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The Band 3 Protein of the Human Red Cell Membrane: A Review

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Band 3 is the predominant polypeptide and the purported mediator of anion transport in the human erythrocyte membrane. Against a background of minor and apparently unrelated polypeptides of similar electrophoretic mobility, and despite apparent heterogeneity in its glycosylation, the bulk of band 3 exhibits uniform and characteristic behavior. This integral glycoprotein appears to exist as a noncovalent dimer of two ~ 93,000-dalton chains which span the membrane asymmetrically. The protein is hydrophobic in its composition and in its behavior in aqueous solution and is best solubilized and purified in detergent. It can be cleaved while membrane-bound into large, topographically defined segments. An integral, outer-surface, 38,000-dalton fragment bears most of the band 3 carbohydrate. A 17,000-dalton, hydrophobic glycopeptide fragment spans the membrane. A ~ 40,000-dalton hydrophilic segment represents the cytoplasmic domain. In vitro, glyceraldehyde 3-P dehydrogenase and aldolase bind reversibly, in a metabolite-sensitive fashion, to this cytoplasmic segment. The cytoplasmic domain also bears the amino terminus of this polypeptide, in contrast to other integral membrane proteins. Recent electron microscopic analysis suggests that the poles of the band 3 molecule can be seen by freeze-etching at the two original membrane surfaces, while freeze-fracture reveals the transmembrane disposition of band 3 dimer particles.

There is strong evidence that band 3 mediates 1:1 anion exchange across the membrane through a conformational cycle while remaining fixed and asymmetrical. Its cytoplasmic pole can be variously perturbed and even excised without a significant alteration of transport function. However, digestion of the outer-surface region leads to inhibition of transport, so that both this segment and the membrane-spanning piece (which is selectively labeled by covalent inhibitors of transport) may be presumed to be involved in transport. Genetic polymorphism has been observed in the structure and immunogenicity of the band 3 polypeptide but this feature has not been related to variation in anion transport or other band 3 activities.

Key words: band 3, integral protein, polypeptide, red blood cell, erythrocyte, transport

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Early high-resolution polyacrylamide gel electrophorograms of human erythrocyte membrane polypeptides in sodium dodecyl sulfate (SDS) revealed a predominant band with a sharp leading edge and diffuse trailing edge, which was variously designated component a, component E, and band 3 [1–8]. Its apparent molecular weight was estimated to be 90,000–100,000, and its prevalence to be $\sim 1 \times 10^6$ copies per ghost membrane [8, 9].

In 1974, Cabantchik and Rothstein [10] demonstrated a close parallel between the inhibition of anion (namely, $^{35}SO_4^{=}$) transport in intact erythrocytes and the rather specific labeling of the band 3 polypeptide. During the ensuing years, numerous structural and functional studies have deepened interest in band 3 and its purported role in anion transport. It is the intent of this review to put this work into perspective.

ISOLATION OF THE BAND 3 POLYPEPTIDE

Band 3 can be assumed to make extensive contact with the apolar core of the membrane lipid bilayer (ie, to be an intrinsic or integral membrane protein) by virtue of the following qualities: 1) It cannot be extracted from the membrane short of dissolving the lipid bilayer itself (eg, with detergents or organic solvents) [5, 24]. 2) Once solubilized, it precipitates in simple aqueous buffers and will eventually aggregate irreversibly in mild detergents such as Triton X-100 [11]; it requires strong detergents such as SDS [1–8] or cetyltrimethylammonium bromide [12] for long-term aqueous solubility. 3) Mild pretreatments of the membrane itself will render band 3 resistant to dissociation in SDS [5]; the molecular basis for this effect is unclear. 4) Band 3 binds considerable Triton X-100 (ie, 0.77 g/g protein), a feature of integral as opposed to nonmembrane proteins, presumably reflecting an extended hydrophobic surface [13]. 5) The amino acid composition of band 3 is distinctly (but not profoundly) enriched in nonpolar residues (Table I; Refs 7, 11, 14). 6) It spans the hydrophobic core of the membrane [15–21] (see also Refs. 9, 22, 23 for review).

Several strategies have been evolved for the purification of this protein.

1. Band 3, and the other integral proteins, can be enriched up to twofold by stripping the membrane of peripheral polypeptides, using denaturing or protein-modifying reagents (eg, mild or strong alkali, lithium diiodosalicylate, or organic mercurials [24]). Some of these treatments appear mild, in that purported transport and liganding activities of the band 3 protein are preserved. (The lipid bilayer may help to stabilize the tertiary structure of integral proteins.)

