Direct NMR Observation of Odorant Binding to Mouse Odorant Receptor MOR244-3*

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Abstract

Mammals are able to perceive and differentiate a great number of structurally diverse odorants through the odorant's interaction with odorant receptors (ORs), proteins found within the cell membrane of olfactory sensory neurons. The natural gas industry has utilized human olfactory sensitivity to sulfur compounds (thiols, sulfides, etc.) to increase the safety of fuel gas transport, storage, and use through the odorization of this product. In the United States mixtures of sulfur compounds are used, but the major constituent of odorant packages is 2-methylpropane-2-thiol, also known as *tert*-butyl mercaptan. It has been fundamentally challenging to understand olfaction and odorization due to the low affinity of odorous ligands to the ORs and the difficulty in expressing a sufficient number of OR proteins. Here, we directly observed the binding of *tert*-butyl mercaptan and another odiferous compound, cis-cyclooctene, to mouse OR MOR244-3 on living cells by saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy. This effort lays the groundwork for resolving molecular mechanisms responsible for ligand binding and resulting signaling, which will, in turn, lead to a clearer understanding of odorant recognition and competition.

Keywords fuel gas; odorant receptor; olfaction; saturation transfer difference nuclear magnetic resonance spectroscopy; thiol

Introduction

For most mammals, olfaction is a vitally important sense that is essential to survival ¹. Conversely in humans, olfaction is often viewed as the least important and least developed sense (from a survival point of view), although its absence or loss can have serious consequences ^{2,3}. For example, olfactory deficits in humans adversely affect hazard avoidance (such as the ability to note the presence of smoke or gas leaks), food selection (and the avoidance of spoiled food), and digestion (especially the initial enzymatic and hormonal preparations for digestion). In spite of the huge range of chemical compounds the olfactory system can detect, not all chemical compounds have an odor. In order for a chemical compound to reach and interact with an odorant receptor (OR), it must have some water solubility, some lipophilicity, a sufficiently high vapor pressure, low or moderate polarity, and a molecular weight no greater than approximately 294 g/mol ^{4,5}.

Odorant mixtures create effects that are both complex and interesting. Odorant suppression, cross-adaptation, and conjugation are among these phenomena. When one component quells the odor of another, it is considered suppression. During cross-adaptation, exposure to one odorant decreases sensitivity to another. When there is strong mutual suppression resulting from the mixing of two odorants (as first described by H. Zwaardemaker) ⁶ it is typically called conjugation. Odorant masking (caused by suppression, cross-adaptation, or conjugation) has profound technological implications in a number of industrial sectors, most prominently in the fuel gas sector. Masking is suspected when a proprietary odorant blend that is added to natural gas can be detected by analytical instrumentation, but cannot be properly detected by an observer with a normal sense of smell. Note that this phenomenon is distinct from odor fade, which more properly describes a decrease in the concentration of an odorant (rather than a decrease, disappearance, or qualitative change in the perception of odor in the absence of a change in absolute concentration). Anecdotal descriptions of masking events in the natural gas industry have persisted for over a decade, with the frequency of such events increasing. There is evidence that the increasing diversity of feedstock streams (to include biogas, coal seam gas, landfill gas, etc.) may play a contributing role ^{7,8}.

The perception of smells is the result of three steps: detection, transduction, and signal integration ⁹. We are particularly interested in detection, as odor masking events likely result from disruption of this step. During detection, the odorants reach the dorsal and dorsoposterior

aspect of the nasal cavity and bind with proteins in order to begin the signal transduction process. Olfactory sensory neurons, located in the olfactory epithelium, are the main cells responsible for the perception of smell^{9,10}. OR proteins are expressed on their surface. These proteins belong to the class A (rhodopsin-like) G protein-coupled receptor (GPCR) family¹¹, and are expressed on both dendrites and axons^{11,12}. Each neuron expresses only one distinct OR^{13,14} from a large gene superfamily with 300-1500 receptors, depending on the species¹². To add to this complexity, the fraction of the available genes expressed varies widely between species. For example, mice express some 1035 putatively functional receptors compared to humans who express approximately 387 receptors¹².

