Bovine odorant-binding protein (bOBP) is a dimeric lipocalin present in large amounts in the respiratory and olfactory nasal mucosa. The structure of bOBP refined at 2.0-Å resolution revealed an elongated volume of electron density inside each buried cavity, indicating the presence of one (or several) naturally occurring copurified ligand(s) (Tegoni et al. (1996) Nat. Struct. Biol. 3, 863–867; Bianchet et al. (1996) Nat. Struct. Biol. 3, 934–939). In the present work, by combining mass spectrometry, x-ray crystallography (1.8-Å resolution), and fluorescence, it has been unambiguously established that natural bOBP contains the racemic form of 1-octen-3-ol. This volatile substance is a typical component of bovine breath and in general of odorous body emanations of humans and animals. The compound 1-octen-3-ol is a typical component of odorous body emanations of humans and animals. The compound 1-octen-3-ol is also an extremely potent olfactory attractant for many insect species, including some parasitic vectors like *Anopheles* (Plasmodium) or *Glossina* (Trypanosoma). For the first time, a function can be assigned to an OBP, with a possible role of bOBP in the ecological relationships between bovine and insect species.

Several isoforms of odorant-binding protein (OBP)\(^1\) have been purified and characterized from the nasal mucosa of many mammalian species (1–5). These 19-kDa soluble proteins are produced in large amounts in seromucous glands of the respiratory and olfactory epithelium and are secreted in the mucus that layers the surface of nasal mucosa (5). OBP's belong to the lipocalin family, which is composed of structurally related soluble proteins that bind different types of small hydrophobic molecules (6, 7). These proteins are usually monomeric and are formed of a nine-strand \(\beta\)-barrel and a C-terminal \(\alpha\)-helix (6, 7). Bovine OBP is a dimer in which the C-terminal domain (residues 125–159) is swapped between the two monomers, a peculiar feature that seems to be specific to the bovine species (8, 9).

An elongated volume of electron density was observed in each of the two internal buried cavities. In the 2.0-Å resolution electron density we assigned this electron density to an unknown molecule of 8–10 non-hydrogen atoms (8), whereas Bianchet et al. (9) tentatively assigned it to a terpenoid compound, citronellyl acetate. Several structures of lipocalins have revealed the presence of specific or nonspecific copurified ligand in their binding sites, such as retinol in retinol-binding protein (10) and pheromones in the major urinary protein (11). The structure of porcine OBP, instead, revealed an empty cavity (12).

The recent characterization of the binding properties of porcine OBP (pOBP) in solution and in the crystal (13) has established that a high degree of hydrophobicity coupled to a molecular mass between 160 and 200 daltons is the main requirement for a ligand to match the \(\beta\)-barrel cavities, independently of its odorous properties, chemical class, and molecular structure. Some of the ligands of pOBP have a very low olfactory threshold (13), whereas others, for instance the toxic 7–11 carbon alkyl aldehydes (14), cannot be considered real odorous compounds (15). The \(K_d\) values, obtained or estimated from direct and competitive equilibrium binding experiments, are in the micromolar range for most of the OBP ligands (1, 13–18). The peculiar binding properties of OBP's with respect to other lipocalins (low ligand specificity and low affinities) and the very high quantities of protein found in nasal epithelium have suggested that OBP's might function as odorant carriers and/or scavengers for olfactory receptors or that they might be involved in endogenous detoxification processes (19). In this latter hypothesis it has been proposed that OBP's might deliver, to the appropriate degradative pathways, some toxic compounds produced during nasal epithelium turnover and inflammatory processes in charge of the nasal mucosa (19). These two hypotheses are not mutually exclusive, and at present, no direct experimental evidence supports or excludes any of them. On the contrary, the present data suggest that the two functions might be carried out in parallel. In the present work, the unambiguous identification of the copurified natural ligand of bovine OBP as the insect attractant 1-octen-3-ol makes it possible to hypothesize a role for bOBP in the ecological relationships between bovine and several insect species.

