Identification of a Third Rat Odorant-binding Protein (OBP3)

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Abstract

From a rat olfactory epithelium cDNA library clones encoding a lipocalin were isolated with sequence identity to the previously described salivary-specific α-2u globulin and the N-terminal region of mouse odorant-binding proteins OBP-III and OBP-IV. *In situ* hybridization showed strong expression in nasal glands displaying a pattern equivalent to rat OBP1. Heterologously expressed protein was evaluated for its binding properties using spectroscopic approaches. The recombinant protein interacted with two fluorescent probes, 1-aminoanthracene (1-AMA) and 1,1′-bis(4-anilino-5-naphthalene)-sulfonic acid. 1-AMA binding was competed by several odorants with high affinity. The thermodynamic parameters of the protein–odorant interaction were determined using isothermal titration calorimetry. Due to its nasal expression and odorant-binding characteristics this protein was designated OBP3.

Introduction

Odorant-binding proteins (OBPs) of vertebrates are supposed to carry hydrophobic odorous molecules through the aqueous compartment of the mucus layer towards the olfactory receptor neurons (Bignetti *et al.*, 1985; Pevsner and Snyder, 1990; Pelosi, 1994, 1996). OBPs are synthesized by lateral and septal glands of the nasal epithelium and secreted into the mucus covering the olfactory epithelium (Avanzini *et al.*, 1987; Ohno *et al.*, 1996; Utsumi *et al.*, 1999). Based on amino acid sequence comparisons OBPs are members of the lipocalin family (Pelosi, 1994; Flower *et al.*, 1993; Flower, 1996). Proteins of this family are characterized by a simple but common folding architecture of eight antiparallel β-strands forming a β-barrel with a deep internal cavity, which is supposed to harbor the binding site for small hydrophobic ligands (Godovac-Zimmermann, 1988; Flower, 1996). Some members of the lipocalins are characterized by a high ligand selectivity, for example the retinol-binding protein interacts only with retinol and a limited spectrum of very related derivatives (Cogan *et al.*, 1976). In contrast, OBPs bind a variety of volatile compounds with micromolar affinity (Cavaggioni *et al.*, 1989; Vincent *et al.*, 2000). Based on these results OBPs are defined as non-specific carriers (Tegoni *et al.*, 2000). However, the discovery of several OBP sub-types in different vertebrate species (Dal Monte *et al.*, 1991; Pes *et al.*, 1992; Felicioli *et al.*, 1993) led to the concept that an OBP subtype may be tuned to a selective ligand spectrum. Detailed studies on recombinant rat OBPs provided evidence for a distinct ligand specificity (Löbel *et al.*, 1998). Binding studies revealed that rat OBP2 interacted only with aliphatic long chain carbonyls and nitriles; the binding properties were strictly based on structural features such as functional group and the aliphatic chain length. In contrast, rat OBP1 displayed binding properties for aromatic heterocyclic odorants.

Here we report the cloning of a third rat OBP related to the family of α-2u globulins. Its binding properties were evaluated by spectroscopic approaches.

Materials and methods

Tissues and reagents

Adult Sprague–Dawley rats were purchased from Charles River (Sulzfeld, Germany). Oligonucleotides were synthesized by Interactiva (Ulm, Germany). The Bluescript SKII vector was from Stratagene. *Escherichia coli* BL21(DE3) was from Novagen (Madison, WI). The expression vector pQE31 and Ni–NTA–agarose were from Qiagen (Hilden, Germany). The fluorescent probes 1-aminoanthracene (1-AMA) and 1-anilino-naphthalene-8-sulfonic acid (1,8-ANS) were from Fluka (Deisenhofen, Germany) and 1,1′-bis(4-anilino-5-naphthalene)-sulfonic acid (bis-ANS) was from Molecular Probes (Eugene, OR). All other chemicals and odorants are commercially available and were of analytical grade.

Screening of a rat olfactory cDNA library and isolation of full-length clones

A digoxigenin (DIG)-labeled probe was prepared from a 500 bp fragment of the mouse *MUP* gene according to the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983) using a random priming DIG labeling kit

(Roche, Mannheim, Germany). This probe was used to screen a cDNA library from rat olfactory mRNA in λNM1149 under low stringency conditions as described previously (Raming *et al.*, 1993). Ten positive phage plaques bearing a 1.2 kb fragment were isolated and subcloned into Bluescript SKII. Sequencing of all the clones was performed using primers T3 and T7 and the ABI prism BigDye ready reaction terminator cycle sequencing kit as recommended by the supplier. Samples were run on a ABI Prism 310 genetic analyser (Perkin Elmer, Foster City, CA). Sequence analysis was performed using the HUSAR 3.0 software package (EMBL, Heidelberg, Germany) based on the sequence analysis package GCG 7.2 (Madison, WI). Sequence homologies were determined using the BLAST algorithm.

