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The red cell band 3 protein: its role in anion transport

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Studies of anion transport across the red blood cell membrane fall generally into two categories: (1) those concerned with the operational characterization of the transport system, largely by kinetic analysis and inhibitor studies; and (2) those concerned with the structure of band 3, a transmembrane peptide identified as the transport protein. The kinetics are consistent with a ping-pong model in which positively charged anion-binding sites can alternate between exposure to the inside and outside compartments but can only shift one position to the other when occupied by an anion. The structural studies on band 3 indicate that only 60% of the peptide is essential for transport. That particular portion is in the form of a dimer consisting of an assembly of membrane-crossing strands (each monomer appears to cross at least five times). The assembly presents its hydrophobic residues toward the interior of the bilayer, but its hydrophilic residues provide an aqueous core. The transport involves a small conformational change in which an anion-binding site (involving positively charged residues) can alternate between positions that are topologically in and topologically out.

Introduction

The Cl^-–HCO_3^- exchange system of the red blood cell membrane is essential for CO_2 transport from tissues to lungs. It has become a much studied model for understanding the role of specific membrane proteins in the transport function because: (*a*) the anion transport protein, band 3, is abundant, constituting 50% of the total membrane protein; (*b*) the transport is a simple equilibrating system, uncomplicated by coupling to other transport systems or to enzyme activities; and (*c*) large quantities of red cells are readily available from blood banks. The literature on band 3 and on anion transport is large, and growing rapidly, but fortunately much material has been compiled in reviews and symposia (Rothstein *et al.* 1976; Cabantchik *et al.* 1978; Gunn 1978; Steck 1978; Tanner 1978; Lassen *et al.* 1980; McCarra & Cantley 1982; Wieth & Brahm 1982). In this presentation some general information concerning band 3 will be briefly summarized, recent structural information on band 3 as a transmembrane peptide will be presented in more detail, and attempts will be made to rationalize its architecture with its transport function. Original literature will only be cited for recent contributions. Other details can be found in the reviews cited above.

General arrangement of band 3 in the bilayer

The name band 3 derives from the fact that when detergent-dissolved red cell membrane proteins are separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and stained, this peptide is located in the third major band from the top, at a location indicating a molecular mass of about 95 kDa. The band is unusually wide, a consequence of the heterogeneity of its carbohydrate, which constitutes about 8% of its total mass. Band 3 is the most

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abundant membrane protein, constituting about 50% of the total, about 10^6 copies per cell. It is an intrinsic transmembrane peptide that has elements within the bilayer but with others exposed at both sides of the membrane. The exposed portions can react with non-permeating reagents or can be cleaved by proteolytic enzymes applied to one side or the other of the membrane. For example, application of chymotrypsin to the outside of the membrane (in the intact cell) results in a single cleavage, and to the inside (in ghosts or inside-out vesicles), in another. As a result three domains can be defined, as illustrated in figure 1. An N-terminal water soluble segment of 42 kDa is located entirely on the cytoplasmic side of the membrane. A middle segment of 17 kDa and the C-terminal segment of 35 kDa are membrane-bound (intrinsic) and can only be removed from the membrane by detergents or by strong organic acids such as acetic or formic. The middle (17 kDa) segment, produced by cleavages at the

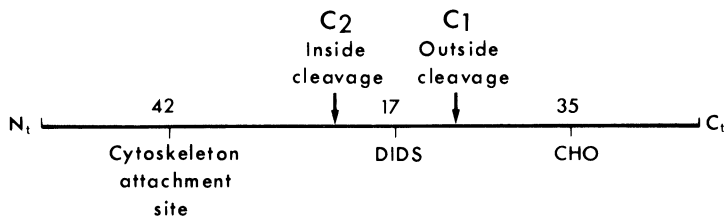

FIGURE 1. Domains of band 3 defined by chymotryptic cleavages at the two sides of the membrane.

two sides, must cross the bilayer. Recent studies indicate that the 35 kDa segment must also cross, for the attachment site of its carbohydrate is exposed at the outside face of the membrane, whereas its two sulphydryl groups are exposed at the inside face (Rao 1979; Ramjeesingh *et al.* 1981*a*).

