0.5% Triton X-100/20 μM butylated hydroxytoluene (pH 7.4)) at room temperature (band 3 (phosphate/Triton)).

Band 3 complexation, substitution of detergent and elution

The band 3 (phosphate/Triton) suspension was

chromatographed at room temperature on a pHMB-Sepharose column (1 mg protein per 1 ml pHMB-Sepharose) at a rate of 0.5 ml/min, in order to allow band 3 complexation. The column was subsequently rinsed with 5 vol. buffer 2 containing 2% octylglucopyranoside and the proteins were eluted with 10 vol. of the same solution, containing 5 mM cysteine. The band 3 octylglucopyranoside suspension was concentrated by ultrafiltration at 5°C.

Reconstitution of band 3 protein into phospholipid vesicles

The lipids asolectin or egg phosphatidylcholine were dissolved first in chloroform/methanol (9 : 1, v/v), dried under N_2, subsequently dissolved in hexane and dried again under an N_2 stream. The whitish film was dissolved in the buffer 2 supplemented with 2% octylglucopyranoside and 20 μM butylated hydroxytoluene by vigorous vortexing and mild sonication on a bath sonicator (Branson 80W). The lipids were subsequently mixed with the concentrated band 3/octylglucopyranoside suspension, and the resulting mixture was dialyzed for 50 h against 1% bovine serum albumin in buffer 2 plus 0.1 mM NaN_3 and a mixture of antiproteases. The turbid suspension resulting after dialysis of band 3 reconstituted vesicles was divided into small aliquots and kept at -70°C until use.

For evaluation of the yield of detergent removed, we omitted the [^{3}H]H_2DIDS labeling of cells. A trace amount of [^{3}H]Triton X-100 was added to the band 3/phosphate/Triton) extract and a trace amount of [^{14}C]octylglucopyranoside was added to the band 3/octylglucopyranoside fraction prior to dialysis.

Isolation and reconstitution of glycophorin and co-reconstitution of glycophorin and band 3

Human glycophorin (blood type A^+ or O^+) was either purchased from Makor Chemicals (Jerusalem) or otherwise prepared from the low ionic strength eluant of the DEAE-cellulose column (Fig. 1). The eluant was chromatographed on a wheat-germ agglutinin-Sepharose affinity column (5 ml gel) prepared as previously described [21], and the detergent substitution and elution with 100 mM *N*-acetylglucosamine (Sigma) in octylglucopyranoside (2%) was carried out similarly to the above band 3 elution.

Reconstitution of either commercial or freshly prepared glycophorin was performed essentially as described for band 3 protein. Co-reconstitution of band 3 protein and glycophorin was performed by mixing equal protein amounts of band 3 protein and glycophorin in octylglucopyranoside, addition of lipids and co-dialysis as described for band 3 protein.

Measurements of anion transport

Measurements of anion transport in reconstituted vesicles were carried out using NBD-taurine as a substrate for band 3 protein as described elsewhere [20]. Briefly, reconstituted vesicles were incubated with 1–2 mM NBD-taurine for 1–2 h at 37°C, to reach transvesicular equilibration, and kept at 5°C. In order to remove extravesicular probe, the vesicles were spun down for 5 min in an Eppendorf microcentrifuge at 5°C. Prior to flux measurements, the residual extravesicular NBD-taurine remaining in the pellet was removed, either by fast filtration through an anion exchange minicolumn (0.5 ml Dowex-citrate in 2 ml disposable syringe at 4°C) or, alternatively, by repeated centrifugation and washings with isotonic ice-cold wash medium (buffer 3 or buffer 4). Flux was initiated by rapid addition of NBD-taurine-loaded vesicles (0.5–2 μg band 3 protein) into a spectrofluorometer cuvette which contained 1.5 ml medium (either buffer 2 or phosphate-buffered saline) and anti-NBD antibody. Flux was monitored in parallel in 2–4 cuvettes from the time of addition of vesicles, and was followed in a quasi-continuous fashion as a time-dependent decrease in fluorescence. In order to reach the value at infinite time (i.e., termination of the experiment), 3 μl aliquots of Nonidet P-40 (10%) were added to the cuvette. The amount of anti-NBD antibodies required for complete quenching of NBD-taurine fluorescence was calibrated prior to flux measurement as previously described [20].