In situ hybridization

Probes were generated from templates using a SP6/T7 *in vitro* transcription system (Roche Diagnostic, Mannheim, Germany). Aliquots of 2 µg of linearized vector were transcribed in the presence of 70 nmol digoxigenin-11-uridine-5′-triphosphate. RNA was precipitated with ethanol and resuspended in 20 ml of *in situ* grade hybridization buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) containing 50% deionized formamide. Rats (>6 weeks old) were killed by CO_2 asphyxiation and decapitated. The lower jaw and top of the skull were carefully removed using a bone cutter (Fine Science Tools, Heidelberg, Germany). Tissues were embedded in Tissue-Tek and frozen on dry ice. Coronal sections of 10 µm were cut on a cryostat at –24°C, adhered to Superfrost microslides and air dried for 2 h. For *in situ* hybridization tissue sections were covered with 10 µl of hybridization solution containing ~3–5 ng DIG-labeled RNA. Hybridization and post-hybridization washes were performed as described earlier (Strotmann *et al.*, 1994).

Construction of the expression plasmid and protein expression

The coding sequence of OBP3 was amplified by PCR using the following primers: OBP3-f/ex/*Bam*HI, 5′-AATATC-*GGATCC*GGAAGAAGCTAGTTTCGAGAGAG-3′, and OBP3-r/ex/*Hin*dIII, 5′-TTGCAT*AAGCTT*CAGGCCTG-GAGACAGCGATC-3′, containing *Bam*HI and *Hin*dIII restriction sites, respectively. The PCR product was double digested with *Bam*HI and *Hin*dIII and subsequently cloned into the corresponding site of expression vector pQE31 (Qiagen, Hilden, Germany) under control of the T5/*lacZ* promotor system. The derived plasmid construct was confirmed by DNA sequencing and transformed into *E. coli* BL21(DE3)/pREP4.

One liter of LB medium with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) was inoculated with an overnight culture of 100 ml in the same medium. Induction was performed when the bacterial culture had reached an A_{600} of 0.5–0.7 with 0.2 mM isopropyl-β-d-thiogalactoside and growth was continued for 3 h. Cells were harvested by centrifugation at 2000 *g* for 15 min and the recombinant OBP3 was purified from the soluble fraction as described previously (Löbel *et al.*, 1998).

Protein analysis and removal of co-purified ligands

The molecular mass of native protein was evaluated by gel filtration on a 2 ×30 Sephacryl S-100 column using a Pharmacia FPLC system; the column was calibrated with BSA, ovalbumin, chymotrypsinogen and cytochrome *c*. The flow rate was 2 ml/min in 50 mM ammonium hydrogen carbonate, pH 7.5, 300 mM NaCl. SDS–PAGE was performed on 15% Laemmli gels (Laemmli, 1970). To remove co-purified ligands the recombinant protein was dialyzed for 2 × 12 h against 100 mM potassium phosphate, pH 7.5, 1 mM 1-octanol in slide-a-lizer chambers with a cut-off of 10 kDa (Pierce, Rockford, IL) and subsequently for 4 × 12 h against 100 mM potassium phosphate, pH 7.5.

Fluorescence binding assay

The fluorescence measurements were performed on a Perkin Elmer LS 50B spectrofluorimeter. Protein concentration was adjusted to 3 µM in 100 mM potassium phosphate, pH 7.5, by measuring absorbance at 280 nm with the calculated extinction coefficient for OBP3 (ε_{280} = 12684/M/cm) according to Magne *et al.* (Magne *et al.*, 1977). The measurements were performed as previously described (Löbel *et al.*, 1998, 2000). The chromophore concentration of 1-AMA was determined by weight and bis-ANS concentration was determined by absorbance at 394 nm (ε_{394} = 16000/M/cm); the probes were dissolved in methanol as 10 mM stock solutions. Fluorescence of 1-AMA was excited at 256 nm and emission was recorded between 420 nm and 600 nm; an emission cut-off filter of 350 nm was used. Fluorescence resonance energy transfer (FRET) between the intrinsic tryptophan (donor) and bis-ANS (acceptor) occurs by excitation at 295 nm; emission was recorded between 300 and 550 nm. Spectra were recorded at 1 nm intervals, at a scan speed of 180 nm/min with four accumulations. The slit widths used for excitation and emission were 5 nm. Binding data for OBP3 were evaluated using the linearization equation according to Stockell (Stockell, 1959). Competitor concentrations causing a decay of fluorescence to half-maximal intensity were determined as IC_{50} values. The apparent K_i values were calculated as

$$K_i = [IC_{50}]/ (1 + [L]/K_d)$$

with [L] being the free chromophore concentration and K_d the dissociation constant of the OBP3–chromophore complex.

