Could a Diiron-Containing Four-Helix-Bundle Protein Have Been a Primitive Oxygen Reductase?

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Dioxygen, which became abundant in the earth's atmosphere approximately 2 – 3 billion years ago,[1] is a molecule with a very high oxidising power. Since dioxygen has a poor reactivity—due to unfavourable thermodynamic and kinetic factors—living organisms could only take advantage of the oxic atmosphere by developing appropriate enzymes that can efficiently utilise it, that is, developing safe high-energy-yielding processes. At the present time, a large variety of proteins that are capable of utilising either dioxygen or its reduced species is known,[2] and their evolution, as well as that of the metabolic pathways they integrate, is a challenging issue. The present work raises the hypothesis that a four-helix-bundle diiron-containing protein may have been an early oxygen reductase operating in the first stages of the transition between the anoxic and the present oxic atmosphere. This protein would have the required features to perform dioxygen chemistry, namely the potential to harbour a binuclear transition metal site. Such a system allows the complete reduction of dioxygen to water and would play an important role in oxygen defence mechanisms in primordial anaerobes. This hypothesis is based on data here reported concerning the oxygen reductase activity of the diiron protein rubrerythrin, together with sequence- and structure-based data of protein phylogeny between this and other diiron proteins. Interestingly, analysis of the available data depicts an evolutionary relationship between such an early system and the alternative oxidases present in extant eukaryotes.

Our knowledge about the diiron carboxylate protein family has expanded considerably in the last years, with an increase in both sequence and structure data.[3, 4] This family comprises the R2 subunit of ribonucleotide reductase (RNR R2), the hydroxylase subunit of the soluble methane monooxygenase (MMOH), (bacterio)ferritin ((B)FR), rubrerythrin (RR), stearoyl-acyl carrier Δ^9 desaturase (Δ^9 desaturase) and hemerythrin (HR). In all cases, carboxylates and oxide or hydroxide ions bridge the diiron site, surrounded by a four-helix-bundle protein fold. These enzymes have quite a large functional diversity, but they have an oxygen-activating step in common that, with the exception of hemerythrin, drives subsequent redox reactions. Plant alternative oxidase (AOX) is a non-energy-transducing terminal oxygen reductase operating in mitochondria and chloroplasts, which is reduced by ubiquinol and catalyses the four-electron reduction of dioxygen to water.[5, 6] Despite numerous efforts, a pure preparation of this protein could never be obtained, thus preventing the elucidation of the chemical properties of its catalytic site. On the basis of sequence comparisons of several alternative oxidases, it has been suggested that they contain a diiron site as a reactive centre in a four-helix-bundle conformation.[7] This hypothesis was recently further analysed by Andersson and Nordlund[8] who revised the initially proposed structural model. These authors, considering additional sequences from alternative oxidases, presented a new model for this protein which likens it even more to the diiron carboxylate proteins.

The amino acid sequence identity between the diiron carboxylate proteins is in general too low to allow the use of a conventional phylogenetic analysis. However, structure-oriented local sequence alignments, comprising only the regions of the four helices aligned according to homologous residues in the metal-binding site, can successfully be used to infer general phylogenetic relationships between these proteins. This approach was followed and the study of more than 50 sequences from diiron carboxylate proteins was undertaken, combining sequence and structure data for the structurally conserved region of the four-helix bundle (Figure 1). These structure-

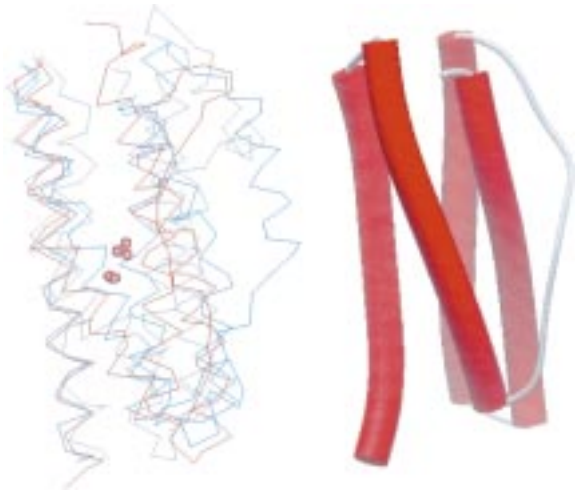

Figure 1. *Superposition of diiron four-helix-bundle protein structures. The structures of rubrerythrin (1RYT, red trace), Δ^9 desaturase (1AFR, grey trace) and methane monooxygenase hydroxylase (1MHY, blue trace) were superimposed using the program Swiss-Pdb Viewer[26] and represented as Cα-wire plots (left). A schematic representation of a typical four-helix bundle is shown on the right. The images were prepared using the program WebLab;[27] the Protein Data Bank (PDB) accession codes are given in brackets.*

