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Preparation and Analysis of Seven Major, Topographically Defined Fragments of Band 3, the Predominant Transmembrane Polypeptide of Human Erythrocyte Membranes†

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ABSTRACT: Band 3 is the ~90 000-dalton membrane-spanning polypeptide believed to facilitate anion transport in the human erythrocyte membrane. Previous studies have shown that digestion of this protein while still membrane-bound generates large, topographically defined, overlapping fragments which account for all or nearly all of its mass. We have now purified seven of these fragments, utilizing selective membrane solubilization, gel filtration, and preparative gel electrophoresis in sodium dodecyl sulfate. Amino acid analysis revealed that fragments derived from the outer surface and membrane-spanning regions of band 3 were distinctly hydrophobic, while cytoplasmic surface segments were relatively polar; these compositional data parallel the solubility in aqueous solutions and the mode of membrane association of the various fragments. Digestion of intact cells with chymotrypsin generated a 38 000-dalton outer-surface and a 55 000-dalton transmembrane fragment. The sums of their apparent molecular weights and of their compositions approximated the band 3 polypeptide, suggesting a single site of cleavage at the extracellular face. Chymotryptic digestion at both membrane surfaces produced a 17 000-dalton transmembrane fragment having the most hydrophobic composition. Mild trypsin digestion released a 41 000-dalton segment from the cytoplasmic pole of band 3, which could be further cleaved by trypsin to yield 22 000- and 16 000-dalton subfragments. Compositional analysis showed that the sum of these two subfragments was nearly the equivalent of the 41 000-dalton tryptic fragment. Furthermore, these data indicate the presence of a small lysine-rich segment near the juxtamembrane region of the 41 000-dalton fragment, which was lost upon further cleavage. The missing segment may overlap with the 17 000-dalton chymotryptic core. A 23 000-dalton fragment cleaved by S-cyanylation from the cytoplasmic terminal region of band 3 had nearly the same composition as the 22 000-dalton tryptic fragment, indicating that these two species are coextensive. Furthermore, end-group analysis suggested the presence of blocked amino termini on both the 22 000- and 23 000-dalton fragments, supporting previous reports that the amino terminus of the band 3 polypeptide is located on the cytoplasmic side of the membrane.

Band 3, an ~90 000-dalton glycoprotein, is the predominant polypeptide of the human erythrocyte membrane, comprising approximately 25% of the protein mass (cf. Steck, 1974). Band 3 is believed to be involved in the facilitated diffusion of anions across this membrane (Rothstein et al., 1976; Zaki et al., 1975; Ho and Guidotti, 1975; Wolosin et al., 1977; Ross and McConnell, 1977). Its disposition as an asymmetric membrane-spanning dimer is appropriate to this function (cf. Steck, 1974; Marchesi et al., 1976).

We recently described the generation of 11 large fragments by the proteolytic digestion of band 3 while still membrane bound and by S-cyanylation of this polypeptide in $NaDodSO_4$[1] (Steck et al., 1976) (see Figure 1). We now report on the purification of seven of these fragments (which represent all or nearly all of the mass of band 3) and their complete amino acid

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[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl phosphofluoridate; PAS, periodic acid–Schiff (stain for glycoproteins).

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analysis. The data confirm and extend our previous ordering of the fragments into a linear and topographic map and provide a basis for sequence analysis of band 3.

A portion of this study was previously reported in a preliminary form (Singh et al., 1977).

Experimental Procedures

Materials. Trypsin (three times crystallized) was obtained from Worthington. Carboxypeptidase A and α-chymotrypsin (type II) were from Sigma. 2-Nitro-5-thiocyanobenzoate (NTCB) was synthesized according to the method of Degani and Patchornik (1971). Fresh solutions of these reagents were prepared just prior to each experiment.

Preparations: Unsealed Ghosts. Unless stipulated, all procedures were performed at 0–5 °C, except that solutions containing $NaDodSO_4$ were kept at room temperature. Human erythrocytes were obtained from units of out-dated bank blood generously provided by the University of Chicago Blood Bank. Each unit of blood (containing approximately 150 mL of cells) was washed by suspension to 1000 mL in 150 mM NaCl–5 mM NaP_i (pH 8) and centrifugation in a Sorvall HS-4 rotor; this treatment was performed four times. The washed cell pellet (~250 mL) was diluted to 3550 mL in 5 mM NaP_i (pH 8); the resulting membranes (ghosts) were pelleted at 13 000 rpm for 20 min in a Sorvall GSA rotor and washed three more times to remove residual hemoglobin (Fairbanks et al., 1971). (Unless indicated, all subsequent washes of membranes utilized these volumes and centrifugation conditions.) Except in the preparation of tryptic fragments, the 5 mM NaP_i buffer was made 0.02% in saponin (Sigma) in the first cycle to promote complete hemolysis.

