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A structural model of the chitin-binding domain of cuticle proteins

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Abstract

The nature of the interaction of insect cuticular proteins and chitin is unknown even though about half of the cuticular proteins sequenced thus far share a consensus region that has been predicted to be the site of chitin binding. We previously predicted the preponderance of β-pleated sheet in the consensus region and proposed its responsibility for the formation of helicoidal cuticle (Iconomidou et al., Insect Biochem. Mol. Biol. 29 (1999) 285). Consequently, we have also verified experimentally the abundance of antiparallel β-pleated sheet in the structure of cuticle proteins (Iconomidou et al., Insect Biochem. Mol. Biol. 31 (2001) 877). In this work, based on sequence and secondary structure similarity of cuticle proteins, and especially that of the consensus motif, to that of bovine plasma retinol binding protein (RBP), we propose by homology modelling an antiparallel β-sheet half-barrel structure as the basic folding motif of cuticle proteins. This folding motif may provide the template for elucidating cuticle protein–chitin interactions in detail and reveal the precise geometrical formation of cuticle's helicoidal architecture. This predicted motif is another example where nature utilizes an almost flat protein surface covered by aromatic side chains to interact with the polysaccharide chains of chitin. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Cuticle proteins; Structural model; Chitin; Protein-carbohydrate interactions; Antiparallel β-pleated sheet half-barrel; Retinol binding protein; Homology modelling

1. Introduction

Cuticle is a complex, bipartite, composite material made of chitin filaments embedded in a proteinaceous matrix (Neville, 1975). It provides stuctural and mechanical support by serving functionally as both skin and skeleton to arthropods (Neville, 1975). It is also a dynamic component of the organism, an in vivo model of protein trafficking, and its materials may be involved intimately and actively in delicately regulated processes of the post-embryonic development (Csikos et al., 1999). However, the interaction of cuticular proteins with chitin fibers and the detailed structure of insect cuticle have not yet been resolved.

Sequence studies on these proteins have revealed that certain sequence motifs occur in proteins from even distantly related species and such conserved motifs have common and important roles for the proper function of cuticle (Andersen et al., 1995). One such motif is the "R&R consensus sequence" first identified by Rebers and Riddiford (1988) in seven cuticular proteins: G-x(8)-G-x(6)-Y-x-A-x-E-x-G-Y-x(7)-P-x(2)-P or a modification of it: G-x(7)-[DEN]-G-x(6)-[FY]-x-A-[DGN]-x(2,3)-G-[FY]-x-[AP]-x(6) (Willis, 1999) (where x represents any amino acid, and the values in parentheses indicate the number of residues). An extension of this motif is a stretch of approximately 68 amino acids which appears to be conserved, the "extended R&R consensus" (Iconomidou et al., 1999).

Rebers and Riddiford (1988) suggested that the original consensus would turn out to be a region of structural importance. Subsequently Andersen et al. (1995) postulated that this motif might be involved in protein/chitin interaction.

Recently, we presented secondary structure prediction and experimental data indicating that β-pleated sheet is most probably the underlying molecular conformation of a large part of this extended R&R consensus, especially the part which contains the R&R consensus itself

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(Iconomidou et al., 1999; Iconomidou et al., 2001). We also proposed that this conformation is most probably involved in β-sheet-chitin chain interactions of the cuticular proteins with the chitin filaments (Iconomidou et al., 1999; Iconomidou et al., 2001). This proposal and experimental findings are in agreement with earlier experimental findings and proposals that β-sheet should be involved in chitin–protein interactions (Fraenkel and Rudall, 1947; Atkins, 1985).

In this work, we detected an unexpected distant sequence similarity of soft cuticle proteins with bovine plasma retinol binding protein (RBP). Retinol binding protein has a β-sheet barrel as its basic structural motif (Zanotti et al., 1994). A large part of this β-sheet barrel is the part similar in sequence to the "extended R&R consensus" (Iconomidou et al., 1999) sequence of "soft" (and of "hard") cuticular proteins. Based on the sequence similarity of RBP with a representative member of the "soft" cuticle proteins, HCCP12, (Binger and Willis, 1994; Iconomidou et al., 1999), we constructed, by homology modelling, a structural model of the "extended R&R consensus". This model has several attractive features to serve as a chitin binding structural motif in cuticle and to provide the basis for elucidating cuticle's overall architecture in detail.