Isothermal titration microcalorimetry (ITC)

Titration experiments were carried out at 30°C with the

isothermal titration microcalorimeter VP-ITC system (Microcal, Northampton, MA). Protein solution was dialyzed twice for 12 h at 4°C against 100 mM potassium phosphate buffer, pH 7.5. Protein samples were sterile filtered and degased directly before carrying out the experiment. Ligand stock solutions were prepared in methanol and diluted 1:100 into the protein dialysis buffer to adjust the final methanol concentration to 1%. The ligand concentration in the syringe was 500 µM. The protein concentration in the calorimetric cell was 30 µM and the content of methanol was adjusted to 1% to minimize the heat dilution effect of the solvent. Injections of ligand solution into the calorimetric cell were carried out at time intervals of 200 s in aliquots of 10 µl. Protein:ligand titration curves were corrected by substraction of the blank titration curves obtained by titration of buffer in the cell with the same ligand solution in the syringe. Analysis of the experimental data was carried out with Origin 5.0 (Microcal). Binding parameters such as number of binding sites (n), the association constant (K_a) and the binding enthalpy (ΔH_b, kJ/mol) were determined as parameters of the fitted experimental binding values.

Results

Cloning, sequence identity and gene expression

In search for additional OBPs a rat olfactory cDNA library was screened with lipocalin-encoding DNA probes, notably a mouse major urinary protein encoding DNA probe.

Ten clones were isolated and sequenced; all gave the same sequence comprising the typical motifs of lipocalins. Therefore, it was considered as a third type of lipocalin expressed in the rat olfactory epithelium and tentatively designated rat OBP3. A search of the database revealed that the same sequence had been reported (EMBL accession no. X14552) and the deduced polypeptide described as salivary protein (Gao *et al.*, 1989). Sequence comparison demonstrated a high level of identity with members of the MUP family, whereas the percentage of identical amino acids with rat OBP1 was determined to be 28% and with rat OBP2 only 18%. Interestingly, the first 32 amino acids of the N-terminal sequence displayed a high degree of identity (~86%) with the two recently described mouse proteins OBP-III and OBP-IV (Pes and Pelosi, 1995).

