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Review

Allergens and their associated small molecule ligands - their dual role in sensitization

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Abstract

Many allergens feature hydrophobic cavities that allow the binding of primarily hydrophobic small molecule ligands. Ligand-binding specificities can be strict or promiscuous. Serum albumins from mammals and birds can assume multiple conformations that facilitate the binding of a broad spectrum of compounds. Pollen and plant food allergens of the family 10 of pathogenesis-related proteins bind a variety of small molecules such as glycosylated flavonoid derivatives, flavonoids, cytokinins, and steroids *in vitro*. However, their natural ligand-binding was reported to be highly specific. Insect and mammalian lipocalins transport odorants, pheromones, catecholamines, and fatty acids with a similar level of specificity, while the food allergen β-lactoglobulin from cow's milk is notably more promiscuous. Non-specific lipid transfer proteins from pollen and plant foods bind a wide variety of lipids, from phospholipids to fatty acids, as well as sterols and prostaglandin B2, aided by the high plasticity and flexibility displayed by their lipid-binding cavities. Ligands increase the stability of allergens to thermal and proteolyticdegradation. They can also act as immunomodulatory agents that favor a Th2 polarization. In summary, ligand-binding allergens expose the immune system to a variety of biologically active compounds whose impact on the sensitization process has not been well studied thus far.

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1. Introduction

The EAACI and WAO nomenclature task force has defined an allergen as an antigen that causes an allergic disease.[1,2] The task force did not further characterize allergens as being either harmless or noxious environmental substances. Such a bias was intentionally avoided based on the fact that allergens can fall into either of these categories, or they may belong to two additional ones defined below. The vast majority of allergens induce the synthesis of specific IgE (sIgE) which depends on the Th2 polarization of naïve T helper cells during a type 2 immune response. While type 1 immune responses that target replicating microbial pathogens have been studied in great detail, elucidation of the mechanisms leading to type 2 immune responses to multicellular parasites, venoms, and allergens is lagging behind. However, this area of research is starting to gain momentum.[3]

The ability of an allergen to initiate the very first steps that will eventually result in a type 2 immune response can be based on its 'rather harmless' interaction with innate immune receptors present on epithelial cells, as has been shown for invertebrate tropomyosins.[4] The interaction of an allergen with a host organism can also result in damage to innate receptors or other constituent parts of its cells. Such has been shown for airborne allergenic proteases from the mold *Alternaria alternata* or from house dust mite, which cause damage to the respiratory epithelium resulting in a type 2 immune response.[5] On the far end of the spectrum, allergenic toxins such as phospholipases A2 present in venoms of stinging and hematophagous insects or predatory animals can cause necrotic cell death that leads to the induction of a strong type 2 response.[6]

There is another category of allergens that requires either components from the matrix[7] or bound ligands[8]to act as adjuvants and whose presence is essential for the induction of a type 2 immune response.[9] Certain lipids from the Brazil nut matrix are necessary for inducing signaling pathways that ultimately result in the synthesis of sIgE against the major Brazil nut allergen Ber e 1, a seed storage 2S albumin.[10] The major birch pollen allergen Bet v 1 by itself neither stimulated dendritic cells *in vitro* nor induced Th2 polarization *in vivo*; it required components from the pollen matrix present in a

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birch pollen extract to manifest a Th2 polarization.[11] Pru p 3, a non-specific lipid transfer protein (nsLTP) and major allergen of peach, harbors a derivative of camptothecin bound to phytosphingosine as a ligand.[12] The phytosphingosine part of the ligand was responsible for the activation of antigen-presenting cells.[13] Moreover, mice exposed to Pru p 3 plus ligand developed more Pru p 3-sIgE than when exposed to Pru p 3 alone.

In addition to their immunological adjuvanticity, ligands can also increase the stability of allergens against proteolytic degradation. The birch pollen-derived E1-phytoprostane, recently identified as another Bet v 1 ligand, was shown to enhance the resistance of Bet v 1to the proteolytic processing by endolysosomal extracts, which was proposed to directly influence its allergenicity.[14] Likewise, the binding of fatty acids by the cockroach allergen Bla g 1 was found to significantly enhance the allergen's thermostability while inhibiting cleavage by cathepsin S, an endosomal protease essential for antigen processing and presentation.[15] These topics are discussed in more details below.

This review summarizes our current knowledge on allergenic proteins that bind small moleculeligands. It also gives an overview on which types of ligands may bind to allergens and what their role in inducing a type 2 response is or might be. Thus, the intention is to indicate new avenues of scientific inquiry into the sensitizing capacities of allergens that are neither damaging nor toxic to host cells by themselves.

Box 1. Major milestone discoveries

- Availability of experimental molecular structures for a range of allergens with hydrophobic cavities[16,17]
- Establishment of biophysical and biochemical methods to study ligand - protein interaction and its impact on local structural changes[18-23]
- Identification of Group 2 house dust mite (HDM) allergens as ligand binding proteins[16,17]
- Discovery of the natural ligand of Bet v 1[24-26]
- Discovery of the natural ligand of Pru p 3[12]

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- Elucidation of the role of the phytosphingosin part of Pru p 3 ligand in the sensitization process[27]
- Discovery of Bla g 1 ligands[15,28]
- Discovery of the natural ligand of Cor a 1[29]
- Allergens from PR-10 family bind broader spectrum of ligands *in vitro* than *in vivo*[25,26,30]
- Ligand enhances proteolytic resistance of Bet v 1 and inhibitsendolysosomal cathepsin S protease activity[14]

2. Serum Albumins

Serum albumins (SAs) are members of a highly-conservedprotein familythat includesnumerous allergens.[31-33]SAsare clinically relevant allergens that originate from animals, where they are major blood components. SAs are also present in milk, muscle, and epithelia. It was demonstrated that ~30% of individuals allergic to animal dander show IgE reactivity towards SAs. [34]A mature SA molecule is composed of approximately 585 amino acid residues that fold into three distinctive domains of similar size and whose structure is stabilized by several disulfide bridges (Figure 1, Equ c 3).[35]

The molecular architecture of SAs allows these molecules to adopt multiple conformations that facilitate simultaneous binding of various ligands (Figure 1),[36] making SAshighly capable small molecule carrier proteins.[37,38] SAs bind many endogenous and exogenous compounds.[39] In addition, SAs play a very important role in the transport of metal cations.[40-42]Structural studies revealedthe presence of many ligand binding sites.Currently,nine fatty acid binding sites have been identified, as well as several sites that are responsible for binding drugs and various metabolites or hormones.[43]

The most relevant allergenic SAs originate from mammals and birds.[32]The allergenic mammalian SAshave very high sequence identities and similarities, cat and dog SAs being the most similar to the human homologue (approximately 83% identities). Even avian SAs display quite high sequence identities (>45%) and similarities (>60%) to human SA (HSA).Therefore, it is not surprising that SAs, due to their high sequence identities, show significant levels of cross-reactivity.[33,41,44]To date, there

