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Purification of Stabilized Band 3 Protein of the Human Erythrocyte Membrane and Its Reconstitution into Liposomes†

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ABSTRACT: The red cell membrane protein, identified on gel electrophoresis as band 3, has been implicated with anion transport. We report here a new rapid procedure for obtaining a stable, functional band 3, essentially free from all other membrane proteins. Red cell ghosts were washed with isotonic saline and solubilized overnight in 0.5% Triton X-100. This extract was applied to a DEAE-cellulose column, and bands 3 and 4.2 and glycophorin (PAS-1) were eluted with high salt concentration. This high-salt fraction was applied to a [[*p*-(chloromercuri)benzamido]ethyl]agarose 4B gel (synthesized according to a modification of the method of Cuatrecasas [Cuatrecasas, P. J. (1971) *Methods Enzymol. 22*, 345–378]) which removed glycophorin and some band 4.2. Pure band 3 was eluted with 0.1 mM cysteine after a low-salt wash. Addition of 15 mM mercaptoethanol immediately after elution was found to prevent protein aggregation. Preparations routinely containing at least 95% band 3 (~1% dimer) and less than 1.5% band 4.2 have been obtained. It should also be noted that this affinity gel can be used to isolate pure glycophorin. Band 3, according to sedimentation analysis on sucrose gradients, existed as a dimer in buffer from which Triton was removed and as a mixture of monomer and dimer in excess Triton. After removal of Triton, band 3 incorporated into liposomes composed of 96% phosphatidylcholine and 4% phosphatidic acid increased sulfate efflux more than 70-fold over that of the control. When 10 μM 4,4′-diisothiocyano-2,2′-stilbenedisulfonate was added to the outside of band 3 containing liposomes, 30–40% of the efflux was inhibited. When the inhibitor was added to the protein before incorporation, nearly complete inhibition was attained. When transport was carried out with no external transportable anions, sulfate efflux was markedly reduced. Influx into liposomes containing band 3 was measured, and a turnover number was calculated which was 24% of the value found for the intact red cell. When stored in 15 mM mercaptoethanol, band 3 remained functional and monomeric for at least a week.

The evidence implicating band 3 of the human erythrocyte membrane as the anion-exchange protein is quite substantial (Knauf, 1979). An important part of this proof has been the isolation and reconstitution of this protein into liposomes and the subsequent demonstration of its ability to mediate anion transport (Rothstein et al., 1975; Ross & McConnell, 1977, 1978). Several laboratories have accomplished this; however, the band 3 preparations in each case have been contaminated with other membrane proteins, notably bands 4.2 and 4.5 and glycophorin (PAS-1). An additional problem has been that isolated band 3 preparations are notoriously unstable and rapidly form high molecular weight aggregates (Yu & Steck,

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1975b).

In order to study the relationship between structure and functionality of the anion-exchange protein, it would be desirable to obtain a pure stabilized band 3 protein which is still functional. In this paper, we report a new quick method for the purification of a functional band 3 protein essentially free from all other contaminants. This method employs the use of a [[*p*-(chloromercuri)benzamido]ethyl]agarose 4B affinity gel, which was synthesized by significant modifications of the method of Cuatrecasas (1971). The *p*-(chloromercuri)-benzoate (pCMB)[1] affinity gel has an additional advantage in that it can also be used to easily isolate pure glycophorin. We also report that band 3 can be stored in low Triton concentrations (0.02–0.05%) in the presence of mercaptoethanol for at least a week and still maintain its ability to increase sulfate efflux when incorporated into liposomes.

Materials and Methods

Materials. L-Cysteine hydrochloride (hydrate), 1,1-diethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride, *p*-(chloromercuri)benzoic acid, dimethyl suberimidate dihydrochloride, L-α-phosphatidylcholine (L-α-lecithin, egg yolk), and L-α-phosphatidic acid (grade I, egg yolk) were obtained from Sigma. Ethylenediamine dihydrochloride was obtained from Eastman. Radioactive sulfate ($Na_2{}^{35}SO_4$, 2 mCi) was purchased from New England Nuclear. Sephadex G-50-300 was purchased from Pharmacia. For the affinity gel, either agarose 4B (Pharmacia) or Bio-Gel A-15m (Bio-Rad) was used.

Analytical Procedures. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. For samples containing Triton X-100, 3% sodium dodecyl sulfate was added to the alkaline copper reagent to prevent the formation of a yellow precipitate (Yu & Steck, 1975a). Protein associated with liposomes was determined by dissolving pelleted liposomes (100000*g* for 2 h) in 10% sodium deoxycholate and 0.01 N NaOH (0.1 mL added to a 0.4-mL sample) and performing a Lowry method with control liposomes (no protein) as a blank. Gel electrophoresis was performed according to the method of Fairbanks et al. (1971), but 7.5% and 4.0% polyacrylamide gels were used instead of 5.6%. Sialic acid was determined according to the method of Warren (1959, 1963) following hydrolysis of protein at 80 °C for 1 h in 0.1 N H_2SO_4.

