

Odorants of Different Chemical Classes Interact with Distinct Odorant Binding Protein Subtypes

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Abstract

The ligand profile for three odorant binding proteins (OBPs) of the rat have been determined using a large number of odorous compounds from different chemical classes. To evaluate the binding spectra of distinct subtypes, all OBPs were produced in *Escherichia coli* as recombinant His-tagged fusion proteins. The individual binding properties of each OBP subtype were analysed using a large array of organic compounds, representing derivatives of aliphatic and aromatic compounds, as well as terpenes, pyrazines and thiazoles, in a competitive spectroscopic binding assay with various fluorescence chromophores as the specific interacting partner for the OBPs. Most of the compounds were identified to interact only with one OBP subtype. But interestingly, a small change, for example in the 2-methyl or 2-ethoxy side chain in the pyrazine and thiazole derivatives to a 2-isobutyl group, caused overlapping binding affinities to rat-OBP1 and rat-OBP3. However, the data strongly support the notion that each OBP subtype displays a characteristic ligand binding profile and interacts with a different subset of exogenous organic compounds in a micromolar range.

Introduction

It is supposed that volatile odorous compounds entering the nasal cavity via the respiratory airstream are transferred across the hydrophilic mucus layer towards the sensory neurons by means of odorant binding proteins (OBPs), which are members of the lipocalin family (Pelosi *et al.*, 1981, 1984; Pevsner and Snyder, 1990; Flower, 1996; Tegoni *et al.*, 2000). These proteins are capable of binding odorants reversibly; however, most of the information concerning their binding properties is based on competition experiments using tritiated bell-pepper odorant 2-isobutyl-3-methoxypyrazine (IBMP) and mixtures of proteins isolated from the nasal mucus (Pelosi *et al.*, 1982; Bignetti *et al.*, 1985; Pevsner *et al.*, 1985). Comparing the structure and binding properties of distinct OBP subtypes should contribute to a more detailed understanding of their physiological roles. The three-dimensional structures of bovine and pig OBPs have been resolved (Tegoni *et al.*, 1996; Spinelli *et al.*, 1998). Whereas the bovine OBP is dimerized by so-called 'domain-swapping', pig OBP occurs in its monomeric form. The structural analysis revealed clear differences between bovine and pig OBP; however, both binding proteins appear to display similar binding properties for several odorous compounds (Pevsner *et al.*, 1990; Dal Monte *et al.*, 1991; Herent *et al.*, 1995). The discovery that different OBP subtypes exist in the nasal mucus of one species (Felicoli *et al.*, 1993; Pes and Pelosi, 1995; Garibotti *et al.*, 1997) implies that OBPs operate as a selective filter in odor pre-selection.

The detailed protein sequence analysis of the three rat-OBPs, described as rat-OBP1, rat-OBP2 and rat-OBP3 (Pevsner *et al.*, 1988; Dear *et al.*, 1991; Löbel *et al.*, 1998, 2001), has shown a low level of sequence identity ($\leq 30\%$) between these three proteins. Comparing rat-OBP sequences with other members of the lipocalin family revealed that rat-OBP1 exhibits sequence motifs of aphrodisin-like OBPs (Vincent *et al.*, 2001), whereas rat-OBP2 can be classified together with tear lipocalins (Löbel *et al.*, 1998) and rat-OBP3 belongs to the family of major urinary proteins (MUP) (Löbel *et al.*, 2001). The sequence diversity and the identification of subclasses in the OBP family has led to the notion that each subtype may be specialized to recognize only a distinct repertoire of odorants. Heterologous expression allows production of a sufficient amount of a defined protein subtype for subsequent binding experiments (Löbel *et al.*, 1998, 2001). The discovery that certain chromophores produce a significant increase in fluorescence emission upon specific interactions with binding proteins has opened new avenues for monitoring the binding of small organic compounds in spectroscopic competition experiments (Kane and Bernlohr, 1996). In previous studies, the search for appropriate indicators of distinct OBP subtypes has led to the identification of 1-anilino-naphthalene-8-sulfonic acid (1,8-ANS) as a high-affinity probe for OBP2 (Löbel *et al.*, 1998). Subsequent studies demonstrated that 1-aminoanthracene (1-AMA) interacts with some OBPs (Paolini *et*

