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Author

Published
2014

Journal Title
FEBS Journal

DOI
https://doi.org/10.1111/febs.12700

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Crystal structure and immunological properties of the first annexin from Schistosoma mansoni – Insights into the structural integrity of the schistosomal tegument

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Keywords
annexin; calcium; parasite–host interactions; schistosomiasis; vaccine design
Abstract

Schistosomiasis is a major parasitic disease of humans, second only to malaria in its global impact. The disease is caused by digenean trematodes that infest the vasculature of their human hosts. These flukes are limited externally by a body wall composed of a syncytial epithelium, the apical membrane of which is a parasitism–adapted dual membrane complex. Annexins are thought to be of integral importance for the stability of this apical membrane system. Here, we present the first structural and immunobiochemical characterisation of an annexin from *Schistosoma mansoni*. The crystal structure of annexin B22 confirms the presence of the previously predicted alpha-helical segment in the II/III linker and reveals a covalently linked head–to–head dimer. From the calcium-bound crystal structure of this protein, canonical type II, type III and B–site positions are occupied, and a novel binding site has been identified. The dimer arrangement observed in the crystal structure suggests the presence of two prominent features, a potential non–canonical membrane binding site and a potential binding groove opposite to the former. Results from transcriptional profiling during development show that annexin B22 expression is correlated with life stages of the parasite that possess the syncytial tegument layer, and ultrastructural localisation by immuno–EM confirms the occurrence of annexins in the tegument of *S. mansoni*. Data from membrane binding and aggregation assays indicate the presence of differential molecular mechanisms and support the hypothesis of annexin B22 providing structural integrity in the tegument.

Database

Coordinates and structure factors have been deposited with the PDB, accession numbers 4mdu, 4mdv.

Introduction

Annexins are widespread throughout the Eukaryota, and members of this protein family have become apparent in molecular studies of diverse parasite taxa, ranging from the diplomonad protist *Giardia* to the neodermatan platyhelminths *Taenia* and *Schistosoma* (reviewed in [1]). These soluble proteins possess a distinctive, highly conserved fold that enables general adapter–like functions at the surface of phospholipid membranes [2]. Concomitantly, annexins possess diverse roles including vesicular transport (endo– and exocytosis), membrane attachment of the cytoskeleton [3] and membrane repair [4]. Whereas the extent and conformation of the N–terminal domain of annexins is variable, the well established annexin fold is characterised by the C–terminal core domain that consists of four repeats (I - IV), folded into a helical bundle and arranged in a cyclic manner (see Figure 1). A slight bend in the core domain gives rise to a convex (“top”) and a concave (“bottom”) side, with the N–terminal domain (if present) extending along the concave side of the molecule.
Structurally, each repeat is made up of five helices (A – E), four of which form a parallel helix–loop–helix bundle. The loop regions, AB and DE, are situated on the convex surface of the molecule that constitutes the canonical membrane binding surface of annexins. The membrane binding can be facilitated, in mammalian annexins, through calcium ions bound between the AB and DE loops (type II sites) and the DE loops (type III sites) of each repeat [5]. In plant annexins, not all repeats are capable of binding calcium ions and direct interactions of exposed basic and hydrophobic residues also engage in membrane binding interactions [6]. The membrane binding mechanisms of $\alpha$–giardins – annexins from *Giardia duodenalis* – have not yet been established in great detail. Due to the varying degree of conservation of type II calcium binding sites among the $\alpha$–giardins, their membrane binding mechanisms may show significant variation. The best characterised member of this family so far is $\alpha$1–giardin which possesses a calcium–mediated and –regulated adsorption behaviour to acidic phospholipid membranes [7].

In the current nomenclature, annexins found in vertebrates, including humans, are classified as ‘A’, annexins from invertebrates, including parasitic helminths, are denoted as ‘B’, those from fungi belong to group ‘C’, plant annexins are ‘D’, and annexins from protists belong to group ‘E’. A recent in–depth analysis of group ‘B’ annexin proteins from a diverse range of invertebrate taxa, including parasites and vector organisms that are harmful to humans and domestic animals, revealed that these proteins separate strongly into clades reflective of the phylogenetic relationships of the organisms in which they are found [8].

Disability–adjusted life years lost to human schistosomiasis in 2010 were measured at 48/100,000, an increase of 20% on measures in 1990 [9]. The disease, one of 17 neglected tropical diseases, is prevalent in rural areas of many tropical nations, where it remains persistently associated with poverty. Disease in schistosomiasis is related to host responses against parasite eggs deposited in the blood vessels surrounding the gut (*Schistosoma mansoni* and *S. japonicum*) or bladder and genital organs (*S. haematobium*), but control is directed towards killing adult worms or the invasive larvae that establish infection. Current control efforts with praziquantel require that the drug must be applied periodically and repeatedly. Moreover, high rates of re–infection after mass treatment limit strategies based on chemotherapy alone. It has thus been suggested that a prophylactic vaccine is the ideal method for sustainable control of schistosomiasis, alone or in combination with anthelmintic drugs [10].

The tegument (body wall) of the intravascular schistosomes is the dominant host interactive tissue of the parasites [10, 11], and the occurrence of many unique components in its proteome as well as its lipidome suggests that schistosomes employ a range of previously unknown and unique molecular mechanisms to survive within their host [12]. This syncytial epidermis interfaces with the host by means of a surface membrane overlaid with a schistosome–specific membrane, the membranocalyx, so that the surface membrane has the appearance of a dual bilayer [13]. The first action of penetrant cercariae (the human–infective larvae) in human skin is to remodel extensively the surface membrane. This cercarial transformation represents the key period of vulnerability for the parasites [10, 11] in which the surface proteins are exposed to immune attack. Although few proteins have been identified in the surface membrane complex of the schistosome tegument [14], annexins, along with schistosome TSP2 and Sm29, are prominent components. Antibody recognition of the latter two of these, TSP2 and Sm29, appears to be related to protective immunity and resistance to schistosomiasis in human populations [10, 15–
Experimental vaccination with recombinant peptides of these two molecules induces strong protection in experimental schistosomiasis, as measured by reduction in adult worm burdens and egg production [17], but as yet, neither produces sterilising immunity nor prolonged immunological memory. Since annexins are proposed to be situated at the junction of the surface membrane and membranocalyx, they are likely to play a substantial role in formation and maintenance of the surface layers.

Using structure-based amino acid sequence alignments, in our recent surveys of novel parasite annexins, we identified a structural feature shared by several (but not all) annexins from parasitic organisms that presents a novel element of those annexins and is thus absent from any of the mammalian annexins [1]. Many schistosome annexins, and those from *T. solium*, *C. elegans* nex-4, as well as α12- and α19-giardin possess an unusually long linker segment between repeats II and III on the concave side of the protein. Whereas the typical length of this linker in annexins is about 10–15 amino acids, the linker peptide found in several parasite annexins comprises between 25 and 38 amino acids. Moreover, secondary structure predictions consistently indicate that this elongated linker region adopts an α-helical structure. Therefore, we hypothesised that these annexins possess an additional α-helical element on the concave side of the molecule, which is generally believed to be distal to the membrane binding side, and may thus provide a target for immunological therapeutics [1]. In this study, we report on the structural and immuno-biological characterisation of the first of any schistosome annexins, annexin B22.