Preparation of [[p-(Chloromercuri)benzamido]ethyl]-agarose 4B. To a 100-mL mixture of washed agarose 4B and water (equivalent volumes) was added 5.0 g of CNBr partially dissolved in 20 mL of water, and the pH was adjusted to and kept at 11.0 with 4 N NaOH. The temperature was maintained at 20 °C by the addition of ice, and after 13 min a large amount of ice was added, and the gel was filtered and washed with 1.5 L of cold water. It should be noted that the washing procedure should take no longer than 2 min since the CNBr-"activated" agarose is unstable. Ethylenediamine dihydrochloride (5.36 g, 40.2 mmol) dissolved in 100 mL of cold water (pH adjusted to 10.0 with 4 N NaOH) was quickly added to the gel, and this was stirred gently at 4–8 °C for 16 h. The gel was then washed with about 4 L of water at room temperature until it was free of the ethylenediamine as determined by the 2,4,6-trinitrobenzenesulfonate color test (Cuatrecasas, 1971). To 50 mL of aminoethylagarose 4B in 90 mL of 40% dimethylformamide (DMF) was added 1.00 g (2.81 mmol) of *p*-(chloromercuri)benzoic acid, and the pH was adjusted to 4.80. 1,1-Diethyl-3-[(dimethylamino)-propyl]carbodiimide (1.35 g; 7.04 mmol) was then added, and the suspension was stirred gently for 1 h at room temperature, maintaining the pH at 4.80 with 1 N HCl. The reaction was allowed to proceed overnight at room temperature. The gel was then filtered, washed with 4 L of 0.1 M $NaHCO_3$, pH 8.80, over a 6–8-h period, and then washed with water. After the washed gel was suspended in 90 mL of 40% DMF, the pCMB coupling procedure was repeated, including the washing steps, as described above. The gel can be stored in water with 0.02% sodium azide at 5 °C for 2–3 months.

Preparation of Ghosts. Fresh human blood collected in tubes containing EDTA as the anticoagulant was used for all ghost preparations. All steps were carried out at 0–5 °C. An equal volume of 0.15 M NaCl and 5 mM Na_2HPO_4, pH 8.0, was added to the blood, and this was centrifuged at 120*g* for 15 min. The supernatant and buffy coat were removed, and the cells were resuspended in 4 volumes of buffer and centrifuged as indicated above. The red cells were then washed twice more but were centrifuged at 480*g* for 15 min. The red cells were hemolyzed in 40 volumes of 5 mM Na_2HPO_4, pH 8.0, and centrifuged at 21000*g* for 20 min. After removal of the supernatant, the ghosts were allowed to move away from the pellet beneath them, and this pellet was then removed. The ghosts were washed 3 times each with 10 volumes of 5 mM Na_2HPO_4, pH 8.0, and centrifuged at 21000*g* for 20 min. After the final washing, the ghosts were milky white in appearance.

High-Salt Wash and Triton X-100 Extraction. Ghosts were suspended in 6 volumes of 0.15 M NaCl and 5 mM Na_2HPO_4, pH 8.0, for 20 min at 0 °C and were centrifuged at 27000*g* for 20 min. The supernatant was removed, and the procedure described above was repeated. The pellet was dissolved in 3 volumes (based on the volume of ghosts) of 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.5, and kept overnight at 0 °C. This mixture was centrifuged at 27000*g* for 20 min; the supernatant was removed and concentrated 4–5 times (Amicon ultrafiltration) before being applied to an ion-exchange column.

Ion-Exchange Chromatography. DEAE-cellulose obtained from Bio-Rad (Cellex D) was prepared for use by washing successively in 10 volumes of 0.5 N KOH, water, 0.4 M KH_2PO_4, water, and 36 mM K_2HPO_4 and 0.5% Triton, pH 7.50. In a typical experiment, 75 mL of Triton extract concentrated (Amicon ultrafiltration, PM 10 membrane) to 15 mL was applied to the Cellex D column (1.7 × 15 cm). The column was washed with 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.50, and some protein eluted at this time. The remaining protein was eluted with 150 mM Na_2HPO_4, 150 mM NaCl, and 0.5% Triton, pH 7.50, and this fraction which included band 3, band 4.2, and glycophorin was applied to the pCMB affinity column.

[[p-(Chloromercuri)benzamido]ethyl]agarose 4B Chromatography. The above fraction was immediately applied to the affinity column (2 × 1 cm) at a flow rate of 30 mL/h. (All subsequent steps were carried out at the maximum flow rate.) The column was washed with 20 mL of 150 mM Na_2HPO_4, 150 mM NaCl, and 0.5% Triton, pH 7.50, and 30 mL of 36 mM Na_2HPO_4 and 0.5% Triton, pH 7.50. Pure band 3 was eluted with 0.1 mM cysteine, 36 mM Na_2HPO_4, and 0.5% Triton, pH 7.50 (prepared just before use), and was collected in approximately 25 mL. This fraction was then concentrated to 6–8 mL by Amicon ultrafiltration (PM 10 membrane) after the immediate addition of 15 mM mercaptoethanol.

[1] Abbreviations used: pCMB, *p*-(chloromercuri)benzoate; EDTA, ethylenediaminetetraacetate; CNBr, cyanogen bromide; DMF, dimethylformamide; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; $NaDodSO_4$, sodium dodecyl sulfate.

