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Three Odorant-binding Proteins from Rabbit Nasal Mucosa

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Abstract

Following the purification of an odorant-binding protein (OBP) from rabbit nasal mucosa, we have identified, purified and partially characterized two additional OBPs from the nasal tissue of the same animal species. OBP-II is a monomer of 21 kDa and isoelectric point 4.2; OBP-III is a dimer with subunits of 23 kDa and isoelectric point 4.8. Like OBP-I, both these new members bind the odorant 2-isobutyl-3-methoxypyrazine. The partial amino acid sequences of the three OBPs, determined by Edman degradation, confirm that they are members of the OBP family, but reveal poor similarity between them. However, higher similarity is found between each OBP and other members of the lipocalin family. In particular, OBP-I is most similar to bovine OBP (55% identity in the N-terminal region), OBP-II is >50% identical, limited to its first 18 amino acids, to mouse OBP-I and porcupine OBP-II, while OBP-III shares 26 out of the first 40 amino acids with major urinary protein (MUP) 4, a member of the mouse salivary proteins. The possible role of these proteins in olfactory transduction is also discussed. **Chem. Senses 22: 383–390, 1997.**

Introduction

Odorant-binding proteins (OBP) are the only proteins of the olfactory system for which binding of odorant molecules has been experimentally demonstrated (Pelosi *et al.*, 1982; Bignetti *et al.*, 1985; Pevsner *et al.*, 1985; Pelosi, 1994, 1996). In vertebrates, they are synthesized by glands of the olfactory or nasal respiratory epithelium and secreted in high concentration in the nasal mucus. During the last decade, amino-acid sequence information and binding data on several OBPs, purified from different vertebrate species (Pevsner *et al.*, 1986; Lee *et al.*, 1987; Dal Monte *et al.*, 1991; Pes *et al.*, 1992; Felicioli *et al.*, 1993; Pes and Pelosi, 1995; Ganni *et al.*, 1997), have been made available and provide the necessary basic information for further understanding the mode of action and the physiological function of OBPs.

The complete amino acid sequences of six OBPs are known (Lee *et al.*, 1987; Pevsner *et al.*, 1988; Tirindelli *et al*, 1989; Dear *et al.*, 1991; Miyawaki *et al.*, 1994), while for the others short sequence segments at or near the amino terminal are known (Pelosi, 1994; Pes and Pelosi, 1995; Ganni *et al.*, 1997). These data indicate that OBPs appear to be poorly conserved during evolution, with similarity values seldom exceeding 50% of identical amino acids.

In recent years evidence has been accumulating that confirms the presence of several classes of OBPs in the same animal species. In the rat two different sequences have been described (Pevsner *et al.*, 1988; Dear *et al.*, 1991), while in the mouse we have been able to purify four different odorant-binding proteins, one of them being a heterodimer

(Pes and Pelosi, 1995), and determine the five relative partial sequences. In the porcupine eight protein species have been isolated and divided, on the basis of their partial amino acid sequences, into two classes (Felicioli *et al.*, 1993; Ganni *et al.*, 1996). Although current information on different types of OBPs in the same animal species is limited, OBPs may be tentatively segregated into sub-classes. Proteins of the same sub-class, purified from different species, show higher similarity than proteins of the same species.

In the rabbit we have described and purified a protein that binds 2-isobutyl-3-methoxypyrazine with a dissociation constant of 0.8 μM and is present, in the native state, as a homodimer of 19 kDa subunits (Dal Monte *et al.*, 1991).

This paper now reports the discovery and purification of two additional OBPs from rabbit nasal tissue and comparative partial sequence information for the three OBPs so far identified.

Materials and methods

Materials

Rabbit heads were kindly provided by a local abbattoir and dissected within an hour after death or kept at –20°C for a few days. Total nasal mucosa (olfactory and respiratory) was used for the experiments. Tritiated 2-isobutyl-3-methoxypyrazine was prepared by tritium exchange, as reported (Pelosi *et al.*, 1981), and had a specific activity, at the time of this work, of about 0.85 Ci/mmol. Mono-P column, Polybuffer, Ampholines and Superose-12 were from Pharmacia/LKB (Uppsala, Sweden). Endoproteinase Glu-C was from Boehringer (Mannheim, Germany). Solvents and reagents for Edman sequential degradation were from Sigma or Aldrich, of 'sequencing' grade. All other chemicals were of reagent grade.

