We have characterized the odorant binding properties of purified bovine odorant-binding protein (OBP) using as a ligand [3H]3,7-dimethyloctan-1-ol ([3H]DMO). A broad variety of odorants, including terpenes, aldehydes, esters, and musks, bind to OBP with affinities of 0.2 to 100 μM. Odorant affinities for OBP correlate most closely with their stimulation of an odorant-sensitive adenyl cyclase as well as hydrophobicity. We also measured the kinetics of binding for the ligands, [3H]DMO and 2-isobutyl-3-[3H]methoxy-pyrazine. Dissociation of both is markedly accelerated in the presence of excess unlabeled ligand. Competition curves of displacers for [3H]DMO binding are shallow, and saturation binding isotherms for [3H]-odorants are curvilinear. These kinetic and equilibrium binding properties suggest that OBP interactions with odorant ligands are negatively cooperative.

Odorant-binding protein (OBP) is a soluble 19-kDa protein localized to the nasal mucosa of several species (1-4). OBP was identified based on its ability to bind radiolabeled odorants including 2-isobutyl-3-[3H]methoxy-pyrazine ([3H]IBMP), [3H]3,7-dimethyloctan-1-ol ([3H]DMO), [3H]amyl acetate, and [3H]methyl/dihydrojasmonate. The correlation between affinities of pyrazine and thiazole odorants for OBP and their detection thresholds (1,5) suggest a physiological role for OBP in olfaction. We have cloned the cDNA for rat OBP (8), and Cavaggioni et al. (9) determined a portion of the amino acid sequence of bovine OBP. Amino acid sequence analysis places OBP in a family of 18-20-kDa soluble proteins that bind and serve as carriers for small lipophilic molecules such as cholesterol, steroids, and retinol (10,11).

To gain further insight into the molecular function of OBP, in the present study we have characterized in detail the binding of a series of odorants to purified bovine OBP, and examined structure-activity relationships. We have also characterized the kinetic properties of [3H]DMO and [3H]IBMP binding to the purified protein.

**MATERIALS AND METHODS**

**Odorants—**[3H]IBMP (14.6 Ci/mmol) was obtained from Du Pont-New England Nuclear. 3,7 Dimethyl[3H]octan-1-ol ([3H]DMO; 57 Ci/mol) was prepared by Amersham Corp. Unlabeled odorants were from International Flavors and Fragrances (Union Beach, NJ) or Fluka Chemical Corp. (Ronkonkoma, NY). Trans-retinal and trans-retinal were from Sigma. Cholesterol was from Avanti Polar Lipids, Inc. (Pelham, AL).

**Preparation of OBP—**Bovine OBP was purified as described (1) from bovine nasal epithelium by sequential ammonium sulfate fractionation, DEAE ion-exchange chromatography and hydroxylapatite chromatography. All odorants used in the binding experiments was >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For estimates of Bmax, we used preparations of OBP that were >99% pure. Final purification was by fast protein liquid chromatography using a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). Proteins were eluted with a linear gradient of 0-0.5 M NaCl in 50 mM Tris-HCl (pH 7.9 at 4°C).

**Equilibrium Binding Assays—**In the standard binding assay (1), 3-10 nM [3H]DMO or [3H]IBMP were incubated with 20-200 ng of bovine OBP in 0.1 ml of 50 mM Tris-HCl (pH 7.7 at 25°C) (buffer A). Specific binding was determined by including 10⁻⁴ M unlabeled odorant in the assay. Specific binding was defined as total minus nonspecific binding. After incubation for 60 min at room temperature, bound ligand was separated from free ligand by vacuum filtration over glass fiber filters precoated with 0.3% polyethyleneimine, as described (1). Radioactivity on the filters was monitored by liquid scintillation spectrometry at 50% counting efficiency.