**Results**

**Crystal structure of annexin B22**

The crystal structure of non-reduced annexin B22 was determined in the monoclinic space group P2₁ with two molecules in the asymmetric unit. Several loop regions are poorly defined in the electron density, in particular the AB loops in repeats I, III and IV, as well as the connection between helices C and D in repeat II. Each monomer possesses the anticipated annexin fold of the core domain (see Figure 1). There is no significant conformational difference between both molecules of the dimer, as reflected by the Ca rms difference of 0.72 Å. The two molecules form an intimate head-to-head dimer with P2₁ symmetry. The buried surface area comprises 955 Å² (~6%) per monomer; since this is considerably larger than the threshold of 800 Å² suggest by Janin [18], this dimer most probably constitutes a physiologically relevant species. Concomitantly, several inter-molecular interactions can be identified in this dimer interface (see Table 1), including eight intermolecular hydrogen bonds, two intermolecular ionic interactions, as well as a covalent bond formed by the non-reduced thiol side chains of Cys173 and Cys173'. Notably, all these interactions occur between repeats II and III of the two molecules. Compared to other members of this protein family, annexin B22 possesses an extended N-terminal domain comprising 36 amino acid residues. The N-terminal peptide from Ser5 to His20 packs against the groove between modules I/IV and II/III on the concave side of the molecule (see Figure 2), with a helical turn in 3₁₀ conformation spanning Ser12 to Ser16 and a subsequent
turn (Asp18 – Gly21). Notably, this segment from Ser12 to Gly21 is embraced by the concave side of the annexin core and the extended linker between repeats II and III. The linker region adopts an α-helical structure from Pro192 to Gly203 which packs against the N-terminal segment.

In the present crystal structures of apo and calcium–bound annexin B22, the electron density of surface-exposed loops is less well defined than in other regions of the protein where close packing of residues is observed. In the apo structure of the protein, the IAB loop (Lys48–Thr52), the link between helices IIC and IID (Glu158), as well as the entire IIIAB loop (Gly224–Glu232) are not resolved in the electron density.

**Calcium binding sites**

Canonical calcium binding sites in annexins are categorised into three types: type II, type III and B sites [19]. The crystal structure of calcium–bound annexin B22 reveals 13 ions bound by the dimer (see Table 2). Clearly, calcium binding is accommodated by ordering of several rather flexible regions, which is evident from the improved electron density in those regions when comparing the apo and the calcium–bound crystal structures, as well as by the positional rms difference of 1.3 Å (calculated for Cα atom positions over the entire structure) between the two structures. In the presence of calcium ions, the IAB loop becomes ordered, due to coordination of two ions. Also, the IIIDE loop undergoes a conformational change due to coordination of a calcium ion. Here, helix IID that only consists of two consecutive helical turns in the absence of calcium, becomes elongated by an additional turn upon coordination of calcium.

The type II calcium binding sites in repeats I, II and IV, as well as the type III site in repeat II are occupied. Additionally, one calcium ion is situated in the B–site of repeat I (’IB’), and another one between the AB and DE loops of repeat IV (’IVABDE’), both in different and non-canonical coordination schemes. The ’IB’ calcium ion is coordinated by the side chain hydroxyl group of Thr52 and by the side chain carboxylate of Glu54 in a bidentate manner. The ’IVABDE’ calcium ion is coordinated by the backbone carboxyl groups of Leu303, Gly304 and Asp344, as well as a water molecule. In both cases, the coordination shells appear unsaturated, since both calcium complexes possess open coordination sites. The thermal displacement (B–) factors for the calcium ions in repeat I and IV are higher than those for the other metal ions, commensurate with the higher B–factors of the IAB and the IVAB loops. Another aspect to consider in this context is the low affinity of annexins for calcium ions in the absence of acidic phospholipid membranes. The low affinity is most likely a further contributing factor to the relatively high B–factors observed for some calcium ions in the present structure.

In the dimer interface, residues Asp126 (helix IIB) and Asp273’ (helix IIID) of the two monomers coordinate a calcium ion in the B–site of repeat II that is close to the non-crystallographic two-fold axis, giving rise to two alternate positions each of which is occupied partially with occupancies of 0.43 and 0.57, respectively.

**Quaternary structure in solution**

Size exclusion chromatography of annexin B22 under varying conditions revealed a monomer–
dimer mixture (see Table 3, Figure S1). In the non-reduced state and in the absence of calcium ions, only about 5% monomeric species were present. Under reducing conditions (0.1 mM DTT), the monomer content increases to about 30%, and the addition of calcium (5 mM) yields the maximal monomer content of about 50%. When tested under a high salt environment (500 mM NaCl), the effect of added calcium seems to be annihilated, and a monomer content of about 30% was detected.

Membrane activity
Annexin B22 shows calcium–dependent binding to acidic phospholipid membranes in both the non-reduced and reduced states (see Figure 3). In order to assess the membrane binding behaviour of the reduced protein, a protein sample kept in buffer containing 0.5 mM DTT was used, and 0.2 mM TCEP was added to the assay buffer. About 15% of reduced annexin B22 bound to membranes in the absence of calcium. The calcium–dependent binding appears to follow a two-phase behaviour: The half-maximal calcium concentration ($c_{1/2}$) for binding to DOPS/DOPC (3:1) membranes is 30 μM (first phase) and 0.36 mM (second phase). The Hill coefficients determined by data fitting for the first phase is ~1, and for the second phase ~7. The maximum binding of 100% was achieved at calcium concentrations above 1 mM. The non-reduced protein displays about 5% calcium–independent binding and about 50% maximum binding, with $c_{1/2}$ (Ca$^{2+}$) of ~40 μM, and a Hill coefficient of ~2.

Reduced and non-reduced protein show membrane aggregation behaviour in the presence, but not in the absence of calcium (see Figure 4).

Expression of annexin B22 in the life cycle
Comparison of the annexin B22 mRNA levels across key life cycle stages revealed a low abundance of transcripts in the developing and mature embryos (eggs and miracidia). Increased transcript abundance was seen in the newly invaded schistosomula with highest abundance in adult males (Figure 5). Surprisingly, transcript levels in adult females were low compared with males.

Immunoreactivity of *S. mansoni* annexins
A mouse polyclonal antibody against annexin B22 was generated and evaluated against recombinantly expressed and purified *S. mansoni* annexin B22 (this study), as well as equal amounts of annexins B5a, B5b, B7a and B30 (unpublished). In the left panel of Figure 6, a separate scan of the gel has been superimposed on the scan of the Western blot in false colours. The location of annexins B5a, B5b, B7a and B30 differs from the residual antibody signal in those lanes. We thus conclude that these residual signals do not correspond to cross-reactivity. Therefore, the anti-annexin B22 antibody mainly recognised recombinant annexin B22, indicating low cross-reactivity against the other *S. mansoni* annexins. Importantly, a band in soluble whole adult protein preparation was recognised by the annexin B22 antiserum, indicating that native annexin B22 can also be detected.

The serum of mice infected with *S. mansoni*, as well as of uninfected mice was then tested by
means of an ELISA for recognition of recombinant annexin B22. Results from this experiment (Figure 6, right panel) show that the folded recombinant protein is indeed recognised by the serum of infected mice. These data indicate that annexin B22 is an immunoreactive protein of *S. mansoni* that can be recognised by the host’s immune system.

**Ultrastructural localisation**

By immuno-EM, annexin B22 was detected in tegument and muscles of adult male schistosomes (Figure 7A–B). Gold particles were detected primarily, but not exclusively, in association with invaginations of the surface membrane and other membrane compartments and vesicular structures, including the elongate bodies, within the tegument. Strong tegument-associated fluorescence emission was observed by fluorescence microscopy (data not shown), corroborating these findings.

