Odorant Binding to Bovine Odorant Binding Protein Detected by Intrinsic Fluorescence

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(Received June 2, 2005; CL-050712)

Odorant binding to bovine odorant binding protein (OBPb) made OBPb structure a little tighter and odorant detection by intrinsic fluorescence possible. For odorant with high affinity such as 3,7-dimethyl-1-octanol (DMO), the binding reaction proceeded in a two-step manner that inferred an existence of a third binding site.

Odorant binding proteins (OBPs) are soluble proteins existing in mammals as well as in insects as their homologs.[1,2] OBP binds a wide range of hydrophobic molecules including odorants.[3,4] Among vertebrate OBPs which have the molecular weight of around 20 kDa and exist as monomer or homodimer depending on OBPs,[1] bovine odorant binding proteins (OBPb) exists as homodimer by the swapping of the α-helical domain of the one monomer over the β-barrel of the second monomer, which is accomplished by a lack of Cys 63 and Cys 155,[5] and hence a disulfide bridge between them (Figure 1a). OBPb is, therefore, considered flexible enough to undergo structural change upon odorant binding. This will enable us to examine odorant binding through intrinsic fluorescence avoiding the use of radioactive ligands as it has three tryptophan residues (Trp 17, Trp 64, and Trp 133).[6] In this paper, we report that odorant binding to OBPb can be measured by Trp fluorescence and provide an insight into how the dimer interface called central pocket (CP) works because the odorant binding-dependent fluorescence change is considered mainly from Trp 64 in CP.

OBPb gene was synthesized by successive elongation of 30 bp synthetic oligos using a polymerase chain reaction (PCR). The complete OBPb gene was then cloned into a GST-fusion vector pGEX6P (Amersham Pharmacia Biotech: APB) (pGEX6P-OBPb). OBPb was expressed in *E. coli* BL21 transfected with pGEX6P-OBPb and purified as described.[7] OBPb was gel-filtrated using Sephadex 75 HR (APB) and stored until use at 4 °C and at a concentration of about 10 μM in a phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and pH 7.2). In experiments, the stored OBPb solution was diluted with PBS to a working concentration of 100 or 200 nM.

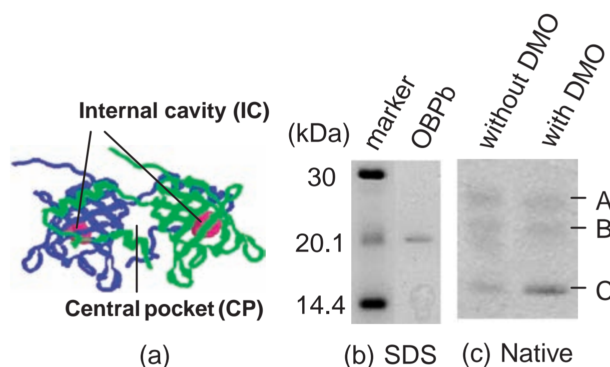

Figure 1. OBPb structure and polyacrylamide gel electrophoresis (PAGE) for OBPb with and without DMO. (a) Structure (PDP ID: 1OBP). (b) SDS-PAGE. Marker: 30 kDa (carbonic anhydrase), 20.1 kDa (trypsin inhibitor) and 14.4 kDa (α-lactalbumin). (c) Native-PAGE.

To examine OBPb size, native and SDS polyacrylamide electrophoresis (PAGE) were performed using PhastSystem (APB). For SDS-PAGE, the samples were added with SDS to 2.5% and 2-mercaptoethanol to 5%, and heated at 100 °C for 5 min and electrophoresed through a 8–25% gradient gel (APB). For sample preparation for native-PAGE, 50 μL of (10 μM each) in PBS was added with 0.5 μL of 10% (v/v) ethanol (final concentration of 0.1% (v/v)) for a reference blank, and 10^{-3} M 3,7-dimethyl-1-octanol (DMO) dissolved in 10% ethanol (final concentration of 10 μM DMO and 0.1% ethanol) and incubated at 25 °C for 1 h. Then, the samples were electrophoresed through a homogeneous 20% gel (APB). Both gels were stained with Coomassie brilliant blue. Proteins were assayed by an assay kit (Biorad) based on the method of Bradford using bovine γ globulin as a standard. SDS-PAGE revealed molecular weight of OBPb to be 20 kDa (Figure 1b), which is a little larger than that reported (19 kDa[8]) because of an attachment of extra residues due to a fusion with GST followed by its digestion. There were three bands on native-PAGE for OBPb (Figure 1c). The upper two bands (A and B) were almost identical between OBPb with and without odorant (DMO) and the smallest one (C) was dependent on odorant. Without DMO, the amount of OBPb in the band C decreased as a result of monomerization. These results show that OBPb in solution exists in rather relaxed forms and exhibits a conformational change to be more compact upon odorant binding possibly because of hydrophobic interaction of odorant molecules with a binding site.