For protein linearity studies, 6 n to 2 μg of OBP were employed in the assay. For odorant inhibition studies, nonradioactive odorants were diluted to 10⁻⁸ M in ethanol and then further diluted in buffer A such that the final ethanol concentration in the binding assay was ≤1%. IC₅₀ values and Hill coefficients were calculated manually or with the equilibrium binding binding data curve-fitting programs EDBA (12) and LIGAND (13).

**Association Kinetics of [3H]-Odorants to Bovine OBP—**Association of [3H]DMO and [3H]IBMP to bovine OBP was examined at 25°C by incubating [3H]-odorant (1-67 nM) with OBP (20-200 ng/tube) for various intervals in triplicate. Association data were fit to the integrated second order rate equation for association (for derivation see Ref. 14):

$$k_{assoc} = \frac{2.303}{t(L_T - R_T) \log R(L - R)}$$

where k, is the rate constant of association for the reaction R + L ⇄ RL, t is the time of incubation, L is the total ligand concentration, R is the total OBP concentration, and RL (or B) is the specifically bound ligand at any given t. In all association experiments, the total bound [3H]-odorant concentration was <10% of the total offered [3H]-odorant concentration.

**Dissociation Kinetics of [3H]-Odorant Binding—**Dissociation of [3H] DMO and [3H]IBMP from bovine OBP was examined at 25°C under conditions in which negligible reassociation of dissociated ligand can occur. [3H]-Odorant was incubated with 50-200 ng of OBP in equilibrium (60 min) as described above. For dissociation by “infinite dilution,” specific binding at equilibrium (B₀) was measured, and then a
Odorant Binding

50-fold excess of buffer A was added and binding measured at intervals from 30 s to 160 min.

For dissociation by dilution plus excess ligand, conditions were identical to the above procedure except that dissociation was in the presence of 50-fold excess buffer A containing 10⁻⁴ M unlabeled odorant (DMO or IBMP). The kinetic rate constant k₂ was calculated as the ratio ln 2/t₁₂ where t₁₂ is the half-time for dissociation from a semilogarithmic plot of time versus B/B₀.

Biophysical Properties of Odorants—Biophysical properties of odorants (see Tables II and III) were provided by Dr. Craig Warren (International Flavors and Fragrances, Union Beach, NJ). Log P, the log of the 1-octanol/water partition coefficient, was predicted by a computer model (15). The molecular volume (in cc/mol) was calculated using Van der Waals radii and bond distances. The volume occupied by an atom is assumed to be a sphere with radius equal to the Van der Waals radius of the atom minus the volume at overlap with adjacent atoms. The volume contributions of attached hydrogen atoms are included in the calculation.

The retention times of odorants were measured as described (16) on two 50-meter fused silica columns (0.32-mm internal diameter, Hewlett-Packard). Odorants were eluted from a carbowax column (0.5 μM non-bonded phase) which tends to retain more volatile, polar compounds and from a methyl silica column (0.5 μM bonded phase) which also retains more volatile molecules. The values for each odorant are dimensionless ratios of elution times.

RESULTS

Structure-Activity Relationships of Ligands in Binding to OBP—Previously we examined relative potencies of a series of pyrazines in binding to bovine OBP and observed a correlation between relative binding affinities for OBP and human detection thresholds of the pyrazines (1). In the present investigation we have examined the binding properties of a broad range of odorant molecules of different chemical classes, including monoterpenes, esters, aldehydes, aromatics, musks,
and jasmine odorants (Figs. 1 and 2). For each odorant 8–15 concentrations were tested for affinities in competing for the binding of [3H]DMO (Tables I and II). The monoterpenes include some of the most pleasant smelling odorants and consist of two isoprene units connected in a head-to-tail orientation with a 2,6-dimethyloctane skeleton. We have evaluated the binding of 27 monoterpenes and other terpenoid compounds (Table I). These vary in affinity over a 300-fold range, with the most potent, DMO, displacing an IC\textsubscript{50} of 0.3 \mu M, while the weakest, including borneol, fail to bind OBP (IC\textsubscript{50} of \geq 100 \mu M). Progressive unsaturation of the monoterpenes is associated with diminished binding affinity as the addition of a double bond in the transformation of 2,6-dimethyloctane, to form 3,7-dimethyl-1-octene, results in a 2-fold decrease in affinity. The addition of a further two double bonds, transforming 3,7-dimethyl-1-octene to neo-allococimene, decreases affinity another 20-fold. Similarly, citronellol differs from DMO only in the addition of a double bond, which decreases affinity about 3-fold. Several of the compounds in this class possess hydroxyl groups at carbon position 1, which do not markedly alter affinity. The optical isomers d-carvone and l-carvone, which have different odor qualities and similar human detection thresholds, have about the same affinity.