2. Gently stripped membranes have been treated secondarily with low concentrations of the nondenaturing detergent Triton X-100 so as to solubilize some of the integral proteins (notably, the sialoglycoproteins) while leaving band 3 in the membranous residue. While these membranes can be resealed for use in transport studies, they appear to contain more protein than just band 3 and more functions than just anion transport activity [25].

3. Band 3 can be solubilized from ghosts at higher concentrations of Triton X-100 (eg, $\geqslant 0.5\%$) or other nondenaturing, nonionic detergents. Purification still requires dissociation of liganded cytoplasmic-surface proteins from band 3 [11]. The protein can then be isolated, still in detergent, by concanavalin A or ricinus communis affinity chromatography [26–30], ion exchange chromatography [11, 31], or immobilization by disulfide-sulfhydryl interchange [32]. (Barratt et al [33] have also isolated band 3 glycoprotein by concanavalin A affinity chromatography in dodecyltrimethylammonium bromide.) It has been observed that band 3 aggregation in mild detergents is significant and increases with

time, temperature, ionic strength, protein concentration, and acidity ([11]; J. Yu and T.L. Steck, unpublished observations). Irreversible adsorption to the chromatography bed frequently leads to reduced yields. Mixed aggregation with other integral proteins in the Triton X-100 extract can reduce purity.

4. Stripped membranes can be dissolved in the denaturing and dissociating detergent SDS and purified by gel filtration [7, 34], hydroxyapatite chromatography [52] or preparative polyacrylamide gel electrophoresis in the same detergent [21, 35]. Aggregation and irreversible binding are not usually a problem, but clean separation from the sialoglycoprotein PAS-1 must be assured. While these preparations are presumably inactive, they are convenient for chemical and even immunologic analysis (see Bhakdi et al [21]).

COMPOSITIONAL ANALYSIS OF THE BAND 3 POLYPEPTIDE

The molecular weight of band 3 has been variously estimated by gel electrophoresis in SDS to be 88,000–105,000 [9]; most recent estimates are compatible with the molecular weight of the polypeptide portion of this glycoprotein being 93,000 ± 3,000 [36]. Electrophoretically, band 3 is characteristically broad and asymmetrical, with a sharp leading edge and a diffuse trailing edge. This dispersion is stable and is not the result of interpeptide associations during electrophoresis [11]. It may be ascribed, at least in part, to variable glycosylation of the polypeptide backbone [11, 26, 28].

There is evidence for multiple minor components, or at least heterogeneous behavior of the polypeptides, in the 90,000- to 100,000-dalton region. There are, for example, three or four minor peripheral polypeptides on the cytoplasmic surface of rabbit erythrocyte membranes which migrate on gel electrophoresis with the bulk of band 3 [37]. A minor trypsin-resistant component in the band 3 region has been observed under some conditions of ghost digestion [21, 38]. Isoelectric focusing has also revealed multiple components in the band 3 region of gels [21, 39]; however, these could as well arise from variations in sialylation or amidation as from heterogeneity in the primary structure of the polypeptides present.

On the other hand, it has been repeatedly observed that the bulk of the band 3 population – perhaps 90% – behaves homogeneously with respect to disulfide cross-linking [36, 40, 41], the binding of glycolytic enzymes [42, 43], selective solubilization [24], and titration with 4,4′-diisothiocyanostilbene-2,2′ disulfonate (DIDS) [44–47]. Those laboratories studying the structure of the band 3 polypeptide by proteolytic dissection, covalent labeling, and partial amino acid sequence analysis thus far have failed to detect significant heterogeneity in individual preparations representing the bulk of the band 3 material [36, 38, 48–51]. For the present discussion, therefore, we shall consider the polypeptide portion of band 3 to be nearly homogeneous.

Our amino acid composition of band 3 is tabulated in column 1 of Table I [35]; similar analyses have been published [11, 14, 21, 34, 48, 51]. It is important that band 3 isolated by a variety of techniques shows uniformity in composition. This may be a reflection of its relative homogeneity. Like many other integral membrane polypeptides, the composition of band 3 is only moderately enriched in hydrophobic and uncharged residues, making its extreme insolubility even more striking.