Fluorescence measurements were carried out either with a Perkin-Elmer spectrofluorimeter equipped with a digital voltmeter and a thermostatically controlled compartment which held four cuvette holders connected on a turret or, alternatively, with a Spex Fluorolog II spectrometer con-

nected to a Datamate microprocessor. Data were analyzed with a Wang PCSII calculator using a non-linear least-squares program based on the Levenberg-Marquardt algorithm [26].

Radioactivity was determined on a Prias liquid scintillation counter (Packard) equipped with a microprocessor for dual-isotope counting.

Results

Isolation and reconstitution

The critical step in the technique of integral membrane protein reconstitution is the efficient removal of detergent and its substitution for lipids [27,28]. In our experience, the most useful detergent for band 3 protein solubilization from membranes is Triton X-100. However, this detergent has a low critical micellar concentration (0.01–0.017%, w/v) [29] and, therefore, cannot be easily removed by presently available techniques without sacrificing substantial amounts of the isolated band 3 polypeptides. On the other hand, the detergent octylglucopyranoside, which is a rather poor solubilizer of band 3 protein from membranes but a good dispersant agent after solubilization, shows relatively higher critical micellar concentration (0.75%, w/v) [30], and thus can be easily removed and substituted for lipids by dialysis [31,32].

The method used in this work for band 3 reconstitution is schematically portrayed in Fig. 1. Virtually all the Triton X-100 could be substituted for octylglucopyranoside, while the band 3 protein remained complexed to the organomercurial-Sepharose matrix. Traces of residual [^{3}H]Triton X-100 (less than 0.0005% final concentration) were not significantly different from background level. After addition of phospholipids (1/16–1/40 (w/w) protein/phospholipid), octylglucopyranoside was removed by dialysis at 5°C, using regular dialysis bags. This step was relatively slow but highly efficient and reproducible and, although it could be expedited by using Spectrapor-2 dialysis bags, we observed a large variability in rates and efficiency of these dialysis bags, depending upon the batch, mode of cleaning and tubing diameter.

Upon removal of octylglucopyranoside, the band 3 lipid suspension became increasingly turbid but showed no visible precipitates. After 15 h,

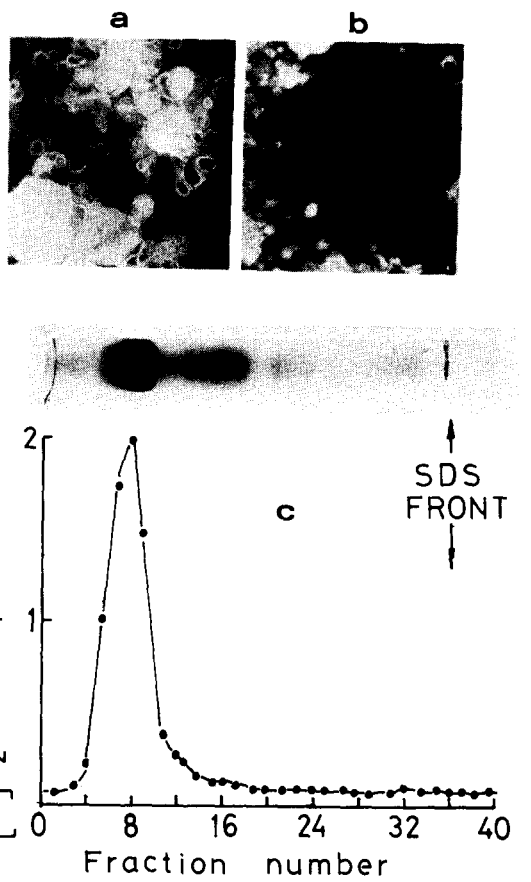

Fig. 2. (a and b). Negative staining of band 3/egg phosphatidylcholine-reconstituted vesicles. Bar denotes 2000 Å. (a) After dialysis (band 3 reconstituted vesicles, Fig. 1). (b) After 1 min sonication of (a). (c) SDS-gel electrophoresis of band 3 reconstituted vesicles from [^{3}H]H$_2$DIDS-labeled cells. Gels were first stained with Coomassie blue (top) and subsequently sliced, dissolved and counted for radioactivity (bottom). Protein loaded on gel: 50 μg. (The average diameter of vesicles in (b) is 200 Å.)