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are eightSAswhose structureshave been determined: bovine (BSA, Bos d 6), canine (CSA, Can f 3), equine (ESA, Equ c 3), feline (FSA, Fel d 2), HSA, goat (GSA), ovine (OSA),and rabbit (RSA).[35,41,45-48]

While the ligand-binding properties of human serum albumin are best studied, there is also a significant body of literature on interactions of small molecule compounds with BSA and ESA.[39,43,49]Structural studies clearly show that SAs bind a broad spectrum of compounds that are biologically active, some of which may affect the human immune system. For example, SAs bind steroid hormones, saturated and unsaturated fatty acids, thyroxine, vitamin D,and flavonoid metabolites, as well as many exogenous compounds like drugs (Table 1).[37-39,43,49-51]It was also shown that small molecule compounds like carbohydrates may bind SAs through the amino groups of lysine residues that are located on the surface of these proteins.[49,52]It was shown that derivatives of carbohydrates, such as the food additive D-mannitol, can form stable covalent derivatives with SAs, leading to the haptenization of these proteins.[52]In fact, D-mannitol is a member of a large group of small molecular compounds that form covalent bonds with proteins like SAs. The interaction of the small molecular compounds with SA does not always require an aqueous solution, but the proteins may be modified by compounds present in air. One such example is the modification of HSA by vapors of hexamethylene diisocyanate leading to immunogenic protein derivatives.[53]

In summary, SAs are able to simultaneously bind many diverse compounds and it can be assumed that some small molecular compounds are always bound to these proteins. Therefore, the human immune system is exposed to SAs that are tightly associated with small molecules that may have immunomodulatory properties. One may speculate that the small molecule binding properties of SAs are responsible for these molecules potentially becoming allergens despite the fact that mammalian SAs are very similar to HSA.[33]

3. PR-10 Allergens

Class 10 pathogenesis-related proteins (PR-10s) are part of a plant's immune defense against pathogens or abiotic stress.[54,55]PR-10s include a wide variety of clinically relevant pollen and food

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allergens that possess a conserved three-dimensional structure.[29,56,57] Typically they contain a hydrophobic cavity which can accommodate different ligands (Table 1).[56]PR-10s are formed by a seven-stranded antiparallel β-sheet and a long C-terminal α-helix enclosed by two shorter helices arranged in a V-shape (Figure 2A).[58] Due to the high structural similarity of PR-10 allergens, IgE antibodies can cause cross-reactivity via a range of different exposure scenarios.[59] Natural PR-10 allergens are usually comprised of a mixture of different isoallergens (>67% amino acid sequence identity) and variants (>90% identity).[60,61] They were shown *in vitro* to bind a variety of small molecules,i.e., flavonoids, cytokinins,and steroids, indicating functions in UV protection, transport of small molecules,and regulation of germination.[25,62-65]

The glycosylated flavonoid derivative quercetin-3-O-sophoroside (Q3OS) was identified as a natural ligand of Bet v 1.0101, the most abundant birch pollen isoallergen.[26] Another quercetin derivative, quercetin-3-O-(2´´-O-β-D-glucopyranosyl)-β-D-galactopyranoside (Q3O-(Glc)-Gal), was recently found to be a natural ligand of Cor a 1, the major hazel allergen.[29] Although the two ligands differ only in the orientation of one OH group in the sugar moiety (glucose vs. galactose) (Figure 2B), Cor a 1 selectively binds its own ligand but not Q3OS and vice versa. Of all isoallergens known, onlyCor a 1.0401 and Bet v 1.0101 showed binding of the identified compounds.[24,26,29]Since non-glycosylated flavonoids are bound rather promiscuously, the varying sugar moieties appear to be responsible for the binding specificity. Interestingly, RNAi-mediated silencing of the Fra a 1 genes in strawberry resulted in white strawberries due to a decrease of different glycosylated metabolites involved in flavonoid biosynthesis.[66] These results suggest that glycosylated flavonoids might also be natural ligands of other PR-10 allergens.

Other PR-10 allergens that were characterized in terms of their interactions with ligands include proteins originating from peanut and kiwi fruit. There are two Ara h 8 isoforms from penaut in the official WHO/IUIS allergen database (www.allergen.org), Ara h 8.01 and Ara h 8.02. Although they share only 55% amino acid sequence identity, both were shown to bind the same small molecule ligands (apigenin, genistein, quercetin, daidzein, progesterone, arachidic acid, palmitic acid, and resveratrol; Table 1).[65] Ara h 8.01 can also bind epicatechin and three molecules of quercetin (Figure

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2) without undergoing an extensivestructural change.In the case of Ara h 8 it was demonstrated that lipid binding increased its thermal and proteolytic stability. [67]Act d 11 from kiwi fruit belongs to the major latex protein/ripening-related protein family (MLP/RRP) and has the same overall fold as Bet v 1.[68,69]The crystal structure of Act d 11 purified from its natural source revealed the presence of an unknown ligand that is likely to contain an indole or a similar ring structure. While the identity of the ligand is still unknown, Act d 11 should be considered a "dressed allergen" that carries small molecule compounds capable of interacting with the human immune system.[63]

IgE binding assays and mediator release assays with Bet v 1 variants showed no increased binding affinities in the presence of ligand.[14,26] Furthermore, no increased activation of dendritic cells could be observed.[14] However, pollen-derived ligands like phytoprostane E1 enhanced the thermostability of Bet v 1 and increased its proteolytic resistance against the endolysosomal proteases cathepsin S and legumain.[14] Preliminary data with Api g 1.0101 from celery indicated that the protease stability of Api g 1.0101 against trypsin was higher in the presence of the flavonoid aglycon apigenin.[70] It has been shown that increased thermostability affects immunogenicity and allergenicity.[71] Furthermore, reduced proteolytic processing of allergens could result in low loading and density of class II MHC peptide complexes, which in turn favors Th2 polarization.[72]

4. Lipocalins

Lipocalin allergens are the most important group of respiratory animal allergens and include a number of allergens with high sensitization rates among allergic individuals.[73] In addition, the lipocalin Bos d 5 is a major allergen in bovine milk, which is the common trigger of food allergy in childhood.[74]Lipocalins form a large protein family and are found in animals, plants, and bacteria. They are small single-domain proteins of 150-180 amino acid residues. The amino acid sequence identities between different lipocalins are low but the fold is conserved, consisting typically of 8 antiparallel β-strands forming a β-barrel, and one α-helix (Figure 3). Inside the β-barrel there is a central cavity which can usually accommodate one ligand (Table 1). The major function of lipocalins is to transport poorly water-soluble ligands.[75,76] Over 20 lipocalin allergens have been characterized, the majority of which are respiratory allergensexcept forthe food allergen Bos d 5.[73]