Removal and Determination of Triton X-100. Triton X-100 was removed from the band 3 preparations by extraction with lecithin/toluene or with Bio-Beads SM2 (Bio-Rad). Protein solution (1 volume) was vortexed for 30 s with 1 volume of a lecithin/toluene (1 mg/mL) solution, and this was centrifuged for 3 min at 4000*g*. The top layer (toluene) was removed by suction, and the above procedure was repeated twice. The remaining toluene was removed by passing nitrogen through the solution (Rothstein et al., 1975). Alternatively, 3.0 mL of protein solution was mixed with 1.5 g of Bio-Beads for 3 h at 4 °C (Holloway, 1973). Prior to use, Bio-Beads were treated with methanol (Holloway, 1973), washed, and equilibrated with 36 mM Na_2HPO_4 and 15 mM mercaptoethanol, pH 7.50. Triton X-100 was determined according to the method of Garewal (1973) with several modifications. To 0.2 mL of sample was added 0.2 mL of 50% ethanol followed by the addition of 0.4 mL of the ammonium cobaltothiocyanate reagent. The mixture was allowed to stand for 5 min, then placed on 0.5 mL of Versilube F50 (Harwick), and sedimented in an Eppendorf centrifuge at 8000*g* for 2 min. The upper phase was removed by suction, and the remaining pellet containing the chromophore was dissolved in 1.0 mL of dimethylformamide (DMF). Excess oil was removed by an additional centrifugation (oil droplets settle to the bottom), and the DMF solution was read at 600 nm. Linear standard curves were obtained between 100 and 1000 μg of Triton X-100. Sulfate efflux from liposomes lacking band 3 protein was increased by the addition of Triton X-100 at concentrations of 0.1% and above (Feinstein et al., 1977). Band 3 preparations incorporated into liposomes contained less than 0.04% Triton.

Incorporation of Band 3 into Liposomes and Measurement of Sulfate Efflux. To a 50-mL round-bottom flask was added 2 mL of chloroform containing 96 mg of lecithin and 4 mg of phosphatidic acid. The flask was rotated under vacuum and a stream of nitrogen to obtain a thin layer coating the flask. Glass beads, 50 μL of radioactive sulfate (10–15 μCi) and 2.0 mL of Triton-removed protein solution (0.5–1.5 mg of protein, 36 mM Na_2HPO_4, 10 mM Na_2SO_4, 5 mM KCl, and 15 mM mercaptoethanol, pH 7.50), were added, and the contents were swirled until the lipids were suspended. The suspension was sonicated for 10 min in a Bransonic 52 bath sonicator, kept overnight at 4 °C, and sonicated one more time for 10 min. The liposomes were passed through a Sephadex G-50 column (1.7 × 45 cm) at 4 °C to remove free radioactivity from trapped radioactivity (Feinstein et al., 1977). About 8 mL of liposomes was collected, and 2-mL aliquots were placed in 0.25-in. dialysis tubing prepared for use by boiling in distilled water for 15 min. The dialysis tubes were placed in screw-capped vials which contained 10 mL of transport buffer (36 mM Na_2HPO_4, 10 mM Na_2SO_4, and 5 mM KCl, pH 7.50). The vials were rotated (10 rotations/min), 0.5-mL aliquots were drawn from the vials at designated times, and 0.5 mL of transport buffer was added to maintain the volume at 10 mL. Transport was carried out in a warm room at 37 °C.

Measurement of Sulfate Influx into Liposomes. Liposomes containing 0.6 mg of band 3, 50 mg of lecithin, and 2 mg of phosphatidic acid were prepared as described above but without trapped radioactive sulfate and without Sephadex G-50 filtration. To 100 μL of liposomes at 37 °C was added radioactive sulfate in 100 μL of transport buffer at 37 °C. The liposomes were shaken in a temperature-controlled shaker bath for the appropriate time, and then the liposomes were immediately put into an ice bath. The liposomes cooled to 0 °C were then added to a 25-mL buret containing 25 mL of Sephadex G-50 equilibrated with the nonexchangeable anion 85 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) at 4 °C, pH 7.50. Liposomes were eluted in 2 min with the same buffer, and a clear separation from free radioactivity was obtained. The trapped radioactive sulfate in the liposomes was then counted.

Rate Zonal Sedimentation Analysis. Linear 5–20% (w/v) sucrose gradients (5 mL) in 36 mM Na_2HPO_4, pH 7.50, were overlaid with 0.5 mL of protein solution (0.50–0.75 mg/mL) and centrifuged for 4.5 h at 49 000 rpm at 5 °C in a Spinco SW 50.1 rotor with the brake off. When band 3 was analyzed, gradients contained 15 mM mercaptoethanol as well. Gradient fractions (0.30 mL) were collected by puncturing the bottom of the tubes and collecting drops forced out by air pressure. The following were used as reference proteins: cytochrome *c* (13 000 daltons), bovine serum albumin (68 000 daltons), yeast alcohol dehydrogenase (150 000 daltons), and catalase (250 000 daltons). Protein was determined with the Bio-Rad reagent.

Cross-Linking of Band 3. Cross-linking was carried out with dimethyl suberimidate (0.67 mg/mL final concentration) in 0.1 M ethanolamine on 50 μg of band 3 protein for 1 h at room temperature. Reaction was stopped by the addition of Na-$DodSO_4$ to a final concentration of 1%. Band 3 was then analyzed on 4% polyacrylamide gels.

