Three-dimensional map of the dimeric membrane domain of the human erythrocyte anion exchanger, Band 3

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The electroneutral exchange of chloride and bicarbonate across the human erythrocyte membrane is facilitated by Band 3, a 911 amino acid glycoprotein consisting of a 43 kDa N-terminal cytosolic domain that binds the cytoskeleton, haemoglobin and glycolytic enzymes and a 52 kDa C-terminal membrane domain that mediates anion transport. Electron microscopy and three-dimensional image reconstruction of negatively stained two-dimensional crystals of the dimeric membrane domain revealed a U-shaped structure with dimensions of 60 x 110 Å, and a thickness of 80 Å. The structure is open on the top and at the sides, with the monomers in close contact at the base. The basal domain is 40 Å thick and probably spans the lipid bilayer. The upper part of the dimer consists of two elongated protrusions measuring 25 x 80 Å in projection, with a thickness of 40 Å. The protrusions form the sides of a canyon, enclosing a wide space that narrows down and converges into a depression at the centre of the dimer on the top of the basal domain. This depression may represent the opening to a transport channel located at the dimer interface. Based on the available protein-chemical data, the two protrusions face the cytosolic side of the membrane and they appear to be dynamic.

Key words: anion transporter/Band 3 protein/electron microscopy/membrane protein structure

Introduction

The human erythrocyte anion exchange protein (Band 3, or AE1) is the major transmembrane glycoprotein of the red blood cell. It mediates the electroneutral exchange of chloride and bicarbonate across the erythrocyte plasma membrane and, as such, plays an essential role in the CO2 transport system of the red cell (Passow, 1986). The protein also provides the binding sites for ankyrin, haemoglobin and glycolytic enzymes (Passow, 1986). Ankyrin binding leads to the attachment of spectrin and other components of the cytoskeleton to the lipid bilayer. The protein also acts as a senescence antigen for aged and damaged red cells (Kay, 1984). Clustering of Band 3 protein in the erythrocyte membrane triggers binding of antibodies to an extracellular part of the protein and causes the removal of the aged or damaged red cells (Turrini et al., 1991). The protein consists of two structurally distinct domains, an N-terminal cytosolic domain (residues 1–360) that contains the sites of cytoskeleton and glycolytic enzyme binding, and a C-terminal membrane domain (residues 361–911) that mediates anion transport (Low, 1986; Jennings, 1989; Reithmeier, 1993; Tanner, 1993). The membrane domain of Band 3 exists as a dimer (Casey and Reithmeier, 1991) with each subunit containing between eight and 14 transmembrane segments (Kopito and Lodish, 1985; Jay and Cantley, 1986; Jennings, 1989; Passow et al., 1992; Reithmeier, 1993). The membrane domain is fully functional as an anion transporter after removal of the cytosolic domain (Lepke and Passow, 1976; Grinstein et al., 1978; Lepke et al., 1992). The protein contains a single site of N-linked glycosylation at asparagine 642; however, enzymatic deglycosylation does not affect the transport function of Band 3 (Casey et al., 1992).

It has been demonstrated by immunochemical and genetic methods that Band 3-related proteins are present in the epithelial cells of kidney, lung and intestine, in liver, brain and heart, in precursor cells of the erythrocytes, as well as in the B and T cells of the immune system (Passow, 1986; Jennings, 1989). All these proteins facilitate anion exchange (AE) and therefore are referred to as AE1, AE2 and AE3 proteins, according to their different gene sequences (Kopito, 1990; Alper, 1991). To date, 13 members of the AE gene family have been cloned and sequenced (Wood, 1992). The size and the function of the cytosolic domain varies, but the membrane domain is highly conserved. Solving the structure of the erythrocyte transporter will provide valuable information concerning the structure of the homologous gene family members.