As the serum used for localisation of annexin B22 was raised in mice, the laboratory hosts of *S. mansoni*, control sections were incubated in pre-immune serum (Figure 7D) or in PBS (Figure 7C) prior to incubation with protein-A gold to ensure that immunoreactivity in labeled sections could be attributed to the immunolocalisation protocol used here and not as a result of adult worms binding serum components *in vivo*. Scattered gold particles were observed in sections incubated in PBS as the primary buffer, and light label was detected in sections incubated in pre-immune serum. In contrast, sections incubated with annexin B22 serum displayed a much higher and consistent localisation pattern in association with membrane compartments, indicating positive immunoreactivity, rather than reaction of the secondary labelling system with host antibodies adsorbed by the parasite while in the host. Furthermore, a mouse serum raised against smooth muscle actin displayed a distinctive localisation pattern in the tegument [20].

**Discussion**

Based on secondary structure predictions and structure–based sequence alignments, we have previously suggested that some Group B and Group E annexins expressed by parasites possess an extended linker peptide between repeats II and III (see Table S1) giving rise to an additional secondary structure element on the concave side of the annexin core domain [1]. The absence of this element in any other non–parasite annexin known to date fuels the idea that this epitope may be of therapeutic utility.

The crystal structure of annexin B22 provides experimental evidence for the unique structural makeup of a parasite annexin with an extended segment linking repeats II and III. Notably, the unique structural feature observed on the concave side of annexin B22 also features particular contributions of the N-terminal domain, which is embraced by the core domain on one side and the helical segment of the extended linker on the other. It is tempting to speculate that other parasite annexins with an extended II/III linker peptide may adopt a very similar conformation. A comparison of the extent of the N-terminal domains for annexins with the unique linker shows that such a fold may be possible for most of them (see Table S1). The assumption that N-terminal peptides with a length considerably less than 36 residues, as in the case of annexin B22, may not be able to span the distance between the N-terminal end of helix IA and the location of
the II/III linker leads to the prediction of a different fold on the concave side of the molecule for the following annexins: AnxB16 (23 residues), AnxB30 (15–18 residues), AnxB32 (21 residues), AnxB33 (4 residues), AnxB34 (22 residues), AnxB35 (16 residues).

Within the conserved annexin fold, annexin B22 shows some conformational variation in loops IIAB and IIIDE which appear to be bent away from their anticipated locations due to the interspersing loop IIAB from the other monomer. Secondly, an insertion of 8 residues at the C-terminal end of helix IIC results in an elongation by one turn. The high thermal displacement factors as well as the poor electron density in many surface-exposed or membrane-binding loops, in particular in the third repeat, suggest high flexibility of these segments. Conformational variation of membrane binding loops has been observed before, namely in annexins A5 (IIAB loop) [21] and the plant annexins (IAB loop) [22]. For annexin B22, we assume that the IIAB loop becomes ordered only in the membrane-bound state.

Annexin B22 and its homologues from *S. japonicum* [8] and *S. bovis* [23] are the only B annexins known to date that possess an exposed cysteine residue in the IIDE loop (Cys173), a position where most other annexins possess a serine residue. In the present crystal structure, Cys173 was found to engage in an inter–molecular disulphide bond that adds to the stabilisation of the unique head–to–head dimer topology with the dimer interface exclusively being located in module II/III. Although the plant annexin 24(Ca32) also crystallised as a head–to–head dimer [24], the dimer interface in that case comprises almost the entire convex surface of both molecules, thus giving rise to a more compact structure of the dimer.

A hallmark feature of annexins is the endonexin sequence MKGXGT–(38)–D/E [25] in the loops of the individual repeats. The sequence MKGXGT is located in the AB loop, and the acidic bidentate amino acid positioned roughly 38 residues downstream is situated in the loop connecting helices D and E. At the level of tertiary structure, this sequence constitutes the type II calcium binding sites of annexins. A second type of calcium binding site in annexins, type III, is not immediately evident from the primary structure. Type III binding sites are situated in the DE loops whereby one or two main chain carbonyl groups and a bidentate carboxylate from helix E coordinate the metal ion together with several water molecules. Recent crystal structures of annexins from group E (α–giardins) [7, 26, 26] revealed that these ancestral annexins possess a variety of different calcium coordination schemes, and several α–giardins lack canonical calcium binding sites, yet can bind calcium in a non–canonical fashion. It has been proposed that the variety of calcium binding sites may represent snapshots of the evolution of canonical metal binding sites in this protein family [7]. Phylogenetically, annexin B22 belongs to the youngest clade of B annexins that exclusively consists of parasitic platyhelminthes [8]. In line with this argument, this annexin engages both canonical and non–canonical calcium binding sites.

The dimer found in the crystal structure suggests severe implications for membrane binding of annexin B22, since large areas of the convex surface with its canonical membrane binding sites are not available for membrane interaction, and the presence of a second molecule in the dimer further presents a significant steric hindrance. However, the results obtained from liposome co–pelleting assays with acidic phospholipid membranes show that the dimeric species is able to bind to membranes in a calcium–dependent fashion, albeit with a maximum binding degree of 50%.
Furthermore immuno-electron microscopy suggested membrane localisation, on the cytoplasmic face. It is possible that the non-occupied I/IV module and its calcium binding loops engage in membrane interactions (see Figure 8). In that case, surface exposed residues from the II/III linker, loop IIIAB and helix IIC are likely to be in close proximity to the membrane surface as well, and may thus directly interact with phospholipids. Intriguingly, the surface representation of the calcium-bound annexin B22 dimer shows a potential dimer–specific non–canonical membrane binding site that could also engage calcium–mediation through the novel binding site (Ca403) in repeat I of both molecules. Notably, the observed Hill coefficient of 2 agrees with the presence of two calcium ions in this putative membrane–binding site. Several polar residues may participate in direct contacts with phospholipids, thus explaining the residual membrane binding in the absence of calcium.

The notion of a non–canonical membrane binding site in the annexin B22 dimer is further fuelled by a prominent groove situated at the opposite side of the dimer (see Figure 8), since this may allow the dimeric annexin B22 to act as an adapter between the membrane on the one hand, and tegument molecules binding to the dimer groove on the other. This groove is constituted principally by helices A and C of repeat IV on both molecules (Figure 1, pink–coloured repeats), with a continuous negatively charged “lining” and slightly positively charged “base”. The width of the groove measures roughly 12 Å and its height is ~25 Å. These properties make it possible to accommodate a structured peptide of suitable electrostatic complementarity.

The membrane binding behaviour of reduced annexin B22 has three different stages:
(i) about 15% bind to the membrane in the absence of calcium;
(ii) up to 0.2 mM calcium, a non–cooperative membrane binding behaviour is observed until about 30% of the protein is located at the membrane, which is followed by
(iii) a phase with high cooperativity (n = 7) in which maximal protein binding of 100% is achieved at about 1 mM calcium.

As is evident from Figure 8, there is a prominent basic patch (mainly at repeat II) at the convex surface of the molecule which most likely gives rise to calcium–independent binding to acidic phospholipid vesicles in stage (i). We hypothesise that one of the calcium binding sites then mediates the calcium–dependent binding observed as stage (ii) until a particular coverage of the membrane surface is achieved. The population of the membrane surface with annexin molecules may either provide a protein platform enabling protein–protein interactions at the membrane or cause some topological changes in the membrane bilayer, thus initiating stage (iii). In this last stage, all calcium binding sites observed in the crystal structure appear to engage in mediation of membrane binding, given the excellent agreement of the experimentally determined Hill coefficient of 7 with the crystallographically observed calcium ions. The apparent availability of the calcium– and membrane–binding capabilities of the I/IV module, even in the non–reduced annexin B22, add to a recurring scheme of repeats I and IV playing a crucial role in annexin–membrane contact. For example, canonical membrane binding sites are mostly absent in plant annexin repeats II and III, and the I/IV module has been shown to carry the calcium–dependent protein–membrane interaction sites in case of cotton annexin Gh1 [22].

