Minireview

Band 3 protein: structure, flexibility and function

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Abstract

The electroneutral exchange of chloride and bicarbonate across the human erythrocyte membrane is facilitated by Band 3, a 911 amino acid glycoprotein. The 43 kDa amino-terminal cytosolic domain binds the cytoskeleton, haemoglobin and glycolytic enzymes. The 52 kDa carboxyl-terminal membrane domain mediates anion transport. The protein is a functional dimer, in which the two subunits probably interact with one another by an allosteric mechanism. It is proposed that the link between the mobile cytoplasmic and the membrane-spanning domains of the protein is flexible, based on recent biochemical, biophysical and structural data. This explains the long-standing puzzle that attachment to the cytoskeletal spectrin and actin does not appear to restrict the rotational movement of the Band 3 protein in the erythrocyte membrane. In the Band 3 isoform from the Southeast Asian Ovalocytes (SAO) this link is altered, resulting a tighter attachment of the cytoskeleton to the plasma membrane and a more rigid red blood cell.

Key words: Band 3 protein; Anion transporter; Membrane protein structure; Cytoskeleton; Southeast Asian Ovalocytosis

1. Introduction

Few proteins have as many diverse important biological functions as the Band 3 protein from the erythrocyte membrane. It serves simultaneously as an anion transporter, an anchor for the cytoskeleton, haemoglobin and glycolytic enzymes, and as a senescence antigen.

Band 3 plays a critical role in the CO_2 transport system, where carbon dioxide is carried as bicarbonate in the blood plasma. Since the solubility of carbon dioxide in the blood is rather low, the CO_2 molecule which diffuses into the erythrocyte through the plasma membrane is converted into HCO_3^- by cytosolic carbonic anhydrase. The bicarbonate anion is then transported out of the cell by the Band 3 protein in exchange for a Cl^- anion [1]. When the blood reaches the lung, the above exchange process is reversed as a result of a CO_2 pressure gradient between the blood and the lung alveoli. In this way, more than two thirds of the CO_2 molecules are transported in the form of HCO_3^-. Each Band 3 protein molecule transports 10^5 pairs of monovalent anions per second. This HCO_3^-/Cl^- exchange is the largest ion-specific flux known for any single cell in the body [2].

Band 3, being the most abundant protein in the erythrocyte plasma membrane (1.2×10^6 copies per cell, roughly 25% of the total membrane protein), also serves as a protein anchor, connecting soluble cytoplasmic proteins and components of the cytoskeleton to the membrane [3]. Spectrin binds to Band 3 via ankyrin and Band 4.1. These interactions with cytoskeletal proteins make Band 3 essential for maintaining cell shape and

mechanical strength [4]. It is also known that the protein provides binding sites for haemoglobin, hemichrome and some glycolytic enzymes [3].

It has become clear in the last decade that Band 3 also has a third function. The protein acts as a senescence antigen for aged and damaged red cells [5,6]. Clustering of Band 3 in the erythrocyte membrane triggers binding of antibodies to an extracellular part of the protein and causes the removal of aged or damaged red cells [7].

Proteolysis and gene sequencing have revealed that Band 3 has two structurally distinct domains [8,9]. The amino-terminal domain (residue 1–360) has a molecular weight of 43 kDa and is cytosolic. The carboxyl-terminal domain (residue 361–911), with a molecular weight of 52 kDa, spans the plasma membrane multiple times [10,11]. The cytosolic domain contains the binding sites for the cytoskeletal proteins and glycolytic enzymes, while the membrane domain mediates anion transport even after the removal of the cytoplasmic domain [12–14]. The membrane domain also contains a single N-linked glycosylation site on its extracellular surface that is not required for transport [15].