Among monoterpenes, higher affinities for OBP show a limited relationship with greater hydrophobicity as measured by the octanol-water partition coefficient, log \( P \) (\( r = 0.67, p < 0.05, n = 25 \)) (Table I). Binding affinities of the terpenoid compounds are not significantly correlated with chemical or physical properties such as molecular weight, molecular volume, and retention times on carbowax or silicon ester columns. The largest terpenoids tested (\( \alpha \)-cedrene, cholesterol, and \( \beta \)-carotene) fail to bind to OBP.

In other series relative affinities for OBP vary from IC\textsubscript{50} values of 0.2 \mu M to numerous odorants which fail to inhibit [3H]DMO binding at 100 \mu M (Table II). High affinity for OBP is not confined to any particular structural class of odorants, as the most potent displacers (with IC\textsubscript{50} values less than 1 \mu M) include monoterpenes, aldehydes, esters, aromatics, a sulfur, and a musk. Furthermore, compounds that fail to inhibit [3H]DMO binding to OBP (with IC\textsubscript{50} Values > 100 \mu M) also include a broad range of structural classes. Relative affinities for OBP are also not related to the hedonic properties of the molecule. For instance, the most potent group includes 2-furfurylmercaptan, which has a sulfurous, noxious odor. By contrast, benzophenone, with a rose-like quality, and musk 89, a woody, pleasant component of perfumes, have high affinities for OBP. Among the substances with low binding affinities, normal butyl alcohol has an unpleasant odorant, while ethyl vanillin, fructone, and aubepine are perceived as pleasant odorants.

As was the case for the monoterpenes, with other chemical classes higher affinity for OBP is associated to a limited extent with greater hydrophobicity. For instance, 2 and 3 hexylpyridine and musk 89, which are potent in competing for [3H]DMO binding, have log \( P \) values of 4–6, whereas phenylethylalcohol, cinnamic aldehyde, and normal butyl alcohol, with poor affinity for OBP, have log \( P \) values of 0.9–1.9. However, there are a number of exceptions as with the terpenes, to this general correlation. 2-Furfuryl mercaptan, one of the displacers with highest affinity, has a log \( P \) value of 1.4. A number of hydrophobic odorants display a modest or low affinity for OBP. For instance, vertenex and ambrettolide, with IC\textsubscript{50} values of 22 and 12 \mu M in competing for [3H]DMO binding, have respective log \( P \) values of 4.2 and 6.5. Because of these exceptions, there is a weak but statistically significant correlation between the log \( P \) values and IC\textsubscript{50} values in inhibiting [3H]DMO binding for non-terpenoid agents (\( r = -0.32, p < 0.05, n = 53 \)). Within smaller structural series there is a trend for hydrophobic ligands to bind with higher affinity to OBP. For example, among six straight-chain aldehydes this correlation is significant (Table II; \( r = -0.87, p < 0.05, n = 6 \)). In a series of homologous pyrazines, we found a general correlation between high affinities for OBP and low sensory detection thresholds (1). A similar general correlation was reported by Topazzini et al. (5) for the binding of cycloalkanethiazoles to bovine OBP. In the present study there is no significant correlation between threshold values for all ligands examined and affinities for OBP. However, since the human detection threshold values for the various odorants have been obtained by different techniques (17, 18) they may not be directly comparable. Also odorant affinities for OBP, which are similar for bovine and rat (7), may differ in humans. Additionally, with such a heterogeneity of chemical structures, one would not anticipate a close relationship between sensory thresholds and binding affinities, since multiple factors determine potencies at sen-
Aldehydes

