

220 million years ago, using amino acid sequences of arginine kinases (Suzuki *et al.*, 1997). Since the sequence identity between Turbinidae and Trochidae myoglobins is very high (84%), the occurrence of IDO-like myoglobin would date back to least this time.

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A Bovine Dander Allergen, Comparative Modeling, and Similarities and Differences in Folding with Related Proteins

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The most important allergenic protein in cow dander and urine is Bos d 2. It is proposed to belong to the family of lipocalins, which are proteins capable of binding small hydrophobic molecules. The allergenic properties of Bos d 2 indicate an interaction between the accessible regions of the native protein and IgE. In this work, a three-dimensional model was created for Bos d 2 by comparative modeling, and features characteristic of outlier lipocalins were observed. The protruding regions of the surface were characterized and used in predicting the possible B-cell epitopes. There is a pocket inside the core and its size is appropriate for small molecules. The model shows a hydrophilic amino acid side chain of glutamic acid 115 on the inner surface of the hole and a phenylalanine as the "gatekeeper" instead of tyrosine, which is common in experimentally modeled lipocalins.

KEY WORDS: Bovine dander allergen; lipocalins; modeling; allergy

1. INTRODUCTION

Although the knowledge of allergens has increased rapidly, it is still unclear why some proteins are allergenic. However, certain common denominators are emerging, e.g., many animal allergens are enzymes or enzyme inhibitors (Thomas, 1993). Another recent observation is that a number of important allergens belong to the lipocalin family of related proteins (Arruda *et al.*, 1995; Gregoire *et al.*, 1996). Dander of several mammalian species contains proteins identified as lipocalin allergens (Ball *et al.*, 1994; Gregoire *et al.*, 1996; Lorusso *et al.*, 1986; Rautianen *et al.*, 1997; Walls and Longbottom, 1985). The most important allergenic protein in cow dander and urine is Bos d 2 (Rautianen *et al.*, 1997;

Ylönen *et al.*, 1992).⁴ An initial sequence analysis suggests that Bos d 2 belongs to the family of lipocalins (Mäntyjärvi *et al.*, 1995).

One characteristic feature of allergens is the interaction of the B-cell epitopes with E antibodies. Structurally, the B-cell epitopes are defined by the accessible regions (Novotny *et al.*, 1986) of the native Bos d 2 and the epitopes provide a structural basis for the triggering of allergic symptoms (Gajhede *et al.*, 1996)).

The lipocalins are molecules characterized by a wide range of different molecular recognition properties: their capability to bind small hydrophobic molecules like pheromones, their binding to specific cell-surface receptors, and their ability to form soluble macromolecular complexes. The different biological functions (e.g., al-

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⁴ Abbreviations: Bos d 2, bovine dander allergen; BDA20, precursor bovine dander allergen 20; MUP, mouse urinary protein; RBP, retinol-binding protein; BBP, bilin-binding protein; OBP—B, odorant-binding protein of bovine; MD, molecular dynamics; SCR, structurally conserved regions.

lergic properties) of lipocalins are likely mediated by one or more of these properties (Flower, 1996), and those functions depend on the 3D structure. The shape and properties of the surface are essential in the formation of soluble macromolecular complexes and in their binding to cell-surface receptors. Thus, it is useful to create a 3D model for Bos d 2, and to use it to characterize the core and to map the protruding regions essential for the allergenicity.

In spite of low identity at the sequence level, the sequential similarity is clearly better and the similarity at the 3D level is usually high (Sivaprasadarao *et al.*, 1993). This together with certain details discussed below convinced us about the possibility to build up a useful model using comparative modeling. For this study the crystal structures of the bovine odorant binding protein (OBP—Bovine; Bianchet *et al.*, 1996; Tegoni *et al.*, 1996), the mouse urinary protein (MUP; Böcskei *et al.*, 1992), the retinol-binding protein (RBP; Newcomer *et al.*, 1984), and the bilin-binding protein (BBP; Huber *et al.*, 1987) all belonging to lipocalins were available. Between these proteins and Bos d 2 the highest identity is to OBP—Bovine protein (31%) and MUP (25%), which are also examples of proteins with low identity (26%; Tegoni *et al.*, 1996), but with rmsd of as good as 1.4 Å for 123 C α -atoms in the central β -barrel region (Bianchet *et al.*, 1996), which is the main structural unit of the lipocalins (Flower, 1996; Sivaprasadarao *et al.*, 1993). In this report we show that the sequence similarity between Bos d 2 and a combination of OBP—Bovine and MUP is partially rather high. The combination sequence is a sequence where the more similar residue is chosen to present and form the new one.