NaOH-Stripped Membranes. Where indicated, packed ghosts (~200 mL) derived from single units of blood were resuspended to 1500 mL in ice-cold 0.1 N NaOH, followed immediately by centrifugation in polyethylene bottles. Pellets (~50 mL) were washed once with phosphate buffer to bring the membranes to pH 7–8. This treatment removes the peripheral proteins and thereby enriches band 3 approximately twofold, so that it comprises roughly 50% of the residual protein (Steck and Yu, 1973).

Solubilization of Band 3 and Integral Polypeptides. Pellets of NaOH-stripped ghosts (with or without digestion) were resuspended to 50–100 mL and dissolved by the addition of dithiothreitol to 0.05% and solid $NaDodSO_4$ to 2% (final). The suspension was warmed to 37 °C for 1 h or longer until optically clear. The solution was concentrated to ~10 mL at room temperature by (a) ultrafiltration (Amicon PM-10 membrane) or (b) dialysis against 10% Carbowax (PEG 20 000 from Fisher) in 0.2% $NaDodSO_4$–0.05% dithiotreitol–0.02% NaN_3. The concentrates were cleared of precipitated lipid by centrifugation. The polypeptide solution was either stored at room temperature or at −20 °C (after freezing in a dry ice–alcohol bath) before further purification.

Isolation of Polypeptides: Band 3. Ghosts derived from single units of blood were stripped of peripheral proteins with 0.1 N NaOH and dissolved in $NaDodSO_4$–dithiothreitol, as described above. The solution (containing approximately 150 mg of protein in 50–100 mL) was concentrated, and band 3 was purified by (a) gel filtration chromatography (agarose A-5m, Sepharose 6B, or Ultrogel AcA22) or (b) preparative electrophoresis on 5% polyacrylamide gels (described below). In both chromatography and electrophoresis, the PAS-positive sialoglycoproteins were eliminated by virtue of their small apparent size.

Chymotryptic 38 000- and 55 000-Dalton Fragments. Each unit of packed, washed erythrocytes was mixed with an equal volume of solution containing 200 μg/mL α-chymotrypsin in 150 mM NaCl–5 mM NaP_i, pH 8, and incubated overnight at room temperature, with shaking (Steck et al., 1976). The addition of 0.05 mM DFP (final) terminated the reaction. The cells were washed three times in the same buffer and treated again with 0.05 mM DFP, and then ghosts prepared in 5 mM NaP_i (pH 8). The membranes were stripped with 0.1 N NaOH and dissolved in $NaDodSO_4$–dithiothreitol. The solution was concentrated, clarified by centrifugation, and chromatographed on Sepharose 6B. Impure fractions of interest were pooled, concentrated by ultrafiltration, and rechromatographed on Ultrogel AcA44. Some preparations were further purified by preparative electrophoresis on a 7.5% polyacrylamide gel.

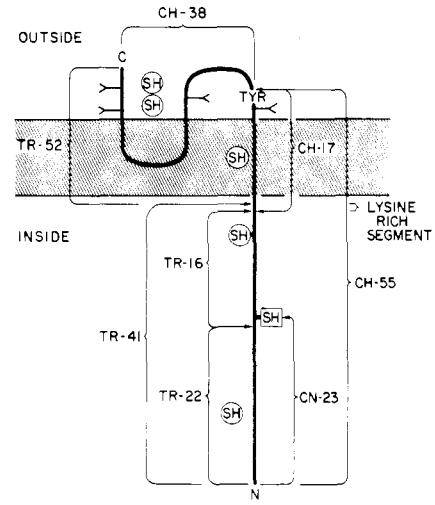

FIGURE 1: A scheme for the disposition of eight major fragments of the band 3 polypeptide. The ordering of the fragments is based on Steck et al. (1976) and the data presented herein. Chymotrypsin (CH) cleavage at the outer surface yields complementary fragments of 55 000 and 38 000 daltons; both are glycosylated and integral (i.e., both penetrate the apolar core of the membrane). A second chymotryptic cleavage at the cytoplasmic surface generates a 17 000-dalton, membrane-spanning glycosylated species. Mild trypsin (TR) digestion cleaves at several cytoplasmic surface loci on band 3, generating a membrane-spanning 52 000-dalton fragment and a water-soluble domain of 41 000-daltons; the latter is further cleaved to 22 000- and 16 000-dalton subfragments, with the loss of a small amount of lysine-rich material. S-Cyanylation (CN) generates a 23 000-dalton species which is nearly coextensive with the 22 000-dalton tryptic fragment. The location of carbohydrate chains (—≺) and cysteines (SH) within each fragment is, at present, indeterminate, except for two cysteine residues in the cytoplasmic domain mapped by cyanylation cleavage (Steck et al., 1976; Fukuda et al, 1977). The orientation of the polypeptide chain is according to Drickamer (1976), Jenkins and Tanner (1977b) and data presented herein; the position of the chain termini (i.e., buried vs. free) is unknown.