To determine the sites of synthesis of OBP3 in the nasal cavity *in situ* hybridization experiments were performed employing a DIG-labeled antisense riboprobe on sections of the rat nasal cavity. As seen in Figure 1A, the secretory glands located in the lateral nasal walls were heavily stained. Reactive cells are located around the root of the incisor, a region that has previously been described as the site of OBP1 expression (Ohno *et al.*, 1996). At higher magnification (Figure 1B) strong signals were detectable in acinar cells; the secretory duct cells and blood vessels were not labeled. No signals were observed in the sensory epithelia throughout the nasal cavity. Using a sense riboprobe no hybridization signals were obtained (data not shown).
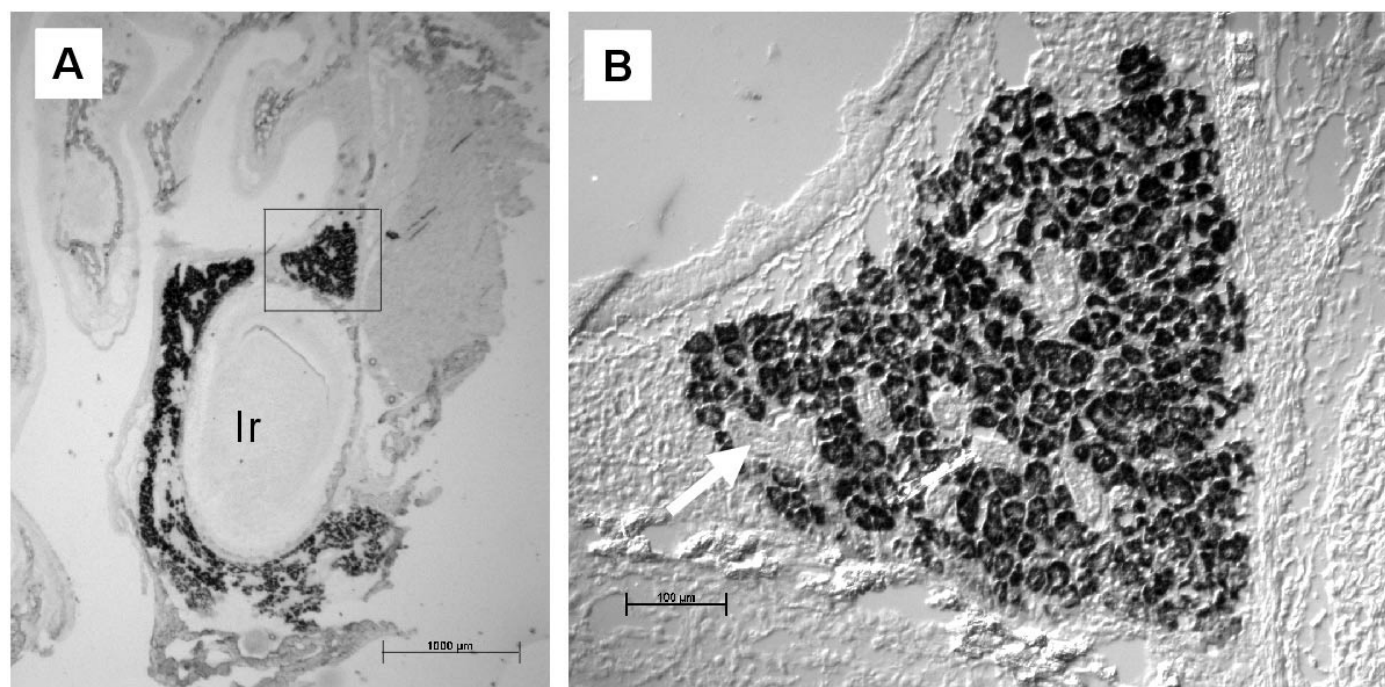

Figure 1 *In situ* hybridization on coronal sections through a rat nose. **(A)** Bright field micrograph of a coronal section through the lateral nasal gland region of the rat nasal cavity hybridized with a DIG-labeled antisense riboprobe. The boxed area is enlarged in **(B)**. Ir, incisor root. **(B)** High magnification of lateral nasal gland cells reactive with the antisense probe. Signals are restricted to acinar cells. The arrow indicates the secretory duct.

Heterologous expression and characterization of OBP3 protein

The OBP3 protein was heterologously expressed in *E.coli* BL2 using the pREP4 vector; optimum yields of soluble protein were obtained by allowing growth at 37°C and after an incubation time of 5 h. Recombinant protein was purified by a single step of Ni–NTA–chelate affinity chromatography which yielded a purity of >98%. About 18 mg of purified product were obtained per liter of bacterial culture. In calibrated gel filtration experiments the recombinant protein migrated with an apparent molecular mass of ~20 kDa; based on the calculated mass of 19.4 kDa, this result indicates that the protein exists in its monomeric form (data not shown). The purified protein exhibited an absorption peak at 340 nm; the intrinsic tryptophan fluorescence spectrum showed a second peak at 440 nm. This observation suggested that small compounds were co-purified with the heterologously expressed protein, however, the contaminants were removed after intensive dialysis, as described in Materials and methods.