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Bos d 5 or β-lactoglobulin occursat thehigh concentration of 0.1 mM in bovine milk.[77,78]The ligand-binding specificity of Bos d 5 is not very high as many different ligands, primarily fatty acids, have been reported for this allergen. The binding affinity for short chain fatty acids is weak ($K_D$sare in mM range) but affinity is higher for long-chain fatty acids ($K_D$sare usually inμM range).[79] A low or moderate binding affinity to Bos d 5 allows dissociation of ligands from the complex after transport. The impact of ligand binding on allergenicity or immunogenicity of Bos d 5 has been investigated in few studies. It has been proposed that retinoic acid or iron-quercetin would bind to T-cell and B-cell receptors and suppress immunogenicity and allergenicity of Bos d 5.[80,81] Based on a study in a mouse model, it was also suggested that ligand-bound Bos d 5 was able to protect against allergic sensitization to unrelated birch pollen allergens.[82]

Crystal structures of at least nine inhalant lipocalin allergens have been determined. The structures for Bos d 2, Can f 2, Can f 4, Can f 6, and Equ c 1 do not contain any ligands but the binding sites have been analyzed.[83-87] The crystal structure of Rat n 1 contains limonene-1,2-epoxide [88] and Mus m 1 harborsa thiazole derivative(Figure 3).[89] All of these structures display a closed binding site, suggesting that a conformational change of the protein is required for ligand association and dissociation.[90] The binding of tyramine and octopamine to Bla g 4 were especially well-characterized by X-ray crystallography (Figure 3)and isothermal titration calorimetry.[91,92] The possible effect of ligand binding to respiratory allergens has not been studied in detail. TheToll-like receptor 4 (TLR4) agonist lipopolysaccharide was suggested to bind to Can f 6 and enhance innate immune signaling.[93]

The role of ligands for the allergenicity of lipocalins requires further investigations. However, some general remarks can be made. The food allergen Bos d 5 seems to have a lower ligand binding specificity and affinity compared to respiratory allergens. The binding affinity of the ligand is an important consideration. Many experiments such as basophil activation tests are performed at nanomolar allergen concentrations. If the allergen concentration is much lower than the$K_D$for ligand binding, the number of complexed proteins and ligands is very low even in the presence of clear

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excess of ligand. The wide range of ligand binding capacity of Bos d 5 may indicate a general transport role for clearance of harmful compounds.[75,94]

5. Non-specific Lipid Transfer Proteins

Non-specific lipid transfer proteins (nsLTPs) belong to the prolamin superfamily and are ubiquitous plant proteins. They have been classified as pathogenesis-related proteins as their expression is upregulated by abiotic and biotic stress.[95-97] nsLTPs are categorized either as LTP1 (approximately 9 kDa) or LTP2 (approximately 7 kDa).nsLTPs are small soluble basic proteins that share a common fold based on fourα-helices stabilized by four conserved disulfide bridges. This compact structure enables nsLTPs to be highly resistant to heat and enzymatic treatment. A common feature of nsLTPs is a tunnel-like hydrophobic cavity with a ligand-binding site. This ligand-binding pocket is able to accommodate and transport a wide variety of lipids, from phospholipids to fatty acids, as well as sterols and prostaglandin B2 (PGB_2), among others (Table 1; Figure 4).[98,99] Accordingly, the lack of ligand specificity of nsLTPs resides in the high plasticity and flexibility of their lipid-binding cavity, which is able to accommodate anything from one or two fatty acids to single- or double-chain lipids.[100]

A considerable number of allergenic nsLTPsfrom pollen and plant foods have been identified. Pru p 3 from peach, the first identified allergenic LTP1, is regarded as a major allergen that affects more than 50% of peach allergic patients, inducing symptoms that range from mild to severe.[101] Pru p 3 displayed preferential binding to unsaturated short chain fatty acids such as oleic acid[27,102,103]andphytosphingosine.[13]A structural model was developed for Jug r 3, the LTP1 from walnut, by using an NMR interaction study and data-driven docking calculations to assess the effect of ligand binding of oleate.[104] Applying the WaterLOGSY NMR method, binding of oleic acid versus stearic acid was compared and the preferred binding of oleic acid was confirmed. Furthermore, the OLE binding sites were identified including the C-terminal region of helix alpha 2, most of helix alpha 3,2 the loop between helices alpha 3 and alpha 4 and the C-terminal loop. Regarding the ligand its hydrophobic tail is inside the cavity, while the carboxylate part is surface exposed. Overall, the internal cavity from Jug r 3 seems to be flexible depending on the ligand binding. In that context the

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C-terminal loop undergoes a conformational change upon lipid binding resulting to a more surface exposed position as compared to unliganded Jug r 3. This local conformational change leads to an increased IgE binding as shown by antibody binding assays and basophil activation tests. These data are in line with reports from Pru p 3, where the same area was previously identified being part of an immunodominant IgE epitope, which was less accessible for IgE antibodies in the apoform, versus the holoform providing increased IgE binding.[104,105]

The prevalence of sIgE to Ole e 7, an LTP1 and minor allergen from *Olea europaea*pollenincreases up to 50% in populations exposed to high levels of olive pollen.[106]Interestingly, oleic acid, an unsaturated fatty acid with a C18 chain, showed the greatest binding affinity to Ole e 7 in accordance with other nsLTPs from maize, peach, apple, hazelnut, and walnut.[27,102,104,107] Ole e 7 preferentially binds negatively-charged phospholipids such as phosphatidylserine and phosphatidylglycerol, with cholesterol able to compete with this binding.[108] While the direct effect of this interaction on allergenicity has yet to be observed, its implications on Ole e 7 interfacial behavior could facilitate sensitization.

Ole e 7,like other aeroallergens, enters the body through the upper airways, reaching the mucosal surface and making contact with two lipid-based barriers: the pulmonary surfactant located on the outer side of the mucus and the luminal plasma membrane of airway epithelial cells. Ole e 7 was shown to adsorb to air-liquid interfaces and effectively interact with pre-formed lipid monolayers composed of negatively charged phospholipids and cholesterol.[108] Ole e 7 seemed to reverse the inhibitory effect on the surface adsorption of the pulmonary surfactant manifested by plasma proteins such as HSA and other clinically relevant allergens like Ole e 1.[109]This suggests that Ole e 7transfers surfactant lipid-components to the interface whose impact on the structure and function of the pulmonary epithelium has yet to be determined.