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Supporting information for this article (complete sequence alignment) is available on the WWW under http://www.chembiochem.com or from the author.

oriented alignments (Figure 2) allow to derive a phylogenetic relation among these proteins (Figure 3). This is confirmed by the high values of the bootstrap analysis which show the robustness of the data and indicate that its inherent variability is within acceptable limits for the definition of groups.[9] Indeed, the fact that these proteins are evolutionarily related is substantiated by structural classifications of protein domains, which gather these proteins under identical fold groups.[25] Taken together, these data suggest that the diiron proteins analysed here are homologous, in the sense of originating by divergence from an unidentified common ancestor: i) Their key catalytic residues are conserved, such as those involved in metal binding; ii) they have a common catalytic function, that is, the activation of molecular oxygen and iii) they share a common fold, involving a four-helix bundle.

The widespread reactivity of the family members towards dioxygen suggests that this may be an ancestral reminiscent feature, which is still conserved in extant proteins but evolving to new specificities through variation and adaptation. Indeed, the diiron site has the ability to perform the required chemistry: breaking of the O–O bond, stabilisation of the intermediate species, and its safe stepwise reduction inside the four-helix bundle. Such a function was conserved through evolution and is still present in the plant alternative oxidase, which reduces oxygen to water.[5] A role for the ancestor protein as an oxygen reductase is suggested on the basis of an essential early oxygen-scavenging mechanism, thus playing an important role during the transition from the anaerobic to the aerobic world.

Rubrerythrin was selected as a model to investigate this activity since it is the simplest available protein of this family. It was first discovered in the sulfate reducer *Desulfovibrio vulgaris*[9] (*Dv*), but rubrerythrins, or rubrerythrin-like proteins, are found in all life kingdoms, including all genomes of anaerobic organisms so far sequenced. The protein is composed of two structural domains: a four-helix bundle which contains a binuclear metal centre, and a rubredoxin-like domain containing a mononuclear iron site. In the particular case of *Dv*, although the "as prepared" (native) protein contains an iron–zinc centre,[10] it has been shown that the overexpressed, iron-reconstituted protein instead accommodates a diiron site.[11] The function of this protein is unknown, although its involvement in oxygen defence processes has been suggested.[12, 13] We investigated the oxygen reduction ability of *Desulfovibrio desulfuricans* ATCC 27774 (*Dd*) rubrerythrin, which, in contrast to the *Dv* protein, contains a diiron site. This protein is indeed capable of the full reduction of oxygen to water. Oxygen uptake by rubrerythrin was measured in two distinct sets of experiments by using two different reductants: i) ascorbate and ii) NADH plus *Dd* soluble extract (Figure 4). Ascorbate can drive oxygen uptake by rubrerythrin (Figure 4, trace a), at a rate of 0.5–0.7 min^{-1}. A control experiment shows that oxygen uptake before addition of rubrerythrin is negligible (Figure 4, trace a). The oxygen consumption rate is unaffected by the external addition of catalase and/or superoxide dismutase (Figure 4, trace b), indicating that neither hydrogen peroxide nor the superoxide anion are intermediates of the reaction. Thus, ascorbate-reduced rubrerythrin fully reduces oxygen to water. The second set of experiments, using NADH plus *Dd* soluble extract to reduce rubrerythrin, aims to investigate if cytoplasmatic NADH oxidation can be linked to oxygen reduction by rubrerythrin. Indeed this is the case: Under the conditions used, oxygen uptake by *Dd* soluble extract and NADH, attributable to spurious oxygen consumption by enzymes present in the extract, is about 30 times slower than that observed after addition of rubrerythrin (Figure 4, trace b). Thus, the soluble extract mediates electron transfer from NADH to rubrerythrin, and rubrerythrin consumes oxygen at a rate of 2 min^{-1}. Once again, catalase and/or superoxide dismutase have no effect on the rates (Figure 4, trace d), confirming that oxygen is reduced to water. In both sets of experiments, the amount of oxygen consumed is greater than the amount of rubrerythrin present in the assay solution, showing that the observed rate is not due to a stoichiometric reaction of the reduced protein with