approx. 80% of the original octylglucopyranoside was removed, and after an additional 35 h only traces of it were detected (less than 0.02%). The resulting material was heterogeneous and rather amorphic (Fig. 2a). After a brief sonication (20–60 s), the vesicle population became more homogeneous in size (200–300 Å diameter) (Fig. 2b). However, a residual population of relatively larger vesicles (over 1000 Å diameter) was present in these preparations, as revealed by negative staining electron microscopy. The vesicles were sealed and unilamellar. When addition of external phospholipids to the band 3 (octylglucopyranoside)

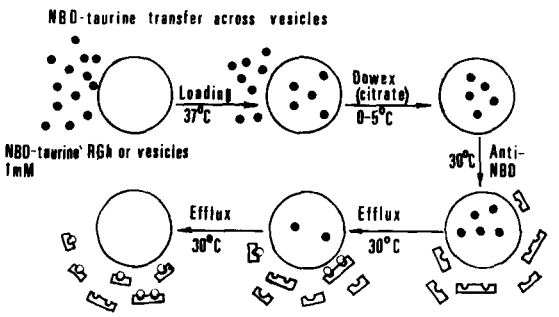

Fig. 3. Schematic representation of the CMTF method. Vesicles were loaded with 1–2 mM NBD-taurine at 37°C and separated from medium at 4°C, either by an anion-exchange minicolumn (Dowex-citrate) or be repeated centrifugations. The substrate loaded vesicles were swiftly transferred to a fluorimeter cuvette containing anti-NBD antibodies as quenchers of extravesicular fluorescence. Fluorescence associated with NBD-taurine decreased with egress of substrate from vesicles to medium. Fluorescence monitoring was conducted in a continuous mode.

suspension (see Fig. 1) was omitted, the final band 3 reconstituted vesicle preparation showed neither visible turbidity nor membranous material in negative staining electron microscopy (not shown). In these conditions the relative amount of phospholipid present in the latter preparation was at the most 40 mol phospholipid/mol band 3 protein as compared to a ratio of 1000–2000 when external phospholipids were added.

The main protein of the band 3 reconstituted vesicle preparation was band 3, as judged by SDS-gel electrophoresis (Fig. 2c). The radioelectrophoretogram of band 3 reconstituted vesicles prepared from [^{3}H]H_2DIDS-labeled cells usually showed no detectable degradation of band 3 polypeptide. In some instances, such as that shown in Fig. 2c, traces of band 4.5 or a minor degradation product of band 3 were detected. However, this represented at the most 3% of the total protein, as observed by Coomassie blue staining of the gel, or 1% of the total label present in the gel. When similar reconstitutions were carried out either with lipids alone or lipids and glycophorin, the reconstituted vesicles after dialysis were also unilamellar and homogeneous in size.

Transport studies

The functional properties of the various vesicles were assessed by the method of CMTF, using the anion substrate analog NBD-taurine and anti-NBD antibodies as extravesicular quencher of fluorescence. The method is schematically portrayed in Fig. 3; details of the technique are given in Materials and Methods. Reconstituted, unsealed vesicles, either containing membrane proteins or not, were loaded with 2 mM NBD-taurine during sonication and by subsequent incubation at 37°C for 2 h.