2. Materials and methods

A popular prediction algorithm, PHD, (Rost, 1996) was used to predict the secondary structure of cuticle proteins (Iconomidou et al., 1999). The algorithm itself produces additional important structural information. When applied to "soft" cuticle proteins it indicated significant structural similarity of this class of proteins to a protein of crystallographically determined crystal structure, that of bovine plasma retinol binding protein (RBP, Protein Data Bank Accession Code 1FEN; Berman et al., 2000; http://www.rcsb.org/pdb/).

At this point, a sensitive alignment of a representative "soft" cuticle protein sequence, HCCP12 (ENTREZ accession number 1169129; Binger and Willis, 1994; Iconomidou et al., 1999) with the sequence of retinol binding protein was produced with CLUSTAL W (Thompson et al., 1994). The BLOSUM 62 similarity matrix was used and all other parameters were the default parameters of CLUSTAL W (Thompson et al., 1994).

A structural model for HCCP12 was then derived by homology modelling, utilizing the WHAT IF program (Vriend, 1990), based on this alignment and using as template the solved structure of retinol binding protein (PDB code: 1FEN; Zanotti et al., 1994). The model was regularized with the WHAT IF regularization options (Vriend, 1990) and optimized employing the GROMOS molecular dynamics software (Van Gunsteren and Berendsen, 1987).

Stereo-pairs were drawn utilizing the program O (Jones et al., 1991) and docking was performed utilizing the program GRAMM (Vakser, 1996).

3. Results

An alignment of the sequence of the "soft" cuticle representative insect cuticular protein HCCP12 with the sequence of retinol binding protein (RBP; PDB code: 1FEN) is shown in Fig. 1. It suggests that HCCP12 exhibits significant sequence similarity to the C-terminal sequence part of RBP. Even more profound is the predicted secondary structure similarity of HCCP12 to the observed (crystallographically) secondary structure of RBP. Predicted secondary structure β-strands and β-turns/or loops align well with observed β-strands and β-turns/or loops of RBP. Significantly, the similarity is more obvious in the part of HCCP12 which contains the "extended R&R consensus", signature of cuticle proteins (Iconomidou et al., 1999; Iconomidou et al., 2001), a feature evolutionarily conserved in several cuticular proteins from "soft" and "hard" cuticles. Identity (of primary structure) is 20% for either the entire HCCP12 (89 residues in entire secreted protein) or the "extended R&R consensus" (66 residues). Conservative substitutions (displayed by light and dark gray colours in Fig. 1) represent 60% (53 out of 89 residues) and 58% (38 out of 66 residues) of the entire HCCP12 sequence and the "extended R&R consensus", respectively (see Fig. 1).

Based on the primary and secondary structure similarity and utilizing the popular homology modelling software WHAT IF (Vriend, 1990), a structural model was constructed for HCCP12 (and consequently for several cuticular proteins of "soft" and "hard" cuticles), using as template the X-ray structure of RBP (Zanotti et al., 1994). The model is presented in Fig. 2. It comprises the C-terminal 66 residues (out of 89 in total) of HCCP12 and corresponds to the "extended R&R consensus" (Iconomidou et al., 1999), the evolutionarily conserved region of "soft" and "hard" cuticle proteins.

Stereo plots of the model of HCCP12, a "soft" cuticle protein and of HCCP66 (Entrez accession number 1169133) and AGCP2b (Entrez accession number 2961110), two "hard" cuticle proteins, are shown in Fig. 3(a, b and c), respectively. These models show conclusively that the "extended R&R consensus" (Iconomidou et al., 1999) of most "soft" and "hard" cuticle proteins may easily adopt the proposed conformation.