Preparation of the extract

The crude extract was prepared by homogenization of total nasal mucosa in 20 mM Tris–HCl pH 7.4 (Tris buffer), using a Polytron homogenizer, followed by centrifugation at 20 000 *g* for 20 min. The clear supernatant was immediately used in the subsequent fractionation steps.

Purification of the binding proteins

In a typical experiment, 20 ml of extract, obtained from total nasal tissue of 10 rabbits, was chromatographed through a 1.5 × 25 cm Whatman DE-52 column. Elution was performed, using a linear 0.1–0.4 M NaCl gradient, in Tris buffer. Each fraction was analysed using 12% SDS–PAGE and assayed for binding activity to the tritiated pyrazine. The fractions containing the proteins of interest were pooled, dialysed against 20 mM Tris buffer, applied to a Mono-Q HR 5/5 anion-exchange column and eluted with a linear 0–0.5 M NaCl gradient, in Tris buffer. Final purification was accomplished by gel filtration through a 1 × 30 cm Superose 12 column, in 50 mM ammonium bicarbonate.

Molecular weights of native proteins were evaluated by gel filtration on a 1 × 30 cm Superose 12 column, using bovine serum albumin, carbonic anhydrase and cytochrome *c* as markers.

Gel electrophoresis

Electrophoresis in denaturing conditions was run on 12% polyacrylamide gel, in the presence of 1% SDS, using a Bio-Rad Mini-Protean II apparatus and according to the procedure of Laemmli (1970).

Isoelectric focusing

Isoelectric focusing was performed in denaturing conditions on 5% polyacrylamide gel, in a gradient of ampholines (pH 3.5–9.5) and using a Bio-Rad Mini IEF Cell, Model 111.

Binding assay

Binding to tritiated 2-isobutyl-3-methoxypyrazine was measured using the filtration assay of Bruns *et al.* (1983), as previously described (Dal Monte *et al*, 1991). Non-specific binding was measured in the presence of a 1000-fold excess of the cold ligand and accounted for <5% of total binding.

Amino acid sequence determination

Partial N-terminal amino acid sequences were determined by direct Edman degradation in a Milligen 6600 apparatus. Samples of purified proteins (200–500 pmol) in ammonium bicarbonate, as obtained from the Superose-12 column, were freeze-dried, solubilized in 5% *N*-methylmorpholine in isopropanol/water 1/1, immobilized on *p*-phenylene-diisothiocyanate (DITC) activated membranes and subjected to automatic sequential degradation.

DITC membranes were prepared by treating glass fibre GF/F disks with 1% 3-aminopropyltrimethoxysilane in toluene at 50°C for 2 h and then, after repetitive washing in

toluene, with 0.2 M DITC in tetrahydrofuran at room temperature for 2 h.

Sequence determinations were generally repeated twice or three times either on the same samples or on samples obtained from different purification batches.

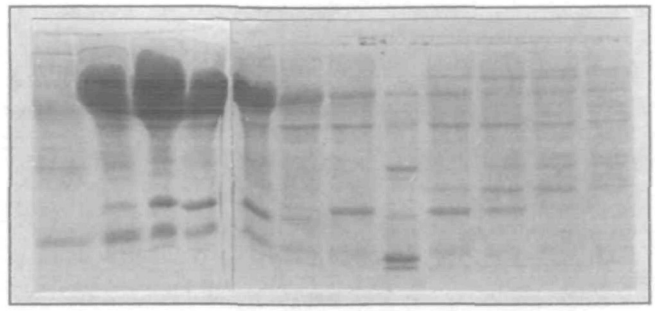
A sample of OBP-I, which was refractory to direct Edman degradation, was digested with endopeptidase Glu-C, by incubating the protein in 50 mM phosphate buffer, pH 7.4, at a ratio of 100:1 with the enzyme, for 30 min at 37°C.The digestion product was subjected to Edman degradation without further purification and afforded a single amino acid sequence.

Results

The observations that led us to the discovery of two additional OBPs were based on measurements of binding activities of protein fractionated by anion-exchange chromatography.

The protocol used for purifying the proteins involved the use of anion-exchange chromatography on DE-52 and Mono-Q to separate the three OBPs from one another, followed by a gel filtration on Superose-12 to yield electrophoretically pure samples of the three proteins.