al., 1998; Briand *et al.*, 2000; Ramoni *et al.*, 2001); for the newly discovered subtype rat-OBP3, 1-AMA has turned out to be the most suitable probe (Löbel *et al.*, 2001). To approach the question of whether each subtype of rat-OBP may be specifically tuned to bind distinct chemical compounds, it was necessary to analyze the binding properties of distinct OBPs for a large variety of odorous compounds and to assess a comprehensive survey of structurally related chemicals. The data of the present study indicate that each of the three rat-OBP subtypes displays a characteristic ligand profile.

Materials and methods

Materials

The expression vectors pQE-30, 31 and 32, and Ni-nitrilotriacetic acid agarose came from Qiagen (Hilden, Germany). The fluorescence probe 1,8-ANS was from Sigma (Deisenhofen, Germany) and 1-AMA was from FLUKA (Deisenhofen, Germany). The odorants were purchased from Sigma (Deisenhofen, Germany) at the highest purity available. All other reagents were of analytical grade.

Protein expression and purification of recombinant OBPs

The expression vector contains an ampicillin-resistance gene and generates a recombinant fusion protein with a (His)₆ tag on the N-terminal part of the protein. The expression was regulated by co-transformation of the pREP4 plasmid, which contains a kanamycin-resistance gene and multiple copies of the *lacI* repressor gene. Induction was performed when absorbance at 600 nm of bacterial cultures reached 0.5–0.7 by adding 0.2 mM isopropyl β-D-thiogalactoside to the medium for 3 h at 37°C. Cells were harvested by centrifugation at 5000 *g* for 15 min, resuspended in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.25 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride, then stored on ice for 1 h. The recombinant OBPs were purified from the soluble fraction as described previously (Löbel *et al.*, 1998, 2001).

Fluorescence competition assay

The fluorescence measurements were performed on a Perkin Elmer LS 50B spectrofluorimeter. Protein concentrations were adjusted to a 50 μM stock solution in 100 mM potassium phosphate, pH 7.5, by measuring the absorbance at 280 nm with the respective extinction coefficient for each OBP subtype according to Magne *et al.* (Magne *et al.*, 1977). The measurements were performed as previously described (Löbel *et al.*, 1998, 2001). The concentration of 1-AMA was determined by weight; the probes were dissolved in methanol to yield a 10 mM stock solution. Fluorescence of 1-AMA was excited at 256 nm and emission was recorded between 420 and 600 nm. Spectra were recorded at 1 nm intervals, with a scan speed of 180 nm/min and four accumulations. The slit width used for excitation and

emission was 5 nm. All odorants used in competition experiments were dissolved in methanol. To avoid solvent-effects in the titration experiments, the final methanol concentration was adjusted to <0.5%. The concentrations of competitor that caused a decay of fluorescence to half-maximal intensity were *IC*₅₀ values. The *K*_i-values were calculated as $K_i = [IC_{50}]/(1 + [L]/K_d)$, where [L] is the free chromophore and *K*_d is the dissociation constant of OBP–chromophore. The *K*_d values for rat-OBP2 and rat-OBP3 were obtained from previous studies (Löbel *et al.*, 1998, 2001), whereas the binding constant of the complex rat-OBP1–1-AMA was calculated from the binding curve using the computer program Origin 5.0 (Microcal, Northampton, MA).

Results

Fluorescence binding experiments

Towards a comparative analysis of all three OBP subtypes, the rat-OBP1 was assessed for interaction with the fluorescence probe 1-AMA. Upon titration of the chromophore to 2 μM recombinant rat-OBP1, the emission maximum of 1-AMA shifted from 542 to 502 nm. The intensity of the emission spectrum was 300-fold increased compared with the free chromophore. Values in the emission maximum were analyzed in the corresponding binding curve (Figure 1). The fluorescence binding parameter of all three rat-OBPs are summarized in Table 1.