Results and Discussion

The major difficulties in purifying a functional band 3 have been (1) the removal of glycophorin and band 4.2 (strongly associated with band 3) in ways that do not denature the protein and (2) the marked tendency of the isolated protein to rapidly aggregate. Yu & Steck (1975a) displaced band 4.2 from band 3 in Triton X-100 extracts by the addition of 3 mM pCMB. Band 3 could then be separated from band 4.2 on an aminoethylcellulose column. Glycophorin could be eluted prior to band 3. Although the purity of the preparation was estimated to be at least 97% with an overall recovery of 15–20%, no attempt to reconstitute the protein to determine functionality was made. The band 3 utilized by Ross & McConnell (1977, 1978) in reconstitution studies was purified on a concanavalin A affinity column (Findlay, 1974) to greater than 90% purity, but contamination with glycophorin and band 4.2 was common. In addition, in order to show reconstituted transport activity, they added glycophorin as well as band 3 to their liposomes. Since their liposomes contained almost 3 times as much glycophorin as band 3 on a mole to mole basis, it is not clear from their studies whether glycophorin as well as band 3 is necessary for anion transport. They did show, however, that glycophorin alone was not responsible for their transport activity. Kahlenberg (1976) obtained band 3 (95% pure) by chromatography on an activated thiol–Sepharose 4B column. However, ghosts were treated with dimethylmaleic anhydride prior to Triton extraction, and this treatment probably inactivates the protein since this amino-reactive reagent has been reported to specifically block anion permeability (Knauf & Rothstein, 1971). No reconstitution studies were reported with their purified preparation.

In the present study, band 3 was purified from ghosts which were first washed with isotonic saline to remove band 6 (glyceraldehyde-3-phosphate dehydrogenase) and then extracted overnight with 0.5% Triton. The Triton extract was concentrated and applied to a DEAE-cellulose column to remove lower molecular weight proteins (mostly in the band 4.5 region). Higher salt concentrations were then used to elute a fraction containing mostly band 3, band 4.2, and glycophorin,

and this fraction was then passed through a [[*p*-(chloromercuri)benzamido]ethyl]agarose 4B affinity column. During this time, glycophorin and some band 4.2 eluted. The column was then washed with the same high-salt buffer in which the protein was applied and with a low-salt buffer before pure band 3 was eluted with 0.1 mM cysteine. Some additional band 3 could be eluted with 50 mM cysteine, but this fraction contained some band 4.2 as well. Upon elution of band 3, 15 mM mercaptoethanol was added immediately as this was found to keep the protein from aggregating. With the above method, pure band 3 could be isolated from the Triton extract in less than 4 h.

It should be pointed out that although pCMB affinity gel can be purchased (Bio-Rad, Affi-Gel 501) or synthesized by the method of Cuatrecasas (1971), both preparations were found to be unsuitable for the purification of band 3 protein because of excessive nonspecific binding of glycophorin probably to spacer not coupled to pCMB. Several modifications of the Cuatrecasas procedure eliminated this problem: (1) The length of the spacer arm was decreased by employing ethylenediamine hydrochloride rather than 3,3′-diaminopropylamine. (2) Less CNBr was used to activate the gel, and the concentration of the spacer was reduced so as to decrease the amount of ethylamino groups on the agarose. (3) Coupling of amino groups with pCMB by carbodiimide activation was carried out twice to ensure complete substitution of free amino groups. The substituted agarose produced in this way did not bind glycophorin at pH 7.50. With Affi-Gel 501 or the Cuatrecasas affinity gel, this could be accomplished only at pH 3.50 (Shami et al., 1977). The success of these modifications was monitored during synthesis of the gel by the trinitrobenzenesulfonate test which determines free amino groups (Cuatrecasas, 1971). The amount of sialic acid contained in band 3 Triton extracts after passage through this gel was determined to see whether these modifications resulted in a preparation essentially free of glycophorin. From the results of three separate experiments, it was determined that there was some residual sialic acid (19–29 nmol/mg of protein). This sialic acid was probably attached to the band 3 protein and not due to a glycophorin contamination since (a) an average molar ratio of sialic acid to protein of 2.2 was obtained, and this is similar to values of sialic acid reported to be found in band 3 protein (Ho & Guidotti, 1975; Fukuda et al., 1978, 1979), and (b) band 3 preparations after $NaDodSO_4$–polyacrylamide gel electrophoresis did not show any staining with the periodic acid–Schiff (PAS) reagent.

It should be noted that this modified affinity gel can also be used to purify glycophorin. In the procedures used above to purify band 3, Triton extracts were applied to the gel in high-salt concentrations (150 mM Na_2HPO_4 and 150 mM NaCl), and some band 4.2 comigrated with the glycophorin. However, if lower salt concentrations were used (36 mM Na_2HPO_4), glycophorin was eluted by itself. A densitometric scan of a gel of pure glycophorin (PAS-1 and PAS-2) isolated in this way and stained for carbohydrate with PAS reagent is shown in Figure 1. Similar gels stained with Coomassie Blue indicated the presence of no other proteins.

A summary of the various fractions obtained during the isolation procedure is shown in Table I. The overall yield of pure band 3 was 25%. When the band 3 preparations were run on 7.5% polyacrylamide gels (Figure 2A,B), stained with Coomassie Blue, and scanned, they routinely contained at least 95% band 3 (~1% dimer) and less than 1.5% band 4.2. Some proteolytic fragments (less than 2.5%) were also seen. These percentages were obtained from gels that were purposely overloaded (50 μg) in order to detect contaminants. The higher molecular weight polypeptide seen on these gels was band 3 dimer and not spectrin since no spectrin was originally extracted with Triton X-100. This was confirmed by running 4% polyacrylamide gels (Figure 2C–F) on which the dimer runs between the monomer and spectrin. The ratio between monomer and dimer remained constant over a period of a week in low Triton buffer in the presence of mercaptoethanol. An amino acid analysis was performed on band 3, and the results are given in Table II.