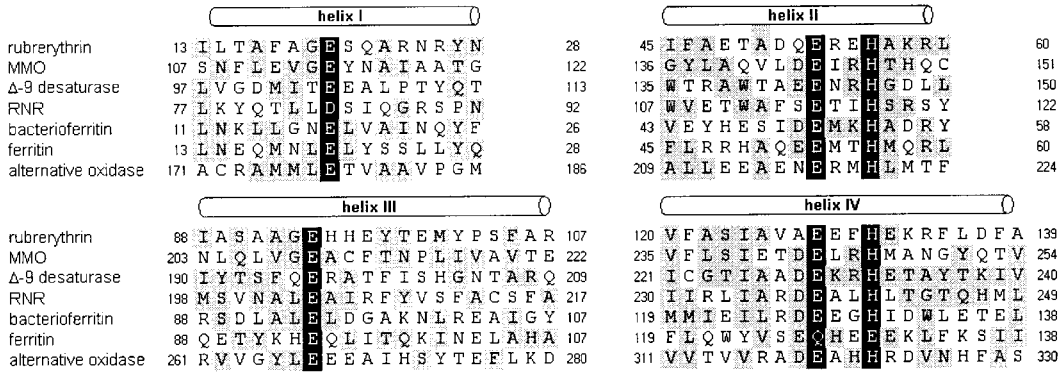

Figure 2. *Structure-oriented multiple sequence alignment of diiron proteins. Details of 7 aligned sequences out of a total alignment comprising 56 sequences are shown, representing members of the family of diiron carboxylate proteins: rubrerythrin* = Desulfovibrius vulgaris *rubrerythrin (P24931), MMO* = Methylococcus capsulatus *(Bath) methane monooxygenase hydroxylase α subunit (P22868), Δ-9 desaturase* = Ricinus communis *stearoyl-acyl carrier protein (P22337), RNR* = Escherichia coli *ribonucleotide reductase (P39452), bacterioferritin* = E. coli *bacterioferritin (P11056), ferritin* = E. coli *ferritin (P23887), alternative oxidase* = Sauromatum guttatum *alternative oxidase (P22185). Black background corresponds to residues involved in diiron site ligation and grey shaded areas indicate conservative amino acid replacements. Sequences were extracted from databanks using NCBI query resources, SWISS-PROT accession numbers are given in brackets. Alignments were made with ClustalW,[23] following recent recommendations,[24] using structural information to select helical regions, followed by manual correction. The shown alignments share 16–31% amino acid similarities.*

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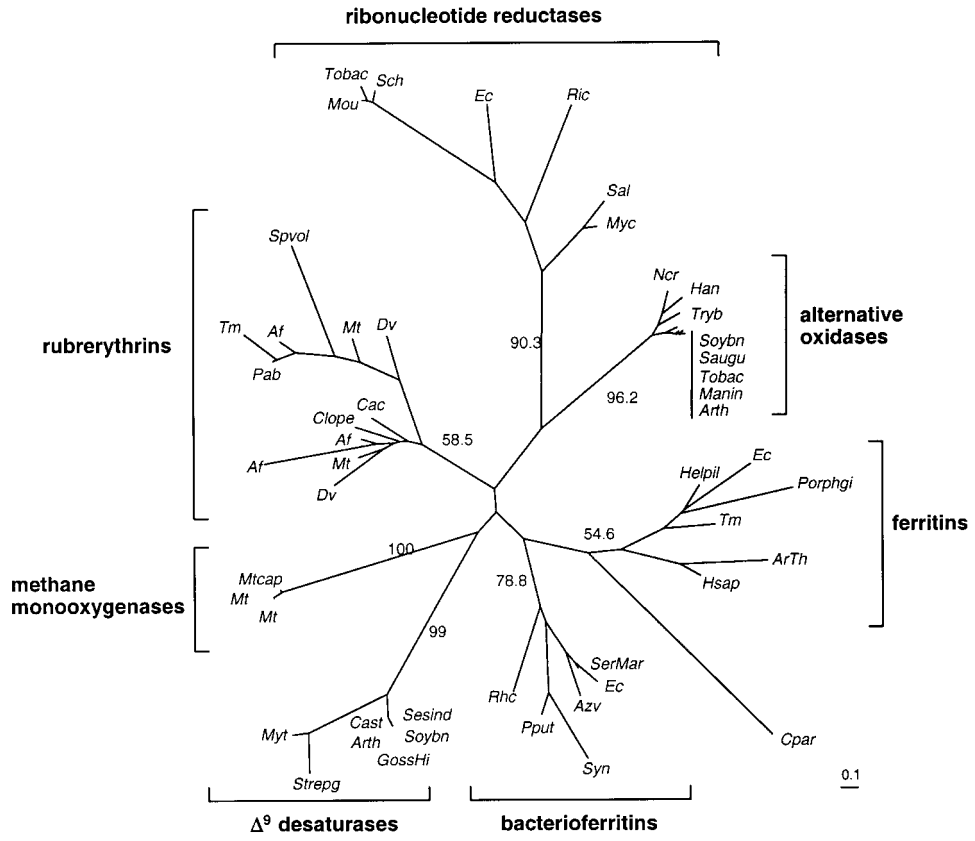