OBP—Bovine has higher similarity than MUP in the barrel region, but its crystal structure differs considerably from MUP and RBP in the tertiary structure and the location of the region formed by helix and the C-terminal region. MUP and RBP are monomeric, but OBP—Bovine forms a dimer (Bianchet *et al.*, 1996; Tegoni *et al.*, 1996). A reason which may explain this is that the region of OBP—Bovine corresponding to the loop 8 is one residue shorter than in MUP, RBP, and Bos d 2. The incomplete loop disturbs the interaction between the strand H and the α -helix, which offers thus a device for the dimerization to the corresponding H strand of the other molecule. The location and folding of the helix and the C-terminal in MUP and RBP but not in OBP—Bovine are also constrained by disulfide bridges (Tegoni *et al.*, 1996). The same loop length of MUP and Bos d 2 and the possibility of a sulfur bridge also in Bos d 2 make MUP a good template for the region formed by the helix and the C-terminal.

2. MATERIALS AND METHODS

The modeling was performed using the Quanta 4.1 software (Molecular Simulations Inc.) running on an IBM RISC 6000 computer. The main templates were the crystallographically determined OBP (entry 1obp) and MUP (entry 1mup) retrieved from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). The sequence of Bos d 2 was aligned against the OBP and MUP by the FASTA method (Lipman and Pearson, 1985). The landmarks in the sequence were used to confirm the reliability of the alignment because of low homology (Taylor, 1988). The final alignment is shown in Fig. 1. The C α coordinates of the residues were copied mainly from OBP—Bovine; the nine N-terminal residues, the loop L3 with residues 62–69, and the C-terminal region from residues 120 to 154 were copied from MUP. The discontinuities in the structure were removed by regularizing. A 1' turn structure of the DG pair in the loop L7 was built up because the DG motif is known to favor type 1' (Sibanda *et al.*, 1989). The side-chain conformations of the identical residues were built to be similar between Bos d 2 and templates. The side-chain coordinates of nonidentical residues excluding prolines were rebuilt up based on the Karplus and Drunbrack rotamer library (Drunbrack and Karplus, 1994) if there was rotamer for the main chain conformation. Otherwise, in a few cases the Ponder rotamer library (Ponder and Richards, 1987) was used.

The model was refined by energy minimization and molecular dynamics using the Charmm force field (Brooks *et al.*, 1983). At the first step Bos d 2 was minimized together with the three water molecules in the core, using the steepest descent method in the vacuum. The COOH of E 115 was protonated because of the core environment, where the pK $_a$ of COOH can be higher than in the pure water. At the second stage, the protein was embedded in the water box with dimensions 49.7, 49.7, and 46.6 Å. After that, the loop L4, the loop L7, the N-terminal, the water molecules, and the side chains were optimized using molecular dynamics at 300 K so that the DG pair of the loop L7 was constrained strongly to the dihedral angles of turn 1'. The system was finally allowed to equilibrate without any constraints for a further 100 psec with coupling to the constant temperature bath of 300 K using the Berendsen algorithm (Berendsen *et al.*, 1984) and 0.1 psec coupling constant.