Competition assay

Competition experiments with a large variety of odorants resulted in the discovery of distinct odorous compounds

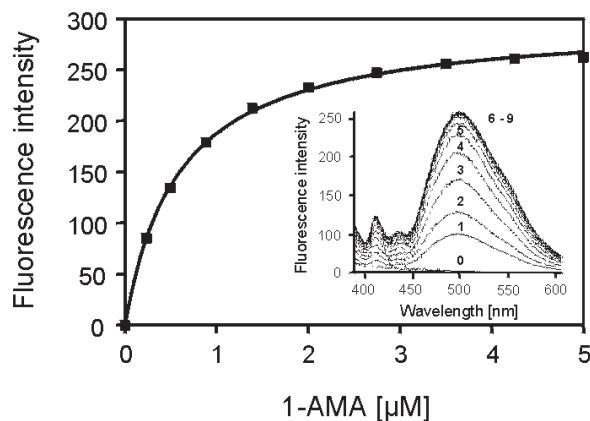


Figure 1 Binding curve of 1-AMA to recombinant OBP1. A solution of 2 μM OBP1 was titrated with increasing concentrations of 1-AMA. The fluorescence was excited at 256 nm, the changes in the emission spectra were recorded between 380 and 600 nm (Inset), and the values of the maximum at 502 nm were analyzed for generating the dose–response curve, which represented means of three determinations. A binding constant of *K*_d = 0.59 μM was determined by fitting the experimental values to a hyperbolic curve with the Origin 5.0 software program. Measurements were carried out in 100 mM potassium phosphate buffer, pH 7.5.

that specifically interacted with one OBP subtype but not with the other two. (–)-Borneol selectively replaced the fluorescent probe from rat-OBP1 but did not affect the interaction of rat-OBP2 and rat-OBP3 with their chromophore (Figure 2). Similarly, the aliphatic compound 1-decanal selectively affected the rat-OBP2–1.8-ANS complex, whereas the thiazol-derivative benzothiazole only quenched the fluorescence of the rat-OBP3–1-AMA complex. These data support previous observations that each OBP subtype may be specifically tuned to exogenous odorants of distinct structural classes (Löbel *et al.*, 1998).

In order to evaluate the spectrum of structurally related compounds that are able to interact with a unique OBP subtype, the fluorescent binding assay was employed assessing multiple derivatives of chemicals representing various structural classes concerning their interaction with the three OBPs. The results of a comprehensive survey are documented in Table 2. In addition, other bicyclic terpenes, such

as (–)- and (+)-camphor, interacted selectively with rat-OBP1, like (–)-borneol. In contrast, all monocyclic terpenes were bound by rat-OBP1 as well as rat-OBP3 with similar affinity, but did not interact with rat-OBP2.

Testing a variety of aromatic compounds revealed the high-affinity interactions of phenol derivatives **10–12**, **14** and **15** with rat-OBP1; the size of the side chain but also its position relative to the hydroxyl groups seems to be relevant. Interestingly, two isopropyl side chains induced a complete shift of the binding characteristic: 2,6-isopropylphenol is not bound by rat-OBP1 but, rather, displayed a high-affinity interaction with rat-OBP3. The novel feature of the derivative may be due to the fact that the hydroxyl group is sterically blocked by the two isopropyl groups in the 2- and 6-positions. The importance of both OH groups and the aliphatic side chain is emphasized by the observation that compounds with a hydroxyl group separated from the aromatic ring by an aliphatic spacer (**13**) or unsubstituted phenol (**9**) and its aldehyde form (**17**) do not display any significant interaction with any of the binding proteins.

Of the large array of aliphatic compounds (**18–26**), including those with a hydroxyl, carbonyl or nitrile group, most interacted strongly and selectively only with rat-OBP2; only an amine form (**24**) displayed low affinity interaction with rat-OBP3.