Figure 3. *Unrooted dendrogram of diiron-containing four-helix-bundle proteins. The dendrogram was obtained from an alignment of 56 sequences (performed as described in the legend of Figure 2) and it was calculated using the neighbour-joining method, removing all alignment positions that contained a gap in any of the analysed sequences and correcting for multiple substitutions (valid for > 97% of the data set). The confidence intervals were calculated using the bootstrap procedure. All calculations were performed using ClustalW.*[23, 24] Ncr = Neurospora crassa, Han = Hansenula anomala, Tryb = Trypanosoma brucei brucei, Soybn = soybean, Saugu = Sauromatum guttatum, Tobac = Nicotiana tabacum, Manin = Mangifera indica, Arth = Arabidopsis thaliana, Ec = Escherichia coli, Helpil = Helicobacter pylorii, Porphgi = Porphiromonas gingivalis, Tm = Thermotoga maritima, Hsap = Homo sapiens, Cpar = Cyanophora paradoxa, Sermar = Serratia marcescens, Azv = Azotobacter vinelandii, Syn = Synechocystis sp., Pput = Pseudomonas putida, Rhc = Rhodobacter capsulatus, Sesind = Sesamum indicum, GossHi = Gossypium hirsutum, Cast = Ricinus communis, Strepg = Streptomyces griseus, Myt = Myxococcus xanthus, Mt = Methanobacterium thermoautotrophicum, Mtcap = Methylococcus capsulatus, Dv = Desulfovibrio vulgaris, Af = Archaeoglobus fulgidus, Clope = Clostridium perfingiens, Cac = Clostridium aceticum, Pab = Pyrococcus abyssii, Spvol = Spirilum volutans, Mou = mouse, Sch = Schizosaccharomyces pombe, Sal = Salmonella typhimurium, Myc = Mycoplasma genitalium.

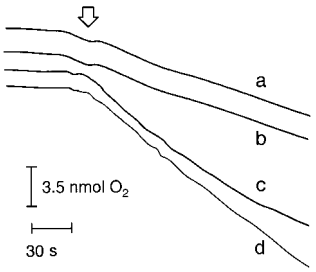

Figure 4. *Oxygen consumption by* Dd *rubrerythrin in the presence of different electron donors. In all traces, the arrow denotes addition of 2.6 nmol of* Dd *rubrerythrin. Trace a: oxygen consumption by rubrerythrin using ascorbate (6 mM) as reductant. The unspecific rate of oxygen consumption prior to addition of rubrerythrin is ten times slower than the one measured afterwards. Trace b: same as trace a, but in the presence of commercial catalase and superoxide dismutase (5 μM each). Trace c: oxygen consumption by rubrerythrin using NADH (1mM) and* Dd *soluble extract (50 μg protein) as reductants. The unspecific rate of oxygen consumption prior to addition of rubrerythrin is ca. 30 times slower than the one measured afterwards. Trace d: same as trace c, but in the presence of commercial catalase and superoxide dismutase (5 μM each). Experiments were performed on an YSI-Clark-type oxygen electrode equipped with a thermally stabilised microcell at 37 °C in 10 mM Tris/HCl (pH 7.6).*