The carbohydrate content of band 3 has been determined by several groups. As can be seen in Table II, there is considerable variation among these reports in both total carbohydrate content and the relative preponderance of the various sugars. These discrepancies could signify that different laboratories have isolated and analyzed subfractions of the

TABLE I. Amino Acid Composition of Band 3 and Five of its Major Fragments (mole percent)

Residue	Band 3	CH-38	CH-55	CH-17	TR-41	CN-23
Lys	3.4	4.2	2.6	3.5	4.3	2.8
His	2.2	2.1	1.8	1.4	3.1	3.8
Arg	5.2	4.8	5.0	4.2	5.2	4.7
	10.8	11.1	9.4	9.1	12.6	11.3
Asx	6.5	6.1	6.4	6.1	8.1	8.0
Glx	11.9	8.9	12.9	9.6	15.3	18.6
	18.4	15.0	19.3	15.7	23.4	26.6
Thr	5.0	5.5	5.5	5.5	5.5	6.4
Ser	6.8	6.9	7.0	7.1	6.0	5.9
Pro	5.7	6.7	5.9	4.9	5.6	5.0
Gly	7.7	7.7	7.8	9.0	6.4	5.6
Ala	7.4	7.5	7.2	7.1	7.6	6.7
½ Cys	0.7	0.7	0.6	0.9	1.2	0.6
	33.3	35.0	34.0	34.5	32.3	30.2
Val	7.6	8.6	6.4	7.3	6.4	5.5
Met	2.2	3.1	2.1	1.3	1.8	2.6
Ile	4.7	6.0	4.5	6.8	3.8	3.0
Leu	13.4	13.4	14.0	12.6	12.4	12.8
Tyr	3.1	1.9	3.0	3.6	2.4	3.1
Phe	5.5	5.4	6.5	7.5	3.8	3.3
Trp	1.2	0.4	0.8	1.8	1.2	1.8
	37.7	38.8	37.3	40.9	31.8	32.1

The identities of the chymotryptic (CH), tryptic (TR), and S-cyanylation (CN) fragments are given in Figure 1 and in the text. Data are recalculated from Steck et al [35].

TABLE II. Carbohydrate Composition of Band 3 (moles/mole band 3)

Residue	A	B	C	D	E	F
Fucose	8.3	2.5	0.75	1.4	4	7
Mannose	8.3	7.2	3.4	4.3	7	5
Galactose	8.3	12	5.2	4.8	24	16
N-acetylglucosamine	7.6	20	5.5	10.0	25	17
N-acetylgalactosamine	0	0	0.87	3.2	4	2
Sialic acids	1.8	–	0.87	0	5	5
Total	34.3	41.7	16.6	23.7	69	52

Data for columns A–F are taken from References 14, 21, 34, 52, 53, and 65, respectively. Values were normalized to moles/mole band 3, assuming that band 3 has a molecular weight of 93,000 and contains 833 amino acid residues [35]. Values for glucose are considered unreliable and have been omitted.

broad band 3 dispersion relatively enriched or deficient in carbohydrate. Generally, galactose and N-acetylglucosamine were found to be most abundant while N-acetylgalactosamine and sialic acids were present in small amounts.

Gahmberg et al [52] have provided evidence that there are two types of oligosaccharide chains, one unusually large and the other of only a few residues. One of each chain per band 3 polypeptide could account for the entire band 3 carbohydrate content. Their analysis also suggests that the major oligosaccharide may be heterogeneous in composition, which might further broaden the band 3 electrophoretic profile.

Individuals possessing the rare blood type En(a–) appear to lack glycophorin (PAS-1 and -2), yet are clinically normal [52, 53]. Band 3 isolated from these variants exhibited a considerable increase in glycosylation, attributable to a longer major oligosaccharide chain [52]. Its composition resembled that of the wild-type band 3, rather than bearing increased sialic acid in compensation for the loss of the major sialoglycoprotein.

Polypeptides in the band 3 region can be phosphorylated by adding $^{32}PO_4$ to intact cells or by incubating unsealed ghosts with γ-^{32}P-ATP (adenosine triphosphate) [9, 23]. The chemical analysis of phosphate in band 3 isolates, however, amounts to no more than 0.2–0.3 mole/mole [43, 54]. Furthermore, the ^{32}P acceptors in the band 3 region are not identical with the bulk of band 3 and some could be minor and unrelated comigrating polypeptides [28, 55, 56]. All of the ^{32}P incorporated in vitro into the band 3 region appears to be associated with a population of integral molecules [56] which are not bound by concanavalin A affinity columns [28]. Minor fractions of band 3 highly enriched in the ^{32}P incorporated in vitro can be isolated by fractional elution from affinity columns of ricinus communis 1 lectin [30].