The mechanism by which Band 3 regulates cell rigidity is not well understood. In addition, the cytoskeleton bound to Band 3 is highly dynamic [3]. How can Band 3 perform its functional role as an anion transporter without interference from the attached cytoskeletal proteins? Recent biochemical, biophysical and structural experiments are throwing light onto these questions. A low resolution (20 Å) three-dimensional structure of the membrane domain of human Band 3 has recently been obtained [16]. In the present minireview, I will summarise the structural information, and correlate it with the experimental data obtained by other methods.

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2. The membrane domain has a canyon-like structure

Two reports on two-dimensional crystallization of Band 3 protein or its membrane domain appeared in 1993 [17,18]. The three-dimensional structure of the membrane domain has been determined to 20 Å resolution by electron microscopy and image reconstruction [16]. The dimeric domain shows a canyon-like structure, consisting of a basal domain and two large protrusions (Fig. 1). The basal domain is roughly 40 Å thick. The two protrusions are 80 Å long and 25 Å wide, with a height of 40 Å. They enclose a space of 55 Å wide at the top, which narrows down to 25 Å at the bottom on the surface of the basal domain. In addition, there is a depression at the dimer interface on each side of the basal domain.

The basal domain has been proposed to be the portion of the protein that spans the lipid bilayer [16]. The two protrusions are believed to represent the loops connecting the trans-membrane fragments in the cytosol and the two termini of the polypeptide. The cross-sectional area of the basal domain is consistent with the 14-helix model of the protein [10,11,19], although a 12-helix model cannot be ruled out, given the limited accuracy in estimating the cross-section from a low-resolution map. The N-linked oligosaccharide molecule that was pre-cleaved for crystallization is not visible in the map. Beppu et al. [20] have recently suggested that the antibody binding site is located on the oligosaccharide molecule, based on evidence from competitive inhibition of antibody binding to the red cells modified with SH-blocking agents. On the other hand, Lutz et al. [21] found that purified, naturally occurring anti-Band 3 antibody binds to polypeptide rather than carbohydrate within Band 3 protein. It remains to be seen which of these two possibilities is correct.

The dimer is believed to be the basic functional unit for anion transport [18]. The depressions at the dimer interface on the two surface of the basal domain may represent the openings of the anion transport channel [16], as suggested in the dimeric cavity model of the protein molecule [22]. In the three-dimensional map, the negative stain partially penetrated into the channel from both side. In the middle of the membrane, the density is the highest, which probably represents the barrier to anion transport. This is consistent with the thin barrier model of anion transport [10]. The protein shown in Fig. 1c is probably in its inward-facing conformation, in which the anion binding site faces the cytoplasmic side and the barrier faces the exterior surface. The depression in the structure on the tentative cytosolic side of the membrane is indeed deeper than the one on the opposite side, as expected for the inward-facing conformation. The inner surface of the canyon may be positively charged and may participate in the anion selection, as originally suggested by Jay and Cantley [22]. It is inter-

esting to note that the same authors even predicted the experiment by which to verify their proposal on the molecular structure of Band 3: 'Information from two-dimensional crystals would be extremely valuable in assessing the proposed dimeric cavity model' [22].

3. Two subunits in the dimer interact allosterically

The dimeric structure with a channel at the dimer interface suggests that allosteric subunit interactions may be involved in the anion transport cycle changing the conformational orientation of anion binding sites [16,18]. Recent experimental evidence from kinetic studies indeed supports the notion of allosteric interactions between the two subunits in the Band 3 dimer [23], as summarised below.

Liu and Knauf [24] showed that binding of eosin-5-maleimide to one Band 3 monomer induced a conformational change in the adjacent monomer in the dimer, by measuring the dependence of reaction rate to the inhibitor concentration. Similarly, covalent labelling of one Band 3 subunit with 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate (H_2DIDS) significantly altered reversible binding of the inhibitor to the second subunit in the dimer [25]. In addition, dual-wavelength spectroscopic experiments showed that intersubunit allosteric hysteresis made a significant contribution to dithionite transport by Band 3 [26].