oxygen. The low activity of oxygen uptake measured for rubrerythrin is not surprising as none of the reductants used is the physiological one (which is so far unknown), and thus a maximal rate could not be determined. Further, the enzyme is capable of reducing oxygen to water, but it is not implied that this is its physiological function—for the present argumentation, it shows that the simplest diiron carboxylate protein known retains catalytic features which are suggested to have been present in an early diiron carboxylate protein. Altogether, these results demonstrate that *Dd* rubrerythrin is able to reduce oxygen to water, and that a redox chain is operating in *Dd*, linking NADH oxidation to O_2 reduction by rubrerythrin with the involvement of a rubrerythrin reductase, possibly the flavoprotein described in ref. [14]. Interestingly, the conserved tyrosine residue in rubrerythrin (Tyr 102 in helix III) which is hydrogen-bonded to a glutamate diiron ligand and may be involved in forming intermediate tyrosine radicals important for the catalytic process, finds an equivalent in the alternative oxidase (Tyr 274 in *Sauromatum guttatum* AOX helix III, Figure 2).[25]

The ability to reduce dioxygen to water has also been reported for other four-helix-bundle diiron proteins, namely MMO[15] RNR and Δ^9 desaturase.[16] Soluble MMO (sMMO) functions as a hydroxylase, oxidase or peroxidase, depending on the availability of the appropriate substrates and on their relative concentrations:[15] i) In the presence of NADH and the reductase component, but in the absence of the hydrocarbon substrate (the so-called uncoupled system), sMMO catalyses the complete reduction of dioxygen to water; ii) MMO may operate as an NADH peroxidase, and it was suggested that this function could be physiologically relevant in the removal of hydrogen peroxide from the cytosol of the aerobe *Methyloccocus capsulatus* (Bath). Also, the homodimeric Δ^9 desaturase, which contains two oxo-bridged diiron sites, was shown to catalyse the reduction of dioxygen to water, possibly by intramolecular electron transfer between diferrous (Fe^{II}) and peroxodiferric (Fe^{III}) centres from different subunits.[16] Since rubrerythrin is also a homodimer, a similar mechanism for complete reduction of dioxygen can possibly occur.

Anaerobic organisms live in reducing environments, so one of the initial consequences of the introduction of oxygen into their habitats is the generation of reactive oxygen species (ROS), as a result of unspecific reactions with existing proteins, metal ions, and metabolites, capable of overcoming the kinetic and thermodynamic barriers for dioxygen reactivity. These reduced molecules are immensely hazardous to the cell[2] and one of the first adaptations to the increase in atmospheric oxygen levels, which were initially very low, must have been the modification and/or utilisation of existing proteins in a way that they could safely and efficiently scavenge these species.

Altogether, the data presented here support our hypothesis that dioxygen reductase activity was a feature present in the common ancestor of the four-helix-bundle diiron proteins, an advantage for early anaerobic organisms thriving in an increasingly aerobic atmosphere. In fact, since oxygen is known to inhibit key enzymes of the anaerobic metabolism, it is justified to propose that processes that remove oxygen itself have arisen as defence mechanisms. Indeed, such a mechanism is much more efficient and safe since it avoids the production of toxic oxygen intermediates, and it is still present in present-day anaerobic organisms. Initially, rubrerythrin-like proteins might have had a dual function, that is, to operate in their original function under anaerobic conditions, but to use oxygen as the electron acceptor when it is present. Indeed, the oxygen reductase activity of rubrerythrin is reminiscent of such a case. Ultimately, a more refined system for oxygen removal would involve regeneration of a cellular substrate, such as NADH, thus preventing metabolic arrest under aerobic conditions and resulting in an indirect energy gain, such as the one described for the anaerobe *D. gigas*.[17–19] The initial adaptation to an oxic atmosphere may not have immediately involved heme-copper oxidases, as Cu^{I} is very poorly soluble under anaerobic conditions. However, in a latter stage and in aerobic organisms, these primordial oxygen-scavenging proteins would be outclassed in efficiency by those enzymes.