It is well-known that band 3 runs as a monomer with a molecular weight of about 93 000 on $NaDodSO_4$–polyacrylamide gels (Steck, 1978), but it may exist in higher molecular weight forms in the absence of $NaDodSO_4$. In order to de-

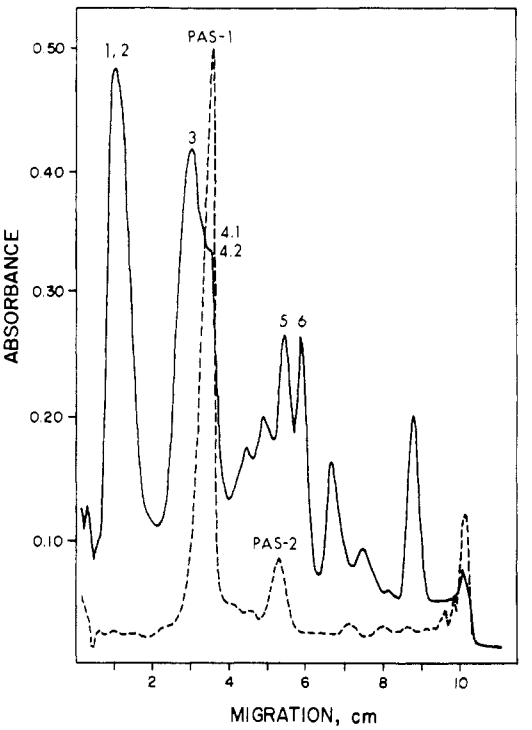

FIGURE 1: Densitometric scans of $NaDodSO_4$–polyacrylamide (7.5%) gels of total red cell membrane protein (—) and glycophorin (---) which was subsequently isolated by passage of a Triton extract of red cell ghosts through the [[*p*-(chloromercuri)benzamido]ethyl]agarose 4B column. Gel containing glycophorin was stained with PAS reagent, and gel containing ghost protein was stained with Coomassie Blue.

Table I: Analysis of Fractions Obtained during the Purification of Band 3

fraction	amount of protein (mg)	principal bands on $NaDodSO_4$–polyacrylamide gel electrophoresis
ghosts (25 mL)	64	all
isotonic saline wash supernatant	7.5	band 6
isotonic saline wash pellet	56.5	all minus band 6
Triton extract	20.8	bands 3, 4.2, and 4.5 and glycophorin
DEAE-cellulose low-salt elution	2.8	band 4.5 and hemoglobin
DEAE-cellulose high-salt elution	12	bands 3 and 4.2 and glycophorin
[[*p*-(chloromercuri)benzamido]ethyl]agarose 4B elution in high salt with no Cys	2.1	band 4.2 and glycophorin
[[*p*-(chloromercuri)benzamido]ethyl]agarose 4B elution in low salt with 0.1 mM Cys	4	band 3

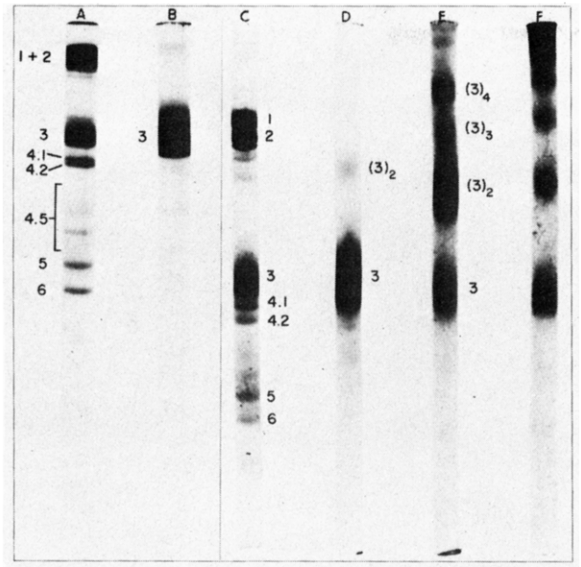

FIGURE 2: $NaDodSO_4$–polyacrylamide (7.5%) gel electrophoresis of (A) red cell ghosts (50 μg) and (B) purified band 3 (50 μg). $NaDodSO_4$–polyacrylamide (4%) gel electrophoresis of (C) red cell ghosts (50 μg), (D) band 3 (50 μg) in Triton-removed buffer (identical pattern seen with band 3 in Triton), (E) band 3 (50 μg) in Triton cross-linked with dimethyl suberimidate, (F) band 3 (50 μg) in Triton-removed buffer cross-linked with dimethyl suberimidate. Cross-linking was carried out for 1 h at 20 °C.