Figure 3(d) displays a stereo plot of the proposed model for HCCP12, complexed with a N-acetyl glucosamine (NAG) tetramer in an extended conformation. It was derived from a "low-resolution" docking experiment of a NAG tetramer to the model of HCCP12, utilizing the docking program GRAMM (Vakser, 1996). The "low-resolution" docking experiment clearly shows that

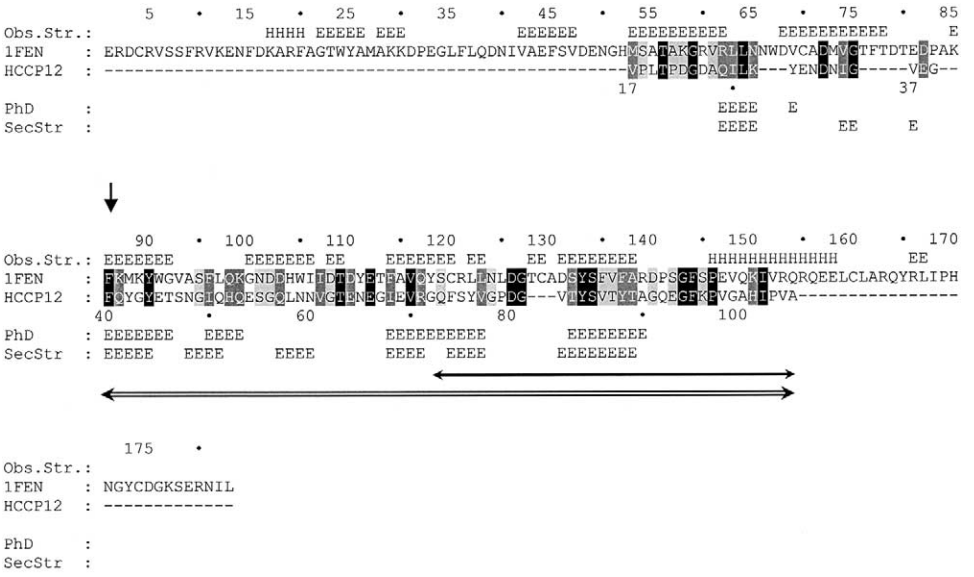

Fig. 1. Alignment of the sequence of the "soft"-cuticle representative insect cuticular protein HCCP12 [ENTREZ accession number 1169129] with that of bovine retinol binding protein (PDB code: 1FEN). This cuticular protein has been associated with cuticles of all three metamorphic stages of *Hyalophora cecropia*. It is a major cuticular protein of larval dorsal abdomen; closely related proteins have been identified in several other species (Binger and Willis, 1994). The sequence of the mature protein is given in the one letter code. It has 89 nominal positions and begins at residue 17 (V) of the unprocessed protein. The numbering at the top is that of retinol binding protein. The names of the proteins are to the left of each sequence. The numbering below HCCP12 is the numbering of the unprocessed HCCP12 protein, to facilitate comparison with Fig. 2. Alignments were created with CLUSTAL W (Thompson et al., 1994) and shading was done with GeneDoc 2.5.0 (Windows 95 version; Nicholas et al., 1997). Black-boxed residues are identical and light and dark gray-boxed residues represent conservative substitutions at the 60% and 80% significance levels, respectively (see also Materials and Methods). The observed (crystallographically) structure of retinol binding protein (PDB code: 1FEN) is shown above its sequence (Obs. Str.). The symbol E represents observed secondary structure of β-strands whereas the symbol H α-helix. Gaps correspond to random coil or β-turns/loops. Predicted secondary structure of HCCP12 according to a popular prediction algorithm, PhD, (Rost, 1996) is displayed below its sequence. Also, predicted secondary structure according to the algorithm SecStr (Hamodrakas, 1988). The symbol E represents predicted secondary structure of β-strands in each case, whereas gaps correspond to random coil or β-turns/loops. The R&R consensus (Rebers and Riddiford, 1988) is underlined, whereas the "extended" R&R consensus (Iconomidou et al., 1999) is doubly underlined below the sequence. The arrow indicates the first residue of the model structure of HCCP12 presented in Fig. 2 (F86 following the numbering of RBP/F40 following the numbering of the complete HCCP12 sequence, including the 16 amino acid signal peptide).

the proposed model for cuticle proteins accommodates, rather comfortably, at least one extended chitin chain.