Fluorescence binding studies

To approach the question of whether OBP3, which is expressed in the nasal glands and thus supposedly appears in the nasal mucus, may in fact act as an odorant-binding
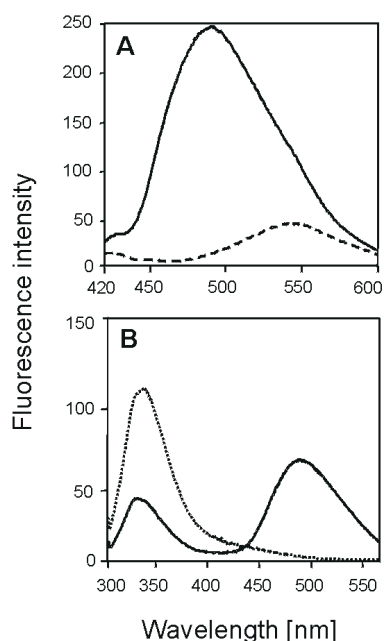
protein, able to bind hydrophobic odorous ligands, in a first approach the hydrophobic fluorescence probes 1-AMA, 1,8-ANS and bis-ANS were employed in spectroscopic binding assays (Paolini *et al.*, 1998). As demonstrated in Figure 2A, in the presence of the protein the emission maximum of 1-AMA shifted from 542 to 490 nm; this shift coincides with a 250-fold increase in intensity. These observations demonstrate an intense interaction of 1-AMA with hydrophobic domains of the OBP3 protein. Exciting the tryptophan fluorescence of the protein at 295 nm elicited a single emission peak at 340 nm. When the protein was pre-equilibrated with bis-ANS the tryptophan emission was reduced and FRET was observed by the emission of bis-ANS at 486 nm (Figure 2B), indicating that bis-ANS also interacts with OBP3. In contrast, the fluorescence properties of 1,8-ANS did not alter significantly in the presence of the protein (data not shown). These data indicate some kind of binding selectivity of OBP3.

To determine the binding affinities of the chromophores titration experiments were performed. In Figure 3A the fluorescence intensity has been plotted versus the chromophore concentration. The data were fitted to a linearized
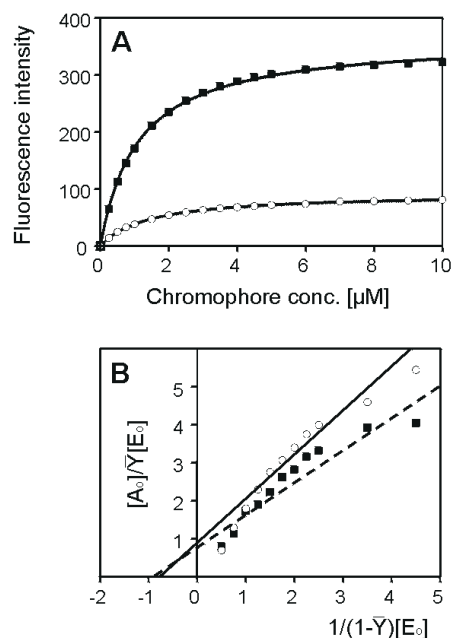

Figure 2 Fluorescence emission spectra of 1-AMA and bis-ANS. **(A)** Spectra of 3 μM 1-AMA pre-mixed with 3 μM OBP3 (solid line) and 1-AMA without protein (dashed line). Fluorescence was excited at 256 nm and spectra were recorded between 420 and 600 nm. **(B)** FRET from OBP3 to bis-ANS. Spectrum of 3 μM OBP3 without bis-ANS (dashed line) and in the presence of 3 μM bis-ANS (solid line). Tryptophan fluorescence of OBP3 was excited at 295 nm and recorded between 300 and 550 nm. All spectra measurements were carried out in 100 mM potassium phosphate buffer, pH 7.5.

Figure 3 Binding curves of 1-AMA and bis-ANS to recombinant OBP3. **(A)** A solution of 3 μM OBP3 was titrated with 1-AMA (filled squares) and bis-ANS (open circles), respectively. The fluorescence was excited at 256 nm for 1-AMA and at 295 nm for bis-ANS, monitoring the FRET of the OBP3 tryptophan to bis-ANS. Concentration-dependent changes in the fluorescence emission maximum were followed at 490 nm for 1-AMA and at 484 nm for bis-ANS. Measurements were carried out in 100 mM potassium phosphate buffer, pH 7.5. **(B)** Linearized form of binding values according to the Stockell equation for 1-AMA (filled squares) and bis-ANS (open circles). Assuming a linear relationship, the slope of the straight line reveals the binding constant of 1-AMA ($K_d = 1.22 \pm 0.09 \, \mu M$), bis-ANS ($K_d = 1.58 \pm 0.11 \, \mu M$) and the number of binding sites. Data are the means of three measurements.

form according to the Stockell equation (Stockell, 1959). Binding affinity constants were calculated from the slopes of the graphs, resulting in similar K_d values of 1.22 ± 0.09 µM for 1-AMA and 1.56 ± 0.11 µM for bis-ANS. For both chromophores the number of binding sites was determined as 1 per protein monomer (Figure 3B).