A first fractionation of a crude extract by anion-exchange chromatography on a DE-52 column indicated the presence of several proteins migrating on with apparent molecular masses in the region of 20 kDa (Figure 1, top). When the fractions containing these small proteins were tested for binding activity to tritiated 2-isobutyl-3-methoxypyrazine, the odorant currently used to detect OBPs, broad peaks of binding were measured. However, in several experiments, repeated in the same conditions and with comparable samples, although the electrophoretic patterns were highly reproducible and consistent, the binding profiles showed some variability. Figure 1 shows the electrophoretic analysis in denaturing conditions of the fractions obtained, together with the binding activity of the same fractions to tritiated 2-isobutyl-3-methoxypyrazine (solid line). A broad peak of radioactivity is associated with fractions 33–41, containing, among others, a protein of 21 kDa. The previously described 19 kDa OBP-I (Dal Monte *et al.*, 1991), eluted in this preparation in fractions 29–33, did not show appreciable binding activity. In another preparation (dotted line of Figure 1, bottom), a peak of binding, corresponding to fraction 33, seems to be due to this protein, while the



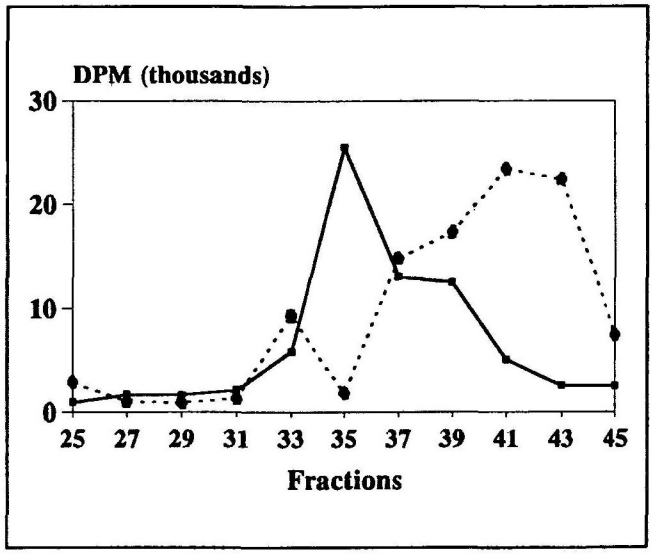


Figure 1 Chromatographic separation of a crude extract of rabbit nasal tissue by anion-exchange on DE-52. The fractions obtained were analysed by 12% SDS–PAGE (top) and assayed for binding activity to tritiated 2-isobutyl-3-methoxypyrazine (bottom, solid line). In a second experiment, performed in the same conditions (SDS–PAGE not shown), the binding assay gave a different pattern (bottom, dotted line), with the main peak of activity associated to a 23 kDa electrophoretic band. Molecular weight markers (M) are, from the top: bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

main binding activity was associated with a third protein species, eluted later in the gradient and migrating with an apparent molecular weight of 23 kDa (fractions 41–43 of Figure 1, top). An exact correspondence between peaks of binding activity and maximal intensities of protein bands was seldom observed. This puzzling phenomenon could be explained by assuming interference from other protein species, still present at this stage in high concentration, or some influence of OBPs on each other.

We prepared purified samples of these three proteins in order to characterize them further.

The 19 kDa OBP-I was obtained electrophoretically pure from fractions 26–33 (Figure 1) by anion-exchange

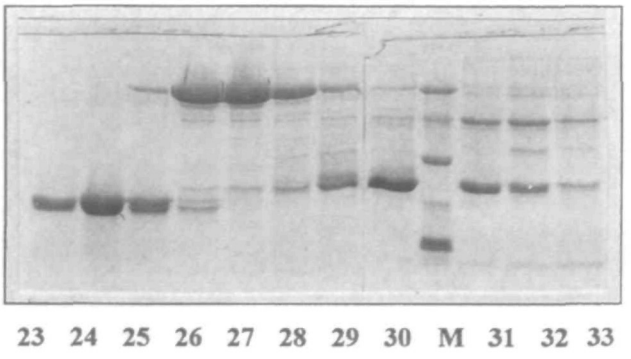


Figure 2 Anion-exchange chromatography on Mono-Q of fractions 37–45, obtained from the DE-52 separation of Figure 1. OBP-II was obtained electrophoretically pure in fractions 23 and 24, while OBP-III (fractions 29–32) still contained major impurities. Molecular weight markers (M) are, from the top: bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