Thus, at an early stage in evolution, a diiron site could catalyse this reaction and allow oxygen scavenging. Evolution led to the diversification of catalytic functions from this original module. Nowadays, rubrerythrins from anaerobes may still play this original role in protecting the organisms upon their exposure to oxic conditions, which could be considered as the refined version of such an early system. In this respect, it is particularly interesting to find a putative rubrerythrin-like protein encoded in the *Cyanophora paradoxa* cyanelle genome.[20] The cyanelle is a cyanobacterium-like structure, considered as a descendant of an ingested cyanobacterium that is an endosymbiont in *C. paradoxa*, and is considered as a possible link to chloroplast evolution.[21, 22] This protein may be the link to the alternative oxidase found in eukaryotes.

The presence of rubrerythrin in all genomes from anaerobic organisms so far sequenced, namely those at the earliest branches of the evolutionary tree, strongly suggests that a rubrerythrin-like protein may have been the ancestor of four-helix-bundle diiron proteins catalysing the reduction of dioxygen to water. Extant proteins may still be crucial in oxygen detoxification by anaerobic organisms.

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Visualization of Annexin I Binding to Calcium-Induced Phosphatidylserine Domains

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Annexins are a family of structurally related eukaryotic proteins that reversibly bind membranes containing anionic phospholipids in a calcium-dependent manner.[1] More than 160 different isoforms have been found in many organisms ranging from mammals to molds.[2] The protein family is defined by its characteristic structure comprising a conserved core made up of four or eight domains of a 70-amino-acid sequence forming five α helices and a variable N-terminal region varying in length and amino acid sequence. The core domains harbor multiple calcium-binding sites, which are all located on the convex side of the molecule.[3] X-ray crystallographic analyses[4–8] and mutagenesis studies[9–13] have shown that the convex site is responsible for initial membrane binding. Calcium ions bound to these sites act as bridges connecting the protein with anionic lipid headgroups. The N-terminal region is thought to be involved in the regulation of different functions of annexins. Although exact physiological functions of annexins have not been identified yet, it has been shown that they participate in a variety of in vitro activities. In particular, some annexins such as annexins I, II, IV and VII can promote membrane aggregation and may thus be involved in cellular endo- and exocytotic pathways. It was shown that annexin I, the protein of interest in our study, is capable of aggregating and even fusing membrane vesicles.[14–16] However, the mechanism of membrane aggregation is still discussed controversially. One model postulates that membrane-bound annexin I molecules form axial dimers prior to interacting with a second membrane, while another model hypothesizes that monomeric bound annexin I interacts with the second membrane.

We utilized scanning force microscopy (SFM) on solid-supported Langmuir – Blodgett (LB) bilayers composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) immobilized on mica (serving as an atomically flat substrate) to directly visualize annexin I binding with high lateral and vertical resolution, thus enabling us to distinguish between the two different models. With this technique height differences in the ångström region as well as morphological changes of the membrane structure and domain formation can be observed in a physiological environment.

Topographic images of an LB bilayer composed of DPPC as the first leaflet and DPPC/DPPS (4:1) representing the uppermost layer pointing to the aqueous phase are mostly featureless, with some defects occurring as dark spots in the SFM images. Addition of a 1 μM annexin I solution in 50 mM Tris (pH 7.4), 1 mM $CaCl_2$ to the bilayer results in the appearance of circular domains with sizes of 3 – 10 μm (Figures 1a and b) that are attributed to specifically adsorbed annexin I. These protein domains occupy (35 ± 3) % of the overall area, exhibit an average height of (3.2 ± 0.3) nm as obtained from a height analysis (Figure 1c), and are stable in a calcium-containing buffer for several hours. The thickness of the annexin layer compares well with the molecular dimension of annexin I as determined by X-ray crystallography, indicating that the protein binds in a monomeric fashion.[4, 17] Recently, Bitto et al., employing X-ray specular reflectivity measurements, also found that annexin I binds as a monomer or monolayer to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE)/1-palmitoly-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (2:5:2) monolayers at the air – water interface. They determined a protein thickness of (3.1 ± 0.2) nm.[18] Remarkably, lateral movement of the protein domains was not observed in the Ca^{2+}-containing buffer within three hours indicating that the lateral mobility of the lipids is low, as is expected for gel-phase lipids at room temperature. Crystallization of the protein, however, did not occur as it was reported for annexin V on a DOPC/DOPS (4:1) membrane immobilized on mica.[19, 20] In

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