A large collection of heterocyclic compounds, including pyrazine and thiazole derivatives (**27–39** and **40–49**) respectively, was assessed. The great majority interacted only with

Table 1 Binding properties of the OBP subtypes to fluorescence probes

	Rat-OBP1	Rat-OBP2	Rat-OBP3
Fluorescence probe	1-AMA	1,8-ANS	1-AMA
Chromophore net charge	positive	negative	positive
Emission maximum (nm)	502	462	480
Binding constant (μM)	0.59 ± 0.03	0.62 ± 0.05	1.22 ± 0.08

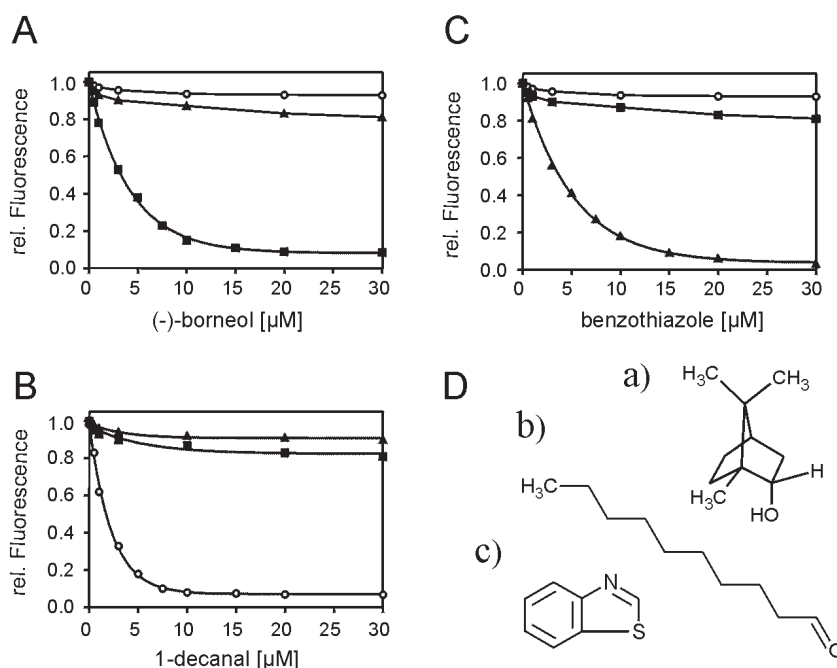


Figure 2 Selective competition of chromophore binding by odorants. Fluorescence of 2 μM pre-equilibrated OBP–chromophore complex was assumed as 1 for OBP1–1-AMA (filled squares), OBP2–1,8-ANS (open circles) and OBP3–1-AMA (filled triangle). Spectra were recorded at 25°C with an excitation wavelength of 256 nm. **(A)** Selective decrease of fluorescence intensity upon titration with (–)-borneol (a) was only observed for OBP1–1-AMA. **(B)** Loss of fluorescence intensity by increasing concentration of 1-decanal (b) was obtained for the complex OBP2–1,8-ANS. **(C)** Decrease of fluorescence upon titration with benzothiazole (c) selectively for OBP3–1-AMA. **(D)** Chemical structure of the odorants used in the competition experiments (a–c).

Table 2 Binding affinities of odorous compounds from different chemical classes to three rat-OBPs