Chymotryptic 17 000-Dalton Fragment. Unsealed ghosts (~100 mL) from individual units of blood were mixed with an equal volume of a solution containing 200 μg/mL α-chymotrypsin in 5 mM NaP_i, pH 8, and stirred for 1 h at 23 °C. The addition of DFP to 0.05 mM and then 10–15 volumes of ice-cold 0.1 N NaOH terminated the reaction. The stripped membranes were pelleted, washed, and dissolved in $NaDodSO_4$–dithiothreitol. The solution was concentrated and clarified by centrifugation. The 17 000-dalton component was isolated by preparative electrophoresis on 7.5% polyacrylamide gel (see below).

Tryptic Fragments. Three interesting fragments, of 41 000-, 22 000-, and 16 000-daltons, are generated by the digestion of isolated membranes with trace levels of trypsin (Figure 1). All three are derived from the cytoplasmic domain of band 3 and are readily released from the membrane (Steck et al., 1976).

Because the 41 000-dalton fragment is readily cleaved to the two smaller species, its yield is enhanced by using low levels of trypsin and by pretreating the membranes with *o*-phenanthroline plus $CuSO_4$, which induces a disulfide cross-link between band 3 protomers in the region cleaved to generate the two smaller fragments. In un-cross-linked membranes and at higher trypsin levels, the 22 000-dalton fragment is formed at high yield; the 16 000-dalton species appears to be further degraded and is invariably recovered at low yield (Steck et al., 1976). The isolation of these three fragments was simplified by stripping the membranes of some peripheral proteins prior to digestion; however, this step had to be most gentle to prevent degradation of the 41 000-dalton component by trypsin.

Unsealed ghosts were prepared without saponin. Glyceraldehyde-3-P dehydrogenase (band 6), aldolase, and other trace, salt-eluted proteins were removed by washing the ghosts with 200 mM NaCl–5 mM NaP_i, pH 8, followed by 5 mM NaP_i, pH 8 (Kant and Steck, 1973; Strapazon and Steck, 1977). Band 3 dimers were disulfide cross-linked by reacting the ghost pellet (~250 mL) for 30 min on ice with an equal volume of *o*-phenanthroline (200 μM) plus $CuSO_4$ (40 μM) in 5 mM NaP_i (pH 8), and then washing with this same buffer alone (Steck, 1972). Bands, 1, 2, and 5 (spectrin and actin; cf. Steck, 1974; Marchesi et al., 1976) were selectively eluted from the membranes by the addition of pre-warmed 0.1 mM EDTA (pH 8) to 3550 mL and incubation at 37 °C for 15 min (Fairbanks et al., 1971). The suspensions were stored overnight at 5 °C and the depleted membranes collected by a 1-h centrifugation at 13 000 rpm.

The membrane vesicles (100–200 mL) were resuspended with an equal volume of 10 mM NaP_i (pH 7.0) and digested with trypsin (0.1–0.5 μg/mL, final) for 1 h on ice, with occasional mixing. The reaction was terminated by the addition of DFP to 0.05 mM. The suspension was made 0.1 N in NaOH (by the addition of 5 N NaOH stock) to assure the release of nonintegral tryptic fragments. The suspension was immediately centrifuged at 18 000 rpm for 30 min in polyethylene tubes (Sorvall SS-34 rotor). The supernatant fraction was collected and recentrifuged to remove the last traces of membrane fragments. The solution was then titrated to pH 4 with glacial acetic acid. After 1 h on ice, the precipitated proteins were collected by centrifugation at 13 000 rpm for 20 min. The precipitate was resuspended in a homogenizer to ~20 mL in 25 mM NaP_i (pH 8) containing 10 mM dithiothreitol; $NaDodSO_4$ was then added to 1% (final concentration). After dissolution, the proteins were concentrated by ultrafiltration and fractionated by chromatography on a 2.5 × 85 cm column of Ultrogel AcA44. Complete purification usually required rechromatography of peak fractions on the same column. Alternatively, the concentrated digest was subjected to a single cycle of preparative electrophoresis on a 7.5% polyacrylamide gel (described below).

Cyanylation Fragment of 23 000 Daltons. (See Jacobson et al., 1973; Degani and Patchornik, 1974; Steck et al., 1976.) Ghosts were stripped of peripheral proteins in 0.1 N NaOH and washed with 5 mM NaP_i, pH 8. The membranes (~100 mL) were dissolved in 50 mM sodium borate (pH 9.0)–2% $NaDodSO_4$. 2-Nitro-5-thiocyanobenzoate was added to a final concentration of 1.0 mM. The solution was incubated for 8–18 h under N_2 in a screw-capped tube at 50 °C. The digest was concentrated by ultrafiltration. Proteins were precipitated by adding to the room temperature sample 10 volumes of ice-cold 2-propanol containing 0.02% 2-mercaptoethanol and incubating on ice for 15 min. The precipitate was collected by centrifugation and washed again with the same volume of 2-propanol–0.02% 2-mercaptoethanol. This procedure leads to quantitative precipitation of the polypeptides, free of $NaDodSO_4$ (Koziarz, 1977). 2-Mercaptoethanol is added to purge the 2-propanol of oxidants which otherwise modify sensitive amino acid side chains. The pellet was then extracted twice with 10 mL of distilled water at 37 °C. This treatment solubilizes most of the 23 000-dalton fragment, but not the hydrophobic contaminants. The preparation was made 1.0% in $NaDodSO_4$ and the 23 000-dalton fragment purified on a 5 × 75 cm Ultrogel AcA44 column (M. K. Singh, T. L. Steck, and H. Köhler, in preparation).