In spite of the large amount of biochemical and biophysical data accumulated during the last 25 years, attempts to elucidate the mechanism of anion transport at the molecular level for Band 3 or related transporters have been hampered by the lack of direct structural information. Dolder et al. have reported two-dimensional (2D) crystals of Band 3. We have also grown 2D crystals of the membrane domain of the Band 3 protein (Wang et al., 1993) which were used for structural analysis by electron microscopy and image reconstruction. The projection maps of negatively stained specimens showed a dimeric structure surrounding a stain-filled depression at the dimer interface (Wang et al., 1993). Here we present the three-dimensional (3D) structure of the membrane domain of Band 3 at 20 Å resolution determined by this technique.

Results

Three-dimensional map

Two-dimensional crystalline sheets were grown from the deglycosylated membrane domain of the human Band 3
protein solubilized in the non-ionic detergent, C12E8 (Wang et al., 1993). These crystals were negatively stained and examined by electron microscopy. Negative stain does not always penetrate well into a hydrophobic sample (Brisson and Unwin, 1985). In the present work, however, there is no continuous stain-excluding layer in the 3D map. This means that the stain has perfused into the membrane lipid, as in the cases of the 2D crystals of bc1 complex (Karlsson et al., 1983), the light-harvesting complex (Kühlbrandt, 1984) and the plant photosystem II (Holzenberg et al., 1993). This appears to be due to the open, mesh-like structure of the crystalline sheets and the presence of residual detergent in the sample. The crystals of the membrane domain of Band 3 have p6 layer group symmetry, with unit cell dimensions \(a = b = 170 \pm 4 \, \text{Å}\). The unit cell contains three homodimers in total, related by a crystallographic three-fold symmetry axis, resulting in a hexameric arrangement of the protein subunits in the 2D crystals. A 3D map at 20 Å resolution was calculated from structure factors obtained by merging 43 electron micrographs recorded in eight different tilt series (0–63°) (Figure 1), with an average phase residual of 29°.

Figure 2 shows a perspective (a) and the top view (b) of the 3D map of the membrane domain of human Band 3. Figures 3 and 4 present the sections of the 3D map vertical and parallel to the membrane plane, respectively. The protein is a dimer with dimensions of \(\sim 60 \times 110 \, \text{Å}\), and a thickness of 80 Å. The dimer is approximately U-shaped; open on the top and at the sides, with the monomers in contact at the lower part, which is referred to as the basal domain (Figure 2a).

The upper part of the dimer consists of two elongated protrusions measuring 25×80 Å in projection (Figure 2b), which extend 40 Å above the surface of the bulkier basal domain (Figures 2a and 3a). The long axis of the upper domain is set at an angle of \(\sim 60°\) with respect to the long axis of the underlying basal domain (Figure 2b). A considerable portion of the upper projection lies above the lipid bilayer (Figure 2b). These two extensions appear as sides of a canyon (Figure 3a), encircling a space which is 55 Å wide at the top (Figure 4a) and narrows to a width of 25 Å at the bottom (Figure 4b). The canyon then converges into a depression on the top of the basal domain which probably indicates an opening to the transport channel (Figures 3a and 4b). The two elongated domains at the top do not contact each other, and therefore are not involved in dimer formation. However, they do participate in crystal contacts (striped areas in Figure 2).

The basal domain of the protein is 40 Å thick, which is sufficient to span the lipid bilayer. The two protein subunits are intimately associated in the basal domain such that the limits of each monomer at the dimer interface cannot be distinguished. This is particularly apparent in the central section of the basal domain shown in Figure 4c. The cross-sectional area of each monomer in the basal domain is \(\sim 1500 \pm 200 \, \text{Å}^2\) (Figure 4c). Since this domain is a high-density feature, its area in the sections is not very sensitive to the choice of contour level. The lower face of the basal domain is rather smooth, with a depression at the dimer interface on the lower side of the structure (Figures 3b and 4d).