Van Dort et al. [27] recently provided in situ structural evidence supporting the existence of ligand-mediated subunit interactions within the oligomeric transporter. They investigated the temperature shift of the calorimetric endotherm (the C-transition) of the membrane domain of Band 3 upon binding by the inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS). A distinctly non-linear correlation was found, implying that the thermal unfolding of the dimeric membrane domain was modulated by allosteric interactions between the subunits.

An allosteric oligomeric arrangement is not unique to Band 3. In fact, many transport proteins are oligomers [28,29]. An oligomeric channel not only offers the potential of regulation by allosteric change, but also has the advantage of being more economical with respect to genetic information [18].

4. The link between the cytoplasmic and the membrane domains is flexible

The cytoskeleton binds to the plasma membrane via Band 3. The mobility of the Band 3 protein itself is, therefore, of importance to the mechanical rigidity of the cytoskeleton and of the whole cell [4]. It has been known for many years that the interaction between the cytosolic
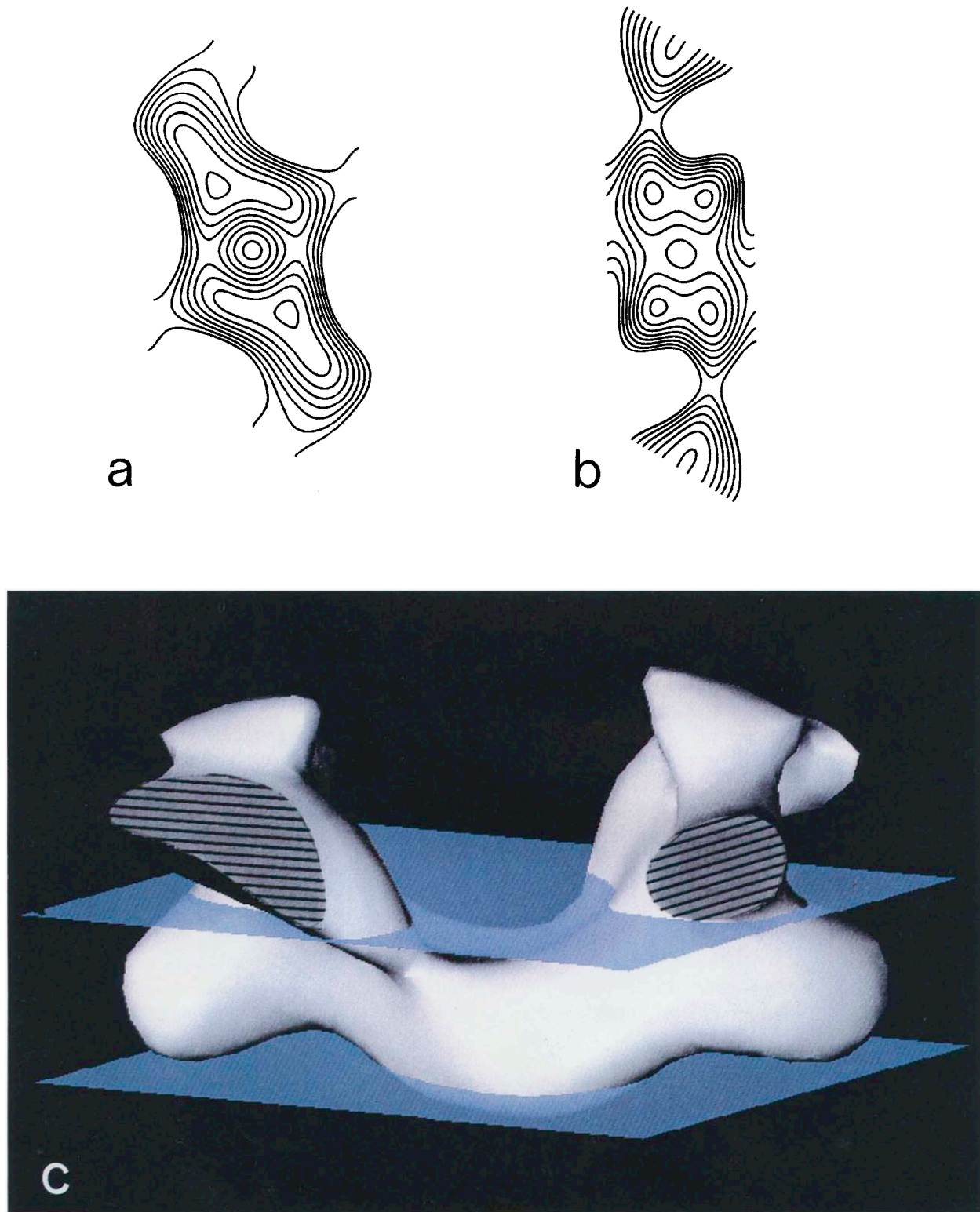
Fig. 1. Structure of the membrane domain of the Band 3 protein determined by electron microscopy and image reconstruction of two-dimensional crystals. (a) Projection map derived from the hexagonal sheets. (b) Projection map from the tubular crystal form. (c) Three-dimensional map determined from the hexagonal sheets. The bulky basal domain is probably embedded in the lipid bilayer, indicated by the two semi-transparent planes. The protrusions above the basal domain are on the cytosolic side of the membrane, forming two sides of a canyon that leads to a depression which is probably the entrance to a pore. Comparing the projection maps and the three-dimensional map, it seems that the protrusions in the cytosol adapt two different conformations in the two crystal forms (from [16,18]).