4. Discussion

The structural model shown in Fig. 2, which is slightly over a half of an antiparallel β-sheet barrel (relative to retinol binding protein), has several attractive features to act as a structural entity interacting with the chitin chains in cuticle.

Firstly, it is an antiparallel β-sheet structure: we have recently experimentally shown that β-sheet predominates in the structure of cuticle proteins (Iconomidou et al., 2001), in agreement to our earlier proposals (Iconomidou et al., 1999) and those of Atkins (1985), Hackman and Goldberg (1979) and Fraenkel and Rudall (1947).

Secondly, the proposed structure has a "cleft" full of conserved aromatic residues (mostly tyrosines and phenylalanines), which are seen to form "flat" hydrophobic surfaces on one "face" of the model structure (Fig. 2). These are the side chains of F40, Y42, Y44 and H52 on the right hand side of the "cleft" and Y76, Y84, Y88 and F95 on the other. The aromatic rings of these residues could well stack against faces of the saccharide rings of chitin (poly-N-acetyl glucosamine) chains. This type of interaction is fairly common in protein–saccharide complexes (Vyas, 1991; Hamodrakas et al., 1997; Tews et al., 1997). It is interesting to note that we had foreseen such interactions from secondary structure prediction alone, two years ago (Iconomidou et al., 1999).

The structures of other types of chitin binding proteins are known and share provocative properties with the structure we propose for HCCP12. For example, recent experimental findings at atomic resolution indicate that an invertebrate chitin-binding protein (tachycitin) and a plant-chitin binding protein (hevein) have a common chitin-binding structural motif, which contains a two stranded antiparallel β-sheet and a helical turn (Suetake et al., 2000). We have modelled a short (7-residue) two turn α-helix at the C-terminus of the "extended R&R consensus" of HCCP12, starting and ending by two proline residues, P97 and P103, almost invariant in all "soft" cuticle proteins (found below P146 and V152 of RBP in Fig. 1). It should be emphasised that this α-helix was not predicted by any of the secondary structure prediction