Due to the head–to–head arrangement of the disulphide–bonded dimer, the canonical membrane
binding sites on module I/IV of each molecule point in opposite directions. We questioned whether dimeric annexin B22 may possess membrane aggregating properties. As shown in Figure 4, membrane aggregation of acidic phospholipid membranes was indeed observed, exclusively in the presence of calcium. However, this property is not specific to the dimer but also seen with monomeric (reduced) annexin B22. One explanation for this result is the ability of annexin B22 forming more than one type of dimeric species. In order to achieve an aggregation–competent dimer, protein–protein interactions are required that position membrane binding sites on each molecule in a non–coplanar fashion. Notably, this does not exclude formation of a non–covalently linked dimer similar to that seen in the crystal structure, since there are several intimate inter–molecular non–covalent interactions possible in the II/III module (see Table 1). However, results obtained from size exclusion experiments indicate that other dimer species may be formed, as evident from the high dimer content under reducing high–salt conditions (see Table 3), calling for a dimer with a rather hydrophobic interaction interface.

Data from this current study indicate that annexin B22 is distinctly associated with parasitism of the mammal host and is preferentially expressed in male parasites. Localisation of this molecule and a similar molecule in S. bovis [23] reveals primarily a tegument–linked distribution, and a distinct association with the dual membrane complex comprising the surface of the adult parasite. This localisation places the annexins as a key mediator of membrane binding in a calcium–dependent or –independent manner. The majority of gold particles in our localisation experiments were located on the cytoplasmic face of the membrane complex. This precludes annexin B22 from a hypothesised “molecular glue” function, whereby the molecule is proposed to bind the two bilayer membrane of the dual apical membrane complex together [14]. Intriguingly, annexin B22 localises to a number of tegument structures, and there is a strong possibility that in its monomeric or dimeric form, this annexin is a pivotal player in membrane fusion events. In localising to the surface invaginations, the sub–cellular distribution of this molecule is reminiscent of that of S. mansoni tetraspanin [20], an important vaccine target linked with membrane remodeling in the tegument [17, 27]. An evaluation of the interactome of the S. mansoni tegument has revealed that annexins are found as interactors of tetraspanins or at least in close vicinity of the tetraspanin web in the tegument [28]. These observations strongly suggest a role for annexins in the complex interactions of the membranes of male parasites.

The fact that annexin B22 is recognised by the serum of infected mice adds to the many characteristics desirable for vaccine development – surface or near–surface location and recognition by host serum. The antibody response observed here opens the possibility that function disruption of this molecule may lead to loss of surface regulatory capacity of adult schistosomes. At this stage, it is not certain that recognition of the molecule might have protective effects; annexins are highly conserved molecules and there might be immunological risks inherent in raising vaccines against the bulk of the molecule. However, the interesting conformation of the disparate linker region and its disposition over the N–terminal domain provides unique structural topographies that could be exploited in vaccines or drugs.

Since the schistosome tegument can be expected to undergo frequent and rapid transformations...
to enable interactions of the worm with the environment, a possible scenario may involve recurrent ruptures in the dual membrane system that would need to occur in a controlled fashion and be quickly executed. Schistosome annexins are pre-disposed to be involved in this process which is highly reminiscent of the membrane repair mechanisms of plasma membranes [29] where homo- and heterogeneous annexin assemblies have recently been shown to build seals in response to the change of calcium levels [30, 31]. Furthermore, annexin B12 from the freshwater metazoan Hydra vulgaris has recently been implicated in specific internalisation of molecules from the environment [32], further supporting our working hypotheses.

Future studies elucidating the schistosomal annexin interactome at the molecular level will provide insights not only into the exciting variation of annexin molecular mechanisms but also into mechanisms responsible for tegument integrity and, thereby, the immune evasion strategies of schistosonmes.

**Materials and methods**

**Cloning of annexin B22**

Annexin B22 (alternative name: annexin (Sm)1 [1, 8]; Smp\_074150; http://www.genedb.org) was amplified from cDNA containing Schistosoma mansoni mixed life cycle stages, using the primers 5’-catgCCATGGCATGGCCAAATATTTCTGAATTT-3’ and 5’-cccCTCGAGTTGTTGATTATATATTCACCCATT-3’. The resulting amplicon was ligated into the pET28a (Novagen, Merck Pty Ltd, Kilsyth, Victoria, Australia) expression vector utilising the NcoI and XhoI restriction sites. DNA sequencing was done using the BigDye chemistry to confirm the sequence of the final construct. Based on comparison of various database entries, there appears to be polymorphism of this gene in *S. mansoni*. Comparison of the translated sequence of our construct with three database sequences highlights three positions of variation in annexin B22: E6E/I9I/T31P (up:Q9XY89), E6G/I9I/T31T (geneDB:Smp\_074150), and E6G/I9I/T31T (gb:XP\_002578586).

**Preparation of recombinant annexin B22**

The DNA of annexin B22 in pET28 was transformed into Escherichia coli BL21(DE3) cells, and a liquid overnight culture in 1 L LB medium with 15 μg mL\(^{-1}\) kanamycin was grown at 37\(^\circ\) C. Protein expression followed an in–house adaptation of the auto–induction protocol described by Studier [33]. A total of 8 L of LB auto–induction medium (15 μg mL\(^{-1}\) kanamycin) were inoculated with the overnight culture. The cells were grown at 37\(^\circ\) C for 4 hrs; then, the temperature was reduced to 18\(^\circ\) C, and incubation continued for 24 hrs.

After harvest, the cells were resuspended (100 mM NaCl, 1 mM EDTA, 20 mM TRIS (pH 8), 0.1% Triton X–100, 1 mM PMSF, 5 mM benzamidinium chloride), and lysed by multiple freeze–thaw cycles and subsequent sonication. The resulting suspension was cleared by ultracentrifugation (100000 \( \times \) g, 30 min, 4\(^\circ\)C). The supernatant from the ultracentrifugation step was then diluted 3–fold with equilibration buffer (100 mM NaCl, 20 mM TRIS, pH 8.0) and
subjected to immobilised metal ion affinity chromatography using Ni$^{2+}$-NTA resin (QIAGEN, Doncaster, Victoria, Australia). The fractions obtained from step elution with 20 mM, 50 mM, 100 mM, 200 mM and 500 mM imidazole in 100 mM NaCl, 20 mM TRIS (pH 8.0) were pooled appropriately, dialysed against 20 mM TRIS (pH 8.0) and purified in a second step by anion exchange chromatography with Q-Sepharose (GE Health). Protein was eluted with a gradient 0–1 M NaCl, 20 mM TRIS (pH 8.0), appropriate fractions were pooled and concentrated by ultrafiltration. The buffer was exchanged to 100 mM NaCl, 20 mM HEPES (pH 7.5) by multiple wash steps in the final ultrafiltration cycles. Reduced protein samples were stored in the same buffer, but including 0.5 mM DTT. All purification steps were monitored by SDS–PAGE.