The medium used in most of the transport studies was buffer 2, similar to that present during dialysis. In some instances, both reconstitution and transport were carried out in phosphate-buffered saline medium. Although the data presented below were obtained in buffer 2, they were very similar to those obtained in phosphate-buffered saline medium. In some experiments unsonicated vesicles were treated with DIDS, a specific inhibitor of red blood cell anion transporter (50 μM at 22°C for 10 min). After removal of excess reagent the vesicles were subjected to sonication as described before.

Typical transport tracings from vesicles recon-

TABLE I

BAND 3 PROTEIN ACTIVITY IN BAND 3/EGG PHOSPHATIDYLCHOLINE RECONSTITUTED VESICLES AND ITS SUSCEPTIBILITY TO INHIBITION BY DIDS AND DNDS

The anion fluxes shown in Fig. 4A were analyzed by non-linear regression analysis according to the two-exponential function (Eqn. 2). The parameters k_1 and k_2 represent the rate constants for the first and second exponential component, P_1 and P_2 represent the associated transport fractions, and r^2 is the determination coefficient.

Treatment	k_1 (min^{-1})($\times 10$)	P_1	k_2 (min^{-1})($\times 10$)	P_2	r^2
Control	4.8 ± 0.30	0.26 ± 0.03	0.43 ± 0.01	0.74 ± 0.007	0.999
$DIDS_{(o)}$	1.9 ± 0.24	0.22 ± 0.01	0.30 ± 0.02	0.78 ± 0.020	0.999
$DNDS_{(o)}$	2.9 ± 0.20	0.20 ± 0.05	0.27 ± 0.05	0.80 ± 0.001	0.996

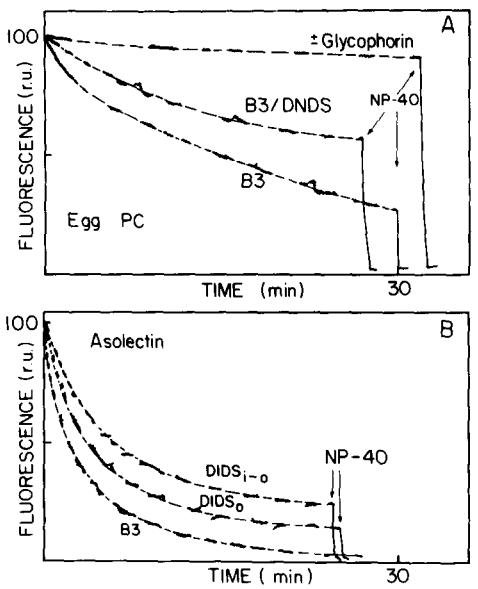

Fig. 4. Efflux profiles of NBD-taurine from reconstituted vesicles. A. Band 3 (B3) and glycophorin were reconstituted with egg phosphatidylcholine (Egg PC) phospholipids at 1 : 15 protein : lipid weight ratio (the ratio refers to each individual protein species), loaded with fluorescent substrate (2 mM NBD-taurine) by sonication and incubation, and analyzed for NBD-taurine transfer. Equivalent amounts of NBD-taurine-loaded vesicles containing band 3 protein, glycophorin or neither of these, were used in this study. The final protein concentrations were 1–2 μg/ml; the medium was buffer 2; and the temperature 30°C. The excitation and emission wavelengths were 478 nm and 550 nm, respectively. The concentration of DNDS in the medium of the indicated system was 20 μM. Fluxes were started at approx. 3-min intervals. In the above profiles they were all shifted to the same zero time. Fluxes were terminated by addition of the detergent Nonidet P-40 (0.2 mg/ml, final concentration). The exponential decay functions based on Eqn. 2 are presented as dashed lines, superimposed on the original fluorescence tracings. The calculated kinetic parameters are given in Table I. B. Band 3 protein was reconstituted with soya-bean phospholipids (asolectin) in a 1 : 40 protein : lipid weight ratio and processed for fluxes as shown in A above, except that the protein concentration was 5 μg/ml and the temperature 35°C. DIDS$_{i-o}$ represents a preparation treated with 20 μM DIDS (37°C) before sealing. DIDS$_o$ represents vesicles treated as in the previous case but after vesicle sealing. The calculated kinetic parameters are shown in Table II.

stituted with either asolectin or egg phosphatidylcholine are shown in Fig. 4 (A and B). The amount of band 3 protein required to obtain a complete flux profile was in the range of 0.5–2 μg protein; the requisite concentration of NBD-taurine was in the range of 50 nM.