Highly potent

Tetradecanal

Undecanal

Moderately potent

Decanal

Nenanal

Heptanal

4-Heptanal

No detectable binding

Pinoacetaldehyde α-Pinylisobutyraldehyde

Coca

Myrrc aldehyde

Agrumen aldehyde

Aubepine

Ethyl vanillin

3,5,5-Trimethylhexanal 2-Methylpropanal

Aromatics

Highly potent

Benzyi isovalerate

Benzophenone

Hexyl cinnamic aldehyde

2-Hexylpyridine 3-Hexylpyridine

Amyl cinnamic aldehyde

Musk 89

Benzyi benzoate

Skatole

Moderately potent

Galaxolide

Cinamic aldehyde

Phenethyl alcohol

Coca

No detectable binding

Methyl isonicotinate

Ethyl vanillin

quinoline 3-Ethyl-4-methylpyridine

Aubepine

4-Methylphenol

FIG. 2—continued

sory odor receptors, including the odorant's volatility and air-
mucus partition coefficient.

An odorant-sensitive adenyl cyclase is thought to be in-
volved in signal transduction for olfaction (19). Previously, we examined the potencies of a homologous series of pyrazines in stimulating adenyl cyclase (20). The correlation between affinities of these pyrazines for OBP (1) and potencies in enhancing the odorant sensitive adenyl cyclase is high, $r = -0.92$ ($p < 0.01, n = 12$). For other substances there is also a significant positive correlation between potencies in stimulating adenyl cyclase and affinities for OBP (Table III) ($r = -0.74, p < 0.01, n = 22$). Odorant affinities for OBP are similar in bovine and rat preparations (5), and effects on adenyl cyclase are similar in rat and frog (20), suggesting that species variations would not preclude attempts to ex-
amine relationships between odorant affinities for bovine OBP and frog adenyl cyclase.

The competition curves of unlabeled ligands inhibiting $[^3H]$ DMO binding are shallow with Hill coefficients ranging be-
tween 0.5 and 1.0 (data not shown). The Hill coefficient for in-
bihition of $[^3H]$ DMO binding by unlabeled DMO is 0.85.

To explore factors accounting for shallow competition curves of ligands for $[^3H]$ DMO binding we generated satu-
rated isotherms of $[^3H]$ DMO and [H]BMP binding to OBP. Previously, we had observed a curvilinear (convex upward) Scatchard plot for $[^3H]$BMP binding consistent with the presence of multiple binding sites or negative cooperativity (1). In the present experiments, we have replicated this find-
ing employing lower concentrations of OBP. Scatchard analy-
sis of the binding of $[^3H]$DMO in the presence of increasing concentrations of unlabeled DMO reveals a biphasic curve (Fig. 3A). Although more than two binding components may be present, a two-site model seems most parsimonious. Analysis for a two-site fit indicates that for $[^3H]$DMO the high affinity component has a $K_D$ of 0.3 μM and a $B_{max}$ of 210 pmol/mg OBP while the low affinity component has a $K_D$ of 97 μM and a $B_{max}$ of 27 nmol/mg OBP. For $[^3H]$BMP binding in the presence of increasing concentrations of unlabeled IBMP, Scatchard analysis for a two-site fit reveals a high affinity component ($K_D = 4.0$ nM, $B_{max} = 48$ pmol/mg OBP) and a low affinity component ($K_D = 2.7$ μM, $B_{max} = 18$ nmol/mg OBP) (Fig. 3B).