and membrane domain of the protein is minimal [30]. Recent spectroscopic and structural data further suggest that the part of the polypeptide connecting the cytoplasmic and the membrane domains of the Band 3 protein is flexible. In other words, there is a hinge between the two domains.

Some spectroscopic evidence is reviewed first. Using polarised delayed luminescence of the intact and the proteolized Band 3, Matayoshi and Jovin [31] found that beside of the two slow-rotating populations of Band 3, there was a highly mobile group of the protein in ghost and intact erythrocyte cells exhibiting a correlation time which is consistent with that expected for a free rotational diffusion of a Band 3 dimer. The mobility of this group was determined purely by viscous interactions within the lipid bilayer and was independent of the presence or absence of the cytoplasmic domain. Using the technique of time-resolved phosphorescence anisotropy, McPherson et al. [32] have also observed that cross-linking of Band 3 via hemichromes caused substantial immobilisation of the membrane domain of Band 3. The motion of the cytoplasmic domain was only partially restricted, suggesting that the cytoplasmic and the membrane domains are independently mobile.

Another line of evidence came from recent structural studies. The dimeric membrane domain of the Band 3 protein was crystallised into two forms of two-dimensional crystals, hexagonal sheets, and helical tubes [18]. Comparing the projection maps and the three-dimensional map obtained from the hexagonal sheets, it is evident that the protrusion of the membrane domain in the cytosol adapts two different conformations in the two crystal forms [16,18]. The protrusion, or part of it, is rotated by about 60° between the two forms. Therefore, the connector (residues 361–403) between the cytoplasmic and the membrane domains of the Band 3 pro-

tein is most likely to be flexible (Fig. 2). The region around residue 360 is poorly conserved among other anion exchanger isomers. There is also a protease sensitive site in the region.

The existence of a flexible hinge between the two domains helps to explain a long-standing puzzle. Cherry et al. [33] observed that the removal of spectrin and actin from the erythrocyte membrane resulted in little or no enhancement of Band 3 rotational mobility, a finding that has been verified since [4,34]. Therefore, these cytoskeletal proteins do not restrict the rotation of Band 3. Similarly, selective proteolysis of ankyrin, or incubating the ghosts at 70°C at which temperature ankyrin was expected to be denatured, had no effect on Band 3 rotation [35]. It should be noted that Cherry et al. [33] measured the rotational mobility of only the membrane domain of Band 3, since the probe for mobility measurement, eosin-5-maleimide, binds to residue Lys-430 in this domain [36].