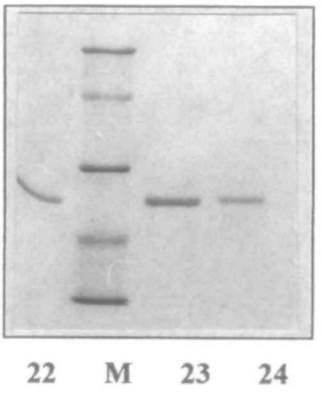


Figure 3 Purification of OBP-III by gel filtration on Superose 12 of fractions 29–32, obtained from the Mono-Q chromatography of Figure 2. SDS–PAGE of the fractions shows that the purified protein is eluted in fractions 22–24. The elution volume corresponds to a molecular weight in native conditions of 45 kDa, indicating the dimeric nature of the protein. Molecular weight markers (M) are, from the top: bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

chromatography on Mono-Q, followed by gel filtration on Superose-12, and used for determination of its amino acid sequence.

The purification of the 21 kDa (OBP-II) and the 23 kDa (OBP-III) proteins was accomplished along similar protocols. Accordingly, fractions 37–45 of the DE-52 chromatography (Figure 1) were again fractionated on a Mono-Q column (Figure 2). This chromatographic step afforded OBP-II electrophoretically pure in fractions 23–24, while OBP-III was finally purified by gel filtration of fractions 22–24 through Superose 12 (Figure 3). A sample of OBP-II was also chromatographed through Superose 12 in order to evaluate its molecular weight in native conditions. We obtained a value of 25 kDa for OBP-II and 45 kDa for OBP-III, indicating that the first protein is a monomer, while the second is present as a homodimer, at least in the conditions of the experiments.

Table 1 Chemical characteristics of the three OBPs so far purified from rabbit nasal mucosa

Protein	Mol. wt (kDa) SDS–PAGE	Mol. wt (kDa) Gel filtration	p*I*
OBP-I	19	34	4.6
OBP-II	21	25	4.2
OBP-III	23	45	4.8

OBP-I and OBP-III are present in solution as homodimers, while OBP-II is a monomer. All three proteins bind reversibly the odorant 2-isobutyl-3-methoxypyrazine. OBP-I had been previously described (Dal Monte *et al.*, 1991).

Isoelectric points of the purified proteins were measured by isoelectric focusing in a gradient of ampholines, as described in Materials and methods. Table 1 summarizes the chemical characteristics of the three OBPs so far purified from the rabbit.

Amino acid sequence determination was attempted on purified samples of the three OBPs by direct Edman degradation. OBP-I was refractory to sequential degradation, indicating the presence of a covalent modification at the terminal amino group, similar to bovine OBP, whose N-terminal is acetylated (Tirindelli *et al.*, 1989). Consequently, a sample of the protein was treated with the proteolytic enzyme Glu-C, that cleaves peptide bonds at the carboxy site of glutamic acid, in the conditions described. The crude product was employed for sequence determination. We obtained a single sequence of 22 amino acids (Figure 4), relative to a region very close to the amino terminal, as indicated by a comparison with the sequences of other OBPs.

The two other proteins had their amino terminal free or only partially blocked, so that we were able to determine the first 18 amino acids for OBP-II and the first 40 residues for OBP-III (Figure 4).

A comparison between any pair of these three sequences shows only poor similarity: OBP-I shares only 24% of its amino acids with OBP-II and 18% with OBP-III, while between OBP-II and OBP-III identity is 41%.

When compared with the sequences of other OBPs and lipocalins involved in chemical communication, interesting homologies become evident (Table 2). Thus, OBP-I is most

Protein	Aminoacid Sequence	No.
rab-OBP-I	-EQLIELVGPWKTVYIVHFNGET-	22
bov-OBP	AQEEEAEQNLSELSGPWRTVYIGSTNPEKIQENGPFRTYFRELV-	
rab-OBP-II	VDPAQVSGXWRTAAIASD-	18
rat-OBP-I	AHHENLDISPSEVNGDWRTLYIVADNVEKVAEGGSLRAYFQHME-	
rab-OBP-III	HSHSEVSQISGEWYSVLLASDHREKIEYNGSMRVFVEYIH-	40
mus-MUP4	EEATSKGQNLNVEKINGEWFSILLASDKREKIEEHGSMRVFVEHIH-	