Kinetics of $[^3H]$Odorant Interactions with OBP—We ex-
ammed the association of $[^3H]$DMO to OBP at 25 °C utilizing four concentrations of $[^3H]$DMO (Fig. 4A). At all four con-
centrations $[^3H]$DMO binding begins to plateau at about 20 min, and half-maximal binding is apparent at 2-10 min. The calculated rate constant for association ($k_+$) under pseudo-
first order conditions is $2.5 \times 10^{5} \text{M}^{-1} \text{min}^{-1}$. The association of $[^3H]$BMP to OBP was examined at a single ligand concentration (3 nM) (Fig. 4B). $[^3H]$BMP binding also reaches plateau by 20 min with a calculated $k_+$ value of $3.6 \times 10^{4} \text{M}^{-1} \text{min}^{-1}$.

We measured the dissociation of $[^3H]$DMO and $[^3H]$BMP, initiating dissociation both by infinite dilution and by adding excess unlabeled ligand (Fig. 5). For binding sites that mani-
fest negative cooperativity, dissociation by infinite dilution in the presence of excess ligand is more rapid than when meas-
In the present study we have explored structure-activity relationships for the binding of OBP of over 80 ligands. OBP interacts with a broad range of markedly different structures. OBP does not display a uniquely high affinity for any single chemical. Among the monoterpenes certain chemical modifications lead to alterations in affinity for OBP. Higher affinity binding is associated to a limited degree with more
lipophilic ligands. However, lipophilicity alone is not a major determinant of affinity for OBP, as a number of relatively hydrophilic odorants display high affinity for OBP and a number of highly lipophilic molecules display low affinity for OBP. Relative potencies of odorants in stimulating adenyl cyclase in olfactory cilia also correlate with affinity for OBP much better than the limited correlation of odorant effects on adenyl cyclase and hydrophobicity (20). The odorant-sensitive adenyl cyclase is thought to be associated with physiologic odorant receptors (19, 20). Thus, our results suggest a structural similarity in the odorant recognition sites of OBP family, little information is available regarding detailed structure-activity relationships for binding to the proteins across a broad series of ligands. Since we have not detected \[^{3}H\] odorant binding to soluble protein from tissues rich in retinol-binding protein such as the liver (1), prominent odorant interactions with retinol-binding protein are not likely. Additionally, we have been unable to demonstrate \[^{3}H\] odorant binding to pure \(\beta\)-lactoglobulin (data not shown), another member of OBP’s superfamily. Unlabeled retinol and retinal interact with retinol-binding protein are not likely. Adenylyl cyclase in olfactory cilia also correlate with affinity for OBP and a broad series of ligands. Since we have not detected \[^{3}H\] odorant binding are consistent with this conclusion. Scatchard plots reveal a molecular mass of 38-40 kDa, indicating that OBP is a pure homodimer. Purified OBP migrates as a single species in two-dimensional gel electrophoresis (data not shown). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a subunit molecular mass of 19 kDa, and gel filtration analyses reveal a molecular mass of 141 kDa. Since OBP is a pure protein, most of these artifactual explanations are improbable. Artifacts associated with properties of four molecularly cloned cDNAs for rat OBP suggest that only subunit is expressed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a subunit molecular mass of 19 kDa, and gel filtration analyses reveal a molecular mass of 38-40 kDa, indicating

The marked acceleration of \[^{3}H\]DM0 and \[^{3}H\]IBMP disassociation elicited by excess unlabeled ligands implies negatively cooperative interactions with OBP. The shallow competition curves of more than 50 ligands competing for \[^{3}H\] odorant to OBP. Values are the percent stimulation of adenylyl cyclase of frog olfactory cilia by substances at 100 \(\mu\)M concentration. The stimulation by citralva is defined as 100%. From Ref. 20.