Vesicles reconstituted from lipid mixtures with or without glycophorin as control for a non-transporter integral membrane protein showed no significant efflux of NBD-taurine. Conversely, band 3 protein-containing vesicles showed typical exponential efflux profiles which were more than an order of magnitude faster than those lacking band 3. These efflux profiles were analyzed by a non-linear regression program [26] to fit one of the two exponential decay functions:

$$F_{(t)} = F_\infty - (F_\infty - F_0)\, e^{-k_1 t} \quad (1)$$

or

$$F_{(t)} = F_\infty - [F_\infty - F_0][P_1 e^{-k_1 t} + P_2 e^{-k_2 t}] \quad (2)$$

where $F_{(t)}$, F_0 and F_∞ are the fluorescence intensities at times t, zero and infinity, respectively, k_1 and k_2 are the respective rate constants, and P_1 and P_2 the fractions of transport corresponding to either k_1 or k_2 (i.e., $P_1 = 1 - P_2$).

In our experience, the analysis of efflux profiles from band 3/egg phosphatidylcholine vesicles as well as from band 3/asolectin vesicles gave statistically better fits when based on the two-exponential (Eqn. 2) rather than on the single exponential decay function (Eqn. 1). The kinetic parameters resulting from these analyses are shown in Tables I and II, respectively. A visual display of the closeness of the best fits obtained by the non-linear regression analysis of experimental data are given in Fig. 4 as dashed lines superimposed on the original fluorescence traces. The component of transport, which displayed in either egg phosphatidylcholine or asolectin the relatively higher rate constant (i.e., P_1), corresponded to 1/4–1/3 of the total transport displayed by band 3 protein. The corresponding rate constants in both systems were about one order of magnitude higher for the fast component.

Using either preparation, the two components of transport showed partial inhibition as a result of covalent DIDS treatment of sealed vesicles (Tables I and II). In these conditions, we assume that inhibition resulted from DIDS modification of only those band 3 polypeptides which were in the

TABLE II

BAND 3 ACTIVITY IN BAND 3/ASOLECTIN RECONSTITUTED VESICLES AND ITS SUSCEPTIBILITY TO INHIBITION BY DIDS

The anion fluxes shown in Fig. 4B were analyzed as described in Table I.

Treatment	k_1 (min^{-1})($\times 10$)	P_1	k_2 (min^{-1})($\times 10$)	P_2	r^2
Control	8.8 ± 1.3	0.37 ± 0.020	1.3 ± 0.13	0.63 ± 0.03	0.99
$DIDS_{(o)}$	4.9 ± 0.4	0.47 ± 0.025	0.93 ± 0.09	0.53 ± 0.03	0.99
$DIDS_{(i)+(o)}$	2.6 ± 0.3	0.54 ± 0.025	0.29 ± 0.20	0.46 ± 0.02	0.99

right-side-out orientation. Similar results were obtained when fluxes from band 3/egg phosphatidylcholine vesicles were carried out in the presence of DNDS (20 μM), a reversibly acting inhibitor of anion transport [2,14,17]. As expected, DIDS treatment of band 3 vesicles, prior to vesicle sealing, elicited a more pronounced inhibition (Table II), thus indicating that at least 70% of the observed NBD-taurine fluxes were of a specific nature. The remaining 30% could be attributed to lack of modification of transport sites brought about either by rearrangement of the protein or by the presence of already sealed vesicles prior to sonication, or else they could be attributed to nonspecific leaks. The fact that the slower component of transport (P_2) showed an apparently lower susceptibility to inhibition, might indicate that k_2 resulted from a combination of specific (i.e., band 3-mediated) as well as nonspecific transport components, both of which displayed similar rate constants. An estimate of the band 3-mediated transport component could be obtained by subtraction of the nonspecific transport rate remaining after treatment of open membranes (i.e., unsealed vesicles) with DIDS (i.e., DIDS i + 0) in Table II. The latter constituted approx. 30% of k_1-control and 22% of k_2-control (Table II). The fraction of band 3-mediated transport susceptible to externally added DIDS was 40% and 60% of the control for k_1 and k_2, respectively (Table II). Similar results were obtained with DNDS, a non-covalent-reacting analog of DIDS (Table I).