The absorption spectrum of band 3 in the nonionic and nonaromatic detergent Ammonyx LO is typical of simple proteins lacking bound chromophores [11].

BAND 3 STRUCTURE

Band 3 spans the erythrocyte membrane asymmetrically [9, 15–23, 35, 50, 51, 58, 59], with its carbohydrate groups confined to the extracellular space [60]. The polypeptides appear to be associated as dimers [40, 41, 61], which correspond in size (~180,000 daltons), prevalence ($\sim 6 \times 10^5$ per ghost), and disposition (integral and transmembrane) to the ~ 70-Å intramembrane particles visualized in the particle-rich faces of freeze-fracture electron micrographs [62]. Band 3 has also been reported to contain 43% α-helix, as judged from its circular dicroism in a nondenaturing detergent [11].

We have cleaved band 3 in situ into large segments representing outer-surface, transmembrane, and cytoplasmic-surface regions (35, 36; Fig 1). Digestion of intact cells with chymotrypsin cleaves band 3 into 38,000- and 55,000-dalton glycopeptide fragments, whose apparent molecular weights and amino acid compositions sum to that of band 3 (Table I). The former would appear to bear the major oligosaccharide unit and the latter, the smaller [52, 65]. Both fragments are firmly integrated into the membrane and are water-insoluble upon isolation [35, 36]. The hydrophobicity index of both of these pieces is approximately that of band 3 itself (Table I), apparently reflecting a balance between those portions in contact with the aqueous compartment and those buried in the bilayer. The 38,000-dalton segment, unlike the complementary 55,000-dalton piece, does not seem to traverse the membrane. It may contribute to the surface projections seen following the freeze-etching of intact red cells previously digested with proteases [63, 64].

Vigorous chymotryptic digestion at both membrane surfaces leaves a 17,000-dalton subfragment of the 55,000-dalton segment spanning the membrane [35, 36]. The 17,000-dalton fragment is the most hydrophobic of the major proteolytic pieces (Table I); it is also irreversibly integrated into the membrane and is highly water-insoluble upon isolation

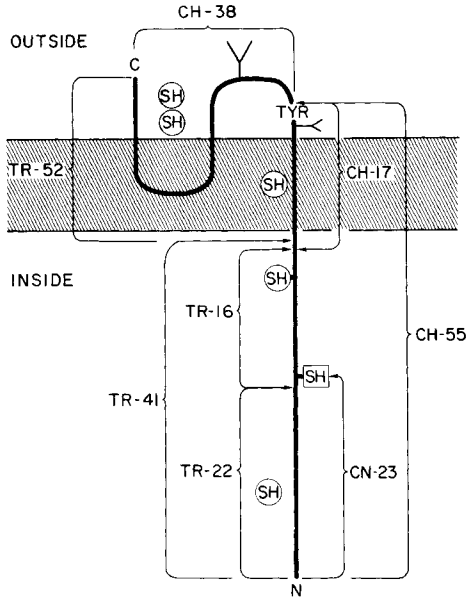

Fig 1. Schematic summary of the disposition of some proteolytic fragments of band 3 in the red cell membrane. Three major, integral fragments are generated by digestion with chymotrypsin (CH). Trypsin (TR) cleaves the polypeptide into a 52,000-dalton integral segment and three major cytoplasmic-surface fragments not anchored in the bilayer. S-cyanylation (CN) of a cysteine residue (SH) cleaves a 23,000-dalton piece from the cytoplasmic-surface, amino-terminal region of band 3. See text for details. Figure adapted from Steck et al [35], with permission.

[35, 65]. These properties render its primary sequence analysis particularly challenging [65]. Since hydrophobic stretches of approximately 20 residues are sufficient to span a lipid bilayer (see, for example, glycophorin [66] or the phage M13 coat protein [67]), this ~ 154-residue segment probably has a complex folding pattern – ie, a globular structure – in the membrane. This would be compatible with the aforementioned freeze-fracture image and with a transport function. Clearly, the position of the several basic residues in the primary structure of this peptide (as well as the other polar residues) will be of great interest.