Figure 4 Partial amino acid sequences of the three rabbit OBPs, compared with those of other OBPs and lipocalins involved in chemical communication. The sequence of OBP-I was obtained after selective hydrolysis with endoproteinase Glu-C, as the amino terminal group was covalently modified. The sequences of rabbit OBP-II and OBP-III are those of the N-terminal Similarity between the three rabbit OBPs is much lower than with other lipocalins Bovine OBP, rat OBP-I and the mouse urinary protein MUP4 were found to be most similar to each of the rabbit OBPs

closely related to bovine OBP, with 55% identity; rat OBP-I and mouse OBP-I are also significantly similar, with ~40% identity, while the similarity with other OBPs is very poor and barely significant. Of interest is the relatively high number of amino acids (eight out of 22) shared with the hamster aphrodisin, a protein with pheromonal activity, present in vaginal discharge (Henzel *et al.*, 1988; Singer and Macrides, 1993). OBP-II seems to be most similar to OBP-I and OBP-II of the mouse and OBP-II of the porcupine, with >50% identical amino acids. A number of other OBPs and lipocalins share significant similarity with this protein. Rabbit OBP-III, instead, seems to be more similar to a group of proteins related to the urinary proteins. These proteins, called major urinary proteins (MUPs), were first described in the urine of mouse (Finlayson *et al.*, 1965) and rat (Dinh *et al.*, 1965) and later in the saliva of the mouse (Shaw *et al*, 1983; Shahan *et al.*, 1987). They are carriers for volatile pheromones (Bacchini *et al.*, 1992; Robertson *et al.*, 1993) and exhibit themselves pheromonal activity. The MUP4, a representative example of the mouse salivary proteins, shares 65% of its amino-terminal residues with OBP-III of rabbit. Other proteins, with more than 50% identity with OBP-III include the mouse OBP-III and OBP-IV, as well as OBP-I of the porcupine.

The rat VEG protein, secreted by the von Ebner gland of circumvallate papillae (Schmale *et al.*, 1990), is much more similar to OBP-II than to the other two rabbit OBPs. This feature is also shown by rat OBP-II, a protein whose similarity with the VEG protein had been already emphasized (Dear *et al.*, 1991).

Table 2 Similarity of the three rabbit OBPs with OBPs of other mammalian species and representative lipocalins involved in chemical communication

	rab OBP-I	*rab* OBP-II	rab OBP-III
bov OBP	55	41	30
rat OBP-I	40	47	22
rat OBP-II	23	41	13
mus OBP-Ia	39	54	36
mus OBP-Ib	38	41	46
mus OBP-II	32	53	28
mus OBP-III	14	29	57
mus OBP-IV	16	33	57
hys OBP-I	18	47	50
hys OBP-II	14	53	37
mus MUP4	14	29	65
ham APHR	36	47	35
rat VEG	14	41	27

The values indicate percent of identical amino acids, relative to the amino-terminal sequences reported in Figure 6. MUP, major urinary protein, APHR, aphrodisin, VEG, von Ebner gland, *bov*, bovine, *mus*, mouse, *hys*, porcupine, *ham*, hamster

Discussion

The data clearly indicate that in the rabbit at least three distinct OBPs are expressed in the nasal tissue. All three proteins show good binding activity toward the odorant 2-isobutyl-3-methoxypyrazine. The partial amino acid sequences, determined for the three isolated polypeptides, indicate that the three proteins belong to the lipocalin family, all presenting the typical 'signature' -G-X-W- near the amino terminal.

Similarity between these OBPs is rather poor. However, when compared with OBPs from other animal species or with other members of the lipocalin family, each exhibits higher similarity values to individual members with specific properties.

The great differences in the amino acid sequences of the three OBP, although limited to their N-terminal region, clearly indicate that they are not the product of single statistical mutations, but derive from distinct genes, that have evolved along divergent lines. Moreover, the sequence similarity with some OBPs of other animal species suggests the existence of subclasses of OBPs, as previously demonstrated only in insect OBPs (Vogt *et al.*, 1991; Breer *et al.*, 1992; Krieger *et al.*, 1993; Pelosi and Maida, 1995). The OBPs of Lepidoptera can be easily segregated into three subtypes, based on the very high similarity values between members of each subclass. In vertebrates OBPs appear to be less conserved than within Lepidoptera species. However, the sequence information, currently being accumulated, indicates that subclasses may exist among the OBPs of vertebrates, with percent of identical amino acids of the order of 50–80% between members of the same class in different animal species and values much lower, down to ≤20% between OBPs of the same species.