**MATERIALS AND METHODS**

**OBP Purification and Extraction of the Ligand**—Single bovine nasal mucosa samples were collected during fall and winter from freshly slaughtered 16–18-month-old males of different geographical origins.
1-Octen-3-ol Is the Natural Ligand of Bovine OBP

(1-Octen-3-ol Is the Natural Ligand of Bovine OBP) OBP was purified according to the procedure previously reported by Bignetti et al. (20). Briefly, purification consists of an ammonium sulfate fractionation of the soluble proteins in the extract of nasal mucosa, followed by two rounds of fast protein liquid anion exchange chromatography (Baker bond and Mono-Q) and a final step of purity of the OBP, which was checked by SDS-polyacrylamide gel electrophoresis, and its functionality was determined in binding tests with 1-aminooacetonic acid (AMA) as fluorescent probe (21–23). The molar absorption coefficient of 47,000 (M⁻¹ cm⁻¹) of OBP dimer at 280 nm was determined according to the Edelhoch method (24) as described by Pace et al. (25). The natural ligand was extracted from samples of native and denatured OBP with different organic solvents (diethyl ether, methylene chloride, and hexane). The protein (a 7.0-mg/ml OBP solution in 20 mM Tris-HCl, pH 7.8) was previously denatured by overnight incubation at room temperature in the presence of 7.5 mM urea. The ligand extraction was performed by vortexing mixtures of OBP-organic solvent in glass tubes (1:1 volumetric ratio), and the organic phases were analyzed by GC/MS. Aliquots of Tris buffer and OBP-organic solvent in glass tubes (1:1 volumetric ratio) were incubated with a fixed amount of OBP (10 mg/ml) at 4 °C and used within 4 days. The influence of the concentration of OBP on the chosing process of OBP was tested and found to be negligible up to 1% v/v, a result in contrast with the behavior of rat OBP reported in Briand et al. (23). In the case of the direct binding, the titration curves were prepared incubating different samples of OBP (0.76 µM dissolved in 20 mM Tris-HCl, pH 7.8, 0.5% ethanol (v/v)) with various amounts of AMA ranging from 0.019 to 10 µM for 24 h at 4 °C. In the case of the competition curves, the OBP samples were incubated with a fixed amount of 2.0 µM AMA and increasing concentrations of 1-octen-3-ol (0.39–50 µM). Fluorescence emission spectra between 450 and 550 nm were recorded at a fixed excitation wavelength of 380 nm using a PerkinElmer LS 50 luminescence spectrometer, and the formation of the bOBP-AMA complex was detected by the increase of the fluorescence emission intensity at 480 nm. The concentration of the complex was evaluated on the basis of a calibration curve obtained by incubating increasing concentrations of OBP (0.076–6 µM) with a saturating amount of OBP (100% binding). The binding and competition curves were analyzed using the nonlinear fitting facility of Sigma Plot 5.0 (Cambridge Soft Corp., Cambridge, MA).

RESULTS

Identification of the Ligand—The bOBP organic extract analyzed by gas chromatography yields a prominent peak (Fig. 1A). Comparison of control and native or denatured bOBP showed a single peak difference in mass spectra. After comparison with the Environmental Protection Agency/National Institutes of Health library, the electronic impact mass spectrum of this compound (ions at m/z 41, 43, 55, 57 (97% base peak), 72, 81, 99, 110) was found to correspond to a C8 monoethenyl alcohol (Fig. 1C). This was confirmed with CI/NH3 data exhibiting ions at m/z 128 and 146 (M + NH4)⁺, leading to a C10H16O formula. Ions at m/z 57 and 72 (McLafferty rearrangement) in the electronic impact mass spectrum and comparison of the retention times of standards indicated the presence of a secondary alcohol and led us to propose the structure of 1-octen-3-ol (Fig. 1C).
Tentative separation of the racemic standard on a chiral gas chromatography column failed. The derivatization of 1-octen-3-ol yielded two peaks in the same gas chromatography conditions (50/50 ratio and electronic impact mass spectra with characteristic ions at \( m/z \) 115 and 127 atomic mass units). The derivatization of the natural compound under identical conditions also yielded two peaks with identical mass, retention time, and intensities (Fig. 1).