Table II: Amino Acid Analysis of Band 3

		Fukuda[a]	Steck[b]	Ho[c]
basic				
Lys	3.3	3.6	3.4	4.7
His	1.9	2.3	2.2	1.9
Arg	5.4	5.3	5.2	4.8
	10.6	11.2	10.8	11.4
acidic				
Asp	6.8	6.9	6.5	7.5
Glu	12.4	11.2	11.9	10.9
	19.2	18.1	18.4	18.4
neutral				
Thr	4.9	5.2	5.0	5.3
Ser	6.4	7.4	6.8	5.8
Pro	6.7	5.6	5.7	5.5
Gly	7.5	7.5	7.7	7.5
Ala	7.2	7.1	7.4	8.3
Cys				
	32.7	32.8	32.6	32.4
hydrophobic				
Val	7.4	6.8	7.6	7.3
Met	2.2	3.2	2.2	1.9
Ile	4.6	4.5	4.7	4.7
Leu	15.2	14.4	13.4	14.1
Tyr	2.4	2.9	3.1	2.50
Phe	5.6	5.8	5.5	5.6
Trp				
	37.4	47.6	36.5	36.1

[a] Fukuda et al. (1978). [b] Steck (1978). [c] Ho & Guidotti (1975).

termine this, band 3 preparations before and after Triton removal by toluene/lecithin extractions (Rothstein et al., 1975) were analyzed by sedimentation on 5–20% sucrose gradients. Band 3 with Triton removed ran with a molecular weight of 320000, which probably reflects a dimer of band 3 complexed with bound detergent. This result agrees with the analysis of Reithmeier (1979), who found that band 3 and bound Triton (0.77 mg of Triton X-100/mg of protein) ran as a complex of 320000, with the molecular weight of the protein dimer alone being 175000. Reithmeier determined the molecular weight of the protein dimer by calculating the amount of bound radioactive Triton and subtracting the detergent molecular weight from 320000. Clarke (1975) also found the same value for the molecular weight by using band 3 prepared by the procedure of Findlay (1974). Isolated band 3 protein prepared according to Yu & Steck (1975a) sedimented in sucrose-density gradients to an apparent $s_{20,w}$ value of 7.6 S (Yu & Steck, 1975b). It was calculated that a 176000 band 3 dimer would have this sedimentation value if its frictional coefficient were 1.3 or it formed a spherical complex with 0.85 mg of Triton X-100/mg of protein (Yu & Steck, 1975b). When band 3 in the presence of excess detergent was analyzed, we obtained two fractions containing band 3 corresponding to molecular weights of 175000 and 290000. The relative percentages of protein found in these fractions were 60% and 40%, respectively. In this case, band 3 was probably present mostly as a complex of monomer (85000–90000 daltons) and bound Triton. The 290000-dalton component corresponds to protein dimer plus bound Triton.

Both of these band 3 solutions were cross-linked with dimethyl suberimidate for 1 h at 20 °C (Davis & Stark, 1970). It was expected that, since band 3 in the Triton-removed solution was initially in a higher molecular form than the band 3 in the excess Triton solution, it should form the higher molecular weight aggregates over the same period of time. After cross-linking and analyzing the protein on 4% $NaDodSO_4$–polyacrylamide gels (Figure 2E,F), it was found that band 3 in the Triton-removed solution formed less dimer, trimer, and tetramer but more aggregates with molecular weights greater than that of the tetramer. It is not known whether the low detergent concentration or the presence of lecithin, which replaced the detergent in the extraction procedure, is responsible for maintaining the band 3 in the dimeric form.

Band 3 with Triton removed either with toluene/lecithin extraction (Rothstein et al., 1975) or with Bio-Beads (Holloway, 1973) was incorporated into liposomes prepared by sonication. Radioactive sulfate was added before the formation of liposomes, and free radioactivity was removed from trapped radioactivity by Sephadex G-50 chromatography at 4 °C (Feinstein et al., 1977). Little transport occurs at this temperature. Liposomes contained 4% phosphatidic acid to prevent aggregation and thereby facilitate the passage through the Sephadex columns. As seen in Figure 3A,B, band 3 incorporated into liposomes greatly increased sulfate efflux at 37 °C over control liposomes containing no protein. If 10 μM DIDS was added to the outside of the liposomes containing band 3, 30–40% of the sulfate efflux could be inhibited. Only a 50% inhibition may be possible since the protein may be oriented in two ways, one with the inhibitory site (normally on the outside in the intact cell) on the inside of the liposome and thereby inaccessible to DIDS. If the protein was kept in the presence of 10 μM DIDS during incorporation and subsequently at 4 °C overnight, complete inhibition was attained. However, this had to be done in the presence of 25 mM cysteine since mercaptoethanol is an effective scavenger for DIDS. Cysteine is slightly less effective than mercaptoethanol in preventing aggregation of band 3.

When liposomes containing band 3 were pelleted by ultracentrifugation, 70% of the protein initially added to the lipids was found to be associated with the liposomes. This percentage was only slightly (~10%) decreased with band 3 treated with DIDS, indicating that the inhibition observed with this compound was not due to altering the protein's ability to associate with the liposomes. The protein could be stored in buffer with