The cytoplasmic aspect of band 3 is several orders of magnitude more sensitive to proteolysis by trypsin, chymotrypsin, papain, and other proteases than is the extracellular pole [16, 36]. The various fragments so generated are readily released from the membrane in a water-soluble form, still associated with one another in a dimeric ensemble [36]. This domain has a composition distinctly more polar than the remainder of band 3, as illustrated in Table I for the largest of the fragments, a 41,000-dalton tryptic peptide. This piece is seen to roughly complement the 17,000-dalton transmembrane segment when they are compared in size and composition to the presumed parent, the 55,000-dalton chymotryptic polypeptide (Fig 1; Table I; Ref 35).

Major fragments of band 3 have also been generated by chemical cleavage at cysteine residues following their S-cyanylation by 2-nitro-5-thiocyanobenzoate (NTCB) [35, 36, 49–51]. In particular, a 23,000-dalton fragment is released from the cytoplasmic terminus of the band 3 chain, adjacent to which is a 12,000-dalton cyanylation fragment. A ~35,000-

dalton segment, apparently the sum of the 23,000 + 12,000 pieces, has also been recovered [49–51]. The composition of the 23,000-dalton fragment is given in Table I.

Drickamer [50, 51] has generated, mapped, and analyzed additional fragments, using S-cyanylation, hydroxylamine, cyanogen bromide, and N-bromosuccinimide to effect chemical cleavage of band 3 molecules previously covalently labeled in various ways from each membrane surface. The outer-surface 38,000-dalton chymotryptic fragment was strikingly unreactive to a variety of probes. In addition, an 11,000-dalton segment of the transmembrane region of band 3 was apparently labeled from both sides of the membrane in a fashion suggesting that this segment looped from the outer surface to the inner and back again.

Tanner and his associates have proposed a somewhat different disposition for the band 3 polypeptide than that shown in Figure 1 [17, 38, 48, 59]. Most notably, an S-shaped structure was suggested, which crosses the bilayer twice and has a large segment of polypeptide duplicated at the two ends of the molecule. My interpretation of these data is that T1 and T2, the two major tryptic fragments which Tanner assumes to be complementary (ie, from different ends of the molecule), are in fact overlapping segments from the same region. I suspect that T2 contains the counterpart of our 17,000-dalton membrane-spanning fragment and is hence a portion of T1 which can be generated in low yield by tryptic digestion of ghosts (see Ref 48 and Fig 4 in Ref 36). Since the readily eluted tryptic fragments we isolate from the cytoplasmic-surface domain of band 3 might have been lost from Tanner's digests during a washing step, and because Tanner's approach was based on autoradiographic analysis of radioiodinated membranes, it is difficult to make a closer comparison of our results at present.

FEATURES OF THE CYTOPLASMIC DOMAIN OF BAND 3

Cross-Linking

Oxidation of ghosts with a variety of reagents, particularly the o-phenanthroline-Cu^{++} chelate complex, converts band 3 into a disulfide-linked dimer [40, 41]. Band 3 probably exists as a stable, noncovalent dimer for these reasons: 1) Dissolution of the membrane in nondenaturing detergents such as Triton X-100 does not reduce the efficiency of the cross-linking reaction despite the profound dilution in local protein concentration attending solubilization [11, 40]. 2) The non-cross-linked and cross-linked forms of Triton X-100-solubilized band 3 have identical sedimentation rates upon sucrose gradient centrifugation [11, 13]. 3) Millisecond flash photochemical cross-linking joins band 3 polypeptides into covalent dimers at a more rapid rate than transient interprotein collisions should allow [61].

The disulfide cross-link has been shown to be confined to the cytoplasmic pole of the polypeptide [36]. Disulfide-cross-linked band 3 can be cleaved by 1 mM CN^- via S-cyanylation to release the same N-terminal 23,000-dalton fragment generated by NTCB [36] (see above). Recently Miyakawa, Takemoto, and Fox [68], using a completely different membrane-impermeable reagent, tartryl-di(glycylazide) (TDGA), also found that band 3 was cross-linked only from the cytoplasmic surface.

At present, there is no evidence as to whether the membrane-integrated portions of the band 3 dimer are intimately apposed.

The Amino Terminus of the Band 3 Polypeptide Is Confined to Its Cytoplasmic Pole

A current concept of the integration of membrane-spanning proteins [69] is an adaptation of the model proposed by Blobel and his colleagues for secreted proteins [70]. The amino terminus of the nascent polypeptide, led by an excisable "signal peptide," is threaded directly from a membrane-bound ribosome through the bilayer into the cisternal space of the endoplasmic reticulum. In this way, the N terminus comes to reside on the outer surface of the plasma membrane. Glycosylation of such proteins would be confined to their amino terminal segment, the carboxyl terminus of the molecule never leaving the cytoplasmic space. This paradigm clearly applies to glycophorin A [66], histocompatibility antigens [71], and perhaps the envelope glycoprotein of vesicular stomatitis virus [72].