Some OBPs are significantly similar to other proteins of the lipocalin family involved in chemical communication. In the rabbit, OBP-III shows highest similarity with members of the mouse urinary proteins (65% of identical amino acids with MUP4), while the two other OBPs do not seem to be significantly related to this class of proteins. This may suggest similar functions for OBPs and urinary proteins, in binding volatile compounds of closely related structures. This idea is supported by the observation that the mouse urinary proteins bind several odorants with specificities similar to OBPs (Cavaggioni *et al.*, 1990). In the mouse we have already observed that two OBPs, purified from nasal tissue (OBP-III and OBP-IV), are very similar (or perhaps identical in one case) to proteins of the MUP family (Pes and Pelosi, 1995). Among these, the closest in sequence to the OBPs are those synthesized by the salivary glands. The physiological function of these proteins, called MUPs only on the basis of their similarity with the MUPs present in the urine, has not been defined. While the MUPs present in the urine have been shown tightly to bind volatile compounds of pheromonal significance (Bacchini *et al.*, 1992; Robertson *et al.*, 1993), for the salivary MUPs no ligand has yet been discovered, although a role for saliva in chemical communication of mice has been reported (Marchlewska-Koj *et al*, 1990). In the pig it is well known that the main sex pheromones, 5α-androst-16-en-3-one and related compounds, are carried in the saliva by specific binding proteins synthesized by the submaxillary gland (Booth and White, 1988; Marchese and P. Pelosi, unpublished data).

It is possible that similar or even identical proteins could be used to transport the same chemical signals both in systems for releasing the chemical messages (urine or saliva) and in those used for perceiving them (olfactory organs). If such a mechanism operates in the mouse, it could also be active in the rabbit. At present, however, there is no indication that rabbits communicate with their saliva or their urine, nor is it known whether carrier proteins are present in these two types of secretions.

The existence of at least three types of OBPs in the rabbit poses again the question of their physiological function. In insects, at least three classes of OBPs have been clearly identified in Lepidoptera (Vogt *et al.*, 1991; Breer *et al.*, 1992; Krieger *et al.*, 1993) and five in *Drosophila* (McKenna *et al.*, 1994; Pikyelni *et al.*, 1994; for review see Pelosi and Maida, 1995). In vertebrates, a similar division of OBPs in sub-classes cannot be easily perceived, owing to the incomplete information available. However, a rather homogeneous group of OBPs includes sequences similar to urinary and salivary proteins of the mouse: rabbit OBP-III, as well as mouse OBP-III and OBP-IV belong to this group, together with OBP-I of the porcupine (Pelosi, 1994; Ganni *et al.*, 1997).

Such diversity may indicate physiologically important discriminating functions towards odour structures, instead of just a passive role of non-selective carrier. Some pathways of olfactory transduction could function, at least for some odours, in a way similar to that used by bacteria for detecting sugars, as previously suggested (Pelosi, 1994, 1996).

The differences in the amino acid sequence between OBPs of the same species may indicate differences in their binding specificity. Ligand-binding data available so far seem not to support this idea; in fact, most of the OBPs of vertebrates exhibit good affinity to the same ligand, 2-isobutyl-3-methoxypyrazine. However, we do not necessarily expect dramatic differences in the spectra of binding between OBPs; discrimination can be easily accomplished with a system using sensors of broad overlapping response spectra, as in the colour vision. The binding pocket of the bovine OBP, the only protein of this family whose

three-dimensional structure has been described (Bianchet *et al.*, 1996; Tegoni *et al.*, 1996), presents a 'smooth' surface, lined with aromatic amino acids, and well agrees with the broad affinity of this protein to several odorant structure. A system based on sensing elements of poor specificity, however, offers the advantage of being responsive to a great number of different stimuli, such as odorant structures.

ACKNOWLEDGEMENTS

We thank Dr Jeff Keen, Department of Biochemistry, University of Leeds, UK, for his invaluable help in sequencing the OBP-I and Dr Krishna C. Persaud, UMIST, Manchester, UK, for helpful discussion and criticism. Rabbit heads were kindly supplied by Cuniavicola Gianro', Montecarlo di Lucca, Italy. Research supported by National Research Council of Italy, Special Project RAISA, Sub-Project no. 4, Paper no. 2688.

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Received March 25, 1996, accepted January 10, 1997