The N-terminal 42 kDa segment provides the attachment site for the cytoskeletal elements of the red cells (spectrin and actin), through specific binding sites for a bridging peptide, ankyrin (bands 2.1, 2.2) (see review by Branton *et al.* 1981). Other cytoplasmic peptides such as glyceraldehyde dehydrogenase, aldolase and haemoglobin can also bind to this part of band 3. On the other hand, the segment seems to play no direct role in anion transport. Vesicles from which it has been cleaved (and discarded) show no loss of anion transport function (Grinstein *et al.* 1978). Both of the membrane-bound segments (17 and 35 kDa) seem, however, to be required for transport (Jennings & Passow 1979; Jennings & Adams 1981; DuPre & Rothstein 1981). The 17 kDa segment contains the binding site for 4,4′-diisothiocyano-2,2′-stilbene disulphonic acid (DIDS), a specific and potent transport inhibitor; cleavage of the 35 kDa segment by papain or by chymotrypsin results in loss of function. In the following sections, the arrangement of these two intrinsic segments will be considered in more detail.

THE 17 kDa SEGMENT

As noted above, the 17 kDa segment (C_1 to C_2 in figure 1) is produced by an outside and inside cleavage by relatively low concentrations of chymotrypsin. If, however, red cell ghosts are subjected to high concentrations of chymotrypsin at low ionic strength, an additional cleavage occurs resulting in a reduction in size of the 17 kDa segment to 15 kDa (Ramjeesingh *et al.* 1980*b*). Both the 17 and 15 kDa segments have two methionine residues cleavable by

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treatment with cyanogen bromide (CNBr). The sequence of cleavage has been followed in detail for the 15 kDa segment (Ramjeesingh *et al.* 1980*a*). In the first cleavage 11 and 4 kDa fragments are produced. In the second, the larger fragment is split into a 7 kDa and a second 4 kDa fragment. The two 4 kDa fragments can be readily distinguished from each other because one contains the binding site for DIDS. The order of the fragments is illustrated in figure 2*a*, with the 7 kDa fragment at the C-terminus of the 15 kDa segment, the DIDS-containing 4 kDa fragment in the middle, and the other 4 kDa fragment at the N-terminal end.

When the 17 kDa segment is cleaved by CNBr, the C-terminal 7 kDa fragment and the DIDS-containing 4 kDa fragment are both produced, but the N-terminal fragment is 6 kDa rather than 4 kDa (figure 2*b*). It is evident, therefore that the 'extra' chymotrypsin cleavage

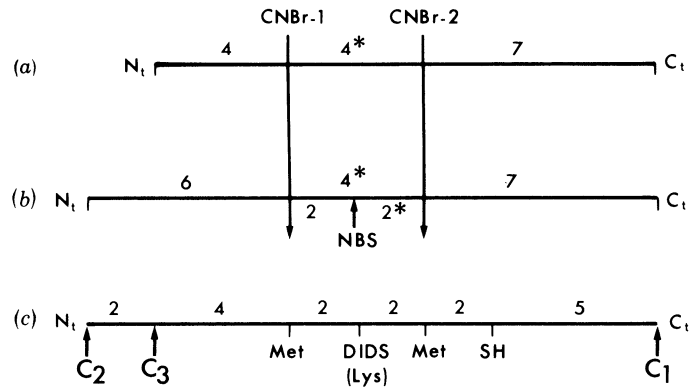

FIGURE 2. Alignment of sites in the 17 kDa segment of band 3: (*a*) cyanogen bromide (CNBr) cleavage of the 15 kDa segment; (*b*) cyanogen bromide cleavage of the 17 kDa segment; (*c*) location of various sites in the 17 kDa segment. Abbreviations are as follows: N_t and C_t, N-terminus and C-terminus; numbers above segments represent sizes of peptide fragments in kilodaltons; * indicates the fragment containing DIDS; CNBr-1 and CNBr-2 are the cyanogen bromide cleavage sites at methionine residues (Met); NBS is the *N*-bromosuccinimide cleavage site; NCTB is the 2-nitro-5-thiocyanobenzoic acid cleavage site at the cysteine residue; C_1, C_2 and C_3 are chymotrypsin cleavage sites; DIDS indicates the DIDS-covalent binding site to a lysine (Lys) residue.

that converts the 17 to the 15 kDa segment must occur about 2 kDa from its N-terminal end (located at the cytoplasmic side of the membrane) (Ramjeesingh *et al.* 1982*a*).