Crystallisation

Initial screening (sitting drop, vapour diffusion) of our large in–house factorial collection with more than 1300 pre–formulated conditions indicated a variety of crystallisation conditions for annexin B22 based on polyethylene glycol (PEG) 400–4000 or 2–methyl–2,4–pentanediol (MPD) at basic pH–levels. Refinement of crystallisation conditions was done using the hanging drop vapour diffusion method, and yielded monoclinic crystals from 12.5% PEG2000, 50 mM TRIS (pH 9.0). All crystallisation experiments were carried out at 16° C.

Since crystals showed only poor X–ray diffraction under cryogenic conditions, dehydration protocols were tried, and a 5 min soak in 32% PEG2000, 50 mM TRIS (pH 9.0) was found to enhance X–ray diffraction from ~9 Å to better than 3 Å. Cryo–preservation of crystals was done by coating with a mixture of Paratone–N and mineral oil (1:1) and flash–freezing in the cryo stream (100 K) or liquid nitrogen.

For derivatisation, heavy atom compounds were mixed into the dehydration solution, and concentrations (1–10 mM) and soaking times (2–60 min) were varied to find optimal conditions for a particular derivative. Crystals of calcium–bound annexin B22 were prepared by including 5 mM CaCl$_2$ in the above dehydration (soaking) procedure.

Data collection, structure solution and refinement

X–ray diffraction data were collected at the in–house diffractometer (Rigaku MicroMax007–HF; R–Axis IV++ detector; Rigaku X–stream cryo equipment), and at the Australian Synchrotron beam line MX1 [34] under cryogenic conditions (T = 100 K). Data sets were indexed with XDS [35], and scaling, truncation and analysis were performed with programs from the CCP4 suite [36]. Attempts to solve the crystal structure by molecular replacement with a library of 155 models [24] derived from 17 annexin and alpha–giardin structures were unsuccessful.

A crystal soaked with PrCl$_3$ yielded a strong anomalous signal and Auto–Rickshaw [37] processing on the EMBL server (http://www.embl-hamburg.de/Auto-Rickshaw/) immediately after data collection indicated that the structure could be solved. Using two MAD data sets (ASM054 peak, 1.378 Å; ASM051 hrem, 0.9538 Å), 16 heavy atom sites were detected by SHELX [38] and initial phases could be derived using SHARP [39]. For phasing statistics, see Table S2; the quality of the experimental electron density is illustrated in Figure 1.

Almost all of the 20 helices for each of the two models in the asymmetric unit could be built into
the experimental map with phases up to 2.6 Å. For model building, cyclic non-crystallographic symmetry averaging using DM and LSQKAB [36] was performed. When about 80% of residues had been built, the model was used in a molecular replacement calculation to phase the high resolution data set ASM058 at 2.2 Å (see Table 4), with which the final model was obtained. Model building was performed with O [40] and Coot [41], and interspersed with computational refinement of atomic positions, grouped and individual B-factors using PHENIX [42]. TLS refinement was included in the final stages with two groups (chain A and chain B). The crystal structure of the calcium-bound protein (data set ASM070) was solved via molecular replacement using the apo protein structure; calcium sites were identified by coinciding peaks in the anomalous density and the difference density. The crystallographic occupancies of the 13 calcium ion sites were optimised during the refinement. The geometry of the final models was scrutinised using MolProbity tools as implemented in PHENIX [42], and parameters of dimer interactions were analysed with PISA [43].

**Size-exclusion chromatography**

Size exclusion chromatography was carried out using a Superose 12 column (GE Healthcare, Rydalmere, New South Wales, Australia) on a BioLogic HPLC system (BioRad, Gladesville, New South Wales, Australia) with an in-line Wyatt miniDAWN TREOS multi-angle light scattering (MALS) detector and a Shimadzu RID–10A refractive index (RI) detector. All experiments were performed at a flow rate of 0.5 mL min⁻¹ with buffers listed in Table 3. Protein samples (final concentration 5 mg mL⁻¹) were incubated with varying additives (EDTA, CaCl₂, DTT; see Table 3) for 30 min before injection. After equilibrating the column, 100 μL of sample were loaded and elution was monitored by UV absorbance at 280 nm, as well as the MALS/RI detectors. Molecular masses of eluting species were analysed using the software Astra (Wyatt, Shimadzu Scientific Instruments, Brisbane, Queensland, Australia). For content estimation, the UV-chromatograms (see Figure S1) were analysed using the program SDAR [44] to determine the area under the elution peaks.

**Protein aggregation assay**

As a validation of the liposome co-pelleting assay, calcium-induced precipitation of annexin B22 was assessed. Protein samples in a total volume of 500 μL of liposome buffer (180 mM sucrose, 50 mM NaCl, 10 mM HEPES, pH 7.5) were prepared with varying concentrations of calcium (0–5 mM) and incubated for 10 min at room temperature. After centrifugation (15000 rpm, 45 min, 4°C), the supernatant was carefully removed. The tubes were washed with 20 μL of 10% SDS, and the samples were subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and analysed densitometrically using the program ImageJ [45]. Results are given in Table S3. Protein aggregation levels are very low and have thus been deemed negligible.

**Liposome co-pelleting assay**

To assess annexin membrane binding properties, we conducted a co-pelleting assay with phospholipid vesicles [46]. The vesicles were prepared from 1,2-dioleoyl-sn-glycero-3-
phosphoserine and 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL, USA) according to the protocol of Reeves and Dowben [47]. The liposome preparations were used at the stage of multi-lamellar vesicles after rehydration with liposome buffer (180 mM sucrose, 50 mM NaCl, 10 mM HEPES, pH 7.5) and collection by centrifugation (15000 rpm, 20 min, 4°C). A total of 0.2 μmol of phospholipids was used for each individual sample (500 μL), composed of 0.6 nmol protein in liposome buffer and varying amounts of calcium (0–5 mM). The same amount of protein was prepared in a control sample used to establish the fraction of bound protein. All samples were centrifuged (15,000 rpm, 45 min, 4°C), and the pellets were resuspended with 20 μL of 10% SDS and subjected to SDS–PAGE. Gels were stained with Coomassie Brilliant Blue and analysed densitometrically using the program ImageJ [45] and fitted with the program SDAR [44]. Assays were conducted with freshly prepared liposomes in triplicate for each calcium concentration. For the data point in the absence of calcium, 1 mM EDTA was added to the sample. When testing the reduced protein, 0.2 mM tris-(2-carboxyethyl)–phosphine (TCEP) were added to the liposome buffer.

**Membrane aggregation assay**

Membrane aggregation was evaluated by monitoring turbidity in a time–dependent fashion. Phospholipid vesicles were prepared and samples (V_i = 500 μL) were constituted as described above. Samples were loaded into a quartz cuvette and the light scattering at 500 nm was monitored in a Jasco V650 UV/Vis spectrophotometer as apparent absorption. After an equilibration phase of 2–5 min, protein (or buffer control) was added to the liposome suspension by rapid mixing, and the apparent absorption monitored for another 20 min.

**Transcriptional profiling of annexin B22 in the life cycle**

Total RNA was obtained from eggs, miracidia, cercariae, schistosomula, cultured as described below, paired adults collected 22 days after infection, and separated adult males and females collected 6 weeks after infection. Schistosomula were transformed artificially and cultured in Basch’s medium [48] for 4 hrs or 4 days, prior to harvesting, or cultured for 4 days in the presence of human red blood cells. Total RNA was extracted using Trizol (Invitrogen) and purified further using an RNeasy column (Qiagen RNeasy Mini Kit). The concentration of purified RNA was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Scoresby, Victoria, Australia). RNA was stored at −80°C until required. A QuantiTect Whole Transcriptome Kit (QIAGEN) was used to synthesise the cDNA from 10 ng – 1 μg of purified total RNA prepared in 5 μL of nuclease–free water. The concentration of cDNA was measured using a Nanodrop ND-1000 spectrophotometer. cDNA was diluted to 50 ng μL⁻¹, aliquoted and stored at −20°C until use.