Two possible explanations have been suggested to account for this surprising effect: (i) the flexibility of the cytoplasmic domain of the Band 3 protein to which the cytoskeleton is attached [32], and (ii) the flexibility of spectrin [37]. The second factor is probably not important. Although spectrin molecule is flexible in solution, the cytoskeletal network formed from spectrin and other molecules will certainly make a relatively tight angular restriction to the Band 3 protein. Here we propose that the lack of restrictions to the membrane domain of Band 3 by the cytoskeleton is, at least in part, due to the presence of the flexible hinge between the cytoplasmic and the membrane domains of the protein. The restriction by the cytoskeleton only extends to the cytoplasmic domain, and the rotational diffusion of the membrane domain is not much affected.

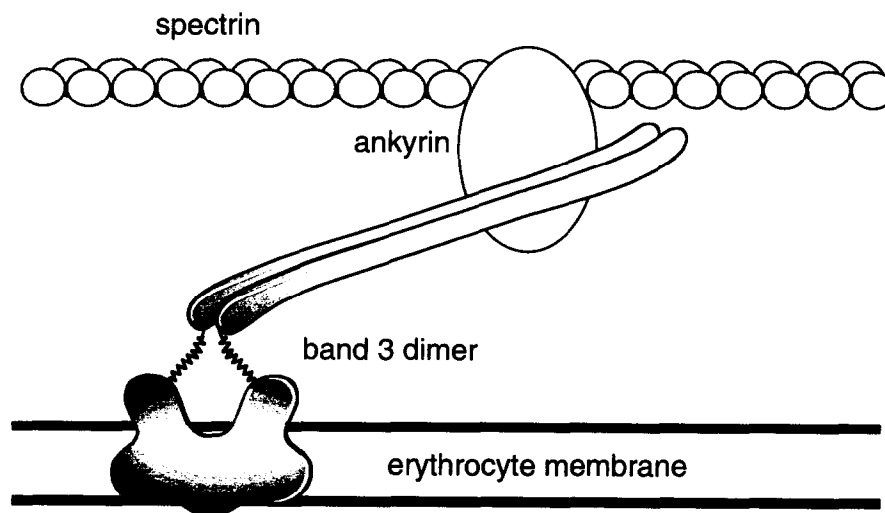Why does the protein possess such a hinge? The cy-

Fig. 2. Schematic picture showing the Band 3 protein and its interactions with the major cytoskeletal components. Spectrin binds to the cytoplasmic domain of Band 3 via ankyrin. The zig-zag lines indicate the suggested flexible link between the cytoplasmic and the membrane domains of Band 3.

tosolic domain of Band 3 is highly elongated, with an axial ratio of 10, implying a molecule with a diameter of 25 Å and a length of more than 200 Å [3]. This domain also has multiple conformations in situ upon binding to the cytoskeleton and to the various enzymes, so that is rather flexible [3,32]. The cytoskeleton also has a high flexibility [4]. In order to avoid undesired interference to anion transport from the cytoskeleton and from the cytoplasmic domain, the Band 3 protein, not surprisingly, possesses a flexible portion that separates those mobile components from the anion transport channel of the protein [16].

If the hinge is so important for the function of the protein, what would happen when it is altered? Would the membrane skeleton become more rigid? Would the protein still transport anions? Nature has actually performed this experiment in the Band 3 isoform in the red cells of humans carrying Southeast Asian Ovalocytosis (SAO).

5. SAO Band 3

SAO is a red blood cell condition marked by altered cell morphology, which is found predominantly in individuals of Southeast Asian decent. Ovalocytic red cells are found to be very rigid, and are resistant to invasion by various strains of malaria parasites in vitro [38,39]. People have been long searching for an explanation at the molecular level.

The underlying molecular defect remained a mystery until Liu et al. [40] made the surprising observation that the ovalocytic phenotype was linked to a structural polymorphism in Band 3, rather than to any change in the cytoskeletal components. It has been further established that a 9-amino-acid deletion near the N-terminal of the membrane domain (residues 400–408) is responsible for the unusual properties of the ovalocytic red cell [41–43].