In conclusion, the interaction of nsLTPs with ligands affects the local protein conformation and in turn the recognition by IgE antibodies, relevant for the allergic effector phase. Whether that ligand interaction is also important for the allergic sensitization phase still remains to be defined, as it was

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also shown that ligand binding may result in an increased susceptibility of nsLTPs to gastroduodenal proteolysis.[110]

6. Group 2 House Dust Mite allergens of the Niemann-Pick type C2

Proteins belonging to the ML superfamily have ML (MD-2-related lipid-recognition) domains, which have been identified in multiple proteins of unknown biological function, associated with interaction with lipids, in plants, animals and fungi. House dust mite (HDM) allergens from group 2 are some of the most important mite allergens, eliciting IgE antibody responses in 70-90% of mite-allergic patients.[111-113] Sensitization to these allergens is associated with the development of allergic diseases, such as asthma, rhinitis and atopic dermatitis. A longitudinal study of the evolution of the IgE response to a panel of twelve HDM allergens during the first two decades of life showed that Der p 2 was the earliest recognized allergen.[113]This study also confirmed the importance of Der p 2 as a major allergen, together with Der p 1 and Der p 23. In addition to its immunogenic properties, Der p 2 can aggravate respiratory airway disease by adjuvant-like activation of lung epithelial cells.[114]

Der p 2 and Der f 2 from the species *Dermatophagoidespteronyssinus* and *D. farinae*, respectively, are small approximately 14 kDa proteins that form a single immunoglobulin-fold domain consisting of two three-stranded antiparallel β-sheets (Figure 5A).[17,115-117] The X-ray crystal (but not the NMR) structure of Der p 2 revealed a large internal hydrophobic cavity, defined by the two β-sheets, that was able to bind lipidic ligands.[17,116] Similarly for Der f 2, the separation and angle between the two sheets in the first NMR and crystal structures ("closed")[118,119] were narrower than those described in a more recent X-ray crystal structure ("open").[120] Der f 2 was shown to bind LPS (lipopolysaccharide) with nanomolar affinity.[16] LPS bound to a cluster of basic residues at one edge of the pocket entrance that attracts negatively charged LPS, with its acyl chains inserted between the β-sheets. To bind LPS, the internal cavity of Der f 2 must open wider than the "open" state described above.[16] Der p 2 also bound LPS, although with low affinity.[121] Recently, both recombinant allergens were reported to bind many lipids promiscuously.[122] They preferentially bound cholesterol among eleven different lipids tested with a liposome pulldown assay.[122]

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Group 2 HDM allergens belong to the ML superfamily of proteins, which bind specific lipids and play important roles in lipid recognition and metabolism.[123] The ML superfamily also includes MD-2, a member of the myeloid differentiation factor-2-related lipid-recognition protein family, and the human cholesterol-binding Niemann-Pick type C2 (NPC2) protein. Hence, the binding of cholesterol by Der p 2[122] is intriguing becausethe structure of Der p 2 is more closely related to the human NPC2 protein than to MD-2.[121,124] As group 2 HDM allergens also share structural homology with MD-2,a study investigated whether they could functionally act as MD-2.[125] MD-2 is the LPS-binding component of TLR4 signaling complex. Association of the MD2-LPS complex to the ectodomain of TLR4 triggers the signaling cascade.[126] Der p 2 was proven to have functional homology with MD-2, by facilitating signaling through direct interactions with the TLR4 complex, and reconstituting LPS-driven TLR4 signaling in the absence of MD-2. Experimental allergic asthma was induced in wild type and MD-2-deficient, but not TLR4-deficient, mice, by airway sensitization and challenge with Der p 2.[125] This study supported the idea of the importance of lipid ligands for the innate immune responses of group 2 mite allergens. While this paper presents a convincing case for Der p 2-MD-2 molecular mimicry, some biochemical studies are suggested for follow up.[127] For example all of the Der p 2 - TLR4 binding experiments were done with immunoprecipitations [125], which does not directly measure affinity. MD-2 has a 0.8 nM affinity for TLR4[128] and Der p 2 lacks the DDD motif important for MD-2 recognition of TLR4.[127] Therefore, it is not clear how Der p 2 could compete for TLR4 in a physiological setting. Second, the mimicry study utilized a Der p 2 Y91A mutant that claimed to abolish LPS binding.[125] This is unlikely to be highly effective as this residue is not highly conserved in mites.[16] Biochemical studies establishing the affinity of Der p 2 binding to TLR4, and the affinity of Der p 2 Y91A binding to LPS would be useful to understand the physiological relevance of the proposed mimicry.

In summary, these studies indicate that various hydrophobic ligands of mite group 2 allergens can be accommodated in the binding pocket via conformational changes,[16,121] highlighting the relevance of molecular flexibility of these allergens.The flexibility of group 2 HDM allergens has also been reported to affect their antigenic surface.[129] Several studies have shown interesting aspects of group 2 HDM allergens related to their capacity to induce adaptive immunity.[130-132] Recently, anti-Der p 2

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human IgE monoclonal antibodies were isolated by hybridoma technology that have the correct pairing of the heavy and light chains as it occurs *in vivo*. Their associated epitopes were identified by immunoassays and NMR analyses.[131]

7. Other Ligand Binding Allergens

HDM group 7 and 13 allergens have low to medium sensitization rates among HDM sensitized individuals.[133] They are known to have lipophilic properties and have been shown to mimic molecules involved in innate immunity.[134,135] The structure of Der p 7 (Figure 5B) was observed to be similar to lipopolysaccharide binding protein (LBP) that interacts with Toll-like receptors after binding lipopolysaccharide and other bacterially-derived lipid ligands.[134] Such lipid binding featuresare common amongst many allergens[136] and allergenicity resulting from functional mimicry of a TLR complex protein has been demonstrated in an animal model.[125]

Rather than binding LPS, Der p 7 binds with weak affinity to the bacterial lipopeptide polymyxin B, a nine-residue cyclic peptide with a lipid tail from the Gram-positive bacterium*Bacillus polymyxa*.[134] The natural ligand for Der p 7 has not yet been identified, but the structural relation to a protein in the TLR pathway and the ability to bind bacterially-derived lipid ligands are suggestive of a mechanism that would lead toward allergic sensitization to these proteins. This remains to be formally demonstrated for Der p 7.

Der p 13 (Figure 5C), which belongs to the fatty acid binding protein (FABP) family, was shown to selectively bind fatty acids and induce airway epithelial cell activation *in vitro* through TLR2-MyD88-NF-kappaB and MAPK-dependent mechanisms.[135]Similar observation weremade for Der p 5.[137]More recently, it was demonstrated that HDM group 13 allergens (specifically Der p 13 and Blo t 13) are sensed by serum amyloid A1 (SAA1) that promotes pulmonary type 2 immunity.[138] SAA1 interacts directly with allergenic mite FABPs, which then activatesthe N-formyl peptide receptor 2 (FPR2). In addition to the direct interaction with SAA1, the authors speculate that, because of its lipid binding properties, Der p 13 can strip away the retinol molecules that typically stabilize SAA1 hexamers. This then drives epithelial cells to release interleukin (IL)-33 in a SAA1-dependent

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manner. It was observed that the SAA1–FPR2–IL-33 axis was upregulated in nasal epithelial cells from patients with chronic rhinosinusitis.[138] From an allergy standpoint, this is curious because Der p 13 is typically considered a minor allergen due to low prevalence of sIgE in mite-allergic patients. However, it is highly expressed at levels on par with the major allergen Der p 2, so it is likely an abundant mite protein.[139] The interpretation of these results appears to be that the binding and adjuvant properties of Der p 13 seem to be more consequential than the ability to stimulate IgE to itself.