In contrast, band 3 exhibits a different pattern. Drickamer [50] has shown that the 23,000-dalton fragment generated from band 3 by S-cyanylation with ^{14}C-NTCB is not radioactively labeled, as is the complementary 71,000-dalton polypeptide. Since every newly generated N terminus should be labeled by this cleavage reaction, the original amino terminus of band 3 would appear to reside in the cytoplasmic, 23,000-dalton piece. Fukuda et al [49] have confirmed this result.

Jenkins and Tanner [48] have adduced complementary evidence favoring the same conclusion. They found that their fragment, 3f (which appears to be very similar to the 55,000-dalton chymotryptic fragment discussed above), shares a blocked amino terminus with band 3. In our view (Fig 1), but not Tanner's, the shared N terminus would be at the cytoplasmic end of band 3. More recently, Steck et al [35] reported that the 23,000-dalton cyanylation fragment and the 22,000-dalton tryptic fragment, both of which map at the cytoplasmic pole of band 3, also have unreactive amino termini, as does the parent band 3 molecule. Thus, four laboratories are in agreement concerning this unexpected orientation of band 3. Furthermore, it seems that the glycosylation of band 3 is confined to its C-terminal section, despite one argument to the contrary [48]. It is interesting that integral intestinal brush border membrane hydrolases may share this "reversed polarity" with band 3 [73]. The mechanism of insertion of these proteins into membranes will obviously be of great interest.

Immunogenicity

Two laboratories have recently reported that rabbit antibodies prepared against band 3 react only with immunodeterminants within the cytoplasmic domain of the molecule [31, 49]. Since immunization was carried out with whole ghosts and with band 3 dissolved in either Triton X-100 (a nondenaturing detergent) or SDS (a denaturing detergent), this specificity cannot be ascribed to the mode of polypeptide preparation. However, evidence for antigenic determinants in the extracellular portion of band 3 has also been reported [21].

Genetic Polymorphism

Mueller and Morrison [74] have observed altered gel electrophoresis patterns in band 3 and certain of its fragments obtained from a small fraction of normal individuals. They ascribe this feature to a ~ 3,000-dalton increase in the size of the cytoplasmic domain of this polypeptide, presumably of genetic origin.

Köhler and associates have recently reported that the 23,000-dalton cytoplasmic-terminal segment of band 3 is both structurally and immunologically heterogeneous among normal human donors [75, 76]. The composition of this fragment showed unexpected variation, particularly in glutamate (or glutamine) content. Furthermore, the amino acid

sequence of a specific pentapeptide from this region varied among normal donors [76]. Finally, mutual inhibition among various 23K preparations in a radioimmunoassay with rabbit anti-23K antisera showed antigenic variation within the donor population [75].

Phosphorylation

Phosphorylation of polypeptides in the band 3 region has been repeatedly observed in intact cell and isolated membranes [9, 23, 54–57]. The phosphate bond is stable – apparently of the ester type. Labeling is not stimulated by cyclic AMP (adenosine monophosphate) in vitro. The phosphate appears to be introduced only from the cytoplasmic surface [77]. Drickamer [50, 51] has found that phosphorylation occurs primarily within 10,000 daltons of the cytoplasmic N terminus of band 3, but that some $^{32}P_i$ can also be detected in fragments ascribed to the center of the polypeptide chain.

Associations

The cytoplasmic pole of band 3 appears to provide binding sites for several other proteins. The most tenaciously bound is the membrane polypeptide termed band 4.2 [42]. This peripheral protein [24], which behaves as a homotetramer in cross-linking studies [40], is of unknown function; unlike the other band 3 ligands discussed below, its binding has not been shown to be reversed by electrolytes or metabolites. Its binding stoichiometry might be as low as one tetramer per 20 band 3 polypeptides [9].

Band 3 provides the membrane binding site for both glyceraldehyde 3-P dehydrogenase (G3PD) [42] and aldolase [43] in vitro; the extent to which these associations occur in the intact cell has yet to be established. The binding reactions are reversible and sensitive to both the elevation of ionic strength and to specific metabolites. The patterns of elution suggest that the associations of these enzymes with band 3 utilize known binding sites for substrates or allosteric effectors. Binding is confined to the cytoplasmic pole of band 3 and has a stoichiometry of one enzyme tetramer per band 3 protomer. There are more than enough copies of band 3 to bind all of the G3PD plus aldolase in the cell. Interestingly, the catalytic activity of aldolase is inhibited reversibly upon binding to ghosts, isolated band 3, or cytoplasmic surface fragments of band 3 [43].

