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Topical Review

Oligomeric Structure and the Anion Transport Function of Human Erythrocyte Band 3 Protein

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Introduction

The recognition that many transport proteins in biological membranes are oligomeric has led to hypotheses about possible roles of subunit interactions in the mechanisms of transport. For example, the transport pathway could consist of the interface between the two halves of a dimer; shifts in the relative positions of the subunits could allow alternate access of a substrate binding site to the two sides of the membrane [32, 133]. There is evidence that the adenine nucleotide translocator of the mitochondrial inner membrane functions by way of concerted interactions between subunits [72], but it is not clear how representative this system is of other transport proteins. No functional role has been established for subunit interactions in the ion transport catalyzed by the (Na,K)-ATPase [76] or the sarcoplasmic reticulum Ca^{++}-ATPase [94]. Another eukaryotic ion transport protein for which considerable structural information is available is the human erythrocyte anion transport protein, band 3. The purpose of this topical review is to summarize the current state of knowledge of band 3 quaternary structure and to discuss the possible relationship between oligomeric structure and the ion transport function of band 3. These topics are prefaced by a brief general review of human band 3 structure and function. The reader is referred elsewhere for detailed discussion of band 3 anion exchange kinetics [12, 21, 38, 39, 48, 59, 73, 93, 122–124, 127], electrical properties [40, 74], conformational changes associated with transport [12, 59, 73, 86, 108], the structure and disposition of the polypeptide chain [11, 29, 60, 68, 73, 85, 90, 112, 119, 139, 144, 155], associations with other proteins [85, 102, 121, 139, 158], interaction with lipids [71, 75], and band 3 from nonhuman red cells [55, 98, 99].

Key Words red cell · chloride · transport · Band 3 protein · oligomer

General Aspects of Band 3 Structure and Function

The physiological mode of band 3 catalyzed anion transport is the one-for-one exchange of Cl and HCO_3. In the systemic capillaries, Cl enters the cells in exchange for intracellular HCO_3; the driving force for this exchange is the slight increase in intracellular HCO_3 that results from the hydration (via carbonic anhydrase) of incoming CO_2. The purpose of the Cl/HCO_3 exchange is to allow HCO_3 to be formed inside the red cell but to be carried to the lungs mainly as plasma HCO_3 [*see* ref. 120]. The pH drop associated with the entry of CO_2 is minimized because the CO_2 hydration is inside the red cell, where hemoglobin is a very effective H^+ buffer. The H^+ formed from the CO_2 also facilitates O_2 release from hemoglobin. The Cl/HCO_3 exchange takes place in reverse in the pulmonary capillaries. The exchange is sufficiently rapid to be complete in the time the red cell spends in the capillary [e.g. ref. 20, 105, 152].

In order to meet the need for rapid anion exchange, the red cell membrane contains a very large number of copies of band 3. It is estimated that band 3 constitutes about 25% of the total red cell membrane protein [35]. Reconstitution experiments with purified band 3 [e.g. 75, 81, 118] have shown that the anion transport requires no other polypeptide. Although glycophorin was included in one of these reconstitutions [118], it is not necessary for anion transport, since En(a-) red cells, which lack glycophorin [44, 142], have normal anion transport [143]. Each band 3 monomer has a single carbohydrate chain attached to an exofacial site in the C-

terminal third of the molecule [30]. The length of the carbohydrate chain is quite variable, the longest containing many repeated galactose-N-acetylglucosamine units [145]. The carbohydrate has no known role in transport.

The band 3 polypeptide consists of two structurally distinct domains. The N-terminus is acetylated [30] and is contained in a water-soluble cytoplasmic domain of molecular weight 43,000, which can be cleaved from the intracellular surface of the membrane by proteolysis under very mild conditions [141]. There appears to be little noncovalent interaction between the cytoplasmic domain and the rest of the molecule [2; *see*, however, ref. 121], and the cytoplasmic domain is not necessary for anion transport [34, 46, 78]. The function of this portion of band 3 is to bind to the membrane skeleton [6, 7, 51]; it also binds to glycolytic enzymes [85, 158] and to hemoglobin [121]. The first 200 residues of the cytoplasmic domain have been sequenced [68].

The remainder of band 3 has molecular weight 52,000 and is hydrophobically associated with the membrane [41, 141]. This membrane domain can be cleaved by chymotrypsin at an extracellular site about 35,000 daltons from the C-terminus [14, 29, 141]. The 35,000-dalton fragment is glycosylated and runs as a diffuse, weakly stained band on SDS polyacrylamide gels [61, 89, 140]. The 35,000-dalton peptide is tightly bound to the membrane [140]; it crosses the membrane at least twice [89, 112, 113, 119] and it contains amino acid residues necessary for anion transport [8, 9, 11, 18, 58, 60, 101].

Between the 35,000-dalton fragment and the cytoplasmic domain is a segment of about 17,000 daltons [46, 141]. This peptide must cross the membrane an odd number of times, since its N-terminus is intracellular and its C-terminus is the extracellular chymotrypsin cleavage site. The 17,000- and 35,000-dalton fragments remain associated noncovalently after extracellular chymotrypsin cleavage [61, 116], and the proteolysis does not cause inhibition of anion transport [14]. A very hydrophobic segment of 37 residues in the middle of the 17,000-dalton fragment has been sequenced [90].

The band 3-mediated anion transport consists almost entirely of an obligatory one-for-one exchange; the Cl conductance through band 3 is about 10,000-fold less than would be expected from $^{36}Cl/Cl$ exchange rates [*see* 73]. The catalytic cycle for the anion exchange is believed to be a "ping-pong" [16] mechanism, in which an intracellular anion binds, is translocated outward, is released, *followed by* the binding and inward translocation of an extracellular anion [48, 59, 73].