The transcription level of annexin B22 at the different life stages was determined by quantitative PCR (qPCR). Primers for annexin B22 and DNA segregation ATPase (TC15682) were designed using Primer3 (http://frodo.wi.mit.edu/) (Table S4) [49], with melting temperatures ranging from 58°C to 60°C. The product size of the amplicon was set between 100 – 200 bp. Each qPCR sample was prepared to a final volume of 20 μL, containing 5 μL (5 ng μL⁻¹) of cDNA, 10 μL of
SYBER® Green (Applied Biosystems, Life Technologies, Mulgrave, Victoria, Australia), 3 μL of nuclease-free water and 1 μL (5 pmol μL⁻¹) each of forward and reverse primers. qPCR settings were identical for all reactions and consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 20 sec, 60°C for 15 sec and 72°C for 15 sec. Amplifications were run in a Rotor Gene (6000) thermal cycler (Corbett Research, Mortlake, New South Wales, Australia). A standard curve was constructed with a mixture of equal volume of each cDNA template from each gene (5 ng μL⁻¹). The average number of gene copies per reaction and standard deviation values were calculated from four normalised cycle threshold values. The data were analysed and quantified using the Rotor Gene 6 software (Corbett Research). A template without cDNA was used as a negative control. All reactions were run as two technical replicates.

**Antibody production**

Polyclonal antiserum was raised against annexin B22 in three week old female ARC Swiss mice. Each mouse was subcutaneously administered 25 μg of recombinant protein, mixed with complete Freund’s adjuvant. The mice were boosted twice at two week intervals via intraperitoneal injection with a further 25 μg of recombinant antigen emulsified in incomplete Freund’s adjuvant. The serum was acquired 10 days after the third immunisation, and stored at -20°C until required.

**Soluble Adult Worm Antigen Preparation (SWAP)**

Approximately one hundred pairs of six week old *S. mansoni* adult worms freshly perfused from Swiss mice were used for adult worm protein extraction. Following perfusion, the worms were washed five times with RPMI 1640 (Invitrogen, Life Technologies, Mulgrave, Victoria, Australia). The worms were homogenised on ice in 20 mM TRIS-HCl (pH 7.4) containing 1 mM EDTA and a protease inhibitor cocktail (Sigma, Castle Hill, Australia). After incubation for 10 minutes at 4°C, the homogenate was centrifuged at 16,000× g for 1 hr at 4°C. The supernatant containing parasites soluble proteins was collected as SWAP.

**Western blot**

Samples of recombinant purified annexin B22 (prepared as above), annexins B5a, B5b, B7a and B30 (unpublished), as well as SWAP were subjected to electrophoresis on a precast Mini-PROTEAN TGX Gel (Biorad) and transferred to a PVDF membrane (Biorad). The membrane was blocked for 1 hr in Odyssey blocking buffer (LI–COR, Lincoln, Nebraska, USA) diluted 1:1 with PBS. The membrane was probed with the polyclonal anti–annexin B22 antibody raised in mice (see above), diluted 1:1000 in the Odyssey blocking buffer with the addition of 0.1% Tween, for 1 hr at 4°C. IRDye 800ce Goat anti–Mouse IgG (LI–COR) was diluted 1:15000 in the blocking buffer with the addition of 0.1% Tween and 0.01% SDS, and the membrane was exposed to this secondary antibody for 1 hr at 4°C. The membrane was scanned using the Odyssey infrared Imager (LI–COR). For scans of the gel and the Western blot, see Figure S2.
Serology

An enzyme–linked immunosorbent assay (ELISA) was used to evaluate the immunological recognition of recombinant annexin B22 by sera of mice experimentally infected with *S. mansoni*. A Nunc Maxisorp 96-well plate was coated overnight with 100 µL of recombinant annexin B22 (5 µg mL⁻¹) in phosphate buffered saline (PBS) at 4°C. The plate was blocked with 300 µL of 2% skim milk in 0.1% PBS supplemented with Tween–20 (PBST) for 1 hr at room temperature with gentle shaking. The plate was then washed three times with 0.1% PBST at 5 min intervals, and finally 100 µL of mouse serum (1:100 dilution in 0.1% PBST) was added. After incubation in the dark at room temperature with gentle shaking for 1 hr, the plate was washed three times with 0.1% PBST, and 100 µL goat anti–mouse IgG–HRP (horseradish peroxidase–conjugated) (Invitrogen) at 1:2000 dilution in 0.1% PBST was added to each well. The plate was incubated in the dark for 1 hr at room temperature with gentle shaking. Following a final wash step with 0.1% PBST, 100 µL dissolved o-phenylenediamine dihydrochloride (OPD tablet, Sigma) was added to each well, and the plate was incubated in the dark for 20 minutes at room temperature. Absorbance measurements at 450 nm were carried out in a Biotek Synergy H4 Multi Mode plate reader (Biotek, Millenium Science, Mulgrave, Victoria, Australia).

Immunolocalisation

Freshly perfused adult parasites were fixed with cold (−20°C) 100% methanol. The fixed parasites were washed in PBS to remove the methanol and embedded in Tissue–Tek Optimal Cutting Temperature (OCT) compound (ProSciTech, Kirwan, Queensland, Australia) and sectioned at 7.0 µm thickness using a cryostat. Sections were blocked overnight with blocking solution (5% low fat skim milk, 1% BSA, 0.02% Tween–20) at 4°C with gentle shaking. After three washes with 0.05% PBST (5 minutes each), sections were incubated for one hour at room temperature with a murine anti–annexin B22 serum diluted 1:100 in 0.05 % PBST. Normal mouse serum was used as a negative control. The sections were washed three times with 0.05% PBST for 5 min each, and incubated with 1:500 PBST–diluted Alexa Fluor 488® goat anti–mouse IgG (H+L) (Life Technologies) for 30 min at room temperature. Sections were washed three times with 0.05% PBST. The slides were air–dried briefly, mounted with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratory, Burlingame, California, USA), and viewed and photographed with an EVOS AMG fluorescence microscope (Olympus, Hamburg, Germany).

High–pressure frozen adult males were cryo–substituted in 0.2% uranyl acetate and 5% water in acetone and embedded in Lowicryl HM20 resin for immunocytochemistry as described [20]. Ultrathin sections (60 nm) were cut onto formvar–carbon coated copper slot grids, and subsequently washed three times in PBS (5 min each) and then blocked for 15 min at room temperature in blocking solution (0.2% BSA, 0.2% fish–skin gelatine, 20 mM glycine, 20% PBS). After blocking, the ultrathin sections were incubated for 30 min at room temperature with anti–annexin B22 serum diluted 1:50 in blocking solution. Following four washes in PBS of 5 min duration each, the ultrathin sections were incubated for 30 min at room temperature with 10 nm Protein–A Gold (Utrecht University) provided as 1:60 dilution in blocking solution. Normal mouse serum was used as a negative control for immunolabeling. Following four washes each in
PBS and MilliQ water (2 min each), the grids were dried briefly on blotting paper. Ultrathin sections were contrasted in uranyl acetate and lead citrate, and then examined and photographed using a JEM 1011 transmission electron microscope (JEOL, Kirwan, Queensland, Australia) operated at 80 kV.