Bla g 1 (Figure 5D),also a lipid-binding protein, is not a major allergen for cockroach allergic patients,[140-142]as is the case for house dust mite (e.g. Der p 1, Der p 2, Der p 23) and birch pollen (e.g. Bet v 1).[143]Bla g 1 is highly expressed in female cockroaches exclusively by midgut cells with a production that is modulated in relation to food intake.[144-146] A role of Bla g 1 in digestion and nutrient absorption has been suggested.[147]Bla g 1-encoding DNA has an interesting genetic structure.[148] Multiple tandem repeats of the gene are found in five different open reading frames, each with some homology to the currently described allergen.[149] It is currently not known whether all of these homologous proteins are allergens. The protein structure of Bla g 1 corresponding to a tandem repeat is spherical with twelve α-helices that enclose a large cavity capable of enclosing up to four diacyl-phospholipids.[15,28] Recombinant Bla g 1 contained a variety of phospholipids whose nature depended on the expression system (Figure 5D), whereas natural Bla g 1 bound a mixture of the fatty acids palmitate, oleate, and stearate. When loaded with these natural fatty acids, Bla g 1 was both more proteolytically resistant and thermally stable than when loaded with lipids with shorter or longer fatty acid chains.[150] The stabilizing effect of the ligands anticorrelated with the generation of known T-cell epitopes using an *in vitro* assay.[15] The implication is that the stabilizing property of the lipids could modulate the generation of T-cell epitopes and subsequent allergenicity. As precedent, isoforms of Bet v 1 that display enhanced stability are able to avoid premature processing in the endosome, yielding a stronger Th2 response than less allergenic variants.[71]This is a distinctly different role for the lipids in stabilizing Bla g 1, as opposed to the immunomodulatory properties of LPS, for example. It is possible that Bla g 1 ligands could also have immunomodulatory properties which have not been fully explored. Lastly, Bla g 1 has a very high affinity for phosphatidylcholine (PC) that exceeds the

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affinity for the natural fatty acids.[15] PC is a major component of lung surfactant so it is possible that Bla g 1 would exchange fatty acids for PC disrupting the natural balance of surfactant in the lung, leading to barrier defects. The delivery of lipid adjuvants and the disruption of natural barriers are both suggested to lead to allergic disease.

Fel d 1, which is the most important cat allergen, has a fold similar to the one observed for uteroglobin.[151] Both proteins possess several helices and an internal cavity that is able to accommodate hydrophobic molecules. It was shown that the cat allergen could bind lipids or steroids.[152]

8. The impact of allergen-bound ligands on the immune response

The fact that several types of allergens bind hydrophobic ligands has prompted the hypothesis that these ligands play a crucial role in inducing the allergic response.[8,153] The binding of lipids to food allergens effects their degradation in the GI tract and their passage through epithelial barriers impacting on the sensitization process.[136] Len c 3, an nsLTP from lentils, otherwise sensitive to heating and digestion, increased its stability when binding lyso-palmitoyl phosphatidylglycerol, a ligand - the authors argue - the allergen picks up during the cooking process.[154] A derivative of the alkaloid camptothecin bound to phytosphingosine was identified as the ligand of the major peach allergen Pru p 3, another nsLTP.[12] The lipid ligand induced the maturation of monocyte-derived dendritic cells and the proliferation of peripheral blood mononuclear cells.[13] The immunological activity of the ligand which resides in the phytosphingosine tail was mediated by Cd1d activation of iNKT cells. In a mouse model of peach anaphylaxis, the complex of Pru p 3 and the ligand induced higher levels of sIgE than the allergen alone.

Detailed studies of the respiratory allergen Bet v 1, the prototypic PR-10 protein, revealed the impact of its ligands on the sensitizing process. A pharmaceutical-grade recombinant Bet v 1was produced in *E. coli* for the formulation of a sublingual tablet to treat birch pollen allergy.[155] This highly purified rBet v 1, free from any ligand, was unable to induce secretion of pro-inflammatory or effector cytokines by human blood dendritic cells or mononuclear cells. Remarkably, Bet v 1-depleted birch

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pollen extract was still able to induce Th2 polarization while purified rBet v 1 did not induce dendritic cell differentiation.[11] Bet v 1 binds various classes of ligands in its large hydrophobic cavity including fatty acids, cytokinins or flavonoids.[156] Immunomodulatory pollen-associated lipid mediators such as leukotriene-like molecules and phytoprostanes when codelivered with the allergen were proposed to play a key role in Th2 polarization,[7] Phytoprostane E1 has also been identified as a new ligand of Bet v 1.[14] This pollen-derived ligand was shown to enhance the proteolytic resistance of Bet v 1 and to inhibit endolysosomal cathepsin protease activity. Diminished proteolytic processing of antigens results in low loading and density of MHCII-peptide complexes which favors Th2 polarization.[72]

9. Conclusions

Allergens belonging to protein families that include SAs, PR-10s, nsLTPs, lipocalins, NCP2, LBPs, and FABPs represent a significant fraction of officially registered allergens. These proteins bind diverse compounds, including fatty acids, lipo-oligosaccharides, phospholipids, oxylipins, catecholamines, trace amines, steroids, flavonoids, and different plant hormones, as well as odorants and pheromones. The studies of the complex mixtures of molecules to which the human body and its immune system are exposed to are just starting. Therefore, we are convinced that not only allergenic proteins allergens but also the small molecular compounds accompanying them play important and still not well-understood roles in allergic sensitization and diseases.

Box 2. Future research perspectives

- More studies on the effect of allergen natural ligands on stability and antigen processing that affects allergenicity
- Identification of small molecules acting as ligands with an impact on the allergic sensitization phase
- Identification of small molecules acting as ligands with an impact on the allergic effector phase
- Identification of small ligand molecules ligands with an intrinsic immunogenic capacity
- Better understanding why isoallergens show different ligand binding preferences

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Author contributions: HB initiated this project. All authors contributed to the writing of the manuscript.

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Table 1. Natural compounds binding to allergens. The table lists only compounds for which experimental confirmation is available.