Band 3 has rather broad substrate specificity. The halides [22], HCO_3 [151], phosphate [24, 52], sulfate [127], borohydride [58], superoxide [82], lithium carbonate [43] and dithionite [124] are all transported, though at widely varying rates. For example, Cl/Cl exchange at pH 7 is about 10,000 times faster than SO_4/SO_4 exchange; as the pH is reduced below 7, chloride transport is inhibited and sulfate transport is accelerated [73]. This apparently results from protonation of a site at or near the anion transport site [57, 93] as first postulated by Gunn [47]. The Cl/Cl exchange flux is inhibited by high Cl concentrations [21]; the exact relationship between the transport site(s) and the site(s) responsible for substrate inhibition is not yet clear [73, 85].

A large number of substances inhibit red cell anion transport, either reversibly or irreversibly [3–5, 13, 18, 19, 25, 69, 90, 107, 132, 152, 159, 162]. Several very potent and useful inhibitors are stilbene disulfonate derivatives, including SITS[1], DIDS, H_2DIDS, DBDS, DNDS, BADS and BIDS. These agents do not penetrate the membrane and can completely inhibit band 3-mediated anion exchange by binding to a site that is accessible from the extracellular water. At least for H_2DIDS [129] and DNDS [38], the inhibition is competitive (with Cl). Intracellular stilbenedisulfonates (e.g., trapped inside resealed ghosts) do not inhibit the transport [67]. Derivatives such as SITS, DIDS, H_2DIDS and BIDS can react covalently with band 3 and irreversibly inhibit anion transport. The amino acid residue

[1] *Abbreviations:*

SITS	4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate
DIDS	4,4′-diisothiocyanostilbene-2,2′-disulfonate
H_2DIDS	4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate
BADS	4-benzamido-4′-aminostilbene-2,2′-disulfonate
BIDS	4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate
DADS	4,4′-diaminostilbene-2,2′-disulfonate
DBDS	4,4′-dibenzamidostilbene-2,2′-disulfonate
$H_2(NBD)_2DS$	4,4′-*bis*-(4-nitro-2,1,3-benzoxadiazolyl)dihydrostilbene-2,2′-disulfonate
DNDS	4,4′-dinitrostilbene-2,2′-disulfonate
NAP-taurine	N-(4-azido-2-nitrophenyl)-2-aminoethane sulfonate
NDS-TEMPO	N-4-(2,2,6,6-tetramethyl-1-oxyl)piperidinyl-N′-4-(4′-nitro-2,2′-stilbenedisulfonate)
SDS PAGE	Polyacrylamide gel electrophoresis in sodium dodecyl sulfate
DIOSPITC	2,6-di-iodosulfophenylisothiocyanate
DTBP	Dimethyl-3,3′-dithiobispropionimidate
DNCO	di-N-(2-nitro-4-azidophenyl)cystamine-S-S-dioxide
$C_{12}E_9$	nona-oxyethyleneglycol *n*-dodecyl ether
$C_{12}E_8$	octa-oxyethyleneglycol *n*-dodecyl ether
EAC	1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide

with which H_2DIDS reacts most rapidly is on the 17,000-dalton chymotryptic fragment [110]. Rothstein and coworkers have shown that the modified residue is a lysine [111] that appears to be located 7000 to 9000 daltons from the C-terminus of the 17,000-dalton fragment [110, 119]. Mawby and Findlay [90] have performed automated Edman degradation on a CNBr fragment that represents the C-terminal 9000 to 11,000 daltons of the 17,000-dalton fragment. The H_2DIDS-binding lysine localized by Rothstein's group should be 2000 to 4000 daltons from the N-terminus of this CNBr peptide. Mawby and Findlay, however, find no lysine in the first 37 cycles. Moreover, the anion transport inhibitor DIOSPITC (an anionic isothiocyanate, as is H_2DIDS) appears to be attached covalently at least from 45 residues from the N-terminus of this peptide [90]. The lysine on the 17,000-dalton fragment with which H_2DIDS reacts covalently is probably not the site to which extracellular transported anions bind. Chloride does not protect this lysine from dinitrophenylation [108], and this lysine can be reductively methylated with no detectable effect on Cl/Cl exchange [58]. The other end of the bound H_2DIDS molecule reacts covalently with a lysine residue on the 35,000-dalton chymotryptic fragment [61]. This lysine is probably associated with the anion transport pathway, since transport is 75% inhibited when it is reductively methylated [58]. The exact location of this lysine in the primary structure is unknown, but is at least 72 residues from the chymotrypsin cleavage site [60].

Wieth and coworkers [8, 9, 152–154] and Zaki [159–161] have shown recently that chemical modification with arginine-selective reagents inhibits band 3-catalyzed anion transport. Bjerrum et al. [9] have demonstrated that a functionally important arginine residue is located in the 35,000-dalton fragment. This arginine is in contact with the extracellular water, since the chemical modification was performed under conditions that favor the phenylglyoxalation of extracellular but not intracellular arginine residues. Wieth and coworkers [152] have also demonstrated the existence of an essential carboxyl group in band 3 (*see below*).