**Discussion**

**Hexameric forms of Band 3**

The membrane domain dimers are related by a crystallographic three-fold symmetry axis, which gives rise to a hexameric arrangement of the protein subunits in the 2D crystals. Hexameric forms of Band 3 have been found recently in some detergent solutions (Dolder et al., 1993; Wong, 1993). When observed under the electron microscope, some of the purified membrane domain also showed a ring-like structure, with a diameter of 150–200 Å (data not shown). These particles were probably also hexamers of the membrane domain. It seems likely that during reconstitution, the protein associates into hexamers first, which then form the 2D lattice. As Band 3 in the native membrane exists as a mixture of dimers and tetramers, the formation of hexamers is likely induced by the solubilization or crystallization conditions. The main contact responsible for the hexameric form occurs between the upper projections while there is little or no contact between basal domains in the crystals.

**The sidedness of the structure**

Figure 5 presents a folding model for the arrangement of the transmembrane segments in Band 3 based on hydrophathy profiles, chemical and antibody labelling and in situ proteolysis (Jennings, 1989; Passow et al., 1992; Wood, 1992; Reithmeier, 1993; Tanner, 1993). In this 14 span model, ~50% of the membrane domain is in the bilayer, 15–18% on the extracellular side and 32–35% on the cytosolic side of the membrane. Comparing this with the density distribution of the molecule in Figure 2, we propose that the basal domain corresponds to the membrane embedded portion of Band 3, although this is to be confirmed experimentally. By implication, the projections in the upper half of the map consist of the cytoplasmic portions of the membrane domain of Band 3, in agreement with the mass distribution in the folding model (Figure 5). A small portion of the cytosolic surface would be
Fig. 2. 3D map of the dimeric transport domain of the Band 3 protein. (a) Perspective view. The bulky basal domain is probably embedded in the lipid bilayer, indicated by the two blue planes which are separated by 35 Å. The longest dimension of the basal domain is ~110 Å. Based on the available biochemical data (see text) we believe that the protrusions on the upper side of the basal domain are on the cytosolic side of the membrane and that the smooth surface on the lower side is extracytosolic. The two domains on the cytosolic side form two sides of a canyon that leads to a depression which is probably the entrance to a pore. Striped areas represent cuts through the map above the putative membrane surface which form protein–protein contacts in the crystals. (b) Top view, showing the open canyon on the cytoplasmic side. The two sides of the canyon narrow down on the surface of the basal domain. The lower part of the protein thought to be embedded in the membrane appears blue. The bar represents 20 Å.
The basal domain which monomers accommodate the pore. This agrees well with the folding model (Figure 5) according to which the exterior surface of Band 3 consists of multiple small loops and only two larger loops of polypeptide. One of the large loops normally contains N-linked carbohydrate. As the crystals were grown from deglycosylated protein, the oligosaccharide chain does not contribute to the density. Our conclusion that this smooth surface probably corresponds to the cell exterior is also supported by the intimate association of the two subunits on the bottom of the structure. It is known that Band 3 can be readily crosslinked by reagents on the outside of the cell (Staros and Kakkad, 1984). The interactions responsible for the dimeric structure of the membrane domain reside in the lipid-embedded portion of the structure. The interactions within the centre of the bilayer appear to be intimate at the present resolution. It is known that dissociation of the dimer into monomers leads to denaturation of the protein (Casey and Reithmeier, 1991), presumably because it requires the disruption of the extensive subunit–subunit interactions in the structure.

The cross-sectional area of the basal domain in the centre of the bilayer is \(\sim 1500 \text{ Å}^2\) per subunit. Bacteriorhodopsin, another transmembrane protein, is composed of seven membrane-spanning \(\alpha\)-helices, with each of the helices occupying \(103 \text{ Å}^2\) on average (Henderson et al., 1990). Assuming the density of the helix packing is similar to that in bacteriorhodopsin, each Band 3 subunit would accommodate 14 transmembrane \(\alpha\)-helices. This estimate is in good agreement with the folding model presented in Figure 5.