The DIDS-containing site can be further localized by cleavage of the middle 4 kDa fragment into two 2 kDa fragments by treatment with *N*-bromosuccinimide (NBS) (Ramjeesingh *et al.* 1980*b*). The DIDS-containing site is found in the fragment toward the C-terminus, as shown in figure 2*b*. The DIDS is covalently linked to the ε-amino group of a lysine residue (Ramjeesingh 1981*b*).

The locations of all identified sites within the 17 kDa segment are summarized in figure 2*c*. All of these have been discussed above except its single cysteine residue, whose location 5 kDa from the C-terminus has been determined by cleavage with 2-nitro-5-thiocyanobenzoic acid (NTCB) (Ramjeesingh *et al.* 1982*b*).

THE 35 kDa C-TERMINAL SEGMENT

The 35 kDa segment (C_1 to C_t in figure 1), produced by chymotrypsin treatment of cells, contains the carbohydrate attachment site. The amount and heterogeneity of the carbohydrate accounts for the poor staining of the segment after its separation by gel electrophoresis

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and for the breadth of the stained band that it forms. It contains two sulphydryl groups that can bind *N*-ethylmaleimide (NEM). These particular sulphydryl groups have the unusual characteristic in that they become cryptic to NEM if the reaction is carried out in ghosts in the absence of reducing agents (Rao 1979).

Although the 35 kDa segment is not susceptible to further chymotrypsin cleavage in intact cells, other cleavages do occur in ghosts exposed to high concentrations of the enzyme at low ionic strength. The 35 kDa segment disappears and the only stainable peptide found in the gels is 8–9 kDa (Ramjeesingh *et al.* 1980*b*; DuPre & Rothstein 1981). This fragment contains the two sulphydryl groups of the parent 35 kDa segment but not its carbohydrate (Ramjeesingh *et al.* 1981*a*). Nevertheless, the vesicles derived from chymotrypsin-treated ghosts contain, in addition to the 8 kDa fragment and the 15 kDa DIDS-containing segment, carbohydrate in an amount and composition consistent with that of intact band 3. The carbohydrate of band 3 has been located in a membrane-bound glycopeptide fragment by the use of labelling tech-

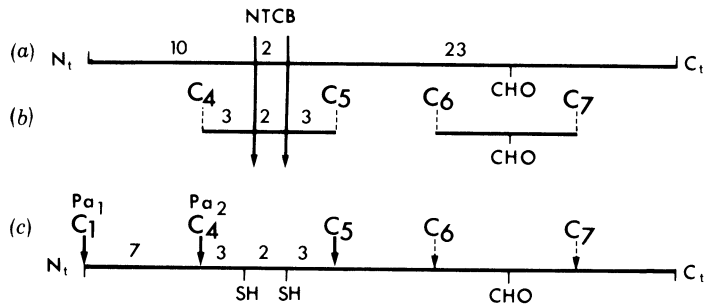

FIGURE 3. Alignment of sites in the 35 kDa C-terminal segment of band 3. (*a*) NTCB cleavage of the 35 kDa segment; (*b*) NTCB cleavage of the 8 kDa segment and tentative location of the glycopeptide fragment; (*c*) location of various sites in the 35 kDa segment. Abbreviations are as in the legend of figure 2 with the following additions: Pa_1 and Pa_2 are papain cleavage sites; CHO represents the carbohydrate attachment site; SH represents the sulphydryl groups of the two cystein residues.

niques involving the coupling of [^{14}C]aniline to its sugar aldehyde groups formed by prior treatment with periodate. It does not form a stainable band, but is spread over a wide molecular mass range (5–20 kDa), its electrophoretic behaviour being dominated by its carbohydrate heterogeneity (Ramjeesingh *et al.* 1982*b*). It can therefore be concluded that during the proteolysis of the ghosts, two intrinsic fragments of the 35 kDa peptide remain in the bilayer, one of 8 kDa and another of undetermined mass, designated CHO-peptide.