Recently hemoglobin was shown to bind to ghosts with a stoichiometry of 1.2–1.3 $\times 10^6$/membrane [78], suggesting that band 3 provides the liganding site. Since this association was only observable in the acidic pH range at low ionic strength, its physiologic significance is unclear.

Branton and his colleagues have presented freeze-fracture evidence that the lateral distribution of the A (PF) face intramembrane particle population (believed to contain principally band 3) is influenced by spectrin [79, 80]. However, a study by Cherry et al [81] on the rotational freedom of band 3 in the intact cell suggested that it is not immobilized, as would be expected if it were bound to an extended spectrin network. Furthermore, Bennett and Branton [82, 83] have reported that the membrane binding site for spectrin is not associated with the bulk of band 3 molecules. The importance of transient interactions between the spectrin-actin system and integral proteins such as band 3 is obviously an important topic for future research.

Ultrastructure

As discussed, a ~40,000-dalton cytoplasmic-surface segment of the band 3 polypeptide can be readily released from the membrane by mild proteolysis [36]. This domain

would amount to 80,000-daltons per band 3 dimer, and should thus be visible in situ in the electron microscope. Recent freeze-etch studies of the true cytoplasmic face of human red cell ghosts and inside-out vesicles have indeed revealed such projections [63, 64]. Their prevalence was compatible with the predicted ~ 600,000-dalton band 3 dimers present per ghost. Furthermore, these projections could be decorated with G3PD, which forms specific associations with band 3 [42]. The projections (and the G3PD decoration) were abolished by mild proteolysis sufficient to cleave away the cytoplasmic aspect of band 3. It is interesting that similar cytoplasmic-surface projections have also been visualized in preparations of Na^+, K^+-ATPase [84], and Ca^{++}-ATPase [85]. This finding can be added to other similarities among these molecules; ie, they are all membrane-spanning oligomeric transport proteins containing disulfide-linkable polypeptides of ~ 10^5 molecular weight [86, 87].

BAND 3 FUNCTION

Several functions have been attributed to the band 3 region of the electrophoretic profile. Bellhorn, Blumenfeld, and Gallop [88] found the erythrocyte acetylcholinesterase (radiolabeled with diisopropylfluorophosphate) in this gel region; however, more recent work has shown that this enzyme, while an integral membrane glycoprotein, is clearly distinguishable from band 3 in its electrophoretic and chromatographic behavior both in Triton X-100 and in SDS [11, 89].

A rather indirect line of evidence, based primarily on the binding of cytochalasin B to the integral proteins of stripped ghost membranes, suggested that glucose transport activity was an attribute of band 3 [90–92]. However, recent studies have clearly identified an integral glycoprotein of 45,000–55,000 daltons as the likely glucose transporter [93–95] (see also the reports of D.C. Sogin and P.C. Hinkle [95a], and of M.A. Zoccoli et al [95b] in this Symposium).

The ~ 10^5-dalton polypeptide of the Na^+,K^+-ATPase has been identified in the band 3 electrophoretic region as the acyl-^{32}P covalent intermediate [96]; however, this polypeptide contributes roughly 10^2 of 10^6 polypeptides to the band 3 zone [97].

Elsewhere in this symposium, both Branton and Sheetz have argued that a minor fraction of the band 3 region which remains associated with spectrin-actin residues following Triton X-100 solubilization of the bulk of band 3 could be the membrane binding site for spectrin. The relationship of this material to the bulk of band 3 therefore warrants careful evaluation.

Osmotic water flow in intact red cells can be inhibited by the sulfhydryl reagent 5,5′-dithio-bis-(2-nitrobenzoate) with a concomitant and highly selective labeling of band 3 [98]. The relationship of water transport to band 3 is thus an intriguing question.