Odor perception is the result of odorant(s) binding to multiple ORs with differing affinities. Modeling methods (ligand-based¹⁵⁻¹⁷, receptor-based^{16,18,19} and dynamic homology modeling¹⁶) can give insight into ligand/protein dynamics. Such techniques become even more powerful when combined with experimental methods, such as site-directed mutagenesis and functional analysis^{13,16}. However, little is known about the odor selectivity of individual ORs and identifying ligands for the majority of ORs remains a daunting challenge. ORs lose their structure, and therefore their functionality, when removed from their natural membrane environment. Therefore, the capability to heterologously express ORs on the cell surface is critical to screening efforts¹¹. Ligand/protein binding can be detected by a cAMP-mediated luciferase reporter gene assay. This assay has important advantages; in particular, ORs can be screened in a high-throughput format to identify odorant ligands and to generate dose response curves^{11,20}. However, this assay is not designed for measuring the competitive binding that is likely associated with odor masking events.

An alternative to the luciferase reporter gene assay is saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy, a method for detecting the interactions of small ligands with large proteins. Figure 1 illustrates the process. During irradiation in the spectral region containing protein signals, but not ligand signals, bound ligands receive saturation transfer from the protein through cross-relaxation based spin diffusion. For weakly binding ligands (dissociation constants from $K_d = 10^{-8}$ to $K_d = 10^{-3}$ mol L⁻¹), saturation transfer decreases the signal measured from free ligands. The difference spectrum (I_{STD}) is obtained by subtracting the spectrum recorded with selective saturation of protein resonances (on-resonance, I_{SAT}) from the reference spectrum recorded without protein saturation (off-resonance, I_0)²¹.

Because the detection is ligand-based, there is no mass limit on the target protein and expensive protein labeling is not required^{21, 22}. Furthermore, as long as the ligand signals do not overlap, this technique is well suited to measuring competitive binding.

STD NMR techniques have detected ligand binding to GPCRs by employing a 2.4×10^4 molar excess of ligand to protein and only 512 scans²²⁻²⁵. These techniques have been applied to determine ligand/protein binding in platelets²⁴ and suspension cells²⁶. For adherent cell lines, membrane preparations were reported to increase signal to noise compared to live cell measurements in studies that examined the binding of natural and artificial sweeteners to a taste receptor²⁷. Despite their challenges, cell suspensions were utilized here due to the potential to ultimately conduct a variety of measurements with the same living cells.

Pursuant to the philosophy that technological problems cannot be effectively addressed until the basic science is understood, we are working to recreate odor masking events with cellular models. Our ultimate aim is to develop techniques to prevent this phenomenon from occurring with fuel gas odorants, and we have focused our research on a well-studied mouse odorant receptor, MOR244-3, which can be functionally expressed at the surface of human Hana3A cells. This OR binds methylthiomethanethiol, a component of male mouse urine, and other structurally related ligands²⁰. Intriguingly, the luciferase reporter gene assay revealed that MOR244-3 did not bind simple thiols. Furthermore, it is not known whether MOR244-3 binds 2-methylpropane-2-thiol, also known as *tert*-butyl mercaptan, the primary constituent of fuel odorant blends. Here, we directly determine whether MOR244-3 binds *tert*-butyl mercaptan by STD NMR of living cells.

Experimental Section

Transfection of Hana3A cells with mouse odorant receptor MOR244-3

Plasmids contained the MOR244-3 open reading frame with a Rho-tag (the first 20 residues of rhodopsin) inserted at the N-terminal of the OR and the receptor-transporting protein 1S (RTP1S). Both were cloned into the pCI mammalian expression vector and transfected into Hana3A cells (modified HEK293T cells) according to standard procedures¹¹. Figure 2 shows cells in which MOR244-3 proteins were labeled by live-cell immunocytochemical staining using mouse monoclonal antibody against rhodopsin. Multiple images were evaluated to determine the transfection efficiency, approximately 40 %.