To examine if certain odorous ligands may be able to displace the chromophore from the binding protein competitive binding assays were performed following the procedure described previously (Löbel *et al.*, 1998). 2-Iso-butyl-3-methoxypyrazine (IBMP), a high affinity ligand for various OBPs (Pelosi, 1994), was used to compete for the protein–chromophore complexes of 1-AMA and bis-ANS. As shown in Figure 4, applying increasing concentrations of IBMP reduced the fluorescence intensity of the OBP3–1-AMA emission spectrum; the half-maximal value (IC_{50}) was determined as 10.1 µM (Table 1). Since the spectrum of the OBP3–bis-ANS complex did not change upon IBMP titration, subsequent displacement experiments were performed using 1-AMA as the chromophore. To assess the binding spectrum of OBP3 odorous compounds which represent several classes of chemical structures were employed in the competition assays. As a first step a different class of heterocyclic compounds was examined, the thiazole derivatives 2-isobutylthiazole and 4,5-dimethylthiazole. It was found that even very low concentrations of both thiazole derivates strongly attenuated the fluorescence
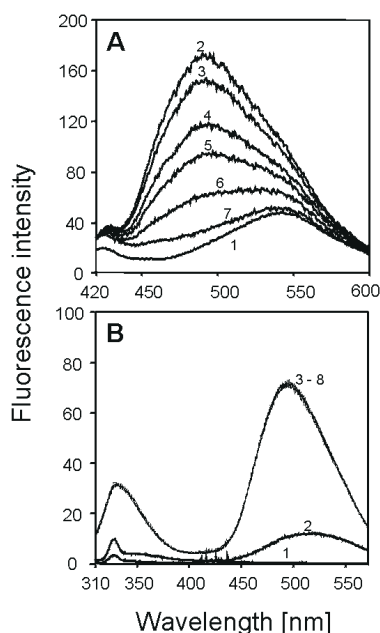
intensity, indicating that these compounds are effective ligands for OBP3 (Figure 5A). In contrast, the unsubstituted thiazole did not significantly alter the OBP3–1-AMA fluorescence. To assess whether the ligand spectrum of OBP3 is tuned to heterocyclic compounds benzene derivatives

Table 1   Binding values of different odorous compounds to OBP3

Chemical class	Odorous compound	$IC_{50} \times 10^{-6}$ (M)[a]	$K_i \times 10^{-6}$ (M)
Heterocycles	2-isobutylthiazole	2.03	0.58
	4,5-dimethylthiazole	6.32	1.80
	thiazole	≥100	
	IBMP	10.10	2.88
Terpenoids	cis-limonene-1,2-epoxide	10.22	2.92
	S-(–)-limonene	8.43	2.41
	(–)-camphor	≥100	
Aromatics	benzaldehyde	≥100	
	benzophenone	≥100	
	Keton-moschus	6.62	1.88

[a]Data are the means of three independent experiments.

Figure 4   Fluorescence quenching upon binding of IBMP. **(A)** Pre-equilibated complex of 3 µM OBP3 and 1-AMA (lane 2) competed by increasing concentrations of IBMP: lane 3, 0.5 µM; lane 4, 1.0 µM; lane 5, 2.0 mM; lane 6, 4.0 µM; lane 7, 8,0 µM. Lane 1, spectrum of 3 µM 1-AMA without protein. Fluorescence was excited at 256 nm. **(B)** Complex of 3 µM protein with 3 µM bis-ANS treated with 1–10 µM IBMP (lanes 3–8). Lanes 1 and 2, spectrum of buffer solution and 3 µM bis-ANS alone. Fluorescence energy transfer was excited at 295 nm.
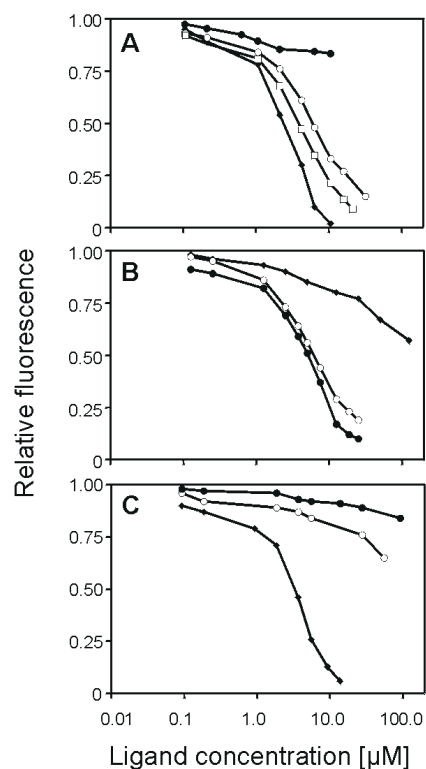