The location of the 8 kDa fragment in the primary sequence of the 35 kDa segment has been determined by use of NTCB to cleave the two cysteine residues that are common to the two peptides (Ramjeesingh *et al.* 1982*b*). In the 35 kDa segment the predominant cleavage products were 10, 1.7 and 23 kDa (figure 3*b*), the last being a very broad band in gels because it contains the carbohydrate of band 3. In the 8 kDa fragment, the predominant products were 3, 1.7 and 3 kDa (figure 3*b*). The 1.7 kDa fragment, common to both peptides, must be the fragment located between the two sulphydryl groups. By aligning the sulphydryl groups as in figure 3, it is possible to locate the various peptide fragments and proteolytic cleavage sites in the primary sequence (figure 3*c*). The two sulphydryl groups are located 10 and 12 kDa from the N-terminal end of the 35 kDa segment. The N-terminal and C-terminal ends of the 8 kDa segment, produced by chymotrypsin cleavage in ghosts, are located 7 and 15 kDa from the N-terminus (designated C_4 and C_5 in figure 3).

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The carbohydrate of band 3 is found in the C-terminal 23 kDa segment (figure 3*a*). As noted, after intensive chymotrypsin treatment of ghosts, the carbohydrate is found in a smaller peptide fragment (figure 3*b*). Its exact size and its precise location are not known, except that it must be on the C-terminal side of C_5 (figure 3*c*).

It is of some interest that papain treatment of intact cells results in two cleavages of band 3 (whereas chymotrypsin produces the single cleavage at C_1). One of the papain cleavages (marked Pa_1) is close to the chymotrypsin cleavage site, C_1 (Jennings & Passow 1979; Jennings & Adams 1981). The second (Pa_2) results in reduction of size in the carbohydrate bearing segment from 35 kDa to 25–30 kDa. These findings are consistent with the conclusion that the Pa_2 cleavage site may be close to C_5 (figure 3*c*). This conclusion is also consistent with the observation that in papain-treated cells converted to ghosts and then retreated with chymotrypsin the 8 kDa segment is recovered intact (Ramjeesingh *et al.* 1982*b*). It is also possible,

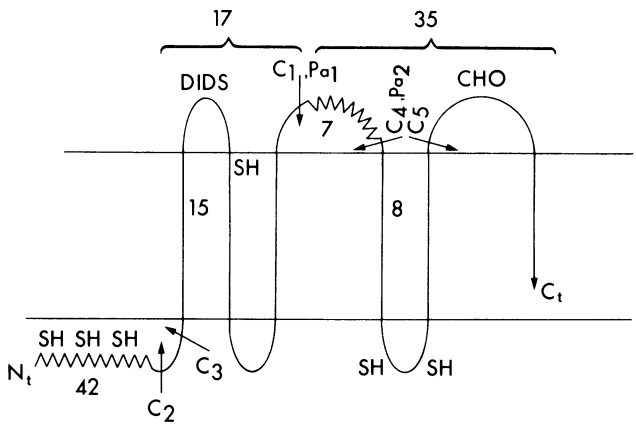

FIGURE 4. A proposed arrangement of band 3 in the bilayer. Abbreviations are as in the legends of figures 2 and 3.

however, that the second papain cleavage results in the loss of about 7 kDa from the C-terminal end of the 35 kDa segment. The location of the C-terminus with respect to the bilayer is not known.

ARRANGEMENT OF BAND 3 WITH RESPECT TO THE BILAYER

Based on the proteolytic cleavages, chemical cleavages and locations of particular sites and residues with respect to the two sides of the membrane, an arrangement of band 3 in the bilayer can be proposed, as illustrated in figure 4. It has already been noted that the 42 kDa N-terminal segment (C_2 to N_t) is entirely exposed on the cytoplasmic face of the membrane, and that it is a soluble peptide that is not hydrophobically associated with the bilayer. In the 17 kDa segment (C_1 to C_2), one chymotrypsin cleavage site (C_1) is produced by proteolysis at the outer face of the membrane and the others (C_2 and C_3) are produced at the inner face. The DIDS-binding site is known to be exposed only to the outside, based on the observations that DIDS, a relatively impermeant molecule, inhibits anion transport (and binds to the site in the 17 kDa segment) when applied to the outside face, but not the cytoplasmic face of the membrane. The S configuration illustrated in figure 4, with three crossings of the membrane, can account for these observations (Ramjeesingh *et al.* 1980*a*; Rothstein & Ramjeesingh 1980) and is consistent with the locations of other inhibitory probes (sulphanilic acid derivatives)