Analytical polyacrylamide gel electrophoresis was used to monitor the purification of all polypeptides. The method was that of Fairbanks et al. (1971), as modified by Steck and Yu (1973). The gels contained 5.0% acrylamide (including 0.19% *N,N'*-methylenebisacrylamide) and 0.2% $NaDodSO_4$.

Preparative Polyacrylamide Gel Electrophoresis. The instrument and procedures were described by Koziarz et al. (1978). The gel column was 5.9 cm in diameter and 12 cm long. The electrophoresis buffer was that of Fairbanks et al. (1971); however, the pH was adjusted to 9.0 and the $NaDodSO_4$ concentration to 0.2%. The gel was prerun with thioglycolate as described (Koziarz et al., 1978).

Gel filtration chromatography was carried out in buffers containing 25 mM NaP_i (pH 8.0), 0.1 or 0.2% $NaDodSO_4$ and 0.02% NaN_3. Units of band 3 and large fragments were purified in 5 cm diameter × 75 cm columns of Bio-Gel A-5m (Bio-Rad) or Sepharose 6B (Pharmacia) in either the ascending or descending mode. Milligram amounts of band 3 were also purified on 1.5 × 40 cm columns of Ultrogel AcA22 (LKB). Smaller fragments were run on columns of Ultrogel AcA44 (LKB), as indicated.

Column fractions were analyzed by their absorbance at 280 nm and by gel electrophoresis in $NaDodSO_4$. Fractions of purified polypeptides were pooled, concentrated by ultrafiltration (Amicon PM-10), dialyzed extensively vs. 0.2% $NaDodSO_4$, frozen in a dry-ice bath, and stored at −20 °C.

Amino Acid Composition. Lyophilized samples of band 3 and its fragments were hydrolyzed in triplicate for 20, 48, and 72 h intervals in 0.5 mL of 5.7 M HCl containing 2 mM phenol and 0.5% 2-mercaptoethanol. Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1956). Tryptophan was measured spectrophotometrically in $NaDodSO_4$ (Koziarz, 1977; Koziarz et al., 1978). Analyses were performed on a Beckman Model 121M amino acid analyzer interfaced with a system AA computing integrator. The stated values for Val, Ile, Leu, and Phe were from the 72 h analysis. The values for Thr and Ser were determined by extrapolation to zero time. Values for the other amino acids were means from the three time points (i.e., nine determinations). The number of residues in each peptide was calculated from its apparent molecular weight in $NaDodSO_4$–polyacrylamide gel electrophoresis (Steck et al., 1976) and the anhydrous molecular weight of each amino acid. The contribution of bound carbohydrate was ignored in these calculations since the molecular weight values employed are based on the mobilities of the fast-moving (nonglycoslyated) peak in the electrophoretic profile.

Results and Discussion

Isolation of Band 3 and Its Fragments. Figure 2 shows representative results of the purification of the polypeptides in question. Since our isolation procedures all relied on molecular sieving in $NaDodSO_4$, trace contamination by species of nearly the same apparent molecular weight was inevitable. However, no other significant contaminants were detected in these overloaded gels. (The slowly migrating component in gel

A is a dimer of band 3 and not a contaminant.) Similarly, these preparations were free of PAS-positive sialoglycoproteins.

Band 3 has been previously prepared by gel filtration in $NaDodSO_4$ by Ho and Guidotti (1975), Furthmayr et al. (1976), Grahmberg et al. (1976), and by Tanner and associates (see Jenkins and Tanner, 1977b) and, in a nondenatured form, on a disulfide liganding column (Kahlenberg, 1976), by concanavalin A affinity chromatography (Findlay, 1974) and by ion-exchange chromatography (Yu and Steck, 1975). Certain large fragments of band 3 have been previously prepared by gel filtration in detergent by Drickamer (1976), Jenkins and Tanner (1977b), and Fukuda et al. (1977).

The recovery of the various polypeptide species varied considerably. Since the source of this protein is not scarce and the options available for purifying hydrophobic membrane polypeptides are limited, we routinely sacrificed yield for purity in our procedures. Typically, the yield of band 3 was ~15 mg per unit of erythrocytes or ~10% (assuming that a unit contains 150 mL of cells and that there is 1 mg of band 3 per mL of cells) (cf. Fairbanks et al., 1971). The 17 000-dalton chymotryptic and the 23 000-dalton cyanylation fragments were recovered at 60–70% yields, taking the molecular weight of band 3 as 91 000 daltons (Steck et al., 1976). The yield of the other species ranged from approximately 25 to 5%, in this order: 55 000-dalton chymotryptic > 22 000-dalton tryptic > 40 000-dalton tryptic > 16 000-dalton tryptic > 38 000-dalton chymotryptic fragments.