Types of Compounds	Ligands	Allergen Family	Allergens	References
Fatty acids	arachidic acid	PR-10	Ara h 8, Bet v 1	25,65
	lauric acid	Lipocalin	Bos d 5	157
	lauric acid	nsLTP	Pru p 3	27,158
	myristic acid	SA	Equ c 3	159
	myristic acid	PR-10	Bet v 1	25
	oleic acid	nsLTP	Cor a 8, Jug r 3, Mal d 3, Ole e 7, Pru p 3, Zea m 14	27,102,104,107,108
	oleic acid	PR-10	Bet v 1	25
	palmitic acid	nsLTP	Pru p 3, Zea m 14	27,160
	palmitic acid	PR-10	Ara h 8, Bet v 1	25,65
	stearic acid	PR-10	Bet v 1	25
	fatty acids (C12 to C22)	Bla g 1	Bla g 1	15,28
	lauric acid	Uteroglobin	Fel d 1	152
Lipooligosaccharides	lipoteichoic acid	Bla g 1	Bla g 1	15
	LPS	NPC2	Der f 2	16
	LPS	NPC2	Der p 2	121
	LPS	nsLTP	Par j 1	161
Lipopeptides	polymyxin B	LBP	Der p 7	134
Oxylipins	phytoprostane B_1	PR-10	Bet v 1	14
	phytoprostane E_1	PR-10	Bet v 1	14
	phytoprostane F_1	PR-10	Bet v 1	14
	ricinoleic acid	nsLTP	Zea m 14	107
Phospholipids	dipalmitoylphosphatidylcholine	nsLTP	Ole e 7	108
	dipalmitoylphosphatidylglycerol	nsLTP	Ole e 7	108
	dipalmitoylphosphatidylserine	nsLTP	Ole e 7	108
	phosphatidylinositol	Bla g 1	Bla g 1	28
	phosphatidylserine	Bla g 1	Bla g 1	28
	phosphatidylcholine	Bla g 1	Bla g 1	28
	phosphotidylethanolamine	Bla g 1	Bla g 1	15,28
	phosphotidylglycerol	Bla g 1	Bla g 1	15,28
Sphingosine derivatives	10-hydroxy-camptothecin linked to	nsLTP	Pru p 3	13

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	phytosphingosine			
Flavonoids and flavonoid derivatives	apigenin	PR-10	Ara h 8, Bet v 1, Que a 1	65
	daidzein	PR-10	Ara h 8, Bet v 1, Que a 1	65
	epicatechin	PR-10	Ara h 8, Bet v 1, Que a 1	65
	genistein	PR-10	Ara h 8, Bet v 1, Que a 1	65
	myricetin	PR-10	Fra a 1	26
	naringenin	PR-10	Bet v 1, Cor a 1	25,63
	quercetin-3-*O*-glucuronide	PR-10	Fra a 1	162
	Q3O-(Glc)-Gal*	PR-10	Cor a 1	29
	quercetin-3-*O*-sophoroside	PR-10	Bet v 1	24
Catecholamines and trace amines	norepinephrine	OBP**	Aed al 2	163
	octopamine	Lipocalin	Bla g 4	92
	tyramine	Lipocalin	Blag 4	92
Odorants and pheromones	limonene-1,2-epoxide***	Lipocalin	Rat n 1	88
	2-(*sec*-butyl)thiazole	Lipocalin	Mus m 1	89
Steroids	cholesterol	NPC2	Der p 2	122
	dehydroergosterol	PR-10	Bet v 1	25
	deoxycholate	PR-10	Bet v 1	64
	testosterone	SA	Equ c 3	39
	stigmasterol	PR-10	Ara h 8, Bet v 1, Cor a 1, Que a 1	65
	progesterone	PR-10	Ara h 8, Cor a 1, Que a 1	65
	androstenone	Uteroglobin	Fel d 1	152
Cytokinins	IPA****	PR-10	Bet v 1	65
	kinetin	PR-10	Bet v 1	25
	zeatin	PR-10	Bet v 1, Pru p 1	65,164
Gibberellins	gibberellin A3	PR-10	Vig r 6	165
Hydroxycinnamic acids	caffeic acid	PR-10	Ara h 8, Cor a 1	65
	ferulic acid	PR-10	Ara h 8, Cor a 1	65
Stilbenoids	resveratrol	PR-10	Ara h 8, Bet v 1, Cor a 1	62,65

*Q3O-(Glc)-Gal - quercetin-3-*O*-(2“-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside

**Odorant Binding Protein

***It is not clear whether the ligand in the Rat n 1 structure (PDB code: 2AG2) was properly identified

****IPA - N-isopentenyladenosine

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Figure legends

Figure 1. Model of equine serum albumin (ESA) in cartoon representation (PDB code: 6XK0). (A) Domain assignment as described by Sugio*et al*.[35] ESA binds simultaneously dexamethasone (DEX; carbon atoms shown as orange spheres), citric acid (CIT; carbon atoms shown as yellow spheres) and myristic acid (MA; carbon atoms shown as purple spheres). [159] (B) Model of ESA with Cys residues and disulfide bridges marked in orange. Position of a single Cys residue, which is not participating in the formation of a disulfide bridge, is marked with a black dashed circle.

Figure 2. Allergens from the PR-10 family and their ligands. (A) Cartoon representation of Bet v 1 (PDB code: 6R3C) with secondary structure elements marked with distinct colors (α-helices in cyan, β-strands in magenta and loops in salmon). (B) Natural ligands of Bet v 1.0101 (Q3OS) and Cor a1.0401 (Q3O-(Glc)-Gal). [29] (C) Crystal structure of Ara h 8.0101 in complex with epicatechin (PDB code: 4MA6). [62] Epicatechin is shown in stick representation. Purple sphere represents Na^+. (D) Crystal structure of Ara h 8.0101 in complex with quercetin (PDB code: 6AWS).

Figure 3. Allergens from the lipocalin family. (A) Binding of lauric acid to Bos d 5 monomer according to the crystal structure (PDB code: 4IB6).[157] Lauric acid is shown in stick representation. (B) Bla g 4 in complex with tyramine (PDB code: 4N7C).[92] (C) Mus m 1 in complex with a pheromone (PDB code: 1MUP).[89] (D) Complex of Rat n 1 with limonene-1,2-epoxide (PDB code: 2A2G).[88]

Figure 4. Representative structures of nsLTPs showing binding to different ligands. (A) Maize nsLTP in complex with α-linolenic acid (PDB code: 1FK6).[107] (B) Wheat nsLTP in complex with 1-myristoylglycero-3-phosphorylcholine (PDB code: 1BWO).[166] (C) Barley nsLTP forming a covalent complex with (12E)-10-oxooctadec-12-enoic acid (PDB code: 3GSH).[167]

Figure 5. Models of ligand binding proteins from less common allergen families.(A) Crystal structure of Der f 2 with polyethylene glycol (PEG; PDB code: 1XWV). While PEG originates from the