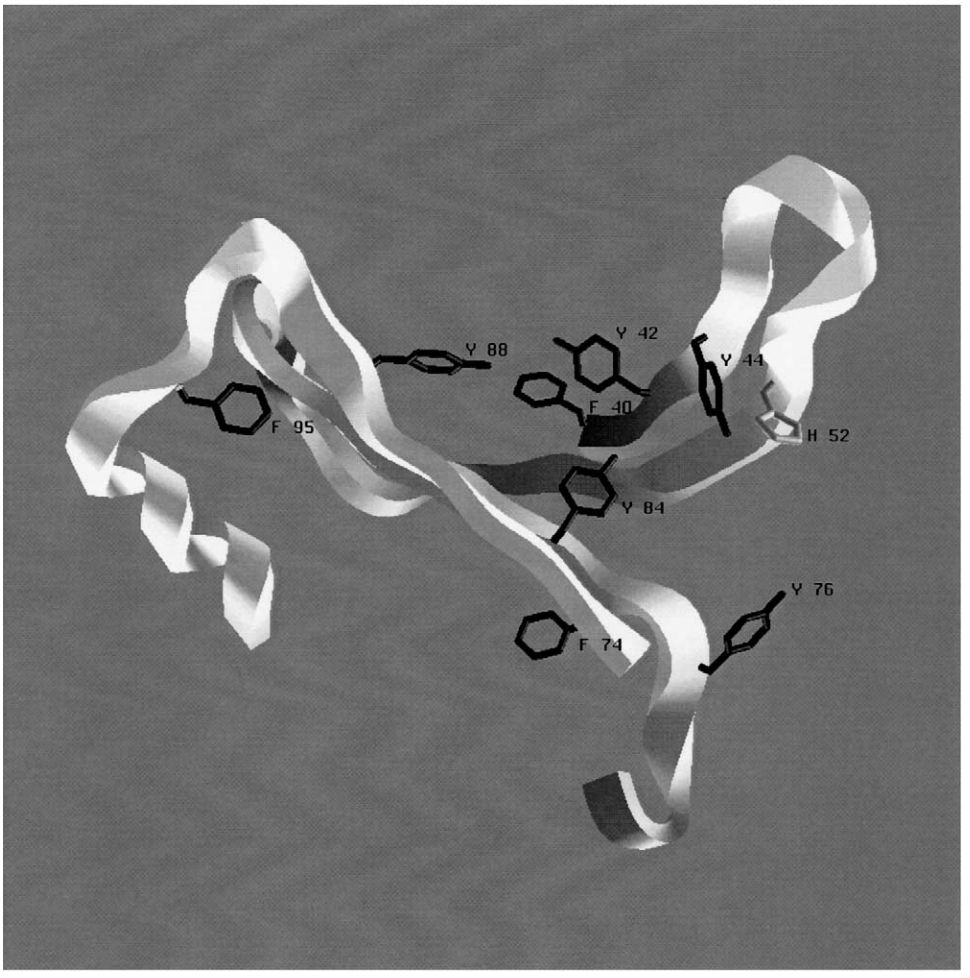

Fig. 2. A ribbon model of cuticle protein structure, displayed using GRASP (Nicholls et al., 1991). The structure of the representative "soft" cuticle protein HCCP12 was modelled on that of bovine retinol binding protein (RBP; PDB code 1FEN, Zanotti et al., 1994) utilizing the program WHAT IF (Vriend, 1990) and the alignment details shown in Fig. 1. The side chains of several aromatic residues are shown and numbered, following the numbering scheme of the unprocessed HCCP12 sequence which begins at residue 17 as VPL. . . . These are: F40, Y42, Y44, H52, F74, Y76, Y84, Y88 and F95. The corresponding positions, following the numbering of the RBP protein are: F86, Y88, Y90, H98, F120, Y122, Y133, Y137 and F144. The model structure has a "cleft" full of aromatic residues, which form "flat" surfaces of aromatic rings (upper side), ideally suited for cuticle protein–chitin chain interactions, and an outer surface (lower side) which should be important for protein–protein interactions in cuticle. The chitin chains (crystallites) are considered as "running" almost perpendicular to the plane of the figure.

algorithms used (Iconomidou et al., 1999). Thus, the C-terminal part of the model, where the "R&R consensus" is located, is reminiscent in some respects of the chitin-binding domain of invertebrate chitin-binding proteins (a two stranded β-sheet followed by a helical turn, Suetake et al., 2000). The two structures, however, cannot be superimposed and show no sequence similarity.

In the case of the bacterial chitinases, three-domain enzymes, which bind and hydrolyze chitin, an immunoglobulin-like module (domain), an antiparallel β-sheet barrel, is postulated to play an important role in "holding" the chitin chain in place to facilitate catalysis. Four conserved tryptophans on the surface of this β-sheet domain are assumed to interact firmly with chitin, "guiding" the long chitin chains towards the catalytic "groove" (Perrakis et al., 1997; Uchiyama et al., 2001).

Also of interest is the nature of protein interactions with cellulose, the major component of plant cell walls, another polymer formed from unmodified glucose monomers, which are polymerized in the same way as in chitin. For all cellulose-binding domains, the proteins contain a planar surface (formed from an antiparallel β-sheet), from which sets of aromatic rings protrude; these, apparently, interact with cellulose (Tormo et al., 1996). Interspersed between these aromatic residues one finds polar residues, which add specificity to the interaction. In addition, polar anchoring residues are observed at the edges of this putative binding surface (Tormo et al., 1996). In general, it appears that carbohydrate-binding modules that bind to crystalline cellulose have a flat ligand-binding surface, whereas carbohydrate-binding modules that interact with single polysaccharide chains contain clefts that accommodate the target carbohydrate (Czjzek et al., 2001 and references therein). Also, a clus-