An integral 52 000-dalton tryptic fragment (see Figure 1) has also been recovered. However, it migrates in the region of the zone 4.5 glycopeptides (cf. Steck et al., 1976) and thus has not been completely purified by the sieving techniques employed here (however, see Jenkins and Tanner, 1977b).

Amino acid composition of band 3 and its chymotryptic fragments is shown in Table I. The composition of band 3 determined here (column 1) resembles previous reports by Ho and Guidotti (1975), Yu and Steck (1975), Furthmayr et al. (1976), and Jenkins and Tanner (1977b). There is a distinct enrichment in hydrophobic residues compared with nonintegral membrane proteins (cf. Steck and Fox, 1972; Capaldi and Vanderkooi, 1972).

Chymotrypsin digestion of intact cells cleaves band 3 into 55 000-dalton and 38 000-dalton fragments; digestion of unsealed ghosts reduces the former to a 17 000-dalton species (see

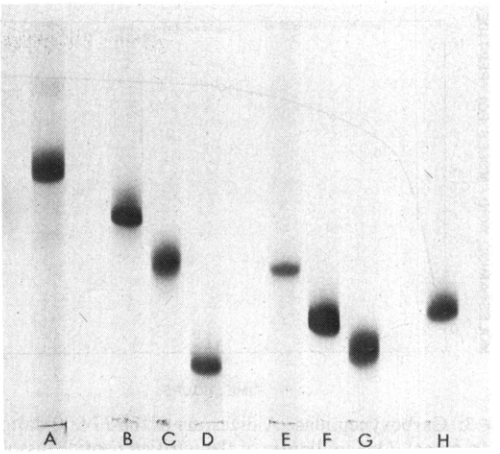

FIGURE 2: Electrophoretic analysis of isolated band 3 and seven defined fragments. Polypeptides were prepared and purified and then analyzed by polyacrylamide gel electrophoresis, with Coomassie blue staining, as described in Experimental Procedures. With the exception of sample E, the gels were overloaded to reveal possible contaminants. Gels: (A) Band 3 (91 000 daltons); (B–D) chymotryptic fragments of 55 000 (B), 38 000 (C), and 17 000 daltons (D); (E–G) tryptic fragments of 41 000 (E), 22 000 (F), and 16 000 daltons (G); (H) cyanylation fragment of 23 000 daltons (molecular weights taken from Steck et al., 1976).

TABLE I: Amino Acid Composition of Band 3 and Three Chymotryptic Fragments.[a]

Amino acid	Band 3 (93 000 daltons)[b]	55 000-dalton fragment	38 000-dalton fragment	17 000-dalton fragment	Σ55 000 + 38 000 dalton fragments
Asx	54.1 ± 1.7	31.5 ± 0.5	21.2	9.3 ± 0.1	52.7
Thr	41.7 ± 3.2	27.0 ± 0.4	19.1	8.5 ± 0.5	46.1
Ser	56.3 ± 5.7	34.4 ± 0.3	23.9	10.9 ± 0.4	58.3
Glx	99.4 ± 7.1	63.7 ± 1.4	30.9	14.7 ± 0.6	94.6
Pro	47.8 ± 5.0	29.1 ± 0.5	23.2	7.5 ± 0.6	52.3
Gly	64.3 ± 2.3	38.6 ± 1.3	26.7	13.8 ± 0.4	65.3
Ala	61.4 ± 5.8	35.2 ± 2.6	26.0	10.9 ± 0.8	61.2
Val	62.9 ± 6.2	31.4 ± 0.8	29.9	11.2 ± 0.6	61.3
Met	18.1 ± 3.7	10.5 ± 1.9	10.8	2.0 ± 0.4	21.3
Ile	38.9 ± 1.4	22.3 ± 1.4	20.9	10.4 ± 0.1	43.2
Leu	111.7 ± 9.0	68.8 ± 8.8	46.5	19.3 ± 2.3	115.3
Tyr	25.9 ± 6.1	14.5 ± 1.1	6.6	5.5 ± 1.2	21.1
Phe	45.8 ± 1.7	32.2 ± 6.6	18.9	11.5 ± 0.7	51.1
His	18.0 ± 0.4	8.7 ± 1.7	7.2	2.1 ± 0.2	15.9
Lys	27.9 ± 0.9	12.7 ± 1.3	14.8	5.3 ± 0.2	27.5
Arg	43.5 ± 4.2	24.5 ± 0.7	16.5	6.5 ± 0.2	41.0
Cys	5.8 ± 0.8	3.0 ± 0.8	2.3	1.4 ± 0.3	5.3
Trp	9.9 ± 4.2	3.8 ± 0.3	1.2	2.8 ± 0.6	5.0
Sum	833.4	491.9	346.6	153.6	838.5
% hydrophobic	37.5	37.3	38.7	40.8	37.9
No. of samples	4	3	1	2	

[a] Band 3 and its chymotryptic fragments were purified and subjected to amino acid analysis as described in the text. Data are expressed as residues per mole ± SD. Hydrophobic residues were taken as Val, Met, Ile, Leu, Tyr, Phe, and Trp (Steck and Fox, 1972). Preparations of band 3 from four donors were analyzed; three individuals were analyzed for the 55 000-dalton species; data on the 38 000-dalton fragment were based on a pool from three donors; 17 000-dalton preparations from two donors were analyzed. [b] Although our best estimate of the molecular weight of the band 3 polypeptide is 91 000 ± 4000 (Steck et al., 1976), we have taken its value as 93 000 in this table to facilitate the comparison of its composition with the sum of the 55 000 + 38 000 fragments.