**Overall Three-dimensional Structure**—As described previously, bOBP is a dimer consisting of 2 × 159 residues at neutral or basic pH and monomerizes at pH values below 4.5 (20). The 2.0-Å resolution structure of bOBP has been reported elsewhere (8). Briefly, each monomer is composed of a lipocalin-type nine-strand \( \beta \)-barrel comprising residues 15–121 (strands 1–8) and residues 145–149 (strand 9) from the other monomer (Fig. 2A). From residue 123 onwards, the topology diverges from the consensus lipocalin fold. The \( \beta \)-barrel is connected by an extended stretch of residues 123–126 to the \( \alpha \)-helix protruding out of the \( \beta \)-barrel and crossing the dimer interface (Fig. 2A). As a consequence, the \( \alpha \)-helix of one monomer is placed close to where the \( \alpha \)-helix of the other monomer would be if bOBP had a classical lipocalin fold, in a special arrangement called domain swapping (33). In the present structure, the two bOBP polypeptidic chains are visible from residues 1–159 and 3–157 for molecules A and B, respectively.

**Internal Cavities and the Buried Ligands**—Inside the \( \beta \)-barrel of each monomer, a large buried cavity of about 407 Å³ is observed (Fig. 2A), at a location similar to that observed in other closed lipocalins, such as the major urinary protein (11). Both cavities contain an elongated patch of electron density map, which has been attributed to an 8–10 non-hydrogen atom linear compound in the structure at 2.0-Å resolution (8).

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**Fig. 1. Gas chromatography-coupled mass spectroscopy of the bOBP extracts.** A, chromatogram of the bOBP extract displaying a prominent peak (arrow). B, derivatization of the compound identified in A to identify the presence and quantity of each enantiomer (see “Material and Methods”). C, mass spectra of the main peak identified by gas chromatography in A (bottom) and of the authentic 1-octen-3-ol (top).
Because of the significant improvements in resolution (1.8 Å), data treatment, map calculation, and refinement procedures, interpretable features came out in the difference maps within the buried cavities. Having determined the nature of the ligand by GC/MS, we could fit a molecule of 1-octen-3-ol in each difference electron density map. After CNS refinement, significant patches of electron density still remained in the difference Fourier maps. The two enantiomers of 1-octen-3-ol were then fitted in the most appropriate conformations, and their occupancies were refined in CNS. The resulting structure contains both enantiomers in a ratio close to 1, a result in accord with the GC/MS data (Fig. 2, C and D). In cavity A, the two isomers have very similar orientations and are quasi-superimposed (Fig. 2C). The aliphatic chains of the two isomers are extended and remain close to each other. The hydroxyl groups point in the same direction, toward an area of the cavity where no hydrogen bond donor is available, however. The positions of the two 1-octen-3-ol enantiomers in the two cavities are slightly different (Fig. 2, C and D), because of a rotation around the ω atom. This movement leaves an empty space in the cavity, between the aliphatic chain of the ligands and the Oγ atom of Thr-38, which is filled by a water molecule hydrogen-bonded to the Oγ atom (Fig. 2E).

The structure of the bOBP-AMA complex reveals two very well ordered AMA molecules in the buried cavities (Fig. 2A). This indicates clearly that these cavities are the general binding site of bOBP, as in pOBP (6, 13), and discredits the existence of a putative third binding pocket previously proposed (8).
The orientation of AMA in the binding pocket is comparable with that of 1-octen-3-ol, with the long axis perpendicular to the axis of the β-barrel (Fig. 2, A and B).

The walls of these cavities are mostly composed of hydrophobic residues. The water-accessible surface area of bOBP alone or with its ligand was calculated for all the residues. The ligands cover 150 Å² of each cavity surface. Residues Phe-36, Phe-89, Asn-103, Tyr-83, and Phe-54 display the larger loss of surface accessibility upon complexation (10–12 Å²) (Table II). The residues involved in the interaction with the ligand are identical in the two cavities and have similar variation of accessibility. Furthermore, all the residues of both cavities are superimposable, including Phe-89, which is found with two different conformations in each cavity (Fig. 2E).