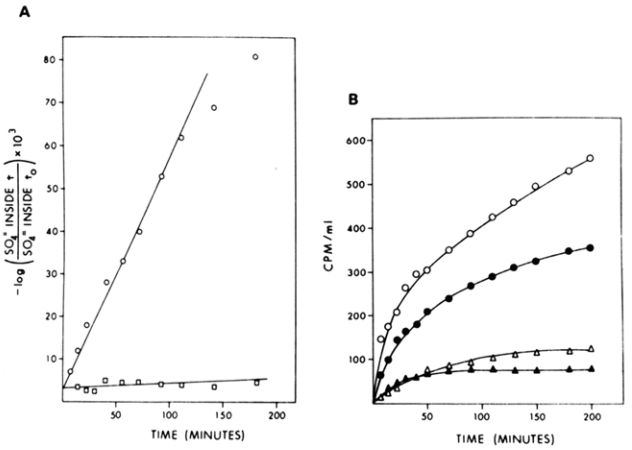

FIGURE 3: (A) Semilog plot of fraction of radioactive sulfate remaining in band 3 (○) and control (□) liposomes vs. time, expressed as $^{35}SO_4^{2-}$ inside at any time t divided by $^{35}SO_4^{2-}$ inside liposomes at time t_0, when the efflux commenced. Comparison of slopes indicates an approximate 70-fold increase in SO_4^{2-} efflux in band 3 liposomes. Efflux was carried out at 37 °C. (B) Sulfate ($^{35}SO_4^{2-}$) efflux from liposomes containing band 3 protein: control liposomes, no band 3 protein (▲); liposomes containing band 3 (1.0 mg of protein, 96 mg of lecithin, and 4 mg of phosphatidic acid) (○); liposomes containing band 3 to which 10 μM DIDS was added (●); liposomes containing band 3 and DIDS added to protein before incorporation into liposomes (△). Efflux was carried out at 37 °C.

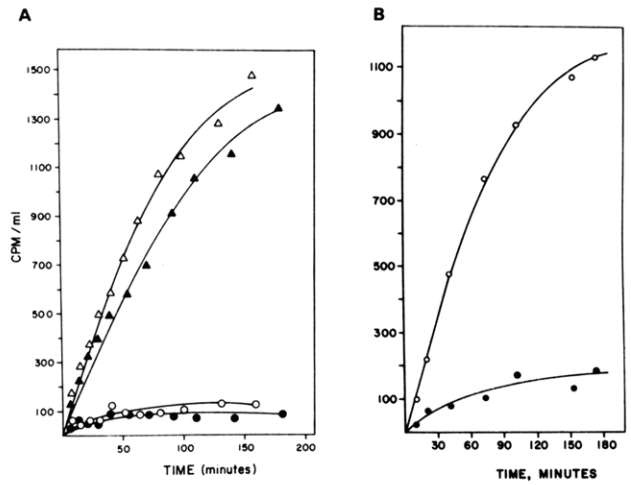

FIGURE 4: (A) Sulfate ($^{35}SO_4^{2-}$) efflux from liposomes containing band 3 protein (1.5 mg): liposomes containing freshly isolated protein (△); control liposomes for this experiment (○); liposomes containing protein (protein stored in 36 mM Na_2HPO_4 and 15 mM mercaptoethanol, pH 7.50, at 0 °C for 7 days) (▲); control liposomes for this experiment (●). (B) Sulfate ($^{35}SO_4^{2-}$) efflux from liposomes containing band 3 protein (1.1 mg): liposomes in regular transport buffer (○); liposomes in buffer with no external transportable anions (●).

Triton removed at 0 °C in the presence of mercaptoethanol and still fully retain transport activity for at least a week (Figure 4A).

When transport was carried out with no external transportable anions (Figure 4B), sulfate efflux was markedly reduced. External salts were replaced with 85 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.50 (Wolosin et al., 1977), by passage through a Sephadex G-50 column equilibrated with this buffer at 4 °C.

When band 3 liposomes were examined by electron microscopy, they were found to be 250–300 Å in size with occasional liposomes of greater size, ranging up to 1000 Å (Figure 5A). It was observed that during efflux experiments only 5–20% of the trapped radioactive sulfate came out of the liposomes. One possible explanation for this is that most of

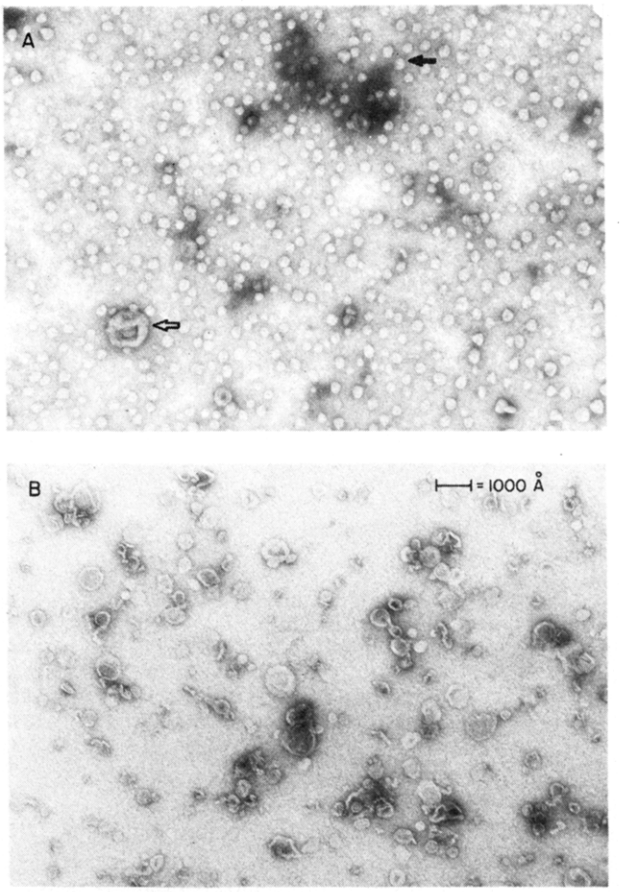

FIGURE 5: (A) Electron micrograph of liposomes containing band 3 protein. Samples were diluted to 30–100 μM in buffer, applied by drops to a formvar-coated grid, negatively stained with 2% uranyl acetate (aqueous), and blotted to a monolayer with pointed filter paper at 4 °C. The samples were photographed in a Hitachi 11-E electron microscope at 75 kV. Sizes of liposomes are approximately 250–300 Å (closed arrow) with occasional liposomes of 800–1000 Å (open arrow). (B) Fraction of band 3 liposomes absorbed by the pCMB column and eluted with 5 mM cysteine; see text for details.