To assess the functional capacity of band 3 polypeptides in the reconstituted state relative to the native state (i.e., in the red blood cell membrane), we computed the specific transfer rates of NBD-taurine in the two different states. Two analytical methods were used for this purpose: method 1 was based on computation of initial efflux rates, v_i (nmol/min), normalized for the amount of band 3 protein (m) (mg) present in the assay, namely,

$$\text{specific transfer rate}_1 = \frac{v_i}{m} \text{(nmol/min per mg)} \quad (3)$$

The initial rates (v_i) were calculated either from profiles such as those shown in Fig. 2, or, alternatively, they were derived directly from continuous profiles stored in the Spex Datamate microprocessor.

The second method (method 2) used to calculate specific transfer rate was based on the computation of the rate of efflux $v = k \cdot s$, where k is the experimental rate constant (Eqn. 2) and s the concentration of NBD-taurine. However, application of this method required normalization of v both for vesicle average dimensions (100 Å radius or 4 pl volume) and for their band 3 protein content (i.e., the average number of band 3 polypeptides per vesicle). Thus:

$$\text{specific transfer rate}_2 = \frac{v}{s_B} \text{(nmol/min per mg)} \quad (4)$$

where s_B is the amount of band 3 (mg) present in 1 liter of vesicles. The specific transfer rate value corresponding to the native system (i.e., red blood cell) was 50 ± 0.5 nmol NBD-taurine/mg band 3 protein per min [19]. Specific transfer rates corresponding to reconstituted egg phosphatidylcholine were taken from six experiments carried out in buffer 2 medium at 37°C and were 39 ± 6 and 24 ± 3 nmol NBD-taurine/mg band 3 protein per min for method 1 and method 2, respectively.

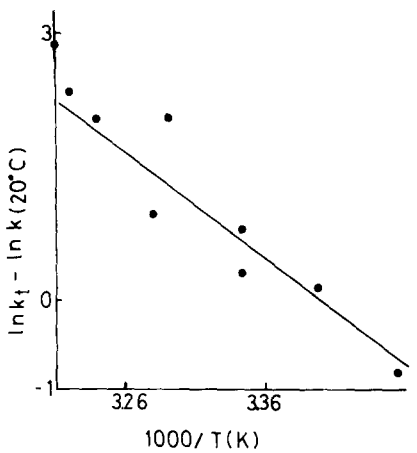

Fig. 5. Energy of activation of band 3 protein-mediated NBD-taurine efflux from band 3/egg phosphatidylcholine calculated from three sets of experiments. Fluxes were carried out in buffer 2 medium in the temperature range of 15–38°C; rate constants ($k_{(t)}$) were normalized with respect to $k_{(t)}$ obtained in each set at 20°C (i.e., $k_{20°C}$). Data analyzed by linear regression gave a value of $E_a = 22 \pm 2$ kcal/mol ($r^2 = 0.922$).

These results clearly indicate that the specific transfer rate values of the native and reconstituted protein are remarkably similar. This is particularly evident with the results based on method 1, which made no assumption regarding vesicle size or band 3 protein density in the vesicles. However, even with method 2, which is subject to gross errors in the estimations of vesicle parameters, results show a major preservation of the functional capacity of band 3 polypeptides in the reconstituted state.

Finally, the rate constants describing band 3 protein-mediated transfer from reconstituted vesicles (band 3/egg phosphatidylcholine) at different temperatures (15–38°C range) were used to determine the activation energy (E_a) of anion transport (Fig. 5). The calculated value of E_a for the reconstituted function was 22 ± 2 kcal/mol ($r^2 = 0.922$ by linear regression analysis). This value was similar to E_a values reported for phosphate fluxes measured in intact cells over the same range of temperatures.