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(Drickamer 1980), cleavages with another proteolytic enzyme, thermolysin (Tanner *et al.* 1980), and with the length of peptide required to cross the bilayer in the form of an ε-helix (about 3 kDa).

Within the 35 kDa segment, the chymotrypsin cleavage site, C_1, is external, the carbohydrate is also exposed on the external side of the membrane, but the two sulphydryl groups are exposed on the cytoplasmic side. The papain cleavage site, Pa_2, presumably close to C_4, is also external. To accommodate these findings, it is proposed that the 8 kDa segment (C_4 to C_5) containing the two sulphydryl groups forms a transmembrane loop, as illustrated in figure 4, and that the segment that can be excised by papain, Pa_1 to Pa_2, is exposed on the external side. The segment of peptide containing the carbohydrate attachment site is also hydrophobically associated with the bilayer. It must be located on the C-terminal side of the 8 kDa peptide, but its exact size and location are not known. It may possibly traverse the membrane.

In the proposed arrangement of band 3, each monomer crosses the membrane at least five times.

Self-associations of band 3 and of its segments

The 15 kDa (C_1 to C_3) and 8 kDa (C_4 to C_5) segments are intrinsic, associated with the bilayer by hydrophobic interactions (Ramjeesingh *et al.* 1980*b*). Nevertheless, amino acid analysis of the segments, and of their various cleavage fragments, indicates amino acid compositions that are not particularly hydrophobic (Ramjeesingh *et al.* 1980*a*, *b*; Rothstein & Ramjeesingh 1980). In fact, the maximum proportion of hydrophobic residues is about 40%, and each fragment contains numbers of charged residues. Their hydrophobic behaviour has been explained by assuming that the membrane-crossing strands form an assembly within the bilayer that presents its hydrophobic residues on its outer face toward the side chains of phospholipids, and its hydrophilic groups either toward its interior to form an aqueous core, or toward the two aqueous solutions at the two sides of the membrane.

Other information supports the concept of such an assembly. For example, under certain conditions DIDS, which normally binds to a site in the 17 kDa segment (C_1 to C_2), can be cross-linked to a site in the 35 kDa segment (C_1 to C_t) (Jennings & Passow 1979). Thus the DIDS-binding site in the 17 kDa segment (figure 4) must be a relatively close neighbour of a site in the 35 kDa segment, both sites being located at the outer face of the membrane. Furthermore, the 17 and 35 kDa segments, extracted in Triton X-100 detergent, are found in the same fraction after filtration on a Sepharose column and they comigrate in a centrifugal field (Reithmeier 1979). The 15 and 8 kDa segments are extracted together in non-ionic detergent, and filter together (Ramjeesingh *et al.* 1980*b*). These findings suggest that the segments are closely associated with each other after extraction into micelles of non-ionic detergent and that they were probably associated with each other in the membrane.

In addition to the intermolecular associations outlined above, substantial evidence has been published indicating that in the intact membrane (Nigg & Cherry 1979) or in Triton X-100 solubilized membranes (Reithmeier 1979) intramolecular associations of band 3 account for its presence as a dimer. Even after cleavage at C_1 and C_2, the 17 and 35 kDa segments extracted by Triton X-100 are present as a dimeric complex (two each of the 17 and 35 kDa segments). Each dimer of band 3, as illustrated in figure 5, may therefore constitute an assembly of ten (or more) membrane-crossing strands.

Although band 3 is structurally a dimer, each monomer contains one DIDS-binding site

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(Jennings & Passow 1979). Given the linear relation between DIDS-binding and inhibition, and the absence of any kinetic evidence of cooperativity (all the data about transport activities are consistent with a 1:1 interaction of substrates and inhibitors with anion-binding sites), it appears as though each monomer of band 3 behaves as an independent transporter. For this reason, the assembly as illustrated in figure 5 is in the form of a dimer, but with one independent functional transport site for each monomer.