The equations of motion were solved using the velocity of the leapfrog algorithm of Verlet (1967). The molecular dynamics integrations were performed with time steps of 2.0 fsec. The calculations were conducted with a nonbonded cutoff value of 9 Å. The nonbonded

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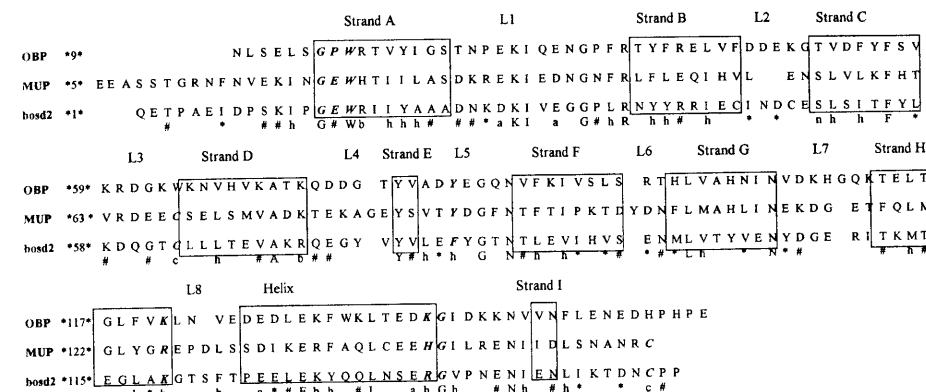


Fig. 1. The alignment of Bos d 2, MUP, and OBP—Bovine. Identical residues in all three proteins are marked by capital letters under the sequence, similar residues in all three proteins are marked by either h (hydrophobic amino acids like V, I, M, A, F, and also Y), a (acidic amino acids like D or E), or b (basic amino acids like K, R, and H) or by n (neutral amino acids like S and T); identity between Bos d 2 and either MUP or OBP—Bovine is marked by #, and similarity between Bos d 2 and either MUP or OBP—Bovine is marked by an asterisk. The landmarks based on the literature in bold and italic.

energies and forces were smoothly truncated using a van der Waals switching function and an electrostatic shifting function. The nonbonded energies were updated at every 20 steps. The SHAKE (Ryckaert *et al.*, 1977) constraint algorithm was used with the tolerance of 0.0004 Å. The simulations in the water box were performed using periodic boundary conditions.

The accessibility of Bos d 2 toward a sphere with radius of 1.0 nm was computed by the Lee and Richards (1971) algorithm under Quanta 4.1. The size of the sphere is comparable to that of antibody-domain dimer (Fv fragment) (Novotny *et al.*, 1986).

3. RESULTS AND DISCUSSION

3.1. Main Structural Features of Bos d 2 and Lipocalins

The sequence of Bos d 2 matches better with the outlier lipocalins than with the kernel lipocalins. The best-matching proteins, the aphrodisin precursor protein (34.4% identity), the bovine odorant-binding protein (OBP—Bovine, 31.4%) and probasin (32.1%) belong to outlier lipocalins, whereas, for example, MUP (24.7%) and retinol-binding protein (below 10%) are typical kernel lipocalins (Flower, 1996).

The 2D representation of the secondary structure of lipocalins has been given by Flower (1996). A β -barrel with eight antiparallel β -strands folding into an orthog-

onal calyx forms the main structural unit of the lipocalins (Flower, 1996; Sivaprasadarao *et al.*, 1993). The lipocalins share sufficient similarity in the form of short characteristic conserved sequence motifs and they are thought to consist of three structurally conserved regions (SCR). The first conserved motif, SCR1, is located near to the N-terminal and it contains a 3_{10} -helix with the adjacent β -strand, which leads to the loop L1 (the notation according to Flower, 1996). The strands F and G with the loop between them form the second conserved motif, SCR2. The third conserved motif, SCR3, is the strand H and a part of the loop linking it to the C-terminal helix. Almost all the lipocalins share the conserved motifs SCR1 and SCR2. Most of the lipocalins contain also the motif SCR3; they are classified as kernel lipocalins. The lipocalins with more divergent sequences, outlier lipocalins, match at the sequence level much less with the three motifs or only with one or two of the motifs (Flower, 1996). The identity between Bos d 2 and MUP, which is a kernel lipocalin, in SCR1 is 40%, in SCR2 is 17.6%, but in SCR3 is 0%. Because of the low identities of the SCR2 and SCR3, Bos d 2 should be classified with the outlier lipocalins.