Discussion

Isolation and reconstitution of transport systems are not only essential for ascribing membrane functions to particular polypeptides but also for understanding their underlying molecular mechanism [27,28]. So as to accomplish this goal, it is necessary to isolate the polypeptides in a reasonably pure and fully functional form. Isolation of red cell membrane polypeptides in general, and of band 3 – the purported anion transporter – in particular, have been widely described in the past [2,8–10]. Several attempts have also been made to assess band 3 protein functional properties in the isolated-reconstituted state [9,12–17]. However, as recently indicated [9,14], the techniques available for isolation of band 3 protein and for determination of its functional properties provided only a semiquantitative picture of the functional status of these polypeptides.

This can be attributed primarily to a critical lack of convenient and reliable tools for assessing the function in reconstituted vesicles. The quantitation of anion transport capacity which has classically been given in terms of normalized rate constants [8,12,14,17] is subject to substantial errors due to heterogeneity in vesicle size and protein density, as well as to a variable component of anion leak which is always present in reconstituted systems [12,14,17]. The latter, particularly important in the case of band 3 protein, arises apparently from the presence of residual amount of detergents in the isolated preparations, and/or from band 3 protein interactions with phospholipids. In the case of glycophorin reconstituted vesicles, the lipid-protein interactions were apparently different as judged by the fact that the nonspecific leaks were markedly smaller than those of reconstituted band 3 vesicles (Fig. 4 and Ref. 34). Therefore the present work has focused on the above problems and on the means to overcome them.

Band 3 protein isolation was accomplished by a modification of previously published procedures (Fig. 1), giving apparently intact polypeptides which show a reasonable degree of purity (more than 98% by densitometry of stained and highly overloaded gels). The only contaminant found occasionally in the system was band 4.5 (Fig. 3), which is claimed by some authors [35,36] to be the genuine red blood cell glucose transporter and by others [37] to be an endogenous band 3 proteolytic product. To what extent the presence of [^{3}H]H_2DIDS label (1–2%) in the band 4.5 area

can be attributed to proteolytic degradation, remains to be explored. Nevertheless, since the contamination in the final band 3 protein preparations, which never exceeded 3% of the total protein, had no apparent effect on the functional properties of the system, we disregarded it in all studies reported here.

In this work we introduced a new method for reconstitution of functional polypeptides from suspensions of Triton X-100, hitherto the most selective and efficient non-ionic detergent for band 3 protein extraction from membranes. The method relied on the complexation of band 3 proteins to solid supports (i.e., ion-exchange and affinity column), which facilitated both elimination of red blood cell lipids and substitution of Triton X-100 for the dialyzable detergent octylglucopyranoside (Fig. 1). In our experience, functional reconstitution of band 3 protein into phospholipid vesicles was best accomplished using octylglucopyranoside. Substitution of this detergent for the more classical cholate or deoxycholate detergents, resulted in vesicles that were extremely leaky to anions. Unlike in previous studies [12,14,15], the phospholipids which provided the most suitable matrix for band 3-mediated function in this work were of a heterogeneous nature, i.e., asolectin (soya-bean phospholipids) or egg phosphatidylcholine. For reasons that are as yet not understood, similar reconstitutions into defined species of synthetic phospholipids of variable chain length and/or degree of saturation were unsuccessful. However, these results are apparently not unique to band 3 polypeptides, as similar observations have been made with other functional membrane systems [27,28]. The vesicles which resulted after mild sonication showed an average radius of 100 Å in negative stain preparations (Fig. 2B).