It is anticipated that the conformational change upon odorant binding accompanies a variation in intrinsic fluorescence, and Trp fluorescence was measured upon odorant binding (Figure 2a). Trp fluorescence intensity decreased with time during illumination and the decrease was not restored even after stopping the illumination. We concluded that a conformational change letting Trp residue be exposed to external water (monomerization in extreme) occurred during the measurement, and odorant binding suppressed the conformational change. Degree of suppression, namely the signal intensity difference between with and without odorant, was odorant dependent, and the higher the affinity of odorant with OBPb was, the larger the signal intensity was (K_D: 0.3 μM for DMO, 1.8 μM for citronellal, and 2.9 μM for geraniol). The signal intensity was also varied dependent on a concentration of odorant, and this means that the signal was a direct product of odorant binding to OBPb. The difference between Trp emissions with and without DMO measured 20 and 30 min after DMO addition were linearly related

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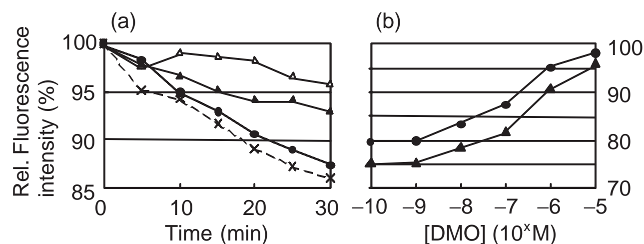

Figure 2. Trp fluorescence from OBPb upon odorant binding. (Continuous illumination-mode) (a) Time-dependent variation. Excitation (Ex) and emission (Em) wavelength were 295 and 340 nm, respectively, and slit widths for Ex and Em were 3 and 5 nm, respectively. Odorants used were DMO, citronellal and geraniol. Concentrations of OBPb and odorants were 200 nM and 10 μM, respectively. Measurement was performed every 5 min for 30 min after the addition of odorant. Fluorescence intensity was normalized by that measured at 0 min and reported in percentil (relative fluorescence intensity). X, none; ●, geraniol; △, DMO; ▲, citronellal. (b) DMO-concentration dependency of the relative fluorescence intensity measured at 20 and 30 min after the addition of odorant. ●, 20 min; ▲, 30 min.

with DMO concentration that ranged from 10^{-9} to 10^{-7} M (Figure 2b). The variation in Trp emission was only applicable to quantification of odorant with high affinity (DMO), but not of odorant with low affinity (geraniol). Continuous illumination to excite OBPb possibly caused OBPb to change its conformation before encountering odorants. A destruction of β-structure caused by a strong light illumination was confirmed by using CD spectroscopy (not shown). To avoid this, samples were taken out of a stock solution and diluted just after odorant addition, Trp emissions were measured at each time point during which interval light illumination was stopped. The binding of geraniol was able to be measured in this manner (Figure 3a). Fluorescence difference indicative of binding for geraniol showed a typical binding character that plateaued at around 30 min after the onset of binding reaction. However, this was not the case for odorants with high and negligible affinity (DMO and cholesterol) (Figure 3b). Fluorescence difference showed a two-step increase for DMO and other odorants with high affinity (e.g., citronellol: $K_D \approx 1.0$ μM or citronellal: $K_D \approx 1.8$ μM; not shown), namely another increase followed the first plateau (i.e., from 3 to 20 min), on the other hand, almost no change for cholesterol and other odorants with negligible affinity (e.g., borneol: $K_D > 100$ μM). Therefore, these again indicated that the relative fluorescence is also responsible for odorant binding and reflects odorant affinity with OBPb. The two-step variation in the relative fluorescence with odorant with high affinity infers that primary odorant binding to a binding site creates another binding site to which additional binding could successively occur. CP other than the suggested binding site called internal cavity (IC) existing in each monomer may serve as the site in which primary odorant binding takes place.

In summary, we described that odorant binding to OBPb was able to be measured by intensity variation in intrinsic Trp fluorescence. The shape of the time-course in Trp fluorescence variation was dependent on odorant's affinity, and OBPb showed

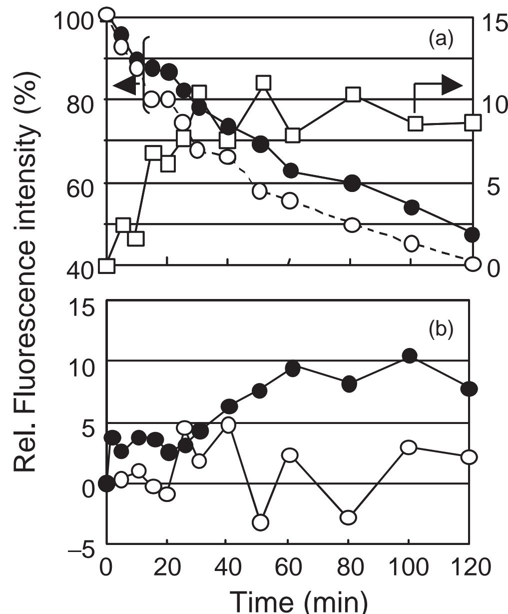

Figure 3. Trp fluorescence from OBPb upon odorant binding. (Discrete illumination-mode) Ex and Em wavelengths were the same as in Figure 2, and slit width was 5 nm for Ex and Em. Concentrations of OBPb and odorants were 100 nM and 10 mM, respectively. Measurement was performed at each time point for 120 min after the addition of odorant. Relative fluorescence intensity was obtained as in Figure 2 and fluorescence difference was calculated by subtracting relative fluorescence intensity without odorant from that with odorant. (a) Relative fluorescence intensity and its difference for geraniol. ○, none; ●, geraniol; □, difference. (b) Relative fluorescence intensity difference for DMO and cholesterol. ●, DMO; ○, cholesterol.

two different binding states when bound with odorant with high affinity such as DMO. This inferred that OBPb has another binding site besides IC, and CP is a possible candidate. To examine if CP functions as a binding site, OBPb-del(eted) is under preparation, in which ten amino acids are deleted (shortened) at the end of the C-terminus that forms CP.

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Published on the web (Advance View) August 13, 2005; DOI 10.1246/cl.2005.1256