The functionally important parts of the assembly are the intrinsic segments (35 and 17 kDa). Thus cleavage at C_2, with the loss of the 42 kDa N-terminal segment (N_t to C_2 in figure 4), does not result in a loss of transport function. After further cleavage at C_1 the 17 and 35 kDa segments remain associated in the membrane and no loss of function results (Grinstein *et al.* 1978). Other cleavages in the 35 kDa segment by papain or chymotrypsin are, however,

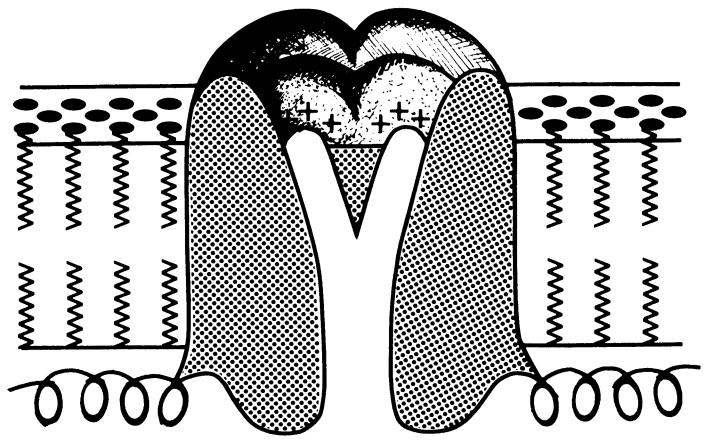

FIGURE 5. A diagrammatic representation of a cross section through band 3 within the bilayer.

inhibitory (Jennings & Passow 1979; DuPre & Rothstein 1981). Furthermore, the covalently bound inhibitor phenylglyoxal is localized in this segment (Wieth *et al.* 1982).

In the model of figure 5, the two DIDS sites of each dimer assembly are shown exposed to the outside, but within a pit or cleft. This location is based on experiments on energy transfer from the DIDS-binding site to the band 3 sulphydryl groups (known to be exposed at the cytoplasmic side of the membrane) indicates a distance of about 4 nm, somewhat less than the thickness of the bilayer (Rao *et al.* 1979). Studies with analogues of DIDS suggest that the two binding sites within each dimer may be about 2.8–4.5 nm apart (McCarra & Cantley 1981).

THE NATURE OF THE TRANSPORT AND OF INHIBITION BY DIDS AND OTHER AGENTS

A simplified kinetic model to account for the many recorded transport characteristics is shown in figure 6*a* and as a protein-structural model in figure 6*b* (Rothstein *et al.* 1976; Cabantchik *et al.* 1978; Rothstein & Ramjeesingh 1980; Rothstein *et al.* 1980). Chloride transport behaves as a saturable, electroneutral (non-conductive), one for one exchange. Transfer of the anion across the membrane requires its interaction with a positively charged transport site (shown as T^+ in figure 6*a*, and represented as a gate in figure 6*b*). The simplified model (figure 6*a*) based on the classical 'carrier' concept is consistent with kinetic information,

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but does not account, for example, for the accessibility of the transport site to DIDS only from the outside. Nor is it consistent with the structure and role of band 3 as the transporting entity. It is inconceivable, for example, given the high rate of anion transport, the fixed orientation of band 3 in the bilayer, and its multiple interactions with lipids, that a part or all of band 3 can oscillate across the total bilayer. It is therefore proposed that the transport occurs via a fixed protein pathway but that a small segment of peptide, represented by the gate of figure 6*b*, can undergo a transition from a topologically 'out' to a topologically 'in' position, by a small rearrangement. The anion can then dissociate and diffuse into the cell. The gate cannot return to the out position unless associated with an anion from the cytoplasmic side. A 1:1 electroneutral exchange is the consequence of this restriction, with the sites alternating between