This 9-amimo-acid deletion is around the cytoplasmic end of the first putative trans-membrane α-helix [11]. How has the SAO Band 3 been changed by this? Detailed analysis on the mutant protein has been done in various laboratories [44–47]. The cytoplasmic domain of SAO Band 3 is structurally and functionally normal, as shown by (i) its calorimetric properties, (ii) native conformational change at low pH, (iii) ability to form an inter-subunit disulphide bond, (iv) affinity and capacity for binding ankyrin and Band 4.1, and (v) kinetics of association with ankyrin [44]. The membrane domain of the SAO Band 3, on the other hand, is characterised by, (i) inability to transport anions [43,44], (ii) inability to bind stilbene disulfonates [44,47], (iii) different calorimetric properties [44], and (iv) different glycosylation pattern [47]. However, ligand-binding sites of a protein usually comprise only relatively small part of its structure. Often just a relatively small, localised structural change in the

protein is sufficient to produce large change in its ligand binding, as has been shown for many proteins [48]. This seems also to be the case for the SAO Band 3 protein. There is strong evidence showing that the membrane domain of the SAO Band 3 does not undergo a gross structural change, namely, (i) both the accessibility to proteolytic enzymes and the proteolytic fragments are the same, (ii) CD spectra show no change [44], (iii) the protein inserts stably into the plasma membrane when expressed in oocytes [46], and (iv) the mutant protein forms homodimers and maybe even heterodimers with the native protein [47]. Taken together, the deletion of nine amino acids at this site near the cytoplasmic end of the membrane domain junction has a profound effect on anion transport and inhibitor binding apparently without dramatic change in the overall structure of the protein.

The translational and rotational diffusion of the SAO band 3 protein were greatly reduced [42,49]. 65% of the protein became immobile, compared with 35% in normal cells [45], resulting in a tighter attachment of the cytoskeleton to the membrane and a highly rigid ovalocyte. Various mechanisms have been proposed to explain this unusual property. The inability to detect any gross structural change in the two domains of the protein has prompted Moriyama et al. [44] and Schofield et al. [45] to suspect that amino acids 400 to 408 form a flexible link between the membrane and cytosolic domain, and that this link has been deleted in the SAO isoform of the protein. With the newly available structural information (Fig. 1) [16,18], this suggestion seems to have gained ground. In Moriyama's' model [44], the deletion of the hinge is proposed to force the cytoplasmic domain to extend rigidly into the spectrin mesh. In this way, it is thought that much of the Band 3 not specifically associated with cytoskeleton components became trapped among them and unable to diffuse laterally. This model, however, cannot explain the reduced rotational diffusion of Band 3. By comparison, in the model proposed by Schofield et al., [45], the adaptation of bound Band 3 to the displacement of cytoskeleton is proposed to take the form of a flexural motion near the membrane surface in the normal red cell. In the Ovalocytic Band 3 it is this hinge that has been deleted. In such a way, the cytoplasmic domain cannot be bent easily, resulting in a stronger resistant shearing-force to any movement of the cytoskeleton.

A slight modification can be made to the above models. The amino acids 400 to 408 are highly conserved in the Band 3 protein [9]. The first membrane spanning α-helix probably starts at Pro-403 [11,19]. Therefore, residues 403 to 408 may not actually account for the entire hinge, although the exact position of the flexible fragment is unknown in the sequence (361–402). Their deletion probably does not eliminate the flexible peptide but rather have changed its conformation to make it become

more rigid. As a result, the cytoplasmic domain may become more firmly attached to the membrane domain therefore also to the plasma membrane.

Since it is found that SAO Band 3 contains a higher proportion of tetramers [47] and the protein even forms long strings of polymers in vivo [50], the change in the oligomeric state probably also plays a role. It would not be surprising find that several mechanisms operate simultaneously in vivo.

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