Preparation of odorants

Tert-butyl mercaptan (TBM) and *cis*-cyclooctene (a previously known MOR244-3 ligand)²⁰ were obtained as pure fluids from a commercial source. It is known that non-adjacent thiols and thioether groups (as well as some other non-sulfurous ligands) require a chelating metal complex for a robust response^{20, 28}. Therefore, CuCl₂ (final concentration of 0.4 mmol/L) was included in each cell sample when TBM was used as the odorant. Due to the low solubility of both TBM and *cis*-cyclooctene, the NMR ERETIC method (Electronic REference To access In vivo Concentrations)²⁹ was used to confirm the final concentrations of odorants dissolved and/or suspended in each sample. For the three replicate binding experiments with TBM, the final concentrations were 1.6 mmol/L, 3.3 mmol/L, and 4.4 mmol/L. For the single binding experiment with *cis*-cyclooctene, the final concentration was 11 mmol/L. For the competitive binding experiment with both TBM and *cis*-cyclooctene, the concentrations were 2.5 mmol/L and 2.0 mmol/L, respectively.

Preparation of NMR samples

NMR samples without cells were prepared by dissolving 20 μ L of odorant in 1 mL of acetone-d₆. Transfected and non-transfected Hana3A cells were grown to confluency in T-75 flasks (approximately 10^7 cells) and harvested by trypsinization and centrifugation at 200 g for 5 min. All preparations were done at room temperature. The cell pellet was resuspended in 400 μ L Hank's Balanced Salt Solution (HBSS). The suspension was then added to NMR tubes containing 100 μ L D₂O, odorant, and CuCl₂ (for experiments with TBM). This preparation provided a cell concentration of approximately 2×10^7 cells per mL. GPCR-expressing cell lines have a receptor density of about 10^6 per cell¹⁹. For a transfection efficiency of 40%, the receptor concentration is 8×10^{12} receptors per mL (approximately 13 nmol / L), which gives a 1.2×10^5 -fold excess of ligand to receptor for the smallest ligand concentration employed. In order to confirm that trypsinization is the best method for the collection of cells for NMR, live/dead staining was performed on cells collected by both trypsinization and scraping. Both methods allowed for the transfer of large amounts of living cells to NMR tubes. Nevertheless, cells collected by trypsinization were more evenly dispersed and, therefore, this was chosen as our preparation method.

NMR data collection and analysis

STD NMR spectra were obtained at 30 °C, with a sweep width of 16 ppm, and acquisition time of 3 s on a 600 MHz spectrometer equipped with a 5 mm inverse double-resonance cryoprobe. Selective protein saturation was achieved by a set of Gaussian pulses of 50 ms length with 9.5 W power level. Forty selective pulses were applied, leading to a saturation time of 2 s. On-resonance irradiation of the protein was performed at -1.0 ppm, and the off-resonance irradiation was set at -20 ppm, where neither protein nor ligand signals were present. The number of scans was 512, preceded by 8 dummy scans. Spectra were subtracted internally by phase cycling after every scan using different memory buffers for on- and off- resonance data.

Results and Discussion

The results of single odorant STD NMR experiments are presented in Figure 3 for cis-cyclooctene and Figure 4 for TBM. Figure 3a shows the NMR spectrum for cis-cyclooctene dissolved in acetone-d₆. Protons corresponding to positions 1, 2, and 3 (see scheme) are located at 5.6, 2.1, and 1.5 ppm, respectively. The solvent produces a peak at 2.05 ppm and the small peak at 2.8 ppm is due to an impurity in the solvent. Figure 3b shows the reference spectrum (I_0) of a suspension containing transfected cells and cis-cyclooctene. Peaks due to cis-cyclooctene are visible. In addition to odorant and solvent peaks, the reference spectrum contains signals between 3.0 ppm and 4.2 ppm. These correspond to glucose (a component of HBSS) and metabolites of the Hana3A cells. There is also a doublet at 1.3 ppm that corresponds to lactate generated by Hana3A metabolism (anaerobic glycolysis).