**Fluorescence Binding Assay with AMA and Competition between AMA and 1-Octen 3-ol**—The fluorescence properties of AMA when surrounded by a hydrophobic environment have already been used to probe binding in pOBP (20) and porcine salivary OBP (34). We have determined the binding affinity of AMA for ligand-depleted bOBP by titrating at 480 nm the decrease of AMA fluorescence at 480 nm was recorded as a function of 1-octen-3-ol concentration (Fig. 3B). The competition curve has a hyperbolic decay with an apparent $K_d$ value of 9.6 μM. The most complete displacement of AMA indicates a stoichiometry of two 1-octen-3-ol molecules per bOBP dimer. Taking into account the stoichiometry of 1.85 AMA molecules per bOBP dimer (data not shown), indicating some degradation of bOBP during ligand extraction. The $K_d$ for AMA for bOBP is 1.0 μM, a value within the range found for most odors toward OBPs (7, 12–16). Because x-ray crystallography experiments indicated that AMA binds in the internal cavities of bOBP described in the previous paragraph, competition of AMA by odors should titrate the same binding site of bOBP, which is the functionally relevant one.

We have determined the binding affinity of 1-octen-3-ol (racemic) by chasing saturating amounts of AMA bound to the internal cavities of bOBP. The decrease of AMA fluorescence at 480 nm was recorded as a function of 1-octen-3-ol concentration (Fig. 3B). The competition curve has a hyperbolic decay with an apparent $K_d$ value of 9.6 μM. The almost complete displacement of AMA indicates a stoichiometry of two 1-octen-3-ol molecules per bOBP dimer. Taking into account the stoichiometry of 1.85 AMA molecules per bOBP dimer (data not shown), indicating some degradation of bOBP during ligand extraction. The $K_d$ for AMA for bOBP is 1.0 μM, a value within the range found for most odors toward OBPs (7, 12–16). Because x-ray crystallography experiments indicated that AMA binds in the internal cavities of bOBP described in the previous paragraph, competition of AMA by odors should titrate the same binding site of bOBP, which is the functionally relevant one.

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**Table II**

| Interactions of the natural ligand, the racemic 1-octen-3-ol, with the residues of bOBP internal cavities |
|-------------------------------------------------|---------------------|
| Cavity B                                        | Cavity A            |
| Ile-22                                         | 8                   |
| Phe-36                                         | 18                  |
| Thr-38                                         | 8                   |
| Phe-40                                         | 11                  |
| Phe-54                                         | 10                  |
| Phe-56                                         | 8                   |
| Val-69                                         | 7                   |
| Ala-81                                         | 4                   |
| Tyr-83                                         | 10                  |
| Asn-87                                         | 7                   |
| Phe-89                                         | 13                  |
| Ala-101                                        | 3                   |
| Asn-103                                        | 17                  |
| Leu-115                                        | 8                   |
| Thr-116                                        | 2                   |
| Gly-117                                        | 2                   |
| Phe-119                                        | 9                   |
| Total                                          | 145                 |
| Cavity B                                       | Cavity A            |
| Phe-36                                         | 18                  |
| Thr-38                                         | 8                   |
| Phe-40                                         | 11                  |
| Phe-54                                         | 10                  |
| Phe-56                                         | 8                   |
| Val-69                                         | 7                   |
| Ala-81                                         | 4                   |
| Tyr-83                                         | 10                  |
| Asn-87                                         | 7                   |
| Phe-89                                         | 13                  |
| Ala-101                                        | 3                   |
| Asn-103                                        | 17                  |
| Leu-115                                        | 8                   |
| Thr-116                                        | 2                   |
| Gly-117                                        | 2                   |
| Phe-119                                        | 9                   |
| Total                                          | 154                 |