Ligands	Rat-OBP1		Rat-OBP2		Rat-OBP3	
	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)
Bicyclic terpenes						
1 (-)-Borneol	4.37	1.25	—	—	—	—
2 (-)-Camphor	4.72	1.35	—	—	—	—
3 (+)-camphor	4.52	1.29	—	—	—	—
Monocyclic terpenes						
4 D(-)-Limonene	3.85	1.07	—	—	8.43	2.41
5 (+)- <i>cis</i> -Limonene-1,2-epoxide	14.13	4.03	—	—	10.22	2.92
6 (+)- <i>trans</i> -Limonene-1,2-epoxide	14.35	3.98	—	—	10.15	2.90
7 (-)-Menthone	6.30	1.75	—	—	7.70	2.21
8 (-)-Menthole	7.30	2.02	—	—	14.30	4.12
Benzene derivatives						
9 Phenol	—	—	—	—	—	—
10 4-Ethylphenol	13.30	3.69	—	—	—	—
11 4-Propylphenol	1.89	0.54	—	—	—	—
12 4-Isopropylphenol	1.37	0.39	—	—	—	—
13 4-Isopropylbenzylalcohol	—	—	—	—	—	—
14 3-Isopropylphenol	1.75	0.52	—	—	—	—
15 2-Isopropylphenol	2.38	0.68	—	—	—	—
16 2,6-Isopropylphenol	—	—	—	—	0.58	0.16
17 Benzaldehyde	—	—	—	—	—	—
Aliphatic derivatives						
18 1-Decanol	—	—	—	—	—	—
19 1-Decanal	—	—	4.35	1.24	—	—
20 Capric acid	—	—	—	—	—	—
21 Myristyl alcohol	—	—	—	—	—	—
22 Myristine aldehyde	—	—	1.70	0.41	—	—
23 Myristic acid	—	—	1.20	0.29	—	—
24 Myristyl amin	—	—	—	—	24.20	7.56
25 3,7-Dimethyl-2,6-octadienenitrile	—	—	8.50	2.42	—	—
26 <i>p</i> - <i>tert</i> -Butyl- α -methyl dihydrocinnamic aldehyde	—	—	6.20	1.77	—	—
Heterocyclic compounds: pyrazines						
27 Pyrazine	—	—	—	—	—	—
28 2-Methylpyrazine	—	—	—	—	24.42	6.97
29 2-Acetylpyrazine	—	—	—	—	—	—
30 2-Methoxypyrazine	—	—	—	—	—	—
31 2-Ethylpyrazine	—	—	—	—	8.75	2.15
32 2,3-Dimethylpyrazine	—	—	—	—	6.30	1.80
33 2,3-Diethylpyrazine	—	—	—	—	5.85	1.67
34 2,3,5-Trimethylpyrazine	—	—	—	—	5.42	1.55
35 2,3,5,6-Tetramethylpyrazine	—	—	—	—	6.80	1.94
36 2,6-Dimethylpyrazine	—	—	—	—	6.30	1.80
37 2,5-Dimethylpyrazine	—	—	—	—	4.62	1.32
38 2-Methyl-3-methoxypyrazine	—	—	—	—	28.20	8.05
39 2-Isobutyl-3-methoxypyrazine	5.67	1.62	—	—	10.10	2.88
Heterocyclic compounds: thiazoles						
40 Thiazole	—	—	—	—	—	—
41 5-Methylthiazole	—	—	—	—	13.45	3.85
42 2-Acetylthiazole	—	—	—	—	16.52	4.72
43 2-Ethoxythiazole	—	—	—	—	—	—
44 2-Isobutylthiazole	4.37	1.25	—	—	2.03	0.58
45 4,5-Methylthiazole	—	—	—	—	6.62	1.88
46 2,4,5-Methylthiazole	—	—	—	—	3.05	0.87
47 Benzothiazole	—	—	—	—	5.84	1.67
48 4-Methyl-5-ethanol-thiazole	—	—	—	—	27.95	7.98
49 4-Methyl-5-acetic acid-ethylester-thiazole	—	—	—	—	—	—

Values are means of 3 independent experiments. Odorant concentrations >50 μ M for half-maximal inhibition were not used for calculating IC_{50} values.

rat-OBP3. Interestingly, the unsubstituted 'lead' compounds pyrazine and thiazole were not bound by any of the OBPs. Substituting the 'lead' compounds with aliphatic side chains led to selective interaction with rat-OBP3; larger-sized or multiple side chains increased the affinity of the compounds (Figure 3). In addition, the hydrophobicity of the side chain influenced the binding; the introduction of an alcohol group in **48** decreased the affinity markedly. Interestingly, by introducing a 2-isobutyl group, a comparable affinity to rat-OBP1 and rat-OBP3 was gained for the respective pyrazine and thiazole derivative (**39** and **44**).

Overall, the data indicate a characteristic ligand profile of each OBP subtype. The binding features of rat-OBP1 and rat-OBP3 seem to be more closely related than rat-OBP2, which seems to be specialized mainly for aliphatic compounds.