The relationship of band 3 to anion transport is discussed extensively by Rothstein [110] elsewhere in this symposium and in other recent reviews [44, 99–101]. Briefly, the major evidence linking band 3 to anion transport is twofold. First, covalent inhibitors of anion exchange, notably stilbene disulfonates such as DIDS, label band 3 rather selectively [10, 14]. Ignoring early discrepancies, complete inhibition occurs when roughly 1.2×10^6 DIDS molecules are bound per cell – ie, one DIDS molecule per band 3 [44–47]. Secondly, partially purified preparations of band 3 confer anion transport activity on lipid vesicles [25, 27]. While neither line of evidence rigorously excludes a minor protein as the anion transporter, no such alternate candidate has been implicated. That band 3 is the anion transporter is thus a strong working hypothesis, consistent with what is known about the structure of this molecule.

The behavior of the anion transporter is that expected of a fixed and asymmetrical membrane-spanning protein like band 3. The kinetic constants for anion transport appear to be different at the two membrane surfaces [102, 103; Gunn RB, Froehlich O, Steck TL: Manuscript in preparation]. Furthermore, mild oxidation, which cross-links band 3, leads to differential changes in the apparent kinetic constants at the two sides of the membrane [102, 103]. Finally, various inhibitors have been shown to act differentially at one membrane surface or the other [101, 103–105].

Papain and pronase digestion at the outer surface of intact red cells inhibits anion transport profoundly [44]. Since the 55,000-dalton membrane-spanning segment of band 3 remains intact following such treatment, it seems reasonable to conclude that some aspect of the degraded 38,000-dalton region is necessary for transport activity. Chymotrypsin digestion of intact cells does not inhibit transport although it cleaves band 3 into 38,000-dalton and 55,000-dalton segments (Fig 1). We may infer that native tertiary structure is retained in the protein mildly cleaved with chymotrypsin but not following the more extensive papain or pronase digestion. Freeze-etch electron micrographs suggest that the outer-surface projections attributable to band 3 dimers survive even vigorous proteolysis of intact cells [63, 64], so that this aspect of the molecule apparently does not fall to pieces upon cleavage.

Recent studies have indicated that two covalent inhibitors of anion flux selectively bind to the 17,000-dalton, membrane-spanning portion of band 3 [50, 106], making this domain a prime target for studies on the structural basis for anion transport.

While the integral outer and membrane-spanning segments of band 3 appear to be involved in anion exchange activity, the 40,000-dalton cytoplasmic-surface domain can be excised without significant effects on transport [103, 106]. The binding of G3PD and aldolase to this portion of the molecule also does not alter transport kinetics significantly [103]. Furthermore, antibodies directed against the 23,000-dalton cytoplasmic terminus of band 3 fail to inhibit anion transport [31].

It is commonly held that the interface between the protomers in oligomeric transport proteins might provide a polar pathway through which solutes traverse the membrane (eg, Singer [107]). This implies that the functional unit of band 3 is the dimer rather than the individual protomers. However, the correlation of the fractional labeling of band 3 polypeptides by DIDS with the fractional inhibition of anion transport is quite linear and has a slope of unity [44–47]. This finding suggests that either 1) each protomer contributes equally and independently to transport activity, 2) there is a very high degree of positive cooperativity in the binding of DIDS to band 3 such that the ligand is essentially added pairwise to dimers, or 3) that DIDS modification of the first protomer of a functional dimer happens to lower the activity of the unit to one-half and that the second bound DIDS molecule completes the inactivation. In the absence of discriminating data, the first alternative seems the most likely. This is not an unreasonable inference, since many soluble oligomeric enzymes are composed of identical, nonintereacting subunits.

How does the anion transporter function? Kinetic evidence suggests a double displacement (ping-pong) mechanism [108]. Presumably, single anions are alternately transported from one compartment to the other in a reciprocating cycle, probably conformational in nature. This hypothesis is consistent with studies of differential inhibitor binding at the two membrane surfaces [101, 110]. For Cl^- and HCO_3^-, the rate constant for such a conformational change would be vastly greater for the substrate-loaded as opposed to the unloaded form of the transporter [101, 109]. Consequently, Cl^- transport and HCO_3^- transport are almost entirely one-for-one; the net movement of these anions,

arising from cycles with an empty return step, would occur about once in 10^5 exchange cycles under conditions where membrane potential gradients are dissipated by a diffusable cation system. This behavior is quite compatible with the purported physiological functions of the red cell anion transport system: 1) the exceedingly rapid 1:1 exchange of Cl^- and HCO_3^- required for efficient CO_2 disposal; 2) the slow net anion movements accompanying rate-limiting cation movements in cell volume adjustment; 3) the slow transport of metabolically important anions, such as phosphate and lactate.

An integrated understanding of band 3 structure and function seems at present to be a distant but increasingly realizable goal.

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