Figure 5   1-AMA binding competition by odorants of several chemical classes. Fluorescence of the complex OBP3–1-AMA (3 µM) was designated 1. **(A)** Heterocycles. Decay of fluorescence as a function of increasing concentrations of IBMP (○), 2-isobutylthiazole (◆), 4,5-dimethylthiazole (□) and thiazole (●). **(B)** Terpeniods. Fluorescence of the complex was decreased by (–)-camphor (◆), S-(–)-limonene (●) and cis-limonene-1,2-epoxide. **(C)** Other compounds. Titration of benzaldehyde (●), geraniol (○) and keton-moschus (◆) to the complex.

were employed, however, neither benzaldehyde nor benzo-phenone were particularly effective in displacing 1-AMA. In contrast, keton-moschus, a musk-like odorant, exhibited a relatively high affinity, with an IC_{50} value of 6.32 μM (Figure 5B). As representatives of non-aromatic compounds the monocyclic terpenes (–)-limonene and *cis*-limonene-1,2-epoxide, as well as the bicyclic compound (–)-camphor, were analyzed. For the monocyclic terpenes IC_{50} values of 8.43 and 10.22 μM, respectively, were obtained, whereas the bicyclic terpene (–)-camphor did not show any significant effect (Figure 5C). A variety of aliphatic compounds, such as geraniol, citralva and linalool, were not able to displace the 1-AMA chromophore (data not shown). These results suggest that a saturated or unsaturated ring structure is a requirement for an appropriate ligand of OBP3.

Titration calorimetry
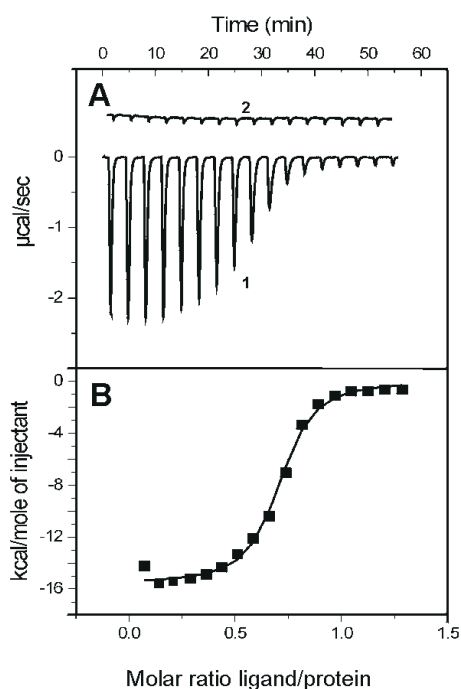
To explore binding of the two most efficient ligands, 2-iso-

Figure 6 ITC curves of OBP3 with 2-isobutylthiazole. **(A)** Raw data. Lane 1, a 500 μM stock solution of 2-isobutylthiazol in the syringe was titrated in 10 μl steps to a 30 μM OBP3 solution in the cell; lane 2, ligand solution in the syringe titrated to buffer solution in the cell. **(B)** The experimental values (filled squares) were fitted to the calorimetric binding isotherm for OBP3:2–isobutylthiazole (solid line). The measurement was carried out in 100 mM potassium phosphate buffer, pH 7.5, adjusted to 1% methanol both in the cell and in the syringe.

butylthiazole and 4,5-dimethylthiazole, ITC was employed in more detail. This approach allows simultaneous determination of thermodynamic parameters, binding stoichiometry and association/dissociation constants of protein–ligand interaction without the need for a labeled compound (Fisher and Singh, 1995; Doyle, 1997). The raw data obtained in a typical titration experiment with 2-isobutylthiazole are displayed in Figure 6A. After integration of the peaks the enthalpy values were plotted as a function of the ligand:protein molar ratio. The data were fitted assuming one binding site per protein monomer (Figure 6B). The resulting binding parameters for 2-isobutylthiazole and 4,5-dimethylthiazole are summarized in Table 2. The heat effect observed during titration indicates an exothermic binding reaction. The Gibbs free energy was calculated to be $\Delta G_b = -36.28$ kJ/mol for binding of 2-isobutylthiazole and $\Delta G_b = -32.58$ kJ/mol for 4,5-dimethylthiazole. The binding constant calculated for 2-isobutylthiazole was $K_d = 0.47$ μM and that for 4,5-dimethylthiazole $K_d = 1.89$ μM. The numbers of binding sites were determined as 0.78 and 0.76, respectively, suggesting a stoichiometry of 1:1 (values <1 indicate the presence of partially inactive protein).