Band 3 Heterogeneity

For the present purpose, our definition of "band 3" includes all human red blood cell membrane integral polypeptides that have apparent M_r (SDS-PAGE) of 90,000 to 105,000 and that are labeled by treatment with low concentrations (<10 μM) of $^3H_2$DIDS. There are minor components (not detectable by protein staining) that migrate with band 3 but that are unrelated to anion transport; these components are detected by iodination (or phosphorylation) and proteolysis of intact cells [65, 115] or by two-dimensional gel electrophoresis [17]. However, the bulk (at least 90%) of the integral protein in band 3 is homogeneous with respect to DIDS (and H_2DIDS) binding and extracellular proteolysis [61, 139].

Band 3 is clearly heterogeneous in its carbohydrate content [e.g. 36, 45, 56, 145]. Much of this heterogeneity can be removed by treating intact cells with an endo-β-galactosidase from *E. freundii* [42], which causes band 3 to migrate as a much sharper band on SDS-PAGE [95]. Even after this partial deglycosylation, band 3 is still broader than bands 1, 2, 4, 5, 6 or 7. This may reflect heterogeneity in endo-β-galactosidase-resistant core oligosaccharides.

Not enough is known about the sequence of band 3 to allow absolute conclusions concerning its homogeneity. Brock et al. [11] have very recently sequenced 72 residues of an integral fragment of human band 3; they report no regions of ambiguous sequence that might suggest microheterogeneity. Kaul et al. [68] have sequenced the N-terminal 201 residues of band 3. Most of this segment is homogeneous, but there is evidence of microheterogeneity in residues 82 to 106.

Ideguchi et al. [54] have recently presented clear evidence that band 3 contains three subspecies of differing isoelectric point. This heterogeneity is not caused by carbohydrate, since the unglycosylated chymotryptic 60,000-dalton fragment also can be separated into three major bands by isoelectric focusing [54]. Although this is consistent with protein heterogeneity, the authors did not address the possibility that band 3 is heterogeneously phosphorylated. The 60,000-dalton fragment is known to be a substrate for phosphorylation [23, 29, 65]. Ross and McConnell [118] had earlier demonstrated that intact band 3 could be separated into components of differing pI; they suggested phosphorylation as one possible cause of the heterogeneity.

An interesting example of human band 3 heterogeneity is the genetic variant identified by Mueller and Morrison [96]. In this variant, the 60,000-dalton proteolytic fragment migrates as a doublet (apparent M_r 60,000 and 63,000) on gradient SDS PAGE in the Laemmli buffer system. The membrane domain of the variant band 3 is normal with respect to anion transport. The only detectable difference between variant and normal is the apparent size of the cytoplasmic domain [96]; the molecular details of this difference are not yet known.

In summary, it is still appropriate to refer to the anion transport protein in human band 3 from most donors as a homogeneous polypeptide, with the understanding that, as more details of band 3 sequence

and structure are established, it is possible that microheterogeneities and further genetic variants will be discovered.

An interesting apparent functional heterogeneity in band 3 has been described by Wieth et al. [153]. A single exposure to phenylglyoxal (PG) inhibits Cl transport in 90% of the band 3, but the remaining 10% is resistant. This 10% can be inhibited by washing and re-exposure to PG. It is not known why some copies of band 3 resist the initial exposure, but one possible explanation is that the aldehyde group on PG forms a Schiff base with an amino group on band 3 near the functionally important arginine residue. This may block irreversible modification of the arginine during the 90-sec exposure. By chance, this could take place in 10% of the copies of band 3. Washing the cells could reverse the Schiff's base, and re-exposure to PG would inhibit 90% of the previously refractory band 3. The PG resistant subset of band 3 is therefore not necessarily a distinct molecular species.

Band 3 Subunit Structure

Freeze-Fracture Studies

It is widely accepted that the intramembranous particles (IMP) observed in freeze-fracture electron microscopy of red cell membranes are composed largely of band 3 [157]. In the fractured membrane, band 3 remains associated mainly with the inner leaflet [33, 149] and thus appears as IMP_p (protoplasmic face). Weinstein et al. [150] have compiled data from several labs on the number of IMP_p per human red cell. The range is 3.6×10^5 to 4.5×10^5. It is of interest to compare this with the number of band 3 polypeptides per cell. The original estimate of this number by Fairbanks et al. [35] is 1.2×10^6, which is based on staining intensity. As discussed in a later section, there is one high-affinity stilbenedisulfonate binding site per band 3 monomer. The number of band 3 subunits per cell can therefore be estimated from DIDS, H_2DIDS or DNDS binding. Data from several labs [38, 39, 77, 130, 151] have shown that there are between 800,000 and 1.2×10^6 high-affinity stibenedisulfonate sites per cell. Fröhlich and Gunn [39] have demonstrated significant variation in the number of sites (correlated with the V_{max} for Cl exchange) among individual donors. Thus, the number of band 3 subunits per cell is very likely between 800,000 and 1.2×10^6, but it is not possible to say exactly how many subunits there are per IMP_p. It is clear, however, that each IMP_p cannot consist of a single band 3 monomer; there simply are not enough particles. The rotory shadow-cast micrographs of Margaritis et al. [88] show distinct heterogeneity in particle size and fine structure, suggesting that some particles could represent dimers and others consist of tetramers of band 3.

Cross-Linking Studies

Since the original work of Steck [138] on the cross-linking of band 3 by Cu^{++}-orthophenanthroline oxidation, considerable further cross-linking data relevant to the state of self-association of band 3 have appeared. The cross-linking methodologies and results on red cells have been reviewed in detail elsewhere [62–64, 91, 109], and the discussion here will emphasize the most recent findings.