**Acknowledgements**

We gratefully acknowledge funding of our laboratories by the National Health and Medical Research Council (AH, MKJ). CYL is supported by a Malaysian Government and Universiti Sains Malaysia ASTS scholarship. We thank Dr S Weeratunga and Dr P Walden for assistance with initial crystallisation experiments, and Dr E Lovas for assistance with electron microscopy. Parts of this research were undertaken on the MX1 beamline at the Australian Synchrotron, Victoria, Australia. The authors acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland.
References


**Supporting information**

Additional supporting information included with this manuscript:

- **Table S1**: Comparison of N-terminal extent of annexins with extended II/III linker peptides
- **Table S2**: Phasing statistics of non-reduced annexin B22
- **Table S3**: Calcium-dependent aggregation of non-reduced annexin B22
- **Table S4**: Primer sequences designed for quantitative PCR
- **Figure S1**: Size exclusion chromatograms of annexin B22 under various conditions
- **Figure S2**: Western blot using the polyclonal anti-annexin B22 antibody
Structure and immunobiochemical characterisation of annexin B22

Figure legends

Figure 1
Top: Cartoon representation of calcium–bound annexin B22 observed in the monoclinic crystal structure. The repeats in core domains of the two molecules are coloured pale green (repeat I), pale blue (repeat II), pale brown (repeat III), and pale pink (repeat IV). The N-terminal domains are coloured dark blue, and the unique II/III linker region is shown in magenta. Calcium ions are rendered as spheres with the following colour coding: orange – type II, yellow – type III, red – novel type. The disulphide bond between Cys173 and Cys173’ is rendered in green and located in the background. Bottom: Stereo image of the experimental density (Pr⁶⁺ MAD phases up to 2.6 Å resolution) contoured at 1σ. The backbone is shown as Cα trace; heavy atoms are drawn as spheres.
Figure prepared with PyMOL [50].

Figure 2
Cα plot of an annexin B22 monomer. The rainbow colour ramp maps from the N-terminus (blue) to the C-terminus (red). The N-terminal peptide (Ser7 - His20; dark blue) packs into the groove between modules I/IV and II/III on the concave side of the molecule, with a helical turn in 3_{10} conformation (Ser12 - Ser16) and a subsequent hairpin turn (Asp18 - Gly21). This N-terminal segment is embraced at the bottom by the unique α-helical segment (Pro192 - Gly203; green) linking repeats II and III. Figure prepared with PyMOL [50].

Figure 3
Calcium-dependent membrane binding of annexin B22 assessed by a liposome co-pelleting assay. The fraction of non-reduced (blue) and reduced (red) annexin B22 bound to multi-lamellar vesicles made of DOPS/DOPC (3:1) was assessed at different calcium concentrations. Data points represent the average of three independent measurements. The solid lines show fits of the Hill equation to the binding data (blue: non-reduced protein, n = 2.4, R² =1.135; green: non-reduced protein, 0 - 0.2 mM, n = 1.1, R² = 1.318; red: reduced protein, 0.1 - 5 mM, n = 7.1, R² = 0.9977).

Figure 4
Liposome aggregation assay. Non-reduced (blue) and reduced (red) annexin B22 causes aggregation of DOPS/DOPC (3:1) liposomes in the presence of calcium, but not in its absence. Results shown are the average of two independent experiments.

Figure 5
Annexin B22 mRNA levels in different stages of the S. mansoni life cycle. Shown are the average
and standard deviation of two technical replica, normalised against mRNA levels of DNA Segregation ATPase.

**Figure 6**
Annexin B22 immunoreactivity. **Left:** The Western blot shows that the polyclonal anti-annexin B22 antibody mainly recognises annexin B22 with low cross-reactivity to the other four recombinant annexins, loaded as same amounts. The blot was scanned with the Odyssey system as described in Materials and Methods. The gel was scanned separately and superimposed on the Western blot image with false colours. The antibody further detects a protein with the same molecular mass as annexin B22 in soluble whole adult protein preparation (Swap). **Right:** Enzyme-linked immunosorbent assay (ELISA). Purified recombinant annexin B22 is probed with sera from mice infected with *S. mansoni*, as well as uninfected mice. The readout obtained for the uninfected serum is at the level of expected background for this assay. Serum from infected mice clearly recognises purified annexin B22. Values shown represent the average and standard deviations of three technical replica.

**Figure 7**
Immunolocalisation of annexin B22 in *S. mansoni* males using protein A–gold transmission electron microscopy. **A** and **B:** Tegument of male labelled with annexin B22. Gold particles are found in association with the apical plasma membrane and its derivatives including surface invaginations. **C:** Controls without primary antibody. Almost no gold particles were observed, indicating that protein A–gold probes do not detect host antibodies that were bound in living tissues of the parasite. **D:** Pre–immune serum, showing low and diffuse label.

Abbreviations: AM–apical membrane complex; BM–basement membranes; My–myofibrils; SI–surface invaginations; Sp–spine.

**Figure 8**
Electrostatic surface representation of calcium–bound annexin B22. The view on the right is rotated by 90° around the horizontal axis. The colour ramp ranges from red (negative charge) to grey (hydrophobic) and blue (positive charge). Molecular solvation was computed through solution of the Poisson–Boltzmann equation with APBS [51]. Figure prepared with PyMOL [50].
## Tables

### Table 1
Intermolecular interactions in the annexin B22 dimer

<table>
<thead>
<tr>
<th>Molecule A</th>
<th>Molecule B</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys173</td>
<td>Cys173</td>
<td>covalent bond</td>
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<tr>
<td>Ca404:D126,D273</td>
<td>Ca404:D126,D273</td>
<td>calcium complex</td>
</tr>
<tr>
<td>Lys169 side chain</td>
<td>Glu177 side chain</td>
<td>ionic interaction</td>
</tr>
<tr>
<td>Glu177 side chain</td>
<td>Lys169 side chain</td>
<td>ionic interaction</td>
</tr>
<tr>
<td>Leu122–CO</td>
<td>Ser271–NH</td>
<td>hydrogen bond</td>
</tr>
<tr>
<td>Gly123–NH</td>
<td>Asp175 side chain</td>
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<tr>
<td>Thr124–NH</td>
<td>Ser271–CO</td>
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<td>Asp175 side chain</td>
<td>Gly123–NH</td>
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<td>Arg227 side chain</td>
<td>Lys155–CO</td>
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<td>Leu122–CO</td>
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<td>Ser271–CO</td>
<td>Thr124–NH</td>
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Table 2
Calcium binding sites observed in the crystal structure of calcium-bound annexin B22