The Band 3 canyon

The prediction of an anion transport pathway consisting of a channel leading to a thin barrier (Passow, 1986; Jennings, 1989; Tanner, 1993) is consistent with the 3D map. We believe that the space between the upper protrusions corresponds to the distal part of the channel. Positive charges on the loops on the cytoplasmic side of the membrane (Figure 5) may be disposed to the inner surface of the canyon, thereby participating in anion selection. The probable location of this narrow pore is reminiscent of gap junctions (Unwin and Zampighi, 1980) and the nicotinic acetylcholine receptor (Brisson and Unwin, 1985) in which a well-resolved channel narrows to an unresolved constriction in the lipid bilayer, although the upper part of Band 3 resembles a canyon rather than the cylindrical channel observed in the other two structures. The depression at the bottom of the canyon at the dimer interface may represent an opening to a transmembrane channel. This may lead to a barrier of anion transport which is buried in the membrane-embedded part of the carrier protein and is unresolved at the present resolution. This is supported by the observation that extensive protease treatment of Band 3 affects but does not completely destroy the anion binding site (Falke et al., 1985). In addition, immobilized inhibitors of anion transport have access to the inhibitor site from the cytoplasmic surface only when attached to a long spacer molecule (Eidelman et al., 1991).

Stillbene disulfonate molecules which have a length of 15–20 Å bind to an external site present in each Band 3 molecule. The distance between the two sites in the dimer is 28–46 Å, as estimated by fluorescence resonance energy transfer (Macara and Cantley, 1981). Recent electron paramagnetic resonance measurements using spin-labelled stilbene disulfonate have shown that the two inhibitor binding sites are not in direct contact but are \(>20\) Å apart (Wojcicki and Beth, 1993). Each subunit must contain an inhibitor binding site that faces the exterior of the cell.
which would appear to be on the lower surface of the membrane domain as seen in Figure 2a.

**The conformational states of the transporter**

Anion transport may proceed by a ping-pong mechanism (Gunn and Fröhlich, 1979; Passow, 1986; Jennings, 1989; Tanner, 1993) whereby a single anion binding site is alternately exposed to one side of the membrane or the other. The binding site has an asymmetric distribution, facing the cell interior in the presence of equimolar anion concentrations (Passow, 1986; Jennings, 1989). In the 2D crystals both sides of the membrane domain are bathed in equimolar chloride solution. Under these substrate conditions, the inward facing conformation of the carrier is predicted to predominate (Passow, 1986; Jennings, 1989). We therefore believe that the 3D map shows the inward facing conformation of Band 3, in which the anion binding site faces the cytoplasmic side and the barrier faces the exterior surface.

Band 3 undergoes dramatic structural changes during the transport process (Passow, 1986; Jennings, 1989; Tanner, 1993). Eosin-maleimide, a non-competitive inhibitor, binds preferentially to the outward facing conformation (Knauf et al., 1993). The binding of stilbene disulfonate inhibitors, which bind from the outside, locks the protein into the outward facing conformation, causing the cytoplasmic surface of the membrane domain to become resistant to proteolysis (Kang et al., 1992).

Two types of 2D crystals have been obtained from the membrane domain of the Band 3 protein, 2D sheets and helical tubes (Wang et al., 1993). The 2D projections suggest that a part of the membrane domain can move, resulting in two different crystal forms (Wang et al., 1993). From the 3D map obtained from the sheets (Figure 2), it appears that the cytosolic portion of the membrane domain, or part of it, can rotate by ~60°, resulting in two different crystal packing forms, and hence different projected structures. It is conceivable that these two elongated flaps on either side of the channel move upon ion binding, inducing a conformational change in the underlying membrane domain which closes the cytoplasmic end of the channel while opening it on the exterior side. Movement of large domains within the membrane may be restricted because of the high viscosity of the lipid bilayer.