Steck's demonstration that band 3 can be cross-linked into a covalent (-S-S-) dimer by mild oxidation of isolated membranes has been confirmed by many investigators [49, 80, 117, 147]. The oxidation produces dimers, trimers and tetramers of band 3. The relative amounts of dimer and tetramer depend on the conditions. For example, Reithmeier and Rao [117] found no detectable tetramer when cross-linking was performed at 0°C on spectrin-depleted membranes, whereas Wang and Richards [147] showed that tetramers are produced at room temperature. In intact cells, mild oxidation with diamide or tetrathionate (both of which, unlike Cu^{++}, can penetrate the membrane) produces band 3 dimers only if the glutathione content of the cells is first lowered by iodoacetate treatment [49].

Membrane-permeant homobifunctional reagents have been used extensively in band 3 cross-linking studies. The imidoester DTBP produces dimers, but not higher oligomers, of band 3 in intact cells [148]. The dimaleimide MMP forms dimers and small amounts of trimer of band 3 [125]. A spin-labeled *bis*-(N-hydroxysuccinimide ester) produces band 3 dimers when reacted with intact cells [156]; reaction with acid-stripped membranes results in band 3 dimers and higher oligomers. The photoactivated homobifunctional cross-linker 4,4′-dithiobiphenylazide produces dimers and tetramers of band 3 in isolated membranes at pH 5.0 to 5.5, but, interestingly, not at neutral pH [92]. At pH 7 to 8, the agent cross-links spectrin but not band 3.

Kiehm and Ji [70] have developed heterobifunctional photoactivated agents that can form covalent cross-links in very short times ($\ll$1 sec). When these agents are applied to isolated red cell membranes, the main band 3 cross-linking product is a dimer, although trimer was reported to form under some conditions. In these experiments the leading (most rapidly migrating) edge of band 3 apparently was not cross-linked; however, in the agarose-acrylam-

ide gel system used, band 4.1 may account for some of the material that runs in the leading edge of band 3 (*see* Fig. 6 of ref. 70). The fact that covalent band 3 dimers are formed within milliseconds is strong evidence that the dimer is not formed by a random collision [64]. The photoactivated reagent DNCO, acting on isolated membranes, can cross-link band 3 almost completely to dimers, with no formation of higher oligomers [53]. Another photoactivated agent, *p*-azidophenylisothiocyanate, also cross-links band 3 to dimers in membrane vesicles depleted of protein other than band 3 [131].

The above cross-linking studies all involve sites on band 3 that are either in the cytoplasmic domain (*see below*) or of unknown location. More recent efforts have been aimed at cross-linking band 3 in intact cells at exofacial sites. Staros and co-workers have synthesized hydrophilic, impermeant homobifunctional reagents that cross-link band 3 to a dimer in intact cells [135–137]. No higher oligomers of band 3 are produced by these agents, under conditions in which well over half the band 3 is cross-linked.

Schweizer et al. [128] have pointed out that the published cross-linking studies do not prove conclusively that band 3 is oligomeric in the intact cell, because the experiments were conducted using isolated membranes or allowed sufficient time for collisional cross-linking to take place. Using an inventive approach, they introduced exogenous amino groups into the band 3 carbohydrate and then attempted to cross-link band 3 in intact cells with a *bis*-imidate reagent that can react with the exogenous amines. At 0 to 4°C, where lateral protein mobility is very limited [37], no cross-linking of the amino group-supplemented band 3 was observed. One possible explanation for the lack of cross-linking in these experiments is that the exogenous amino groups were introduced into galactose residues in the band 3 carbohydrate, which has quite variable length. Thus, the amino groups on adjacent subunits would probably not be the same distance from the membrane surface. This consideration cannot completely explain the results, however, since the same procedure is reported [128] to produce extensive band 3 cross-links in spectrin-free vesicles that bud spontaneously from *in vitro* aged red cells.

Optical Probes

Two kinds of optical studies on isolated red cell membranes have provided strong evidence that band 3 is not monomeric *in situ*. Nigg and Cherry [104] performed transient absorption anisotropy measurements on membranes containing band 3 labeled on the membrane domain with eosin maleimide. The time course of the relaxation of the absorption anisotropy is directly related to the rotational mobility of the fluorophore. Nigg and Cherry found that cross-linking band 3 to a covalent dimer by Cu^{++}-orthophenanthroline did not lower the rotational mobility of the bound eosin. Thus, the band 3, prior to the covalent cross-linking, was apparently associated as dimers or higher oligomers. This interpretation of the data assumes that the cytoplasmic and membrane domains of band 3 can not rotate independently. This assumption is probably valid, since removal of the cytoplasmic domain by mild proteolysis increases the rotational mobility of the membrane domain [103]. The latter studies are deserving of comment in relation to band 3 oligomeric structure, since removal of the cytoplasmic domain strongly increases the mobility of as much as 40% of the band 3. Since only 10 to 15% of the band 3 protomers are directly bound to ankyrin [6, 7], fewer than 40% of the presumed band 3 dimers can be immobilized by a direct interaction with ankyrin. The data of Nigg and Cherry [103] can therefore be interpreted as support for the existence of higher ($n > 2$) oligomers of band 3. This interpretation, however, is complicated by the fact that other (nonankyrin) proteins, such as hemoglobin and glyceraldehyde-3-phosphate dehydrogenase, are known to bind to band 3 cytoplasmic domain [85, 121, 158]. Mühlebach and Cherry [97], in their recent studies of the effects of cholesterol on the rotational mobility of the membrane domain of band 3, have presented evidence for the existence of oligomers larger than tetramers. These experiments were performed on membranes lacking the band 3 cytoplasmic domain, and it is possible that the large ($n > 4$) oligomers formed as a result of the increased lateral mobility of the membrane domain.