<table>
<thead>
<tr>
<th>Site</th>
<th>Molecule</th>
<th>Classification</th>
<th>Coordination distances</th>
<th>Coordination no</th>
<th>Occupancy Mol A / Mol B</th>
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<tbody>
<tr>
<td>Ca401</td>
<td>A, B</td>
<td>type II</td>
<td>Met47–CO (2.5), Gly49–CO (2.8), Gly51–CO (2.5), Asp91 (2.4), H2O (2.7)</td>
<td>5</td>
<td>1.0 / 1.0</td>
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<td>Ca402</td>
<td>A, B</td>
<td>type II</td>
<td>Met119–CO (2.4), Gly121–CO (2.4), Gly123–CO (2.5), Glu173 (2.4), H2O (2.6)</td>
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<td>1.0 / 0.96</td>
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<tr>
<td>Ca403</td>
<td>A, B</td>
<td>type III</td>
<td>Lys169–CO (2.6), Thr172–CO (2.5), Glu177 (2.4)</td>
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<td>1.0 / 1.0</td>
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<tr>
<td>Ca404</td>
<td>A, B</td>
<td>dimer interface B site</td>
<td>Asp126 (2.4), Asp273 (2.9), Asp273′ (2.4), H2O (2.6), H2O (2.6)</td>
<td>6</td>
<td>0.47 / 0.0</td>
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<tr>
<td>Ca404′</td>
<td>A, B</td>
<td>dimer interface B site</td>
<td>Asp126′ (2.4), Asp273′ (2.5), Asp273 (2.3), H2O (2.6), H2O (2.6)</td>
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<tr>
<td>Ca405</td>
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<td>type II</td>
<td>Met300–CO (2.6), Gly302–CO (3.2), Leu303–CO (2.5), Asp346 (2.4, 2.4), H2O (3.7)</td>
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<td>Ca406</td>
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<td>novel</td>
<td>Gly304–CO (2.8), Asp344–CO (2.5), H2O (2.7)</td>
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<td>B site</td>
<td>Thr52–CO (2.6), Glu54 (2.5, 3.2)</td>
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<td>1.0 / 0.0</td>
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Distances are given in Å.
Table 3
Annexin B22 quaternary structure in solution determined by size exclusion chromatography

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Buffer</th>
<th>Protein sample</th>
<th>$x$(Monomer)</th>
<th>$x$(Dimer)</th>
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</thead>
<tbody>
<tr>
<td>Non-reducing, no calcium</td>
<td>100 mM NaCl, 0.5 mM EDTA, 20 mM HEPES 7.5</td>
<td>annexin B22, non-reduced</td>
<td>0.05</td>
<td>0.95</td>
</tr>
<tr>
<td>Reducing, no calcium</td>
<td>100 mM NaCl, 0.5 mM EDTA, 0.1 mM DTT, 20 mM HEPES 7.5</td>
<td>annexin B22, 0.5 mM DTT</td>
<td>0.32</td>
<td>0.68</td>
</tr>
<tr>
<td>Reducing, with calcium</td>
<td>100 mM NaCl, 5 mM CaCl$_2$, 0.1 mM DTT, 20 mM HEPES 7.5</td>
<td>annexin B22, 0.5 mM DTT</td>
<td>0.52</td>
<td>0.48</td>
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<tr>
<td>Reducing, with calcium and high salt</td>
<td>500 mM NaCl, 5 mM CaCl$_2$, 0.1 mM DTT, 20 mM HEPES 7.5</td>
<td>annexin B22, 0.5 mM DTT</td>
<td>0.30</td>
<td>0.70</td>
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</tbody>
</table>

$x$ is the molar ratio of specified oligomer compared to total amount of protein. The size exclusion chromatograms for the listed conditions are shown in Figure S1.
Table 4
Data collection and refinement statistics for non-reduced annexin B22

<table>
<thead>
<tr>
<th>Data set</th>
<th>ASM058</th>
<th>ASM070</th>
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<tbody>
<tr>
<td>Protein</td>
<td>apo annexin B22</td>
<td>calcium-bound annexin B22</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Mol #1</td>
</tr>
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</table>

**Data collection**

<p>| | | |</p>
<table>
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<td>X-ray source</td>
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<td>AS-MX1</td>
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<td>Detector</td>
<td>ADSC Quantum</td>
<td>ADSC Quantum</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.3378</td>
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<td>Space group</td>
<td>P2_1</td>
<td>P2_1</td>
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<tr>
<td>Cell dimensions</td>
<td>67.7 Å, 87.8 Å, 68.9 Å, 111.2°</td>
<td>67.8 Å, 88.5 Å, 68.5 Å, 111.2°</td>
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<tr>
<td>Max. resolution (Å)</td>
<td>2.2 (2.32 - 2.20)</td>
<td>2.5 (2.64 - 2.50)</td>
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<tr>
<td>Wilson B-factor (Å^2)</td>
<td>39.4</td>
<td>70.9</td>
</tr>
<tr>
<td>No of unique reflections</td>
<td>38248 (5549)</td>
<td>26180 (3791)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.6 (7.6)</td>
<td>3.8 (3.6)</td>
</tr>
<tr>
<td>Completeness</td>
<td>0.999 (1.0)</td>
<td>0.998 (0.995)</td>
</tr>
<tr>
<td>( R_{\text{sym}} )</td>
<td>0.081 (0.606)</td>
<td>0.041 (0.386)</td>
</tr>
</tbody>
</table>

**Refinement**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>No of reflections in working / test set</td>
<td>38225 / 1884</td>
<td>24239 / 1302</td>
</tr>
<tr>
<td>No of non-H protein atoms</td>
<td>5734</td>
<td>2846</td>
</tr>
<tr>
<td>No of water molecules</td>
<td>188</td>
<td>117</td>
</tr>
<tr>
<td>No of ions</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>
### Structure and Immunobiochemical Characterisation of Annexin B22

<table>
<thead>
<tr>
<th>Average B-factor ($\AA^2$)</th>
<th>Protein</th>
<th>44.8</th>
<th>47.0</th>
<th>42.9</th>
<th>67.1</th>
<th>69.7</th>
<th>71.43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td></td>
<td>42.2</td>
<td></td>
<td></td>
<td>57.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>83.3</td>
<td>95.4</td>
<td>89.6</td>
</tr>
<tr>
<td>rmsd B-factor for bonded atoms ($\AA^2$)</td>
<td>5.19</td>
<td>5.07</td>
<td>5.31</td>
<td>5.66</td>
<td>9.29</td>
<td></td>
<td>8.92</td>
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<tr>
<td>rmsd bond lengths (Å)</td>
<td>0.007</td>
<td></td>
<td></td>
<td></td>
<td>0.011</td>
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<tr>
<td>rmsd bond angles (°)</td>
<td>1.039</td>
<td></td>
<td></td>
<td></td>
<td>1.188</td>
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<tr>
<td>MolProbity analysis(^c)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
<td></td>
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</tr>
<tr>
<td>Ramachandran favoured (%)</td>
<td>95.4</td>
<td></td>
<td></td>
<td></td>
<td>95.6</td>
<td></td>
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<tr>
<td>Rotamer outliers (%)</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
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<tr>
<td>C-beta outliers</td>
<td>0</td>
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<td></td>
<td>1</td>
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<tr>
<td>Clashscore</td>
<td>7.90</td>
<td></td>
<td></td>
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<td>12.54</td>
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<tr>
<td>Overall score</td>
<td>2.15</td>
<td></td>
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<td></td>
<td>2.30</td>
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<tr>
<td>R-factor(^d)</td>
<td>0.190 (0.255)</td>
<td></td>
<td></td>
<td></td>
<td>0.190 (0.298)</td>
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</tr>
<tr>
<td>$R_{free}$(^e)</td>
<td>0.244 (0.337)</td>
<td></td>
<td></td>
<td></td>
<td>0.257 (0.366)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses refer to the last resolution shell.

\(^a\) $R_{sym} = \Sigma |I - \langle I\rangle| / \Sigma I$, where $I$ is the observed intensity, and $\langle I\rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections after rejections.

\(^b\) Residues numbers are offset by +2 compared to the amino acid sequence of annexin B22 due to two additional N-terminal residues of the recombinant construct.

\(^c\) MolProbity analysis as implemented in PHENIX [42].

\(^d\) $R$-factor = $\Sigma ||F_o| - |F_c|| / \Sigma |F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively.

\(^e\) $R_{free}$ defined in [52].