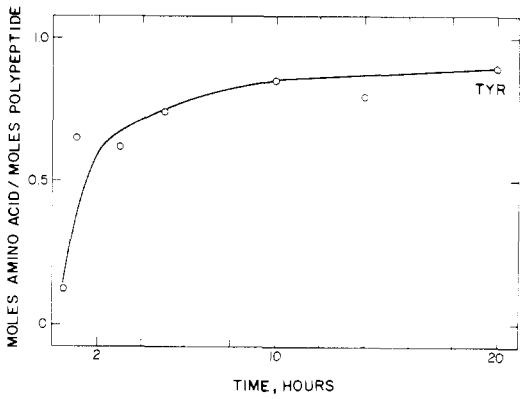

FIGURE 3: Carboxypeptidase A digestion of the 17 000-dalton chymotryptic fragment. One milligram of the purified peptide dissolved in 1.0 mL of 0.1 M NH_4HCO_3 (pH 8.1)–0.2% $NaDodSO_4$ was incubated with 10 μg of carboxypeptidase A for 20 h at 37 °C. At intervals, 0.1-mL aliquots were removed, mixed with 0.1 mL of glacial acetic acid to terminate the reaction, dried under nitrogen, and quantitated on the amino acid analyzer. Free tyrosine was the only amino acid to increase in a time-dependent and enzyme-dependent fashion.

Figure 1 for the disposition of these pieces). The 55 000- and 38 000-dalton segments (Table I, columns 2 and 3) are approximately as hydrophobic in composition as band 3, while the membrane-spanning 17 000-dalton fragment (column 4) is even more so. The hydrophobic composition of band 3 and the three chymotryptic peptides correlates with their integral association with the membrane (Steck et al., 1976) and their insolubility in aqueous media lacking detergents.

That the 17 000-dalton fragment is more hydrophobic than the two others is not surprising, in that the 38 000-dalton tract, bearing most of the carbohydrate in the molecule (Jenkins and Tanner, 1977b; C. A. Bush and T. L. Steck, unpublished), must communicate extensively with the extracellular aqueous space (Steck and Dawson, 1974), while the 55 000-dalton species contains a water-soluble, ~41 000-dalton polar domain (vide infra). The content of polar (especially basic) residues in the 17 000-dalton transmembrane segment is of particular interest, since it may bear on both the integration of this region into the membrane and on its role in the transport of anions. It is this segment of band 3 which specifically binds two different covalent inhibitors of anion flux (Drickamer, 1976; S. Grinstein et al., 1977).

The 55 000- and 38 000-dalton fragments are generated by limited digestion of intact erythrocytes by chymotrypsin. Since they appear to be complementary by dissection studies and their apparent molecular weights sum to that of band 3, it is likely that they result from the cleavage of the parent polypeptide at a single site. Strong support for this premise is derived from column 5 in Table I: the sum of the individual amino acid residues in these two fragments closely approximates the composition of band 3 (average absolute difference = 7.7%, if tryptophan is excluded).

The cleavage site which generates the 55 000 and 38 000-dalton fragments can be assigned to a portion of band 3 which projects into the extracellular aqueous compartment, where chymotrypsin can reach it. Furthermore, both resultant fragments bear carbohydrate (cf. Steck et al., 1976; Jenkins and Tanner, 1977b), an indication of hydrophilic disposition. Because both fragments are also strongly bound to the membrane (Steck et al., 1976), we envision that the extracellular site of chymotryptic cleavage resides in a hydrophilic region between two domains integrated in the membrane, as shown in Figure 1.

We previously inferred that the 17 000-dalton and 55 000-dalton chymotryptic fragments shared a common site of cleavage at the extracellular membrane surface (Steck et al., 1976). Drickamer (1976) subsequently identified this site on the 55 000-dalton piece as carboxy-terminal tyrosine. We have performed a similar analysis on the 17 000-dalton segment (Figure 3). Digestion with carboxypeptidase A in $NaDodSO_4$ released only tyrosine in a time-dependent and enzyme-dependent fashion, approaching 1 mol of Tyr/mol of fragment early in the 20-h incubation. This finding strengthens the view that 55 000- and 17 000-dalton chymotryptic fragments coterminate at their carboxyl ends and that the amino terminus of band 3 is in the cytoplasmic surface domain. The findings of Jenkins and Tanner (1977b) that a thermolysin digestion product which closely resembles the 55 000-dalton chymotryptic fragment in other ways also bears a COOH-terminal tyrosine suggest that it may also be generated by cleavage at the same, highly restricted site.