Other evidence against a monomeric structure of band 3 *in situ* has been provided by the fluorescence studies of Dissing et al. [26] and Macara and Cantley [83], who used resonance energy transfer to estimate the distance between pairs of fluorophores, each bound on a separate band 3 subunit. If 1.2×10^6 band 3 monomers were distributed evenly over the 140 μm^2 area of the membrane, the center-to-center distance between monomers would be over 100 Å. The measured distance between the bound fluorophores is 30 to 50 Å, suggesting that the band 3 in the labeled membranes must be oligomeric (*see below*).

Isolated Band 3

Yu and Steck [158] and Clarke [15] first showed that human band 3 forms a stable noncovalent dimer in Triton X-100. Macara and Cantley [85] have re-

ported an interesting experiment that demonstrates how stable this dimer is. They prepared one set of band 3 dimers (in Triton X-100) from cells that had been labeled covalently with BIDS. Another preparation of dimers was from cells labeled on band 3 with eosin maleimide. The two preparations were mixed, and no exchange of partners was detected after 24 hr at room temperature. Nakashima et al. [100] performed polyacrylamide gel electrophoresis in nonionic detergents ($C_{12}E_9$ and Triton X-100) and showed that purified human band 3 migrates as distinct dimeric and tetrameric species. No monomer was detected. Since well-defined bands were observed at the expected positions of dimer and tetramer, with no material between the two bands, it appears that no significant dimer ↔ tetramer interconversion took place during the 16-hr electrophoresis at 4°C. The human band 3 as isolated by Lukacovic et al. [81], however, is a mixture of monomer and stable dimer in Triton X-100.

Pappert and Schubert [106] have recently shown that, in contrast to the above results, reversible monomer ↔ dimer ↔ tetramer associations can take place in freshly prepared purified band 3 in nonionic detergent, but that, with time, stable dimers formed. The dimer formation could be retarded by reducing agent, but the stable dimer, once formed, was not dissociated by reduction of disulfides. The details of the time-dependent change in band 3 that results in stable dimerization are not known. Pappert and Schubert fit their sedimentation data quantitatively to a monomer ↔ dimer ↔ tetramer equilibrium model [*see also* 28] and calculated the weight percent of band 3 in each state of association. The proportion of tetramer increases continuously as the total band 3 concentration is raised, until, at the highest concentrations studied in $C_{12}E_9$ (700 μg/ml), about 25% of the band 3 is calculated to be tetrameric. The association constant for tetramer formation is higher in Ammonyx LO: at 300 μg/ml, about 40% of band 3 is tetrameric. As others have pointed out previously [e.g. 85, 139], the band 3 concentration in the membrane is enormous compared with the concentrations normally used in studies of band 3 in detergent. If 10^6 band 3 molecules are confined to a 100-Å-thick layer of 140 μm^2 area, the concentration in the membrane is over 100 mg/ml, or nearly 1000-fold larger than those used by Pappert and Schubert. Thus, if the association constants for band 3 tetramerization in detergent are at all close to those in the membrane, band 3 would be expected to be mainly tetrameric *in situ*.

In summary, the experimental evidence to date virtually excludes the possibility that band 3 is entirely monomeric *in situ*, although monomers may possibly exist transiently as the result of dissociation of dimers or tetramers. Several lines of evidence suggest, but have not yet proven, that some or all of band 3 is tetrameric in the membrane. If band 3 is a tetramer, then the tetramer does not have fourfold rotational symmetry, since it appears to consist of a pair of dimers. The tetramers and dimers may be interconvertible in the membrane, although nothing is known about the time scale of this process, and the steady-state proportions of dimer and tetramer are uncertain.

Relation to Anion Transport

Anion Exchange Kinetics

The kinetics of band 3-catalyzed anion exchange have been studied in considerable detail [e.g. 21, 48, 73, 122–124], and it is generally agreed that the kinetics imply that there must be at least two anion binding sites per functional unit of transport. The first is a substrate site, and the second is a site that is responsible for self-inhibition of anion transport at high substrate concentrations [21]. Although it is possible to interpret this self-inhibition in terms of interactions between subunits, it is also possible that both sites are on the same subunit.

Irreversible Stilbenedisulfonate Binding

Irreversible attachment of increasing amounts of DIDS or H_2DIDS to the red cell membrane causes an increasing inhibition of band 3 catalyzed transport of a variety of anions, to a maximum inhibition of over 99% for chloride and bicarbonate [151]. Significantly, the relationship between the amount of irreversibly bound inhibitor and the percent inhibition of transport is strictly linear between 0 and over 90% inhibition. This linear relation has been observed in several laboratories, and the quality of the data is quite high [50, 77, 130, 151]. The stoichiometry of H_2DIDS binding at 100% inhibition was calculated by two groups to be one H_2DIDS per band 3 subunit [77, 130]. Jennings and Passow [61] showed directly that each subunit can bind one H_2DIDS molecule, since all copies of band 3 can be covalently cross-linked between the 60,000- and 35,000-dalton chymotryptic fragments by low concentrations of H_2DIDS. Thus, band 3 does not exhibit "half of the sites" reactivity with respect to DIDS and H_2DIDS.