The remaining spectra in Figure 3 are difference spectra ($I_0 - I_{SAT}$) and are scaled identically. Figure 3c shows the STD spectrum of transfected cells plus cis-cyclooctene. Peaks due to the buffer have been subtracted out and are no longer visible. Three peaks corresponding to the protons of cis-cyclooctene are clearly visible and are labeled. These peaks indicate the binding of cis-cyclooctene. Figure 3 also shows the STD spectra of two controls. Figure 3d shows the STD spectrum of transfected cells without cis-cyclooctene. The absence of the peaks (5.6 ppm, 2.1 ppm, and 1.5 ppm) seen in Figure 3c indicates that the binding was not due to the cells interacting with the buffer solution. In addition, these peaks are not seen in Figure 3e, which shows the STD spectrum of non-transfected cells with cis-cyclooctene. These results

clearly indicate that the binding seen in Figure 3c is a result of the cells being transfected to express the odorant receptor MOR244-3 and not a general response of Hana3A cells to the cis-cyclooctene.

Figure 4a shows the NMR spectrum for TBM dissolved in acetone-d₆. Again, peaks due to the protons in acetone and a solvent impurity are located at 2.05 ppm and 2.8 ppm, respectively. Protons corresponding to position 1 in TBM (see scheme) are located at 1.4 ppm. Figure 4b shows the reference spectrum (I_0) of a suspension containing transfected cells, TBM and CuCl₂. Again, multiple peaks located between 3.0 ppm and 4.2 ppm are due to glucose and Hana3A metabolites and the doublet located at 1.3 ppm is due to lactate.

The remaining spectra in Figure 4 are difference spectra ($I_0 - I_{SAT}$) and are scaled identically. Figure 4c shows the STD spectrum of transfected cells plus TBM and CuCl₂. One peak (1.4 ppm) corresponding to the protons of TBM is clearly visible and is labeled. This peak indicates the binding of TBM. Figure 4 also shows the STD spectra of two controls. Figure 4d shows the STD spectrum of transfected cells without TBM. The absence of the peak (1.4 ppm) seen in Figure 4c indicates that the binding was not due to the cells interacting with the buffer solution. In addition, this peak is not seen in Figure 4e, which shows the STD spectrum of non-transfected Hana3A cells with TBM and CuCl₂. These results clearly indicate that the binding seen in Figure 4c is a result of the cells being transfected to express the odorant receptor MOR244-3 and not a general response of the Hana3A cells to the TBM and CuCl₂.

One critical consideration for STD NMR studies of ligand binding to membrane-bound proteins in whole cells is the choice of on-resonance (-1.0 ppm) and off-resonance (-20 ppm) saturation points. In particular, the -1.0 ppm on-resonance saturation point might be close enough to odorant signals at 1.4 ppm and 1.5 ppm for a small amount of accidental transfer. To investigate this possibility, we measured the excitation profile of our Gaussian pulse. Observation of the control spectra allows us to say that, if present at all, this transfer is quite weak and accidental transfer can be considered negligible. An additional concern is that the -20 ppm off-resonance saturation point might accidentally saturate the ligand by excitation of the lipid bilayer. This seems unlikely because the control STD spectra containing cells and ligand (Figures 3e and 4e) were flat, with no indication of ligand saturation. To investigate this possibility further, we examined saturation through the amide protons (on-resonance at 10 ppm, off-resonance at -100 ppm and 1280 scans). Supplementary Data Figure S1 demonstrates similar

binding topography to Figure 4c, though the peak is weaker. This indicates that saturation transfer to the odorant was not due to excitation of the lipid bilayer but truly reflects ligand binding.