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solution used during crystallization, its presence reveals a large cavity that is used by Der p 2 to bind hydrophobic ligands.[120] (B) Structure of Der p 7 (PDB code: 3H4Z).[134] This allergen was shown to bind the bacterial lipopeptide polymyxin B. (C) Solution structure of Der f 13 (PDB code: 2A0A).[168] Der f 13 is a member of the fatty acid binding protein family. (D) Structure of Bla g 1 in complex with lipids including phosphatidic acid.[28]

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Conflicts of Interest

Dr. Chew reports grants from the National University of Singapore, Singapore Ministry of Education Academic Research Fund, Singapore Immunology Network, National Medical Research Council (NMRC) (Singapore), Biomedical Research Council (BMRC) (Singapore), and the Agency for Science Technology and Research (A*STAR) (Singapore), during the conduct of the study; and consulting fees from Sime Darby Technology Centre; First Resources Ltd; Genting Plantation, and Olam International, outside the submitted work. Dr. Pomésis employed by Indoor Biotechnologies, Inc. and is the contact PI of the 2R01AI077653-10A1 Award from the NIH/NIAID that funds research presented in this article. She declares that her research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.Other authors declare that they have no conflicts of interest. Dr. Rouvinen reports contract research between University of Eastern Finland and Desentum Ltd, outside the submitted work. Dr Rouvinen is also a shareholder in Desentum Ltd.

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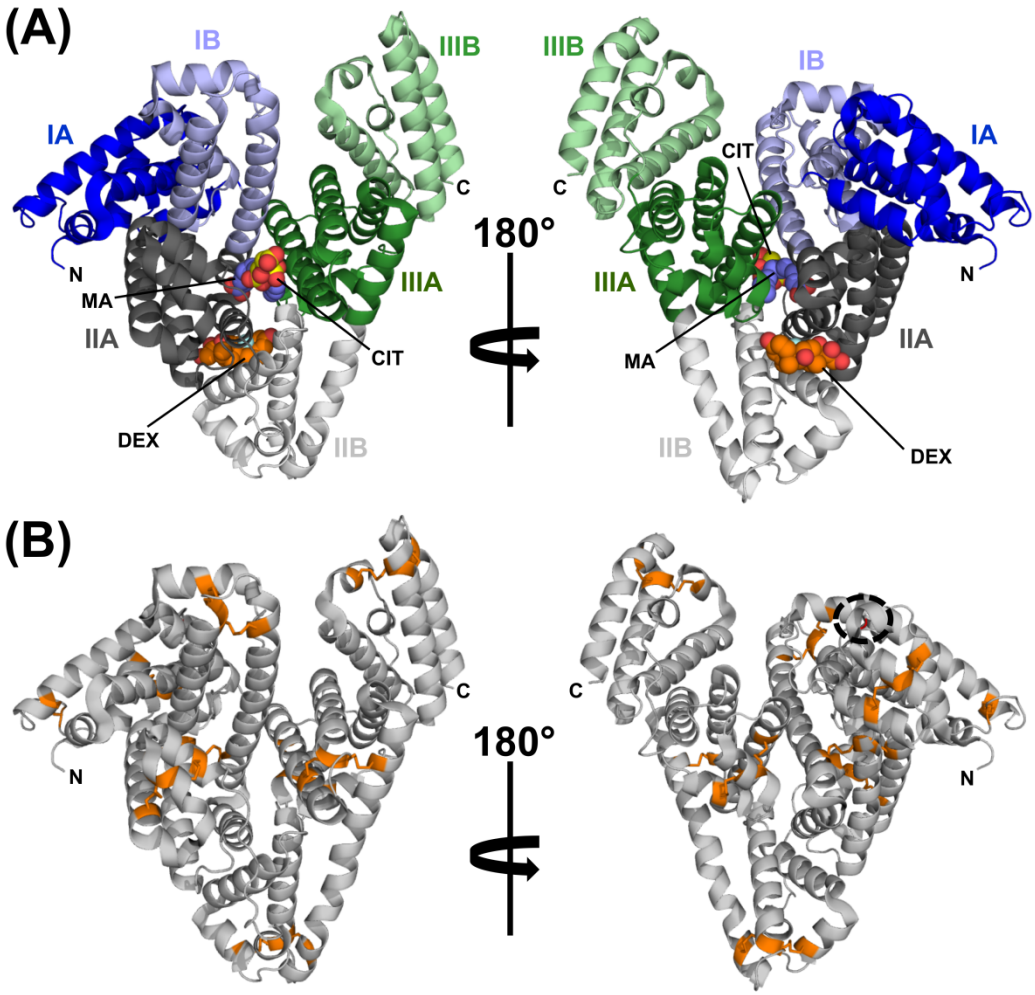

Figure 1

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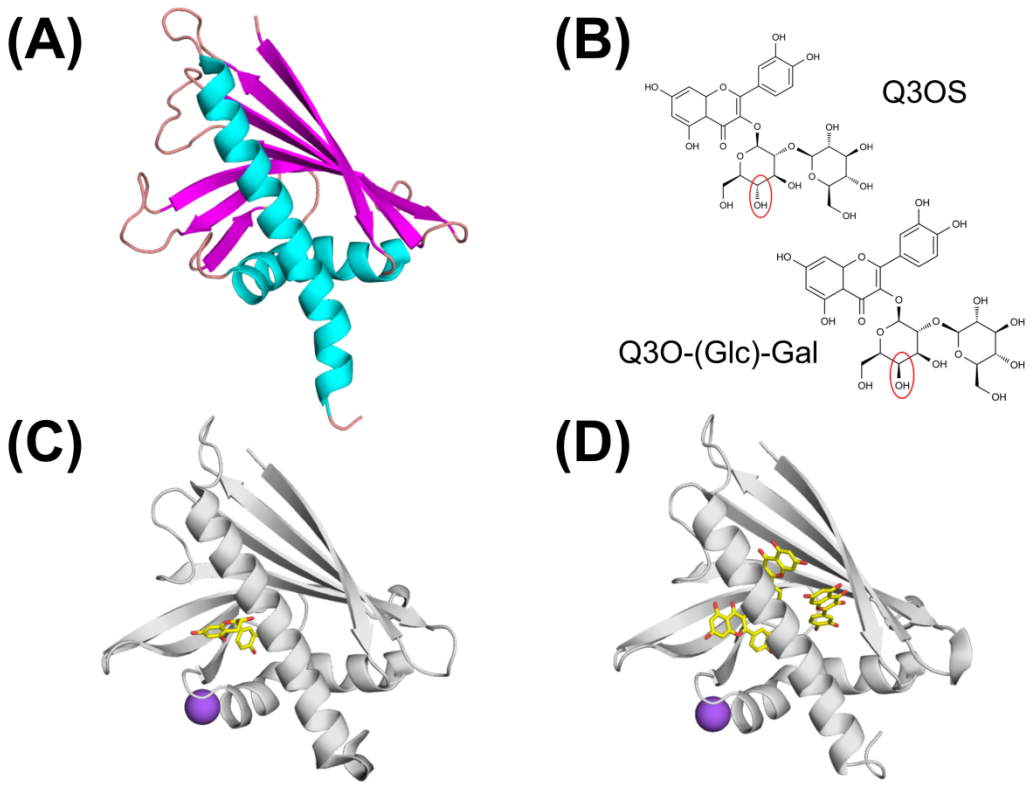