By itself, this linear relation between DIDS (or H_2DIDS) binding and transport inhibition very nearly disproves the hypothesis that both halves of

Table. Reversible stilbenedisulfonate binding to erythrocyte membranes

Stilbenedisulfonate	Conditions	Result	Ref.
DBDS	Membranes, 5 mM citrate, pH 7, 23°C	No cooperativity	114
DBDS	Membranes 28.5 mM citrate, pH 7.4, 25°C	Strong negative cooperativity	27
DBDS	Membranes, 5 to 28.5 mM citrate, pH 7.4, 25°C	Moderate negative cooperativity at the highest citrate concentration	146
DNDS	Intact cells, 2 to 150 mM Cl, pH 7.8, 0°C	No cooperativity	38
NDS-TEMPO	Resealed ghosts, 40 to 120 mM citrate, 0 to 120 mM Cl, pH 7.6, 20°C	No cooperativity at concentrations up to $10 \times K_D$	126
BADS	Membranes, 28.5 mM citrate, pH 7.4, 23°C	No cooperativity, some low-affinity binding unrelated to band 3	79
$H_2(NBD)_2DS$	2.8 or 28 mM citrate, pH 7.4, 25°C	Slight evidence of negative cooperativity at 28.5 mM citrate	83

the band 3 dimer must be functional in order for anion transport to take place. One alternative explanation would be that binding of DIDS exhibits very strong positive cooperativity, i.e. DIDS binding to one subunit strongly facilitates binding to the other [139]. Such positive cooperativity for DIDS is very unlikely, as shown by studies on reversibly binding analogs (*see below*).

Another possibility [139] is that the catalytic unit is actually the dimer and that one bound DIDS per dimer lowers the transport rate by 50%. A second bound DIDS, according to this model, completely inactivates the dimer. This model has not been proven incorrect, but it is not likely that the single bound DIDS would inhibit a dimeric transporting unit by 50% for both Cl at 0°C [151] and SO_4 at 30 to 37°C [77, 130].

Reversible Stilbenedisulfonate Binding

Many stilbenedisulfonate derivatives that bind reversibly to band 3 have been prepared. All of these are potent inhibitors of red cell inorganic anion transport and bind to a site on band 3 that is probably identical to the site for DIDS and H_2DIDS. The binding of stilbenedisulfonate appears to be competitive with anion binding to the outward-facing substrate site [38, 39, 129]. Several binding studies have been carried out with these inhibitors (*see* Table). The data are simplest to interpret for the least bulky compound, DNDS. Fröhlich [38] has shown that DNDS binds to a single class of sites, indicating that binding of DNDS to one band 3 protomer does not affect the DNDS affinity for the other subunit(s) of a dimer or tetramer. Schnell et al. [126] synthesized a spin-labeled stilbenedisulfonate, NDS-TEMPO, one end of which is rather bulky. Both in the presence and absence of transportable anions, the binding of NDS-TEMPO to the membrane is a simple hyperbolic function of the free concentration over the low-to-moderate concentration range. At concentrations above 20 μM, "systematic deviations became apparent" which have not yet been studied in detail. Still, the range over which the binding shows no detectable cooperativity extends to occupancy of well over 75% of the band 3 protomers. Lieberman and Reithmeier [79] have recently characterized the reversible binding of BADS to band 3 in intact membranes; the binding exhibits no detectable negative cooperativity, although there is a low-affinity binding that is probably unrelated to band 3. Macara and Cantley [84] had earlier reported detectable negative cooperativity in BADS binding to red cell membranes. It is not clear how much of this is caused by low-affinity binding to sites not on band 3.

Kampmann et al. [66] recently tested the idea that H_2DIDS binding to one subunit affects the interaction of H_2DIDS with adjacent subunits. They measured the kinetics of the intramolecular crosslink formed between the two band 3 chymotryptic fragments by H_2DIDS. The kinetics are independent of the percent of band 3 polypeptides occupied by H_2DIDS. There is thus no evidence that the presence of H_2DIDS on one subunit affects the covalent reaction of H_2DIDS with neighboring subunits.

In 1979, Dix et al. [27] presented evidence that the bulky, symmetrical stilbenedisulfonate DBDS binds with high affinity to only one site per band 3 *dimer*. More recently, Verkman et al. [146] showed that the negative cooperativity in DBDS binding is not as pronounced as was indicated by the original Dix et al. [27] data. Nonetheless, Scatchard plots

and double reciprocal plots of the binding data show pronounced deviations from linearity except at low ionic strength [*see also* 114]. The data of Barzilay et al. [5] on the inhibition of SO_4 transport by DBDS also show evidence of negative cooperativity. The DBDS binding studies of Dix et al. [27] and Verkman et al. [146] were performed in a citrate medium, i.e. in the absence of transportable anions. However, in the presence of Cl, which competitively lowers the apparent DBDS affinity, there is no detectable negative cooperativity in the DBDS binding (J.A. Dix, *personal communication*). The detailed mechanism of this effect of Cl is not yet clear, but the result shows that DBDS binding shows negative cooperativity only under certain conditions (high ionic strength citrate medium).

Macara and Cantley [83] have provided further evidence for negative cooperativity in stilbenedisulfonate binding to band 3. When 80% of the band 3 subunits are covalently occupied by BIDS, the affinity for the reversible binding of $H_2(NBD)_2DS$ to the unoccupied monomers is much lower than the binding affinity to native band 3. It is significant that the data on the quenching of BIDS fluorescence by $H_2(NBD)_2DS$ can be explained much more readily by tetrameric band 3 than by dimers alone.