The results presented in Figures 3 and 4 demonstrate that MOR244-3 heterologously expressed by living Hana3A cells binds both cis-cyclooctene (as expected) and TBM. Since cis-cyclooctene provided peaks outside the region where glucose and other buffer compounds had peaks, its binding was straightforward to detect. By contrast, TBM was more challenging, as its sole peak (1.4 ppm) was very close to the lactate doublet (1.3 ppm). Interestingly, the lactate doublet appeared in one of the difference spectra (Figure 3c), suggesting that it might also be a weak-binding ligand, likely of a surface protein (not the OR). However, this finding was not consistent for all samples and may indicate that this doublet is the result of partial saturation by the Gaussian pulse train, or the result of lactate concentration increasing during the experiment. In the reference spectra, the lactate doublet grew with time as the cells released more lactate, providing additional evidence that cells remained alive throughout the NMR experiments. This observation demonstrates the importance of interweaving the acquisition of reference spectra with saturation transfer spectra.

The amplification factor (A_{STD}) is obtained by multiplying the relative STD effect of a given signal (the intensity of a signal in the difference spectrum divided by its intensity in the reference spectrum, I_{STD}/I_0) with the molar ratio of ligand in excess relative to the protein, $[odorant]/[OR]$.

$$A_{STD} = \frac{I_{STD}}{I_0} \times \frac{[odorant]}{[OR]} \quad (1)$$

For the three independent experiments with TBM, I_{STD}/I_0 was equal to 0.002 with a relative standard deviation of 13%. A_{STD} values were 280, 460, and 630 and increased with TBM concentration. This could reflect binding kinetics, but since the proteins are most likely saturated with ligand at concentrations of 1.5 mmol/L or greater, the A_{STD} values should be equivalent. In this scenario, the relative standard deviation is 39%. Four sources of uncertainty were expected to impact the peak integrals in our experiments: slight spectral deviations between the on-resonance and off-resonance spectrum that result in slight imperfections in subtraction; repeatability in the concentration of odorant receptor due to variation in transfection rate and cell density; baseline drift; and peak overlap. Although odorant concentrations determined by the ERETIC method should be accurate to less than 0.1 mmol/L, it should be noted that the ERETIC

reference spectrum and the STD reference spectrum had different resolutions, which led to an increase in the uncertainty for this value.

For an odorant such as cis-cyclooctene, which contains three proton environments, there are three different A_{STD} values. Their relative magnitude provides information about the binding configuration within the OR protein. This is because protons located closer to the binding site should receive a greater transfer of energy during the “on resonance” saturation. Therefore, the peak from that proton compared to its peak in the reference spectrum is smaller relative to protons located further from the binding site. For the difference spectrum shown in Figure 3c, A_{STD} for protons at position 1 is 69% larger than A_{STD} for protons at position 2 and 81% larger than A_{STD} for protons at position 3. Modeling studies can be used to further understand binding configuration.

The results of one competitive binding STD NMR experiment are presented in Figure 5, which shows three difference spectra ($I_O - I_{SAT}$). Without odorants, no peaks are observed (Figure 5a). After adding TBM to the cell suspension, its binding was confirmed (Figure 5b) before cis-cyclooctene was added (Figure 5c). The presence of all the peaks corresponding to the odorants indicates that both odorants bind to the odorant receptor MOR344-2 and receive saturation transfer. Examining the difference spectra, it appears that adding cis-cyclooctene decreased the binding of TBM, based on the smaller peak at 1.4 ppm in Figure 5c vs. Figure 5b. This should reduce the relative STD effect for TBM binding. However, quantification of this effect is not possible. Integration of the peak areas for peak 3 and peak 4 is unreliable due to both noise and to overlap in the peaks for cis-cyclooctene and TBM.