3.2. The Alignment

The secondary structures are shown in the alignment as named according to Flower (1996). The com-

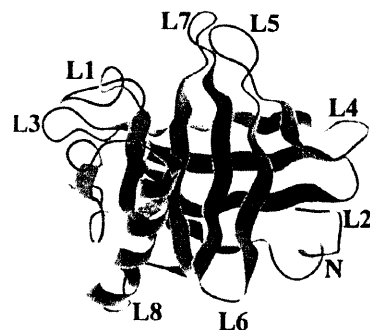


Fig. 2. The overall folding of Bos d 2. The protruding regions are shown by letters. N, N-terminal; C, C-terminal; loops are marked based on the Flower notation.

parison of the sequences of Bos d 2 and MUP and OBP—Bovin was done by finding apparent similarities based on the following rules: residues are (1) identical in all three proteins, (2) similar in all three proteins, (3) identical between Bos d 2 and either MUP or OBP—Bovin, and (4) similar between Bos d 2 and either MUP or OBP—Bovin. If at least one of the rules was satisfied it was regarded as apparent similarity. This analysis showed high apparent similarity concerning the main secondary structures. Apparent similarities are 81.8% in the strand A, 84.6% in the loop L1 (13 residues), 50.0% in the strand B, 62.5% in the strand C, 44.4% in the strand D, 100% in the strand E, 75.0% in the strand F, 62.5% in the strand G, 66.7% in the strand H, 71.4% in the helix, and 75.0% in the last strand of the C-terminal. Some landmarks which are commonly found in lipocalins are given in bold and italic in the alignment (Fig. 1), like the most conserved sequence motif in lipocalins, the GXW triplet. The tryptophan is completely invariant in the motif and the region has a similar characteristic conformation in all the lipocalins. The tryptophan has also been proposed to play a role in the triggering mechanism of the ligand binding (Sivarasadao *et al.*, 1993). Further, a sulfur bridge has been found in some lipocalins (Böcskei *et al.*, 1992; Newcomer *et al.*, 1984) and a sequence comparison reveals the possibility of a sulfur bridge between C(63) and C(154) in Bos d 2, and similarly with MUP, but not with OBP—Bovin. Bos d 2 has F(82) as the so-called gatekeeper. The phenomenon is not unique; sequence analysis reveals phenylalanine as a gatekeeper also in aphrodisin. It has been proposed (Huber *et al.*, 1987) for BBP that the ligand can leave the pocket only

if the tyrosine aromatic ring swings out. In the crystal structure the hydroxyl group forms a hydrogen bond with the ligand (Huber *et al.*, 1987) or the water (Böcskei *et al.*, 1992). A detailed analysis shows that there is a conserved basic residue (K in Bos d 2 and OBP—bovin and R in MUP) in the end of the strand H. This position is occupied by K or R not only in lipocalins, but throughout the calycin protein superfamily, which consists of lipocalins, fatty acid-binding proteins, and avidins (Flower, 1995). The helix of Bos d 2, like those of the other lipocalins, have many of the attributes of a good helix. There are glycines as the C-cap for Bos d 2 [G(139)], OBP—Bovin, and MUP (Fig. 1), as in 34% of helices, and a basic residue as a stop signal just before the C-cap (Richardson and Richardson, 1988).

Modeling of the β -barrel region is based mainly on OBP—Bovin because of the better homology. However, the length of the loop 8 and the existence of sulfurs in correct positions (sulfur bridge) suggest that the C-terminal of Bos d 2 is similar to MUP and RBP. It has been shown that the location of the C-terminal in MUP and RBP is constrained by the sulfur bridge and stabilized by the elongated loop 8 (Tegoni *et al.*, 1996). On the basis of the sequence comparison both of them seem to exist also in Bos d 2. The C-terminal of OBP—Bovin participates in dimerization (Tegoni *et al.*, 1996). Bos d 2 is a monomer, suggesting that the C-terminal is not located in the same way as in OBP—Bovin. However, OBP—Bovin is also a monomer at acidic conditions, behaving as a globular protein in chromatography, suggesting that it is fully folded (Tegoni *et al.*, 1996), perhaps in the same way as RBP, MUP, and Bos d 2.