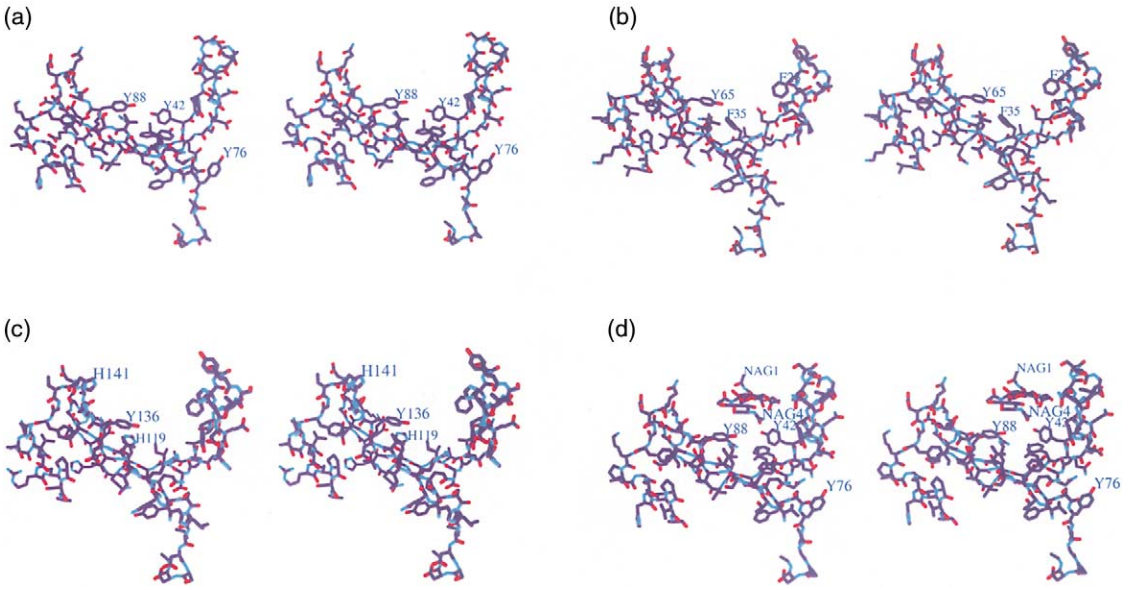

Fig. 3. (a) A stereo pair, drawn with the program O (Jones et al., 1991) of "soft" cuticle protein HCCP12 [ENTREZ accession number 1169129]. The numbering scheme used is that of the unprocessed protein and is similar to the numbering of Fig. 1 and Fig. 2. (b) A stereo pair, drawn with the program O (Jones et al., 1991) of "hard" cuticle protein HCCP66 [ENTREZ accession number 1169133]. The numbering scheme used is that of the unprocessed protein. The terminal Ile83 residue could not be modelled and is not shown. (c) A stereo pair, drawn with the program O (Jones et al., 1991) of "hard" cuticle protein AGCP2b [ENTREZ accession number 2961110]. The numbering scheme used is that of the unprocessed protein. His140 and the terminal Val155 residues could not be modelled and are not shown. (d) A stereo pair, drawn with the program O (Jones et al., 1991) of a complex of the "soft" cuticle protein HCCP12 [ENTREZ accession number 1169129] with a N-acetyl glucosamine (NAG) tetramer in an extended conformation. The complex was derived from a "low-resolution" docking experiment of a NAG tetramer, in an extended conformation, with the model of HCCP12 (Fig. 2, Fig. 3(a) utilizing the docking program GRAMM (Vakser, 1996) and the default parameters of the program.

ter of aromatic residues, which form a planar surface on an antiparallel β-pleated sheet, appears to be the chitin binding domain of the anti-fungal protein AFP1 from *Streptomyces tendae*, which was recently determined from NMR-solution studies (Campos-Olivas et al., 2001).

Therefore, apparently, there are several solutions in nature whereby chitin binds to protein and in all cases, β-sheet and surface aromatic residues appear to be significant (see also Iconomidou et al., 1999).

Thirdly, the model structure proposed here (Fig. 2) adopts an almost ideal "shape" to interact and "cover" the chitin crystallites (fibres, rods) in insect cuticle. It is very instructive in this respect to remember the model proposed by Blackwell, some twenty years ago (Fig. 6 of Blackwell and Weih, 1980), of a protein sheath consisting of a 6_1 helix of protein subunits, covering in a continuous way the chitin microfibrils in cuticle.

Fourthly, the outer surface of the antiparallel half β-barrel, of the structure proposed here, has almost ideal properties to promote regular three-dimensional packing of chitin crystallites covered by protein β-sheets, for the formation of the helicoidal structure of cuticle: we suggested almost twenty years ago (and verified later experimentally) that β-sheets dictate the formation of helicoidal silkmoth chorion architecture (see Hamodrakas, 1992 and references therein).

The half-barrel structure proposed for HCCP12 and applicable to other cuticular proteins was based on "homology" modelling with bovine retinol binding protein. Yet we emphasize that there is compelling evidence that HCCP12 and bovine retinol binding protein are not homologous; they are not members of the same protein family. Bovine retinol binding protein belongs to the lipocalin family of proteins. The family is defined by a β-barrel-like structure consisting of 8 anti-parallel β-strands; it has three recognizable domains, the first well conserved (Akerstrom et al., 2000). Lipocalins have cysteine residues that form intra-molecular disulfide bonds (Flower et al., 2000). Our alignment does not include a match to this first domain and most cuticular proteins lack cysteines (Andersen et al., 1995). Lipocalins are found in arthropods. Relevant examples are insecticyanin from lepidoptera (reviewed in Flower et al., 2000) and crustacyanin from lobster cuticle (Cianci et al., 2001). Both sequence and X-ray analyses established the identification of these proteins as typical lipocalins. Interestingly, insecticyanin and crustacyanin are present in cuticle (Riddiford et al., 1990; Cianci et al., 2001). They lack however, the Rebers and Riddiford (R&R) consensus found in HCCP12 and over half of the cuticular proteins identified to date (Andersen et al., 1995). It is an extended form of this consensus that is the major contributor to the half-barrel structure we now

propose for the conformation of cuticular proteins. The internal cavity in the barrel of insecticyanins and crustacyanin is occupied by pigment, whereas, the "groove" in the half-barrel structure proposed for insect cuticular proteins is available to accommodate chitin chains. Thus, while lipocalins and R&R consensus-bearing cuticular proteins do not appear to be related, the lipocalins have proven informative for modelling the structure of these cuticular proteins.

The model structure shown in Fig. 2 has already been experimentally tested indirectly (Rebers and Willis, 2001), without any prior knowledge by the authors of the model structure proposed here. AGCP2b, a "hard" cuticle protein from the mosquito *Anopheles gambiae* was synthesized, purified and shown to bind to chitin beads. A fusion protein was then made with 65 amino acid residues of the "extended R&R consensus" of AGCP2b fused to glutathione-S-transferase (GST). The fusion protein, called GST+65, was found to bind to chitin beads, whereas GST alone did not. A "mutant" of this fusion protein, called GST+65.YF, which had Y88 and F95 replaced by alanines, showed no binding to chitin beads (Rebers and Willis, 2001; these residues correspond to F137 and F144 of RBP in Fig. 1 and Y88 and F95 in Fig. 2). This, apparently, indicates the importance of these two aromatic residues of the "R&R consensus" for binding to chitin, in excellent agreement to the proposed model. Another "mutant" of this fusion protein that also failed to bind to chitin was called GST+65.RD (Rebers and Willis, 2001). Here, alanines were substituted for two residues conserved in proteins from hard cuticles. In Fig. 1 they correspond to S55 and Q57 found below RBP residues 101 and 103. These residues flank G56 that is conserved in position in the "extended consensus" of cuticle proteins from all hard and many soft cuticles (Iconomidou et al., 1999). According to the proposed model, the side chains of these two polar residues point away from the hydrophobic "cleft" and should not participate in chitin binding. However, it should be noticed that the conserved G56 is located at a sharp turn, at the end of the second β-strand (in the vicinity of H52 of Fig. 2). The alteration of two polar residues by two alanines may result in destruction of this turn and to improper folding, thus, leading to a structure not capable of binding chitin.

The proposed model might be the guide of such mutation and chitin binding experiments and there lies its potential value to act as a model for unravelling, through carefully designed experiments, effectively all chitin–cuticle protein interactions in detail.

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