Figure 2

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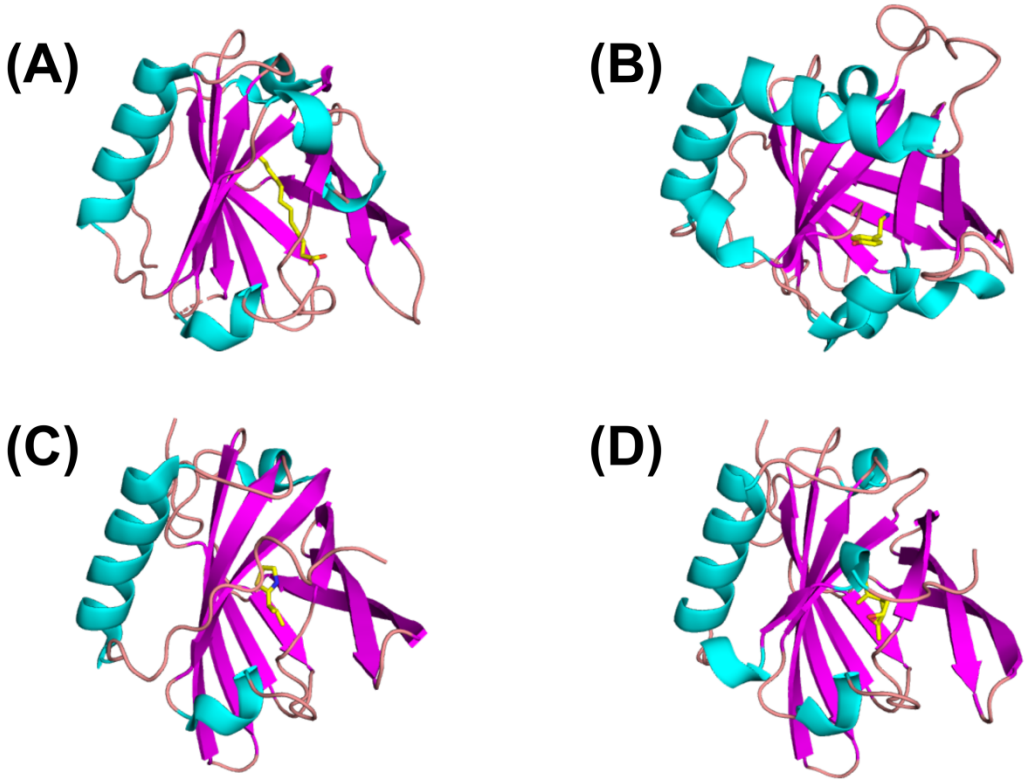

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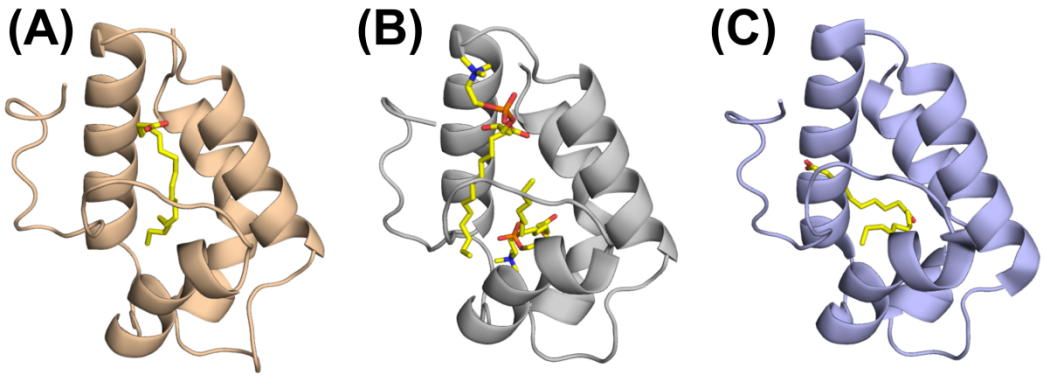

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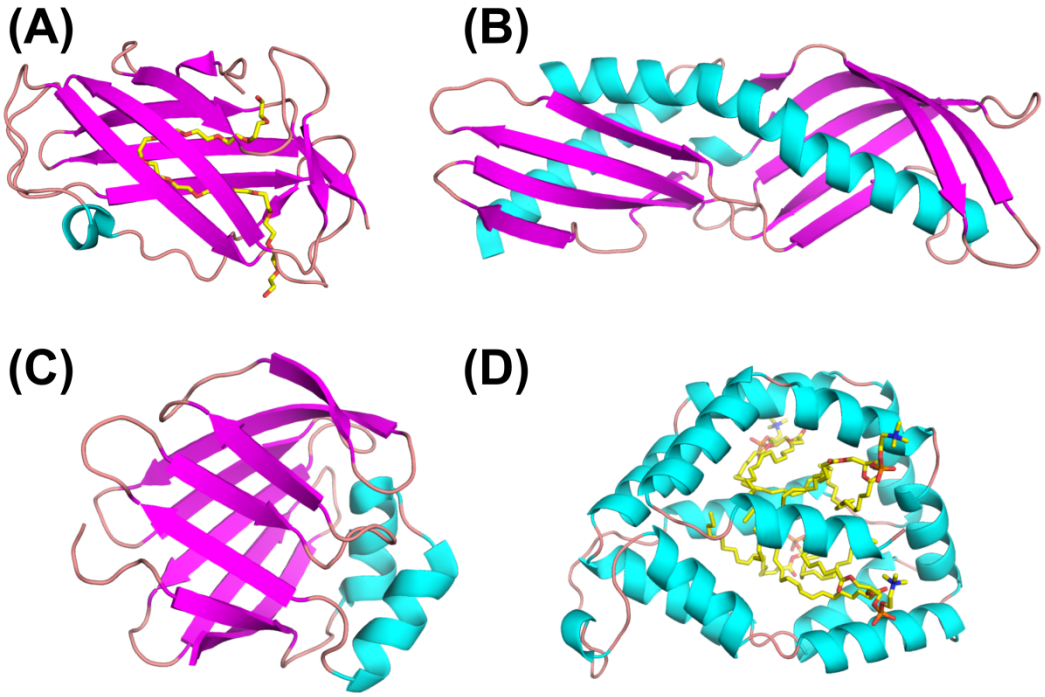

Figure 5

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