In other systems reported to display negative cooperativity (22-27), the binding of ligands to receptor sites is generally measured in crude membrane preparations. In these instances, apparent negative cooperativity could be accounted for by incomplete washing, more than one population of binding protein, small amounts of nonspecific, rapidly dissociating low affinity sites, the non-equivalence of labeled (often iodinated) ligand and unlabeled ligand, polymerization of hormone ligands, unstirred layers, and ligand-like interactions causing steric or electrostatic hindrance (26, 28-30). Since OBP is a pure protein, most of these artificial explanations are improbable. Artifacts associated with properties of the ligands are unlikely, since we have obtained closely similar results utilizing \[^{3}H\]IBMP and \[^{3}H\]DM0, which are structurally dissimilar.

The curvature of a Scatchard plot, revealing apparent high and low affinity compartments, might reflect binding of a ligand to different proteins. Several lines of evidence indicate that OBP is a pure homodimer. Purified OBP migrates as a single species in two-dimensional gel electrophoresis (data not shown). Sequence analysis of four molecularly cloned cDNAs for rat OBP suggests that only subunit is expressed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a subunit molecular mass of 19 kDa, and gel filtration analyses reveal a molecular mass of 38-40 kDa, indicating

Odorant Binding

FIG. 3. Scatchard analysis of $^3$H-ligand binding to purified bovine OBP at equilibrium. Incubations included fixed amounts of OBP (3–50 ng) and $^3$H-ligand (3–15 nM) with varying concentrations of unlabeled ligand (5 nM–100 μM). A, $[\text{H}]$DMO binding. B, $[\text{H}]$IBMP binding. See “Materials and Methods” for details.

FIG. 4. Association kinetics of $^3$H-ligand binding to purified bovine OBP. A, binding of $[\text{H}]$DMO at 1, 3, 17, or 67 nM at time points from 2 to 60 min. Each tube contained 50 ng of OBP. B, binding of 3 nM $[\text{H}]$IBMP to 50 ng of purified bovine OBP. Data presented are from a typical experiment, performed in triplicate, which was replicated three times.

FIG. 5. Dissociation kinetics of $^3$H-ligand binding to purified bovine OBP. Fifty ng of OBP were incubated with 5 nM $[\text{H}]$DMO or $[\text{H}]$IBMP for 45 min at 22°C. Dissociation was initiated by the addition of 4.9 ml of 50 mM Tris-HCl (pH 7.7 at 25°C) in the absence (“infinite dilution”) or presence (“excess ligand”) of 10⁻⁷ M nonradioactive ligand in the buffer. A, $[\text{H}]$DMO dissociation by infinite dilution (open squares) or excess ligand using nonradioactive DMO (diamonds) or IBMP (open squares). B, $[\text{H}]$IBMP dissociation by infinite dilution (diamonds) or excess ligand using IBMP (open squares). Data presented are from a typical experiment, performed in triplicate, which was replicated three times.

that mature OBP exists as a homodimer (1, 2). In studies employing several ligands, $[\text{H}]$amylacetate, $[\text{H}]$DMO, $[\text{H}]$methylthiylhydrojasmonate and $[\text{H}]$IBMP (1, 2), we have found a ratio of one ligand molecule/homodimer (7). After interaction of a ligand with a site on one subunit of OBP, negative cooperativity may diminish the likelihood of a second molecule binding to the other subunit. Alternatively, the two monomers may contribute to create a single binding site only in the dimer. The enzymes tyrosyl-tRNA synthetase (31) and transaldolase (32) provide other examples of negatively cooperative interactions between ligands and proteins, in which a ligand binds to only one of two identical monomers in a homodimer.

It is unclear what role negative cooperativity plays in OBP function. Several lines of evidence suggest a role for OBP as a carrier, possibly delivering odorants to receptor sites on olfactory cilia (3, 6, 8). Perhaps negative cooperativity facilitates release of odorants from OBP to interact with receptors. OBP may also participate in the concentration of odorants from ambient air into nasal mucus (3, 7), a process in which negative cooperativity would regulate how much odorant is accommodated in the mucus.

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