Conclusions

ORs are involved in the sensation of a wide variety of chemically and structurally diverse odorant molecules. We have directly observed the binding of *tert*-butyl mercaptan and cis-cyclooctene to the mouse OR MOR244-3 in live cells, including the simultaneous binding of these odorants. Our results show that STD NMR methods can be used to monitor specific ligand/protein binding interactions. These methods have the benefit of not being an end-point, and various concentrations and/or different odorants can be added to the same live cell samples. STD NMR methods will allow us to measure thermodynamic and kinetic properties, such as the dissociation constants (K_d) for various odorants, to monitor for competitive binding, and to

monitor samples for the deactivation of particular ORs. In addition, STD NMR methods provide valuable data for the identification and mapping of ligand binding sites and offer an instrumental tool for resolving molecular mechanisms responsible for ligand binding and resulting signaling, which in turn, leads to a deeper understanding of odorization.

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Figure Captions:

Fig. 1 A scheme illustrating the use of STD NMR to monitor interactions between a membrane receptor and a ligand; (a) ‘on resonance’ saturation during ligand binding and (b) ‘off resonance’ saturation where the protein is not perturbed (odorant and receptor are blue throughout indicating no selective saturation). Saturation selectively applied to the receptor is rapidly transferred by spin diffusion throughout the receptor (indicated by the red receptor). This saturation is also transferred to any bound ligands (odorant becomes red after binding the red receptor and having saturation transferred to it). As weakly binding ligands are bound and released, the pool of unsaturated ligand decreases and leads to $I_{SAT} < I_O$.

Fig. 2 Evidence that the OR MOR244-3 is expressed at the surface of Hana3A cells was obtained by live cell immunocytochemical labeling of mouse monoclonal anti-rhodopsin antibody (4D2). Nontransfected (a, d) and transfected (b, e) samples have similar cell density in the transmission images (a, b), but clear differences in fluorescence intensity around individual cells (d, e). Imaging at higher magnification (c, f) reveals discrete points of high fluorescence intensity suggesting surface protein expression. Nuclear label in (f) is Hoechst 33342.

Fig. 3 NMR spectra examining the binding of cis-cyclooctene to the odorant receptor MOR244-3. (a) NMR spectrum of cis-cyclooctene in acetone-d₆ (b) Reference spectrum of transfected cells in HBSS/D₂O with cis-cyclooctene (c) STD spectrum of transfected cells in HBSS/D₂O with cis-cyclooctene (d) STD spectrum of transfected cells in HBSS/D₂O with no odorant (e) STD spectrum of non-transfected cells in HBSS/D₂O with cis-cyclooctene

Fig. 4 NMR spectra examining the binding of TBM to the odorant receptor MOR244-3. (a) NMR spectrum of TBM in acetone-d₆ (b) Reference spectrum of transfected cells in HBSS/D₂O with TBM and CuCl₂ (c) STD spectrum of transfected cells in HBSS/D₂O with TBM and CuCl₂ (d) STD spectrum of transfected cells in HBSS/D₂O with no odorant (e) STD spectrum of non-transfected cells in HBSS/D₂O with TBM and CuCl₂.

Fig. 5 NMR spectra examining the simultaneous binding of cis-cyclooctene and TBM to the odorant receptor MOR244-3. (a) STD spectrum of transfected cells in HBSS/D₂O with no odorants (b) STD spectrum of transfected cells in HBSS/D₂O with TBM and CuCl₂ (c) STD spectrum of transfected cells with cis-cyclooctene, TBM and CuCl₂.

Saturation Transfer Difference Nuclear Magnetic Resonance (STD NMR) Spectroscopy for the Detection of Binding of Odorant Ligands to Odorant Receptors

a) receptor-specific "on" saturation

*Large molecular weight, low concentration G-protein coupled receptor are NMR invisible

b) "off" resonance saturation

*100 to 24,000-fold excess of ligand to protein: NMR visible upon reversible binding (K_D from 10^{-8} to 10^{-3} mol/L)