Although BIDS binding to 80% of the band 3 protomers lowers the $H_2(NBD)_2DS$ affinity for the remaining sites, it does not affect the K_I for inhibition of phosphate transport by NAP-taurine [84]. It also does not affect the K_m for SO_4 transport [83]. A related experiment has been performed by Gunn (*personal communication*), who inhibited about 70% of the band 3 subunits irreversibly with DIDS and measured the extracellular Br concentration dependence of the Cl/Br exchange flux in intact cells. As in native cells, the flux is inhibited by high Br concentrations. Therefore, neither the apparent substrate affinity nor substrate inhibition is affected by inactivating half or more of the band 3 subunits irreversibly with BIDS or DIDS. This indicates that the transport characteristics of a given subunit are not affected by irreversible inactivation of the adjacent subunit(s).

In summary, the binding of most stilbenedisulfonates to band 3 subunits does not exhibit detectable negative cooperativity. For the bulky, symmetric stilbenedisulfonate derivatives DBDS and $H_2(NBD)_2DS$, occupancy of one subunit does lower the affinity for the other subunits of the dimer under some conditions. It is worth emphasizing that although covalently bound BIDS interferes with binding of $H_2(NBD)_2DS$ to adjacent subunits [83], the reversibly binding BIDS analog, BADS, displays no detectable negative cooperativity in binding to band 3 [79]. This suggests that the binding site on each monomer has room to accommodate the entire BADS (or BIDS) molecule, but that the larger derivatives DBDS and $H_2(NBD)_2DS$ cannot bind to band 3 without having steric effects on adjacent subunits. These presumed steric effects, at least for DBDS, are not evident at low ionic strength [114, 146] or in the presence of Cl (J.A. Dix, *personal communication*).

Single (or Half) Turnover Cl Efflux

The above studies on inhibitor binding to band 3 and transport inhibition kinetics indicate that anion transport by a given subunit is not affected by irreversible inactivation of adjacent subunits. This notion is supported by the kinetic studies of Jennings [59], who measured ^{36}Cl efflux from resealed red cell ghosts into media that contained virtually no rapidly penetrating anions. The ghosts initially contained about 3×10^6 Cl ions, and lost about 0.9×10^6 ions in the first few seconds of the efflux. The rapid Cl efflux appears to represent the outward transport events (binding, translocation, release) of a single catalytic cycle. Since the number of Cl ions rapidly leaving each ghost is close to the number of band 3 protomers, the stoichiometry of the complete catalytic cycle is most likely one anion moving outward and one moving inward per protomer. Inhibition of about half the protomers irreversibly with DIDS lowers the number of rapidly effluxing Cl ions by about 50%. Thus, the "half-turnover" Cl efflux on a particular band 3 subunit is not detectably affected by irreversible inhibition of an adjacent subunit.

Negative Cooperativity Induced by Arginine Modification

Two laboratories have recently shown that red cell anion transport can be inhibited by treating red cells (or resealed ghosts) with arginine-selective reagents such as phenylglyoxal and cyclohexanedione [8, 9, 152, 153, 159–161]. Maximal inhibition of transport is over 90%, and, for phenylglyoxal, inhibition is associated with the modification of a residue in the 35,000-dalton chymotryptic fragment [8, 9]. The arginine modification does not prevent stilbenedisulfonate binding, but it changes the characteristics of the binding. In resealed ghosts in which Cl transport had been maximally inhibited with phenylglyoxal, irreversible DIDS binding is considerably (12-fold at low DIDS concentrations) slower than in control ghosts. The phenylglyoxal treatment also lowers the affinity for reversible DIDS binding to all copies of band 3. Significantly, in ghosts that have been phenylglyoxalated on virtually all copies of

band 3, the total irreversible DIDS binding at low DIDS concentrations is one DIDS per band 3 *dimer* [9, 152]. The same is true for H_2DIDS binding to cells previously modified with cyclohexanedione [159]. Thus, the DIDS binding to the arginine-modified band 3 exhibits strong negative cooperativity. The detailed mechanism of this altered binding is not yet known.

Another indication of possible interaction between band 3 subunits is provided by the recent work of Wieth and coworkers [152], who characterized the inhibition of band 3 by the water-soluble carbodiimide EAC. The time course of inactivation of Cl/Cl exchange by EAC is biphasic. There is rapid inhibition of 50% of the flux (and a 50% drop in high-affinity DIDS binding sites), followed by a much slower inactivation of the other 50% of the flux. This indicates that EAC bound to one subunit inhibits transport by that subunit but does not inhibit the function of adjacent subunits. The bound EAC does, however, strongly retard EAC binding to the neighboring subunit of the dimer.

Evidence that Monomeric Band 3 Cannot Bind Stilbenedisulfonates

An interesting recent paper by Boodhoo and Reithmeier [10] addressed the question of whether or not isolated, monomeric band 3 can still bind stilbenedisulfonates with high affinity. They isolated band 3 in the nonionic detergent $C_{12}E_8$, in which the band 3 is dimeric. The dimers were immobilized on derivatized Sepharose under conditions that allowed one monomer per dimer to be attached directly to the Sepharose. Using dodecyl sulfate or guanidinium to dissociate the dimers, they showed that monomeric band 3, after removal of the denaturant, could not bind the stilbenedisulfonate BADS. When the monomers were released from the column, dimers reformed, each of which could bind two BADS molecules with high affinity. This result indicates that the dimeric structure of band 3 is required for stilbenedisulfonate binding, even though the stoichiometry of the binding is one stilbenedisulfonate per protomer. This could mean that the binding site itself is close to the interface between subunits, or that interactions between subunits are necessary for the native conformation of the protein.

Molecular Details of the Band 3 Dimer

The above data strongly suggest that, in the native red cell membrane, band 3 is associated as dimers, which, themselves, probably associate further into tetramers. Nothing is known about the details of the structure of the tetramer, but some information is now available about the monomer-monomer contacts in the band 3 dimer.