The amino acid composition of four fragments derived from the cytoplasmic domain of band 3 is shown in Table II. These polypeptides are all considerably less hydrophobic than those in Table I. These fragments can also be released from the membrane without its dissolution and are soluble in aqueous media without detergents (see Experimental Procedures for their preparation, and Steck et al., 1976). We therefore consider the cytoplasmic pole to be a hydrophilic domain of a hydrophobic protein.

It is clear that, under our conditions, cyanylation does not cleave band 3 at every cysteine residue (Figure 1 and Tables I and II), but predominantly at a locus ~23 000-daltons from the cytoplasmic end of the band 3 polypeptide (Steck et al., 1976). Drickamer (1976) has also drawn this conclusion. Furthermore, Drickamer provided evidence suggesting that this peptide contains the amino terminus of the band 3 molecule; the data of Jenkins and Tanner (1977b) are consistent with this conclusion. We have repeatedly failed to identify a free amino-terminal residue in the 23 000-dalton fragment by both Edman degradation and dansylation (M. K. Singh, H. Kumar, T. L. Steck, and H. Köhler, unpublished data). These findings suggest that the blocked amino terminus of band 3 (Tanner and Boxer, 1972; Ho and Guidotti, 1975; Jenkins and Tanner, 1977b; H. Köhler and T. L. Steck, reported by Yu and Steck, 1975) resides in this fragment.

The 23 000-dalton cyanylation fragment and the 22 000-dalton tryptic fragment appear to be nearly coextensive when analyzed by digestion studies (Steck et al., 1976). Amino acid compositional analysis (Table II, columns 1 and 2) demonstrates that these two fragments are indeed closely similar, with only Ser and Glx differing by two or more residues between them. We have also subjected approximately 500 nmol of the 22 000-dalton tryptic fragment to automated Edman degradation. No significant amounts of any phenylthiohydantoin-amino acids were detected in ten cycles. These data strengthen the premise that the 22 000-dalton tryptic fragment and 23 000-dalton cyanylation fragment are not only coextensive, but coterminate at the blocked amino terminus of the band 3 polypeptide.

From previous digestion studies, we inferred that the 22 000- and 16 000-dalton tryptic fragments were complementary and together accounted for the bulk of 41 000-dalton peptide (Steck et al., 1976). Amino acid analysis bears out this hypothesis; the sum of the content of the two smaller tryptic pieces closely resembles the composition of the 41 000-dalton fragment (Table II; compare columns 4 and 5). However, both the composite size (38 000-daltons) and composition of the 22 000 and 16 000-dalton pieces fall short of equalling the

TABLE II: Amino Acid Composition of Four Cytoplasmic Surface Fragments.[a]

Amino acid	23 000-dalton cyanylation	22 000-dalton tryptic	16 000-dalton tryptic	41 000-dalton tryptic	Σ22 000 + 16 000 dalton fragments
Asx	16.4 ± 0.9	17.2 ± 0.7	9.1 ± 0.5	29.5 ± 1.1	26.2
Thr	13.0 ± 0.6	11.7 ± 0.8	6.4 ± 0.1	19.9 ± 1.2	18.1
Ser	12.1 ± 0.9	9.6 ± 1.7	9.3 ± 0.1	21.8 ± 1.3	18.9
Glx	38.0 ± 5.5	33.5 ± 1.7	24.0 ± 1.5	55.8 ± 8.0	57.5
Pro	10.2 ± 0.5	9.0 ± 1.0	10.8 ± 0.3	20.3 ± 0.5	19.9
Gly	11.4 ± 0.7	11.6 ± 0.7	10.5 ± 0.6	23.4 ± 0.4	22.0
Ala	13.7 ± 0.6	15.4 ± 0.7	11.3 ± 0.4	27.6 ± 0.7	26.8
Val	11.3 ± 1.1	12.1 ± 0.5	9.7 ± 0.7	23.2 ± 0.2	21.7
Met	5.3 ± 0.6	5.0 ± 1.5	2.8 ± 0.4	6.6 ± 0.1	7.8
Ile	6.1 ± 1.5	5.8 ± 0.6	4.4 ± 0.5	13.7 ± 0.6	10.0
Leu	26.1 ± 2.8	24.3 ± 4.0	20.6 ± 0.1	45.1 ± 1.9	44.8
Tyr	6.3 ± 2.0	5.3 ± 0.6	3.5 ± 0.3	8.9 ± 1.8	8.8
Phe	6.7 ± 1.3	6.6 ± 0.8	6.3 ± 0.7	13.9 ± 0.7	12.9
His	7.8 ± 0.6	7.7 ± 0.5	4.2 ± 0.2	11.4 ± 0.8	11.9
Lys	5.8 ± 0.8	6.3 ± 1.1	2.5 ± 0.3	15.7 ± 2.1	8.7
Arg	9.6 ± 0.5	10.2 ± 0.4	7.1 ± 0.3	18.8 ± 0.01	17.3
Cys	1.3 ± 0.04	1.0 ± 0.1	1.4 ± 0.1	4.3 ± 1.4	2.5
Trp	3.6 ± 0.9	2.4 ± 1.1	0.3 ± 0.2	4.5 ± 0.9	2.7
Sum	204.7	194.7	144.2	364.4	388.5
% hydrophobic	31.9	31.5	33.0	31.9	32.1
No. of samples	8	3	3	2	