The average protein density in the final preparation was estimated as 1–2 band 3 monomers per vesicle, assuming a 1 : 2000 protein-to-lipid molar ratio, and the average area of either 40 $Å^2$ occupied by a phospholipid molecule or $2 \cdot 10^4$ $Å^2$ occupied by a band 3 dimer. However, at present, the distribution of protein in vesicles, let alone the state of band 3 polymerization and its relevance to the function, cannot be specified. These points have recently been emphasized in the reconstitution of the Ca^{2+}-ATPase [38] which showed that excess addition of phospholipids to protein/detergent mixtures did not result in the dilution of the protein upon reconstitution but, rather, in formation of protein-free vesicles. In the present study, an approximately equal fraction of band 3 polypeptides showed right-side-out orientation and some inside-out orientation, as observed from the susceptibility of the reconstituted function to inhibition by non-penetrating agents such as DIDS or DNDS (Figs. 4A and B).

The most critical test for evaluating a reconstituted transport system relies on the determination of its functional capacity relative to that of the native protein. Previous approaches to that question relied mainly on the use of rate constants obtained from experimental profiles of anion transfer across vesicles and their normalization for surface/volume and for band 3 protein density in the membrane matrix [12]. Although a similar approach was also used in the present study (Table II, method 2), it should be noted that the estimations of those normalization factors are subject to large errors due to the heterogeneity of band 3 protein density and distribution of vesicle size.

In order to obtain a more reliable measure for the functional capacity of the reconstituted system, we computed the specific transfer rate values from the initial transfer rates (v_i) provided by the CMTF profiles normalized only for band 3 protein present in the assay. In our experience, it was only with the CMTF method that the initial transfer rates could be measured with the requisite degree of accuracy. The advantages of this methodology reside in its high sensitivity, in terms of time resolution, and amount of assayed material. Moreover, the fact that the fluxes were monitored in a continuous fashion, enabled, on the one hand, the use of only 0.5–2 μg band 3 protein per flux assay but, more importantly, it enabled the accumulation and storage of an infinitely large number of experimental points for analytical purposes.

The reconstituted band 3 protein showed functional specificity for the fluorescent substrate analog NBD-taurine but not to other NBD-derivatives such as NBD-alanine (not shown). Specificity was also observed in the susceptibility of the reconstituted function to specific inhibitors of anion transport such as DIDS and DNDS (Figs. 4A and 4B). Moreover, the fact that reconstituted vesicles

containing phospholipids, either alone or together with glycophorin, failed to mediate NBD-taurine transfer, provided additional support for the role of band 3 protein in mediating anion transfer in reconstituted vesicles.

The results demonstrate a high degree (more than 80%) of functional preservation of anion transport in the reconstituted band 3/egg phosphatidylcholine. This indicates that, on the average, band 3 polypeptides have fully retained their transport capacity in the reconstituted state. This capacity was equally expressed in either low ionic strength, buffer 2 medium or in the higher ionic strength phosphate-buffered saline medium. Specific transfer rate values for the native system are available in phosphate-buffered saline but not in buffer 2, because reliable kinetic data at such low ionic strength are not easily attainable with the intact system (i.e., cells or ghosts). In contrast to a previous report [16], the present work shows that glycophorin, co-reconstituted with either phospholipids alone or with band 3 protein and phospholipids, had no demonstrable effect on the anion transport capacity.

Finally, the functional capacity of the reconstituted system was assessed in terms of its temperature dependence. According to a recent study [39,40], tracer NBD-taurine transport profiles reflect, to a large extent, properties of the anion transport system as determined by the predominant anion present in the medium. This was indicated also to be the case for E_a, the energy of activation of anion transport. In the present work, the calculated E_a of 22 ± 2 kcal/mol found with the reconstituted band 3 protein in phosphate medium (15–38°C range) (Fig. 5), was in the range of published values of E_a for phosphate exchange in the intact system, i.e., 28 [42], 29 [43] and 20 Kcal/mol [33]. This finding agrees qualitatively with recent reports on the similarity of E_a of sulfate exchange in native and reconstituted band 3 [12,14,15,17]. This suggests that, with the present method of reconstitution into the aforementioned mixture of phospholipids, the isolated band 3 protein has largely preserved the functional properties that are characteristic of the native state.

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