**DISCUSSION**

Identification of lipocalin-bound compounds based on mass spectroscopy coupled to x-ray structure determination has already been successful in the past, for example in the case of the major urinary protein, where four pheromonal components were identified as copurified ligands (11). This procedure failed, however, with aphrodisin, another pheromonal lipocalin (35). In the present study, by using the same type of procedure, we have identified a unique compound, the racemic 1-octen-3-ol, as the naturally occurring copurified ligand of bOBP. Furthermore, both $R$ and $S$ isomers were found to match nicely the electron density maps of the bOBP natural ligand at 1.8-Å resolution. The contacts between the ligand and amino acid residues of the β-barrel cavities have been characterized at the atomic level. The x-ray structure of the fluorescent probe AMA has revealed that it binds in the same cavity as 1-octen-3-ol. Although several reports of OBP fluorescence titration with AMA are available in the literature, we provide here the first direct evidence that AMA binds in the lipocalin internal pocket. This gives more weight to fluorescence experiments based on ligand displacement, such as those described here. All these

**Fig. 3. Fluorescence titration of the bOBP binding site.** A, fluorescence titration curve of bOBP with AMA. Before the experiment, the natural ligand was removed from the protein by urea denaturation and organic solvent extraction (see “Materials and Methods” for details). B, competition curve between 1-octen-3-ol and AMA on bOBP. The concentration of 1-octen-3-ol is reported versus the residual concentration of AMA bound to bOBP (see “Materials and Methods” for details).
results converge to unambiguously identify 1-octen-3-ol (R,S) as the natural copurified ligand of bOBP.

Previous studies in solution (1–5) and the recent structural characterization of porcine OBPs binding properties (13) have shown that a high degree of hydrophobicity coupled to a molecular mass between 160 and 200 daltons is the main requirement for a ligand to fit the β-barrel cavities of OBPs, irrespective of the chemical class, subtypes, and molecular structure. These compounds can be odoriferous chemical messengers that strongly contribute to mediate the relationship between an individual animal and its environment, in food search, mating, etc. (36), or toxic compounds such as natural and synthetic pollutants (19).

Moreover, among all potential ligands of OBPs, two groups can be distinguished: exogenous compounds inhaled from the environment and endogenous compounds produced by the animal. The endogenous compounds can be produced in the naso-mucosa itself as consequence of physiological epithelium turnover, inflammation, and injuries resulting from the action of physical (temperature and humidity), chemical (pollutants and oxidants passing through respiratory airways), and biological (parasites, bacteria, and viruses) aggression. They can also be produced and released by organs (lungs, stomach, bloodstream, etc.) and flow through the nasal cavity as breath components. In this context, it is particularly striking to observe that 1-octen-3-ol has an endogenous origin in bovine species and in ruminants in general (37). It is produced in large quantities from fatty acids by lipoxigenases (38) probably associated to the ruminal microflora and then released in the environment as a component of the breath of the animal (37).

It has also been demonstrated that 1-octen-3-ol has a relevant role in the olfactory chemoreception-driven behavior of many worldwide insect species, including parasite vehicles like Anopheles and Glossina (38, 39). These insects express in their antennae two receptors specific for the breath components 1-octen-3-ol and carbon dioxide, respectively. Their simultaneous stimulation drives host-seeking behavior toward bovines through their breath components (37). The identification of 1-octen-3-ol as the bOBP naturally observed ligand suggests that bOBP might be used by bovines to remove parts of 1-octen-3-ol from the breath flowing through the nasal cavities and to make them less appealing for several insect species. This would result in a general decrease of the number of insect bites and furthermore might partially protect the animal from parasitosis and infectious diseases carried by these insect vectors.

This hypothesis on the possible role of OBPs in the ecological relationships between bovine and insect species must be limited, at present, to the case of bovine species, because only in this case has the presence and the role of the breath component 1-octen-3-ol in the ecological relationships with insects been extensively documented. Besides, this function should not be taken as an alternative to the other roles previously proposed, such as that of a binder of exogenous compounds. All the possible putative roles of OBPs in mammalian physiology, toward either endogenous or exogenous compounds, are compatible with their above-mentioned capacity to bind several classes of small molecules, with their stability, and with their abundant production and presence in nasal mucosa.

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