[a] The four fragments were purified and analyzed as described in the text. Data are expressed as residues per mole ± SD. Hydrophobic residues were as listed in Table I. Each sample analyzed was from a different donor.

41 000-dalton peptide. Of the apparent deficit of 25 residues, seven are attributable to Lys, and another 3–4 each to Asx, Ile, and Ser. It is not surprising that the missing material is rich in lysine, a target of tryptic digestion.

We argue that the peptide(s) not accounted for probably are derived from a segment between 16 000-dalton tryptic fragment and the membrane-integrated domain, as follows (see Figure 1). No residues could have been lost beyond the amino terminus of the blocked 22 000-dalton tryptic fragment, if it contains the NH_2 terminus of band 3. Furthermore, the 22 000-dalton tryptic segment has the same number of Lys and Arg residues as the overlapping 23 000-dalton cyanylation fragment (Table II). This finding suggests that no Lys or Arg residues fall between the carboxyl termini of the 22 000-dalton and the 23 000-dalton pieces. Since the 16 000-dalton tryptic fragment overlaps the carboxyl terminus of the 23 000-dalton cyanylation fragment (Steck et al., 1976), no material could have been lost from that juncture.

The size and disposition of the 41 000-dalton tryptic fragment determined in previous digestion studies led to the inference that this species represented essentially all of the cytoplasmic domain of band 3 and therefore roughly complemented both the outer surface plus transmembrane segments generated by chymotrypsin (38 000 + 17 000 = 55 000 daltons) and the integral tryptic fragment of 52 000 daltons (Steck et al., 1976), each of which accounts for the remainder of the 91 000-dalton band 3 polypeptide (see Figure 1). Indeed, the sum of the compositions of the 38 000-, 17 000-, and 41 000-dalton fragments roughly approximated that of band 3 (not shown). A more interesting comparison was made between the 41 000-dalton tryptic species and the difference between the 55 000- and 17 000-dalton chymotryptic fragments, since both should represent the bulk of the cytoplasmic domain of band 3 (Figure 1). The data in Tables I and II indicate that these two segments are, in fact, quite similar. There is, however, an excess of ~25 residues in the 41 000-dalton tryptic piece; these are primarily Lys (8), Asx (7), Glx (7), and His (5). Thus, an overlap may exist between the 17 000-dalton chymotryptic fragment and the 41 000-dalton tryptic fragment which is highly enriched in charged residues. This overlap may be in the vicinity of a putative lysine-rich ~25 residue segment lost when the 41 000-dalton segment was further cleaved by trypsin to 22 000- and 16 000-dalton pieces (vide supra). These data imply that several of the 5 lysine residues of the 17 000-dalton chymotryptic core segment may lie at its cytoplasmic pole, a prediction of possible relevance to our understanding of the role of band 3 in anion transport.

Calculations of overlaps or missing segments based on the comparison of three sets of compositional data obviously have an appreciable statistical error. However, the two computations predicting a lysine-rich segment near the juxta-cytoplasmic membrane surface appear to be significant. Sample contaminations could also contribute to the computed discrepancies among fragment compositions, but these would have to be sizable, and contaminants were not evident in Figure 2. Errors in the estimation of molecular weights would not lead to the prediction of regions of unusual composition, such as a lysine-rich segment. In any case, these calculations are viewed as merely suggesting hypotheses for which the isolation and characterization of the putative regions will provide definitive information.

Our compositional and chain-terminal analysis of seven principal fragments of the band 3 polypeptide corroborates and extends their ordering deduced from analytical labeling and digestion studies (Steck et al., 1976; Drickamer, 1976). As before (Steck et al., 1976), we find no evidence for heterogeneity of the polypeptide chain of band 3, isolated from single donors. Our formulation is in only partial agreement with that of Jenkins and Tanner (1975, 1977a,b). A major discrepancy between our findings may arise from the failure of Jenkins and Tanner to recover and identify the water-soluble fragments released from the cytoplasmic domain of band 3 following digestion with trypsin. We take their tryptic fragment, T2, to be an integral subfragment of peptide T1 (the equivalent of our

52 000-dalton tryptic species), perhaps contaminated by peptides cleaved from other cytoplasmic surface constituents.

Acknowledgments

We thank Benita Ramos and Susanna Rudofsky for their expert technical assistance and Dr. Pedro Wahrmann for his contribution to the early phases of this study.

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