Discussion

The results of this study indicate that each of the three OBP subtypes display a unique ligand binding profile. Previous studies suggested that an OBP may bind most of the odorous compounds (Pevsner *et al.*, 1990); however, these analyses were based mainly on protein preparations purified from nasal mucus, which do not allow a clear discrimination between various subtypes. In this study recombinant proteins were utilized to assess distinct subtypes for their binding properties. Improving the spectroscopic analysis approach applicable to all three OBP subtypes allowed the monitoring under comparable experimental conditions of a multiplicity of odorous compounds representing various odorants. The data indicate that each chemical class can be roughly assigned to a distinct OBP subtype. Systematically investigating a comprehensive spectrum of chemical classes differing only slightly in side-chain substitutions reveals the importance of the degree of methylation, e.g. of the thiazole derivatives, in increasing the binding affinity to rat-OBP3. Furthermore, the analysis of a large repertoire of heterocyclic compounds underlined clearly that only derivatives with an isobutyl side chain are able to interact with both rat-OBP1 and rat-OBP3, whereas most of the other heterocyclic compounds exhibit affinity to only rat-OBP3. Thus, the OBP subtypes analyzed in this study seem to be specialized for interacting with exogenous compounds. This may be in contrast to other OBPs; in a recent study it was found that a bovine OBP binds 1-octen-3-ol, a compound produced endogenously (Ramoni *et al.*, 2001).

The presence of several OBP subtypes suggests a more specific role of these proteins in the perireceptor events of olfaction than acting as a general unspecific carrier for all hydrophobic compounds (Pes and Pelosi, 1995; Garibotti *et al.*, 1997). Based on the diversity of OBPs, it seems conceivable that they are involved in preselecting those volatile compounds that are biologically relevant to finally interact with the olfactory sensory cells, suggesting a role

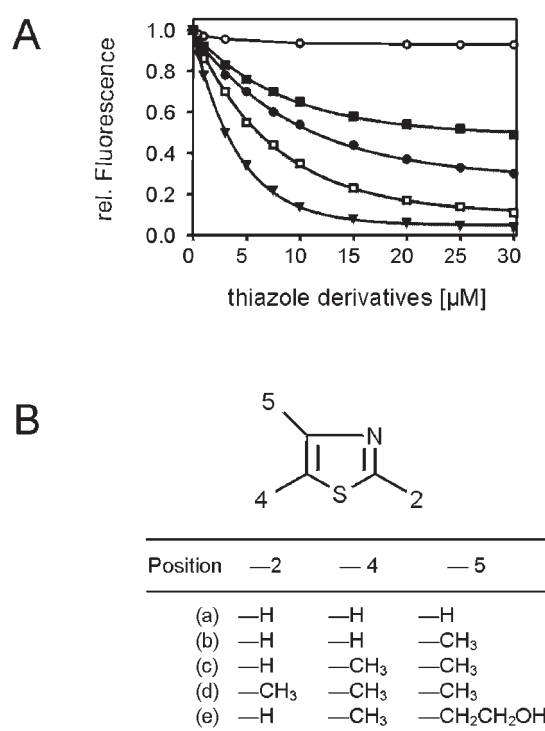


Figure 3 Competition binding assay of OBP3-1-AMA complex with various thiazole derivatives. **(A)** Fluorescence of pre-equilibrated OBP3-1-AMA complex was assumed as 1 in the maximum of fluorescence emission at 480 nm. The concentration of OBP3 and 1-AMA was 3 μM. The decrease in fluorescence intensity was plotted as a function of competitor concentration. Spectra were recorded at 25°C with an excitation wavelength of 256 nm. Unsubstituted thiazole, open circle; 5-methylthiazole, solid circle; 4,5-dimethylthiazole, open squares; 2,4,5-trimethylthiazole, filled triangles; 4-methyl-5-ethanol-thiazole, solid squares. **(B)** Schematic drawing of the 'lead' compound thiazole; side chain substitutions of the derivatives are summarized in the table.

of OBPs as a specific filter rather than a passive shuttle protein for odorants in the mucus layer of the olfactory epithelium. Furthermore, the idea that OBPs themselves may be involved in receptor activation is supported by recent observations demonstrating that related lipocalins, such as aphrodisin or mouse MUPs, are able to trigger signalling processes in chemosensory cells (Kroner *et al.*, 1996). The latest identification of LIMR, a lipocalin-interacting membrane receptor for tear lipocalin (Wojnar *et al.*, 2001), may substantiate the notion that OBPs could interact with membrane receptor proteins in the olfactory epithelium. The structural diversity of OBPs and the rather restricted binding profile of each subtype demonstrated in this study will support further efforts towards a more complete understanding of the OBP function.

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