Discussion

A third lipocalin sub-type expressed in the rat nasal glands has been identified. Based on its primary structure it shows only weak homology to the previously characterized rat odorant-binding proteins OBP1 and OBP2, but is more closely related to the lipocalin sub-class α-2u globulins, which comprises ~20 different sub-types (McFadyen *et al.*, 1999). Although these proteins have previously been thought to be expressed only in male rat liver, they have recently also been found in salivary glands (Shahan *et al.*, 1987; Gao *et al.*, 1989; Laperche *et al.*, 1993). The notion that the same or very similar lipocalin types may be expressed in different tissues is further supported by the observation that N-terminal sequences of OBPs isolated from mouse nasal mucus share significant similarity with the primary structure of α-2u globulins (Pes and Pelosi, 1995).

Binding assays revealed that OBP3 displays a ligand specificity which differs from that of the two other rat OBPs. OBP3 interacted with the two fluorescent ligands, 1-AMA and bis-ANS, but not with 1.8-ANS, which has recently been shown to interact quite specifically with OBP2. Moreover, the two active fluorescent probes 1-AMA and bis-ANS apparently bound to different sites on OBP3. Whereas

Table 2 Thermodynamic parameters of thiazole ligands to OBP3 at 30°C according to ITC

Compound	n	$K_d \times 10^{-6}$ (M)	ΔH_b (kJ/mol)	$T\Delta S_b$ (kJ/mol)	ΔG_b (kJ/mol)
2-Isobutylthiazole	0.78 ± 0.009	0.47 ± 0.04	−65.19 ± 0.3	−28.91	−36.28
4,5-Dimethylthiazole	0.76 ± 0.012	1.89 ± 0.09	−52.75 ± 0.4	−20.17	−32.58

1-AMA was competitively replaced by distinct odorous compounds, replacement of bis-ANS was not observed by any of the compounds employed. The observation that 1-AMA binding was competitively inhibited only by compounds with a ring structure suggests that a quite specific binding site was monitored by 1-AMA and implies that bis-ANS apparently interacted with a different hydrophobic site. Whether both probes may interact with distinct domains of the protein, e.g. within the cavity formed by the eight β-barrels or on the outside of the protein, is unclear. It is interesting to note that an additional binding site for hydrophobic ligands is formed at the central interface of the two monomers upon dimerization of bovine OBP (Tegoni et al., 1996). However, the dimerization of bovine OBP seems to be based on unique structural features of this protein; formation of OBP3 dimers has not been observed during this study. Previously it was reported for β-lactoglobulin that two indedendent binding sites for different ligands exist simultaneously on the protein monomer (Narayan and Berliner, 1997; Wu et al., 1999). For this reason it might be conceivable that bis-ANS interacts with a hydrophobic surface cleft on the outside of OBP3, as identified for retinoic acid on β-lactoglobulin (Lange et al., 1998). Since these structural elements are characteristic for all lipocalins (Flower, 1996), it cannot be excluded that bis-ANS binding may be a general feature of lipocalins. These considerations imply the need for alternative assays for monitoring the interaction of odorous hydrophobic compounds with nasal lipocalins without the necessity of preformed OBP–chromophore complexes. Therefore, ITC was employed in appropriate experiments to monitor the interaction of odorants with OBP3. The calculated binding data determined from ITC measurements (Figure 6 and Table 2) correspond very precisely to the K_i values obtained from the competitive fluorescence measurements (Table 1). Moreover, the affinity of 2-isobutylthiazole and 4,5-dimethylthiazole can now be expressed by the Gibbs free energy; the values of –36.28 kJ/mol for 2-isobutylthiazole and –32.58 kJ/mol for 4,5-dimethylthiazole are about four times lower than the ΔG_b values of the interaction of biotin with streptavidin, with a molar binding affinity of 10^{-12} (Klumb et al., 1998), but in the same range as for the interaction of IBMP with pig OBP and of oleic acid with fatty acid-binding protein (Burkhard et al., 1995; Burova et al., 1999).

The coexistence of at least three distinct lipocalin sub-types in the nasal mucus supports the view that each of these proteins may display a characteristic ligand-binding specificity. This notion seems to be supported by the data obtained from preliminary binding assays (Löbel et al., 1998; this study). However, the binding properties of individual OBP sub-types has to be assessed in much more detail under the same experimental conditions and using advanced technical approaches. Studies along this line are in progress.

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