the liposomes did not contain band 3, and, therefore, the sulfate trapped in them could not be transported. Based on an estimate of 30 000 phospholipid molecules per vesicle (Enoch & Strittmatter, 1979), it can be calculated that only one band 3 dimer would be found per vesicle. If some vesicles contained more than one protein dimer, other vesicles would contain no protein at all. In an attempt to separate liposomes with protein from liposomes without protein, band 3 liposomes were passed through the pCMB affinity gel. About 2% of the liposomes containing about 50% of the protein were absorbed on the gel, and they were eluted with 5 mM cysteine. When electron micrographs of this fraction were inspected, a continuum of liposome sizes from 250 to 900 Å was observed (Figure 5B). Therefore, it is not possible to say into which size liposome band 3 has preferentially incorporated.

We have observed, as have Sze & Solomon (1979), that using the dialysis tube method for determining transport rates introduces an extra compartment into the analysis since the rate of movement of ions across the tube is not instantaneous. Therefore, we used a different method to determine a turnover number for purified, reconstituted band 3. Sulfate influx into liposomes was measured by incubating liposomes (with and without band 3) with radioactive sulfate and then removing the free radioactivity in the medium from the trapped radioactivity in the liposomes by gel filtration. A similar procedure has been used by Wolosin et al. (1977). Transport was ter-

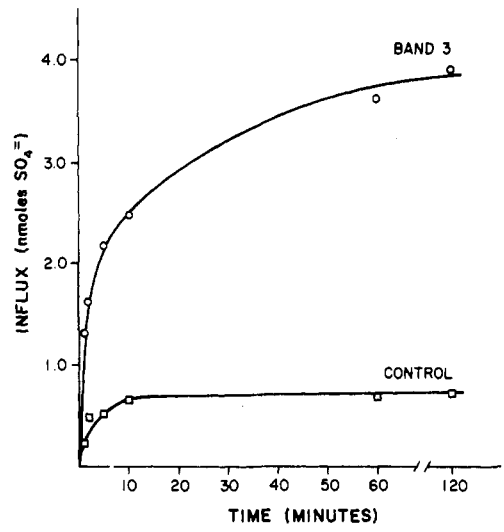

FIGURE 6: Sulfate influx into liposomes containing band 3 protein (○) and into liposomes containing no protein (□). Influx was carried out at 37 °C, pH 7.50, with a 10 mM sulfate concentration.

minated by placing the liposomes in an ice bath, and any possible loss of radioactivity from the liposomes during the gel filtration was minimized by running the columns at 4 °C and by equilibrating the columns with 85 mM Tes, which does not exchange with sulfate. A typical result is shown in Figure 6. Using the value of sulfate taken up by the band 3 liposomes in 1 min and subtracting the value for control liposomes, we calculate an influx rate of 53 nmol of sulfate (mg of protein)$^{-1}$ min^{-1} at 37 °C, pH 7.50, at a concentration of 10 mM sulfate. This is based on a 70% incorporation of band 3 (see above). From this, a turnover number of 0.08 sulfate s^{-1} (band 3 monomer)$^{-1}$ is obtained. From the analysis of Schnell et al. (1977) and the number of band 3 monomer molecules per cell (Knauf, 1979), the turnover number for band 3 in the intact erythrocyte is 0.34 sulfate s^{-1} site^{-1}. This value was determined by corrections for the effect of pH and sulfate concentration from the data and calculations of Schnell et al. (1977). Schnell et al. (1977) determined that maximal sulfate flux in erythrocyte ghosts at 37 °C in 23 mM potassium phosphate buffer, pH 7.3, was 4.76×10^{-6} mol min^{-1} (g of cells)$^{-1}$. Half-saturation occurred at 40 mM sulfate. Assuming a linear relationship between flux and sulfate concentration over the range of 0–40 mM sulfate, we calculate that at 10 mM sulfate the flux would be 333 nmol/mg of band 3 protein: This is based on 1.2×10^6 band 3 monomers/cell, 1.0×10^{10} cells/g of cells, and a molecular weight of 90 000 for the band 3 monomer. With a correction for pH according to Schnell et al. (Δ log sulfate flux = $-0.86 \times 0.2\Delta$pH), the red cell ghost sulfate flux would be 333/1.48 = 225 nmol/mg of band 3 at pH 7.5. Sulfate flux in our reconstituted liposomes is therefore 24% of that in the red cell ghosts at the same pH and sulfate concentration.

In conclusion, this study describes an efficient method for the rapid isolation and stabilization of a pure preparation of band 3 protein. In addition, since this study demonstrates (a) a highly active transport ability of the purest band 3 preparation incorporated into liposomes reported to date in the literature and (b) inhibition by DIDS, it provides further significant proof that band 3 is the anion-transport protein of the red cell. We believe that this method for isolation and stabilization of band 3 protein should also facilitate studies on the properties of the protein in solution.

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