One function of the N-terminus of the membrane domain is to act as a linker to the large cytoplasmic N-terminal domain which was removed by proteolysis. There is evidence that the cytosolic domain of Band 3 is highly asymmetrical, with a length of >200 Å (Low, 1986). This domain also has multiple conformations in situ upon binding to the cytoskeleton and to the various enzymes (Low, 1986). It is, therefore, not surprising that the membrane domain of the protein possesses a flexible link that separates the elongated and mobile cytosolic domain from the anion transport part of the protein to avoid undesired interference to anion transport from the cytoskel- eton. When the link is removed or altered, the cytosolic domain is probably more firmly attached to the membrane domain, and therefore also to the membrane (Wang, 1994).
Comparison with other ion channels and transporters

Band 3 is a member of a multi-gene family of anion exchangers that are involved in intracellular pH regulation and cell growth and differentiation (Reithmeier, 1993). Since the membrane domains of these proteins are highly conserved, the structure we have determined also likely represents the structure of the homologous AE proteins. Cation exchangers (e.g. Na+/H+ and Na+/Ca2+ exchangers) have a similar membrane topology to Band 3 (Figure 5), suggesting that they too may have a similar transmembrane structure.

The overall shape of the transport domain of the Band 3 protein is entirely different from that of any known structure of other membrane proteins, including those involved in ion transport, such as bacteriorhodopsin (Henderson et al., 1990) and Ca2+-ATPase (Toyoshima et al., 1993), or channel formation, such as the nicotinic acetylcholine receptor (Unwin, 1993), the gap junction (Unwin and Zampighi, 1980) and bacterial porins (Weiss et al., 1991). The unique structure of Band 3 must reflect its function which is to transport ions across a membrane at a very high rate. This rate is closer to that of an ion channel than that characteristic of a typical transporter.

Materials and methods

Protein purification and crystallization

The membrane domain of human Band 3 was prepared by mild trypsin treatment (5 mg/ml, 0°C, 1 h) of ghost membranes followed by alkaline stripping, solubilization with 1% octaethylene glycol n-dodecyl ether (C12E8) and ion exchange chromatography (Casey et al., 1989). The purified protein was deglycosylated using N-glycanase F as described previously (Casey et al., 1992). Crystalline sheets of the membrane domain of Band 3 were grown by reconstitution with an equal weight of dimyristoyl phosphatidylcholine and 5% (w/w) cholesterol in 100 mM NaCl, 10 mM MgCl2, 5 mM sodium phosphate, pH 8.0, 10% PEG 200 as described (Wang et al., 1993).

Electron microscopy and image processing

Crystals were negatively stained with 2% uranyl acetate and examined by electron microscopy. Low-dose micrographs were recorded on a JEOL 2000EX electron microscope operated at 200 kV and at a nominal magnification of 40 000×. Optical diffraction of electron micrographs indicated structure factors to 20 Å resolution. About 500 electron micrographs were recorded from crystals tilted at different tilt angles (0-63°). Eight tilt series with a total of 43 images were processed. Seventeen of these images were taken at high tilt angles (>40°), ensuring an even sampling of reciprocal space. Areas of 1024×1024 or 2048×2048 image points, depending on the size of the crystal, were digitized on a Perkin-Elmer 1010M microdensitometer with a step size of 20 μm that was equivalent to 5 Å spacing on the crystal. Electron micrographs were processed by established methods (Amos et al., 1982) on a VAX computer cluster. Lattice distortions were corrected as described by Henderson et al. (1986). An area of 100×100 pixels at the centre of the image, containing 10–20 unit cells depending on the tilt angle, was used as the reference and only one round of correction was performed. The unbending step increased the signal/noise ratio, hence the number of reflections with high IQs. Lattice lines were fitted by the method of Agard (1983) with a sampling interval of 1/300 Å in the z direction. The merged data set contains 32 independent lattice lines, yielding 301 unique Fourier components to 20 Å resolution (Figure 1). The resolution in the z direction is between 25 and 30 Å. A 3D map was calculated using the CCP4 crystallographic programs (Evans, 1991). The map was contoured to include a molecular mass of 53 kDa per monomer, and was displayed using the AVS program on a Starlient computer.

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References

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