It is well established that the cysteine residues responsible for covalent (-S-S-) dimer formation by mild oxidation are in the cytoplasmic domain [117, 141]. Of the three cysteines in this part of band 3, no one particular residue is much more likely than the others to participate in the intermolecular disulfide [117]. The isolated cytoplasmic domain itself cannot be cross-linked into disulfide dimers [117] but it has been shown to self-associate as a noncovalent dimer [1]. This suggests that different intermolecular interactions exist in the isolated cytoplasmic domain than are operative *in situ*.

The isolated membrane-bound domain of band 3 has been shown to exist as a dimer in nonionic detergent [116]. It is of interest to determine what segments of the primary structure are involved in the subunit interactions in the membrane domain. This question has been addressed in a recent paper by Staros and Kakkad [136], who cross-linked band 3 to covalent dimers, using impermeant reagents that react with the membrane domain at the extracellular surface. Subsequent chymotrypsin treatment of the cells revealed that no cross-links were between pairs of 60,000-dalton segments. Instead, an intermolecular cross-link was formed between a 60,000-dalton chymotryptic fragment of one subunit and a 35,000-dalton fragment of another subunit. It is not known whether intermolecular cross-links also formed between 35,000-dalton fragments on adjacent subunits. This work shows that the dimer is probably arranged such that there are close contacts between the extracellular portions of the 17,000- and 35,000-dalton segments of neighboring subunits [*see also* 87], but there is no evidence of contact between neighboring 17,000-dalton fragments. This conclusion is supported by the finding of Dubbelman et al. [31] that protoporphyrin-sensitized photooxidation causes extensive cross-linking of red cell membrane protein, but not the 17,000-dalton fragment of band 3.

In contrast to the reagents used by Staros and Khakkad [136], H_2DIDS cross-links the two chymotryptic fragments of the same band 3 subunit. This is demonstrated by treating cells with H_2DIDS at alkaline pH, without chymotrypsin pretreatment. No covalent dimers are formed [61] even though the two segments of band 3 have been covalently cross-linked. As expected, subsequent chymotrypsin treatment of the cells does not change the electrophoretic mobility of the band 3. To demonstrate that the bound H_2DIDS does not prevent the chymotrypsin cleavage, we isolated band 3 from H_2DIDS/chymotrypsin-treated cells. Carboxypeptidase di-

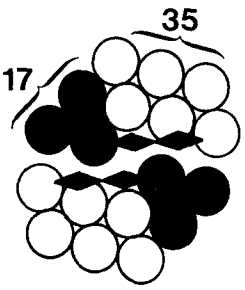

Fig. Hypothetical arrangement of the membrane-bound portion of the band 3 dimer, viewed from above the membrane. Circles represent cross-sections of membrane-spanning segments. Filled circles are in the 17,000-dalton fragment; open circles are in the 35,000-dalton fragment

gestion and Edman degradation showed that the chymotrypsin cleavage had taken place (M.L. Jennings, *unpublished*). Thus, the H_2DIDS must have cross-linked the chymotryptic fragments of the same subunit, prior to the chymotrypsin cleavage.

The Figure is a representation of one of several possible structures of the membrane-bound portion of the band 3 dimer, viewed from above the membrane. Bound stilbenedisulfonate is represented by the double rhombus, and each subunit consists of nine membrane-crossing segments. Three crossing segments are postulated to be in the 17,000-dalton chymotryptic peptide (i.e. the membrane-bound portion of the 60,000-dalton fragment) as suggested by its size. The 35,000-dalton fragment is shown as containing six crossing segments, but the exact number is not known. The stilbenedisulfonate site contains segments from both fragments [60, 79] and, as indicated by Staros and Kakkad [136], the 17,000-dalton fragments of adjacent subunits are not in close contact. The stilbenedisulfonate site is shown as being near the interface between the subunits [84]. This would explain the negative cooperativity in the binding of some stilbenedisulfonates under certain conditions (*see above*), but other explanations are also possible. It is by no means proven that the site is near the interface. In any case, the stilbenedisulfonate binding site does appear to depend on subunit-subunit contacts [10]. The nature of the interface between subunits is not certain. It could contain a hydrophilic channel [134] as well as hydrophobic protein-protein contacts. The role of lipid in the subunit interface is unknown.

The ideas represented in the Figure have some experimental support, but many other configurations are consistent with the data. At this point it is not useful to suggest a similarly detailed model of the band 3 tetramer. Staros and Kakkad [136] have presented the novel hypothesis that the tetramer is formed by the following two sets of interactions: dimerization of the membrane domains, and associations between cytoplasmic domains of adjacent dimers, to form the dimer of dimers. There is no experimental evidence for this arrangement, but the hypothesis should be possible to test, using combinations of extracellular and cytoplasmic cross-linkers.

Conclusions

Evidence from many laboratories using several different techniques strongly suggests that, in the intact red cell, band 3 exists as dimers which can associate with other dimers to form tetramers. The kinetics of anion transport inhibition by stilbenedisulfonates indicate that irreversible inhibition of one subunit does not detectably affect anion transport by the other subunit. This does not imply that monomeric band 3 could necessarily transport anions; the native conformation of each subunit may require stabilizing interactions with another subunit, as indicated by the recent work of Boodhoo and Reithmeier [10]. A more detailed understanding of the structure of the band 3 dimer/tetramer will require information on which specific segments of the primary structure are involved in subunit-subunit contact. The combination of chemical cross-linking with proteolysis [136] is a promising approach to this problem.

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