3.3. Model of Bos d 2

The secondary structures concerning the main β -barrel and the α -helix region are preserved well during the simulation. The rms deviation was ca. 2.2 Å for all the atoms and ca. 1.7 Å for the C_α -atoms and slowly reaches an apparent plateau. The rms deviation of similar types of residues between the final structure of Bos d 2 and the X-ray structure of MUP was 1.1 Å. During the dynamics calculation, the radius of gyration varied only slightly around 16.83 Å. For the last structures, the most of the ϕ - ψ combinations were in the allowed region with only four exceptions.

A cartoon model of Bos d 2 after the total dynamics and minimization is shown in Fig. 2. It shows the global folds and the protruding regions of Bos d 2. The core is occupied mainly by hydrophobic and neutral hydrophilic residues and the modeling leads to a structure where the

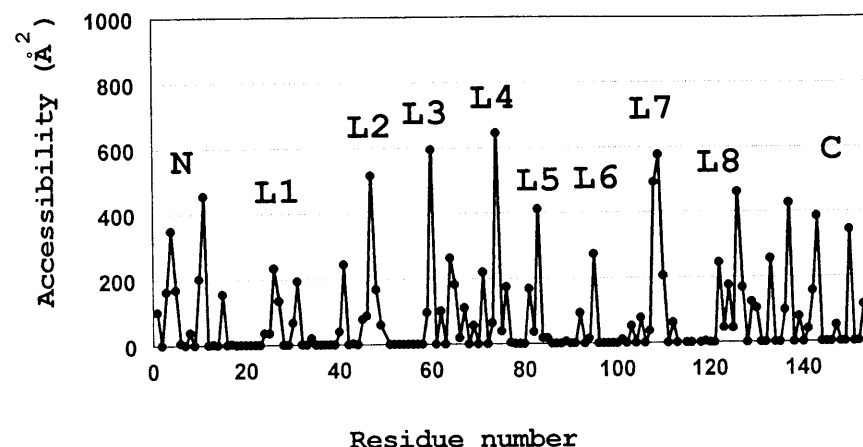


Fig. 3. Accessibility of residues of Bos d 2 (after dynamics and minimization) computed by the Lee and Richards algorithm using 1.0-nm sphere radius.

side chain of E(115) in the strand H is oriented toward the core, so that the carboxylic group sticks into the pocket forming a natural docking site for the ligand. The pocket inside is appropriate for small organic molecules.

3.4. B-Cell Epitopes

The accessible regions can be responsible for the contact with immunoglobulins and it may be possible to influence the allergenic properties of Bos d 2 by mutating these regions. The most probable B-cell epitopes can be deduced by comparison of the accessible regions of MUP and Bos d 2. They are both allergens (Lorusso *et al.*, 1986; Rautiainen *et al.*, 1997), in contrast to OBP—Bovin, which has not been proved to be either allergenic or nonallergenic.

The plot of accessibility versus residue number of MUP is not shown, but is very similar to that of Bos d 2 in Fig. 3. The contact area of IgE can be 700 Å² (Davies *et al.*, 1990), which means that some loops or regions can simultaneously contact with IgE if they are spatially close enough. The model (Fig. 2) shows five such regions: the L1–L3–C-terminal region, the L5–L7 region, the L2–L4–N-terminal region, the L6–L8 region, and the α -helix region. The most accessible regions are the loops L5–L7 and L2–L4. However, at the sequence level the loops L2, L4, and L6 differ most between MUP and Bos d 2, suggesting that these regions are less likely