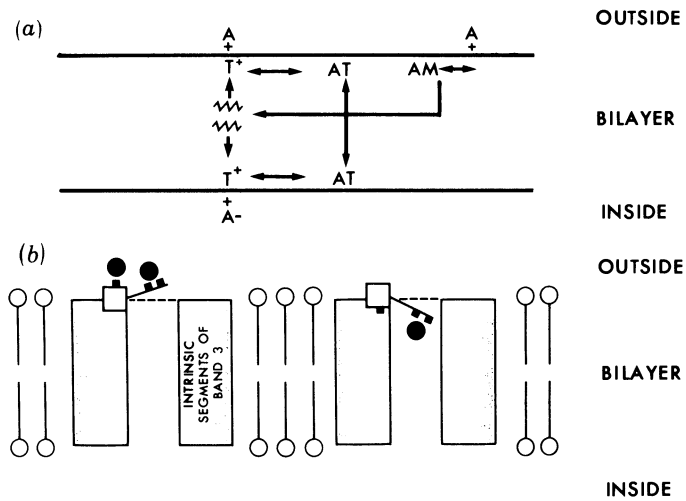

FIGURE 6. A functional model for anion transport. (*a*) A kinetic model with T representing the transport site and M the modifier site; (*b*) the model accommodated to band 3 structure, with the dotted line representing a diffusion barrier, the gate the transport site, and the square the modifier site.

inward-facing and outward-facing, displaying a ping-pong mode of kinetic behaviour. A predictable characteristic of the proposed mechanism is that various steady-state distributions of inward-facing and outward-facing sites can be induced by anion gradients or by the use of permeant and less permeant anions on the two sides of the membrane. The predicted kinetic consequences of such distributions are borne out by experimentation (Gunn & Fröhlich 1979; Knauf 1979; Knauf *et al.* 1980; Jennings 1982; Fröhlich 1982). The sites can also be completely and permanently 'recruited' and 'trapped' at one side or the other with inhibitors that can react covalently with the transport sites (Grinstein *et al.* 1979). Thus DIDS can 'capture' the sites at the outside of the membrane. They then become inaccessible to interaction with the photoaffinity probe *N*-(4-azido-2-nitrophenyl)2-aminoethylsulphonic acid (NAP-taurine) at the inner face of the membrane. The *trans*-effect of DIDS on NAP-taurine binding to band 3 supports the proposed mechanism.

Another anion-binding site exposed only at the outside is the modifier site (figure 6). Its affinity for Cl^- is considerably less than that of the transport site, but its affinity for organic anions such as NAP-taurine is much higher (Cabantchik *et al.* 1978; Knauf 1979). Although the modifier site does not transport, it is functionally linked to the transport site. Binding of anions to this site results in a non-competitive inhibition. Furthermore, if the transport sites

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are recruited to the inward-facing direction, the modifier site becomes inaccessible to probes such as NAP-taurine (Knauf *et al.* 1980).

In this short paper it is not possible to deal with a number of second-order complexities of the transport system related to its transport behaviour toward divalent anions, its asymmetries, its proton titratable groups, and its capacity to allow a small fraction of conductive Cl^- flux. These topics are discussed in some detail elsewhere (Gunn 1978; Cousin & Motais 1979; Gunn & Fröhlich 1979; Knauf 1979; Passow *et al.* 1980*a*, *b*; McCarra & Cantley 1981, 1982; Fröhlich 1982; Fröhlich *et al.* 1982; Jennings 1982; Knauf *et al.* 1982; Wieth & Bjerrum 1982; Wieth & Brahm 1982).

Transport in relation to band 3 structure

In terms of protein structure, the model in figure 6*b* assumes that the assembly of the intrinsic portions (the membrane-crossing strands) of band 3 provides an aqueous channel across the membrane. It is not, however, an open channel because the conductive flow of Cl^- is very low (the electrical resistance of the membrane is very high). Thus there must be a barrier to free diffusion within the peptide structure, across which the exchanges of Cl^- must occur. The model further assumes that the exchanges involve a conformational change; that at least two conformational states (sites topologically out or topologically in, represented by the gate's being on the outward or inward side of the barrier) must exist in equilibrium with each other, with equilibration spontaneous, but only occurring when a Cl^- ion is bound to the transport site. The turnover of Cl^- through the system is very fast, $2 \times 10^5\ s^{-1}$, about that of the fastest enzyme reaction, suggesting that the change in conformation involves a *small* perturbation with movements over *small* distances.

The gate mechanism of figure 6*b* is one simple representation of the conformational change. Other more complex variants have been proposed to account for additional information such as asymmetries and the small but important conductive fluxes. It is also possible that the anion-binding site is stationary and that the barrier moves with respect to it, and that allosteric relations between elements of the transport system may be involved in its operation (Knauf 1979; Knauf *et al.* 1980; Passow *et al.* 1980*a*, *b*; Fröhlich *et al.* 1982; McCarra & Cantley 1982). Our information about the architecture of the transport site and protein assembly is is much too primitive to specify detailed molecular mechanisms.

Structural complexity of the transport and modifier sites can be inferred from a consideration of the interactions of DIDS, NAP-taurine and other inhibitors (Cabantchik *et al.* 1978; Motais & Cousin 1978; Knauf 1979; McCarra & Cantley 1982; Wieth *et al.* 1982). The initial interaction of DIDS with the membrane (and the only one for analogues of DIDS with no covalent binding groups) is reversible. The binding affinity is very high because of chelation of its two sulphonic acid groups with positively charged ligands of membrane, because of contributions of hydrophobic interactions between the benzene rings and membrane ligands, and because of electronic interactions. A secondary, slower reaction (highly temperature dependant) is covalent, between one of the isothiocyano groups of DIDS and an ε-amino group of a lysine residue. It can be concluded that the binding site must be complex, involving in addition to hydrophobic areas at least three positively charged groups. Multiple, positively charged, proton-dissociating groups related to transport have also been inferred from pH effects on transport (Cabantchik *et al.* 1978; Knauf 1979; Passow *et al.* 1980*a*, *b*; McCarra & Cantley 1982; Wieth & Brahm 1982).

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The interaction of NAP-taurine, a photoaffinity probe for anion transport, also occurs in two steps, a reversible binding involving the interaction of the sulphonic acid group with a positively charged ligand together with hydrophobic associations of the benzene ring. Upon exposure to light, a secondary covalent reaction occurs, a consequence of conversion of the azido group to a nitrene, which can then react with any number of ligands (Cabantchik *et al.* 1978; Knauf 1979).

Although, as noted, kinetic analysis indicates two distinct mechanisms of inhibition for DIDS and NAP-taurine applied to the outer surface of the membrane, the former by interaction with the transport site and the latter with the modifier site, the binding of the probes is not mutually exclusive. Prior interaction with DIDS prevents NAP-taurine binding and vice versa. It therefore seems likely that a single complex site may provide the binding ligands for both probes, a part of the site acting as the transport site and another part as the modifier site (Cabantchik *et al.* 1978; Knauf 1979; McCarra & Cantley 1982). It should be noted that the inhibitory action of both NAP-taurine and DIDS is related to interaction of their sulphonic acid groups, whereas the covalent interaction involves the azido or isothiocyano groups, some distance away. Thus the covalent attachment sites located in the 17 kDa segment (C_1 to C_2 in figure 1) are not the actual locations of the transport and modifier sites, but are neighbouring sites. Given the complex folded structure of band 3 (figure 4), such neighbouring ligands may be at some distance from each other in the linear structure, associated with other membrane-crossing strands that make up the assembly. The conformational changes associated with transport may reflect changes in the spatial relations of the membrane-crossing strands with respect to each other.

This concept of a functional assembly is supported by a number of experiments demonstrating that the binding of DIDS to its specific binding site results in measurable changes related to other parts of band 3 at some distance, such as its interactions with lipids (determined by shifts in temperature transitions) (Snow *et al.* 1978), locations of tryptophan residues in relation to the bilayer (Kleinfeld *et al.* 1982), and binding of haemoglobin to the cytoplasmic part of band 3 (N_t to C_2 in figures 1 and 4) (Salhany *et al.* 1980).

It is clear that although a great deal of information is now available about anion transport and band 3 structure, many questions concerning the molecular nature of this apparently simple transport system still remain to be answered.

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