to be the cause of allergic symptoms. Also the N-terminal is close to these regions, is partly disordered, and has only a low sequential similarity between the two allergens. This means that the most probable B-cell epitopes are the L5–L7, L1–L3–C-terminal, and the helix regions. When Bos d 2 and MUP are compared seven of the eight accessible residues are similar in L1, none of three in L3, three of five in L5, one of four in L7, four of eight in the C-terminal, and six of eight in the helix. The most similar accessible regions are thus the loop L1, the α -helix, and the C-terminal. These are also structurally similar in MUP and Bos d 2. A sequence comparison reveals that these regions are quite similar also in major horse allergen (Gregoire *et al.*, 1996) sequentially and probably also structurally. Because of the characteristic unusual loop structure and the high accessibility similarity between Bos d 2 and MUP, the L1–L3–C region or a part of it is a potential and attractive choice for B-cell epitope.

4. CONCLUSIONS

Many common features of Bos d 2 with the outlier lipocalins lead to conclusion that Bos d 2 belongs to outlier lipocalins rather than to kernel lipocalins. In spite of uncertainties in the modeling, the model contains many interesting details and provides a good starting point for understanding the allergenic properties of Bos

d 2, offering an opportunity for immune response modulation with recombinant derivatives with reduced allergenic properties.

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Podophyllotoxin and Nocodazole Counter the Effect of IKP104 on Tubulin Decay

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Tubulin, the subunit protein of microtubules, undergoes a time-dependent loss of functional properties known as decay. We have previously shown that the drug 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone (IKP104) accelerates decay, but that in the presence of colchicine, IKP104 becomes a stabilizer of tubulin. To see if this is due to conformational effects specific to colchicine or simply to occupancy at the colchicine site, we examined the effects of nocodazole and podophyllotoxin, two well-known competitive inhibitors of colchicine for binding to tubulin, on IKP104's acceleration of decay. We found that podophyllotoxin abolished IKP104's accelerating effect and, like colchicine, turned it into a stabilizer of tubulin. Nocodazole's effects were similar to those of podophyllotoxin and colchicine, in that it abolished IKP104-induced enhancement of decay; however, in the presence of nocodazole, IKP104 caused little or no stabilization of tubulin. Since colchicine, nocodazole, and podophyllotoxin have very different interactions with tubulin, but all inhibit the IKP104-induced enhancement of decay, our findings suggest that this inhibition arises from occupancy of the colchicine site rather than from a direct conformational effect of these two drugs.

KEY WORDS: Tubulin; microtubule; IKP104; podophyllotoxin; BisANS; sulfhydryl groups.

1. INTRODUCTION

Microtubules, organelles performing a variety of critical functions, are composed of the protein tubulin, a heterodimer of two subunits called α and β (Dustin, 1984). The tubulin molecule is unusual in that it is prone to decay, a process whereby it loses its ability to assemble and to bind to ligands such as colchicine (Wilson, 1970). Decay is accompanied by an increased exposure of sulfhydryl groups and hydrophobic areas (Ludueña and Roach, 1981a; Prasad et al., 1986). The decay process

is strongly influenced by a variety of structurally different ligands which bind to tubulin and inhibit microtubule assembly (Ludueña and Roach, 1991). Of these ligands, one group (colchicine, podophyllotoxin, vinblastine, phomopsin A, and ustiloxin A, among others) inhibit decay (Ludueña and Roach, 1981a; Prasad et al., 1986; Ludueña et al., 1989, 1992, 1994). Others (maytansine and spongistatin 3) have no effect on decay (Prasad et al., 1986; Ludueña et al., 1995a). Some ligands, such as halichondrin B, accelerate decay (Ludueña et al., 1993).

Of the tubulin ligands, the one whose effect is most complex is 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone (IKP104).⁵ IKP104 binds very tightly to tubulin, probably at the vinblastine site (Mizuhashi et al., 1991,

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⁵ Abbreviations: BisANS, bis-5,5'[(8-*N*-phenyl)aminonaphthalene-1-sulfonic acid]; IKP104, 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone.