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Multimodal Ligand Binding Studies of Human and Mouse G-coupled Taste Receptors to Correlate with their Species-Specific Sweetness Properties

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Key words: Sweet taste receptor, ligand binding, G-coupled protein receptors (GPCRs), saturation transfer difference (STD), nuclear magnetic resonance spectroscopy (NMR), differential scanning calorimetry (DSC), circular dichorism (CD) spectroscopy

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Abstract

Taste signaling is a complex process that is linked to obesity and its associated metabolic syndromes. The sweet taste is mediated through a heterodimeric G protein coupled receptor (GPRC) in a species-specific manner and at multi-tissue specific levels. The sweet receptor recognizes a large number of ligands with structural and functional diversities to modulate different amplitudes of downstream signaling pathway(s). The human sweet-taste receptor has been extremely difficult to study by biophysical methods due to inadequate methods for producing large homogeneous quantities of the taste-receptor protein and a lack of reliable in vitro assays to precisely measure productive ligand binding modes leading to activity upon their interactions with the receptor protein. We report a multimodal high throughput assays to monitor ligand binding, receptor stability and conformational changes to model the molecular interactions between ligand-receptor. We applied saturation transfer difference nuclear magnetic resonance spectroscopy (STD-NMR) complemented by differential scanning calorimetry (DSC), circular dichroism (CD) spectroscopy, and intrinsic fluorescence spectroscopy (IF) to characterize binding interactions. Our method using complementary NMR and biophysical analysis is advantageous to study the mechanism of ligand binding and signaling processes in other GPCRs.

Keywords

Heterodimeric G protein coupled receptor · saturation transfer difference nuclear magnetic resonance spectroscopy · differential scanning calorimetry · circular dichroism · intrinsic fluorescence spectroscopy

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INTRODUCTION

Human sweet taste receptor is a heterodimeric complex of the proteins hT1R2 and hT1R3. The complex is a member of the G-protein-coupled receptor class (GPCR) which share a common design of a seven transmembrane heptahelical domain with an extracellular N-terminus and intracellular C-terminus as well as a signaling pathway controlled by heterotrimeric G-proteins that stimulate the synthesis of intracellular second messengers such as cyclic AMP, inositol phosphate and Ca^{2+} ions. It has been further grouped as a class C glutamate receptor, of which there are 4 other families: Class A rhodopsin family, class B secretin family, class D adhesion family and class E frizzles/smoothened family. The class C GPCR share a common structure of a large amino-terminal domain (ATD) which serves as the principle ligand-binding domain, followed by a short cysteine-rich region (CRR) tied the transmembrane domain (TMD) and intercellular C-terminal domain [1],[2].

The sweet-taste receptor has been shown to bind a large ensemble of molecules such as sugars, artificial sweeteners, sweet-tasting proteins and some D-amino acids that mediate the sweet taste response (Fig 1A). Regions of the complex that bind specific ligands include the ATD of hT1R2: non-caloric sweeteners aspartame, neotame, sucralose and monellin, a sweet-tasting protein; the ATD of T1R3: cyclamate, neohesperidin dihydrochalcone and lactisole; and the ATD and CRR of hT1R3: sweet-tasting proteins brazzein and neoculin [3-7]. Both proteins have been shown to bind natural sugars glucose and sucrose with distinct affinities even though the individual contributions of each protein to the interaction are unknown.

One of the major difficulties in studying the molecular details of the function of this complex has been the lack of a reliable method for producing large quantities of purified proteins

using recombinant technology. The mouse versions of T1R2 ATD and T1R3 ATD have been successfully produced, but only in small quantities and as fusion proteins [8]. Recently, human (h)T1R3 ATD was purified and characterized [9]. Here we describe multi-modal screen methodologies that are required for complex heterodimeric sweet taste receptor. First we will describe, procedure for producing both highly purified proteins for human (h) or mouse (m) fusion protein SUMO-T1R2 ATD and protease cut T1R2 ATD [10]. Gel filtration chromatography demonstrated that the protein exists in a dimeric configuration. Second, we describe several complementary methods for the study of the constructs and their interaction with small ligands and their secondary conformational changes that have elicit a sweet-taste response in human taste panel tests [11] and by the heterologous calcium assay in over expressing HEK211 cells in culture [12]. We show by circular dichroism spectroscopy a decrease in the overall α-helical content of the construct upon binding to ligands that have elicit a sweet-taste response in human taste panel studies [11] and by the heterologous calcium assay in culture [12]. In addition, there appears to be an overall decrease in thermal stability in the tertiary structure of the SUMO-hT1R2 ATD fusion protein upon binding of neotame, a small sweet-taste inducing molecule. Saturation transfer difference spectra confirmed that molecules eliciting a CD response also gave positive difference spectra. Finally, we show that circular dichroism spectroscopy and saturation transfer difference spectroscopy can be useful in studying allosteric effects of one sweet-taste responding molecule over another, as described by the heterologous calcium assay for sweet-taste response [12]. In this work, we demonstrate that we can study the highly purified sweet-taste protein and it's binding of target molecules using biophysical methods.

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RESULTS

We report here the results of experiments using multimodal biophysical techniques that probe the interaction of human and mouse T1R2 ATD with small ligands that are known to elicit a sweet-taste response. The production and purification of functional sweet-taste receptor proteins has been a challenge due to its large size and number of cysteines that need to be oxidized in the right form in order to be functionally correct.

Protein production and purification of ATDs. We report the successful construction and conditions that allow for the study of functionally relevant proteins. The constructs used in this study were from extracellular amino terminal domains (ATDs) of the receptor and contain ligand binding sites (figure 1A). The ATD region of the protein was cloned into both a HIS tag vector with a TEV cleavage site and a SUMO vector. The proteins were purified to near homogeneity as described by the 12% SDS-PAGE profiles (figure 1B). The protein was in the form of a homodimer from either construct as determined by molecular weight standards from gel filtration column (figure 1C).

Figure 1.

A)

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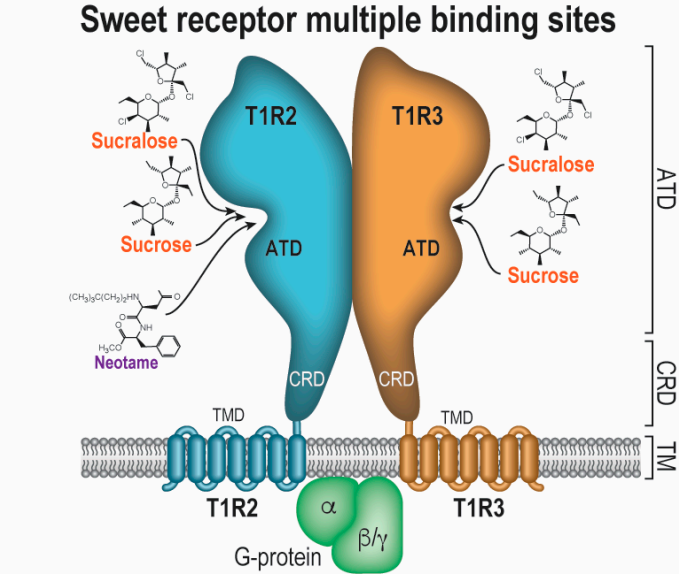

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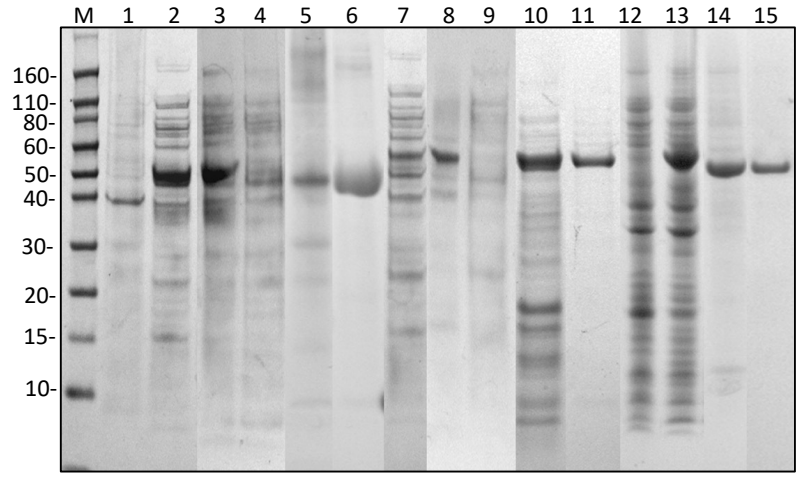

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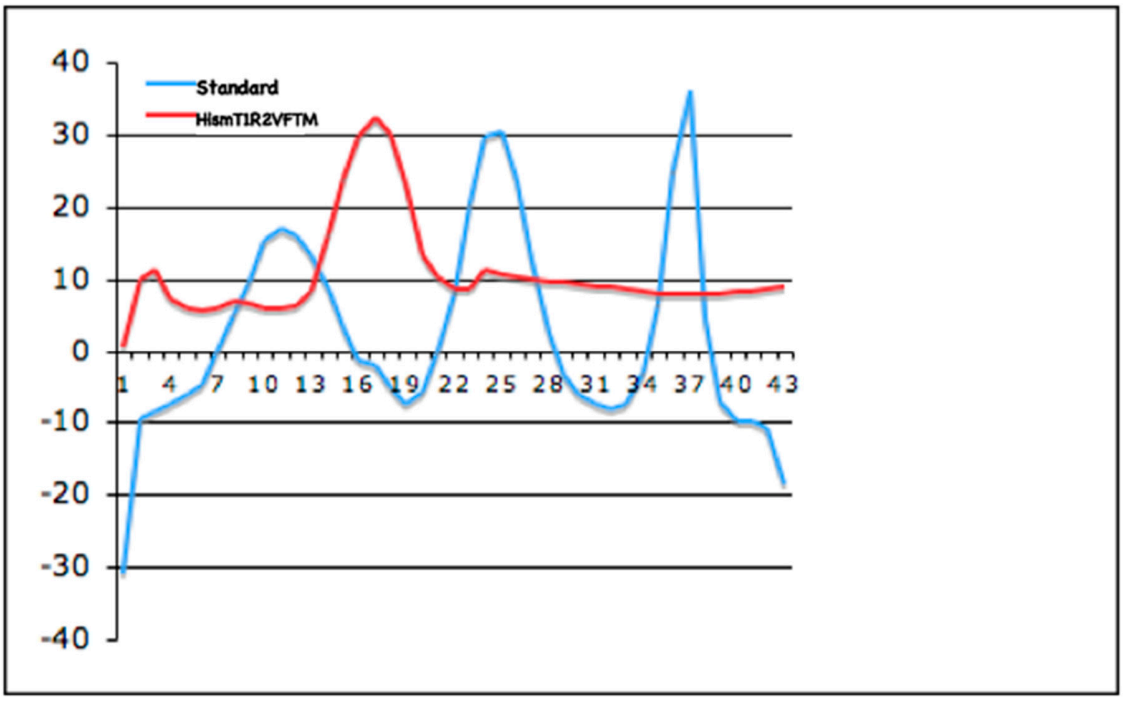

Figure 1. A) Model of sweet receptor and its proposed interaction sites with sweet ligands. B) SDS-PAGE of the expression and purification of human and mouse proteins. Lanes shown are: M. Novex Sharp Pertained Protein Standard (kDa); 1. –IPTG, total proteins; 2. +IPTG, total cell proteins containing His-TEV-ATD-hT1R2 (~56 kDa); 3. Pellet: His-ATD-hT1R2; 4. Supernatant: His-ATD-hT1R2; 5. Refolded His-ATD-hT1R2; 6. FPLC purified His-ATD-hT1R2; 7. +IPTG, total proteins, His-TEV-ATD-mT1R2 (~63 kDa); 8. Pellet of His-ATD-mT1R2+IPTG; 9. Supernatant of His-ATD-mT1R2; 10.Refolded His-ATD-mR2; 11. Purified His-ATD-mT1R2, by FPLC; 12. -IPTG; 13. +IPTG, His-Sumo-TEV-ATD-hT1R2; 14. cut His-Sumo-Tev-ATD-hR2 (ATD-hT1R2, ~54 kDa and His-Sumo-Tev, ~12.5 kda); 15. FPLC purified ATD-hT1R2. C) Tev protease cleaved ATD protein peaks off the Superdex 200 prep grade FPLC column.

Ligand binding to the receptor T1R2 and T1R3 ATDs results in secondary structural changes.

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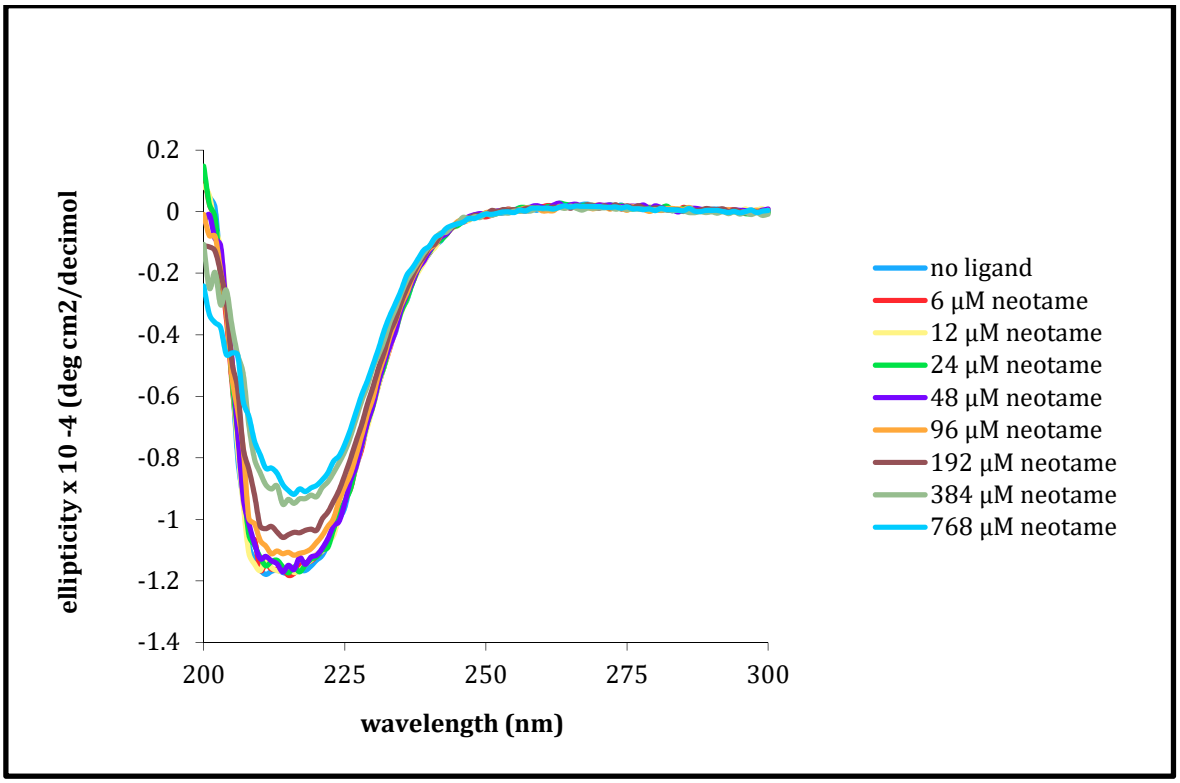
We used CD to monitor changes in the ATD secondary structure after addition of relevant ligands concentrations to ATD. There was a small but measurable increase in the overall molar ellipticity values upon addition of neotame (human specific binding ligand) resulting in a loss of about 5% of α-helix on the protein. Neotame showed binding to the His-hT1R2 ATD by CD (figure 2A). Changes in ellipticity at 209 and 219 nm with increasing concentrations of neotame and titrations with different concentrations of neotame are shown in figure 2B. On the other hand, non-sweet monosodium glutamate (MSG), produced no change in the CD profiles especially at 209 and 219 nm values (figure 2C).

Figure 2.

A)



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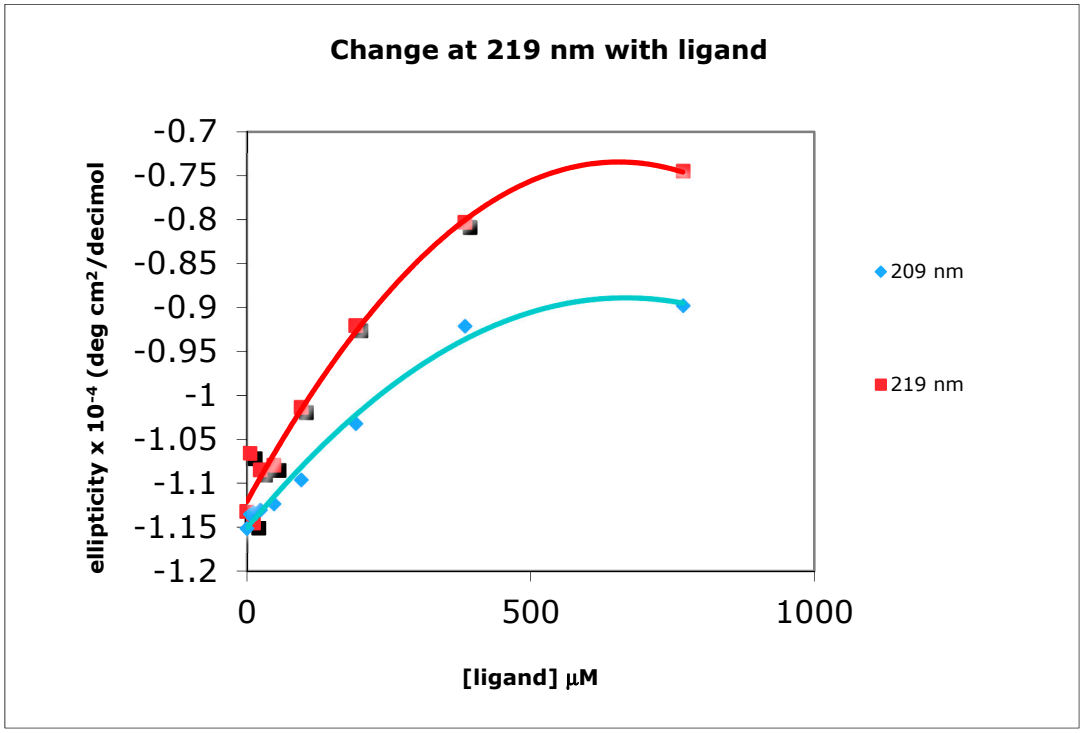

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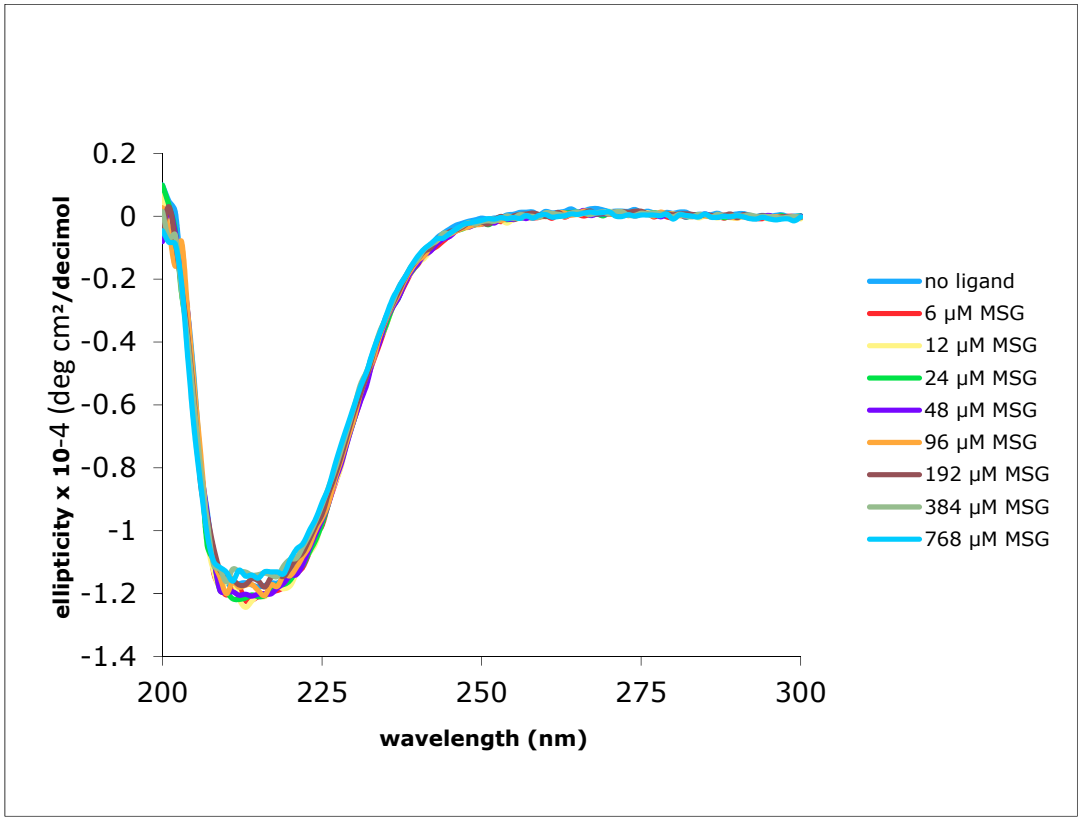

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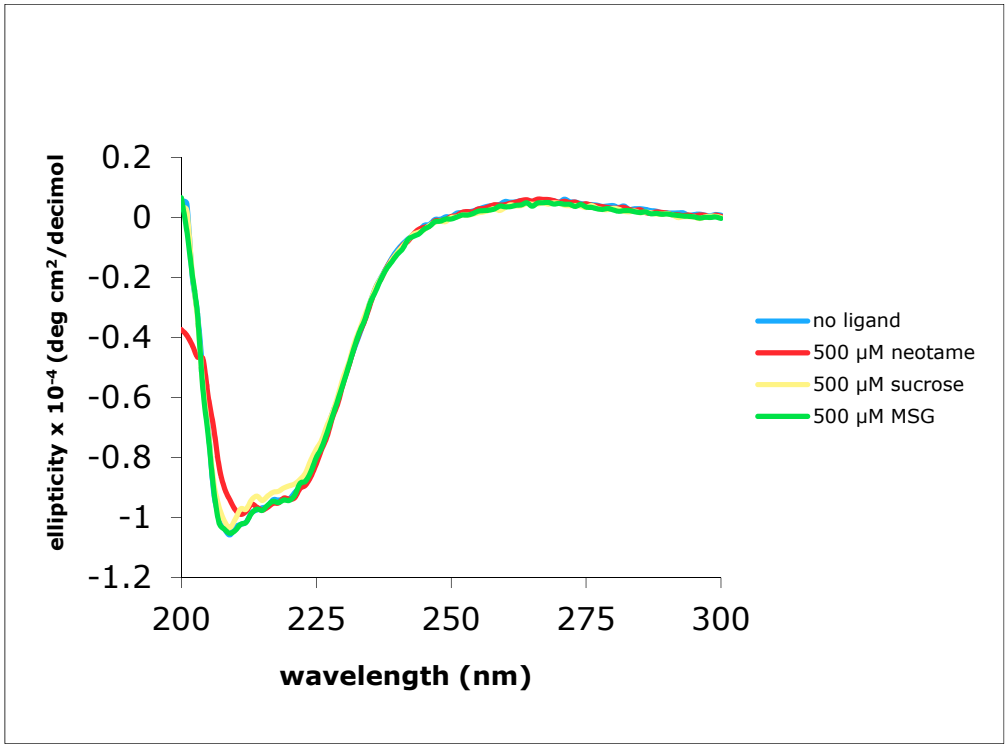

Figure 2. Circular dichroism spectra of His tagged human and mouse T1R2 ATD ± ligands. Panel A) human T1R2 ATD ± neotame, Panel B) changes in ellipticity at 209 and 219 nm with increasing concentrations of neotame; panel C) human T1R2 ATD ± MSG; panel D) addition of saturating concentrations of either neotame, sucralose or MSG to mouse T1R2 ATD.

Figure 2D demonstrates that when mouse His-T1R2 ATD was substituted for the human counterpart, neotame at a concentration (500 µM) affected a no loss of ellipticity decrease compared to the human protein (compare figure 2A and 2D). Sucrose also showed a small decrease in ellipticity upon binding to the mouse protein as with the human protein. Whereas MSG, the negative control, showed no change in ellipticity as was expected. A summary of measured K_d values for other sweeteners are reported in Table 1.

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Table 1. K_D values measured by CD.

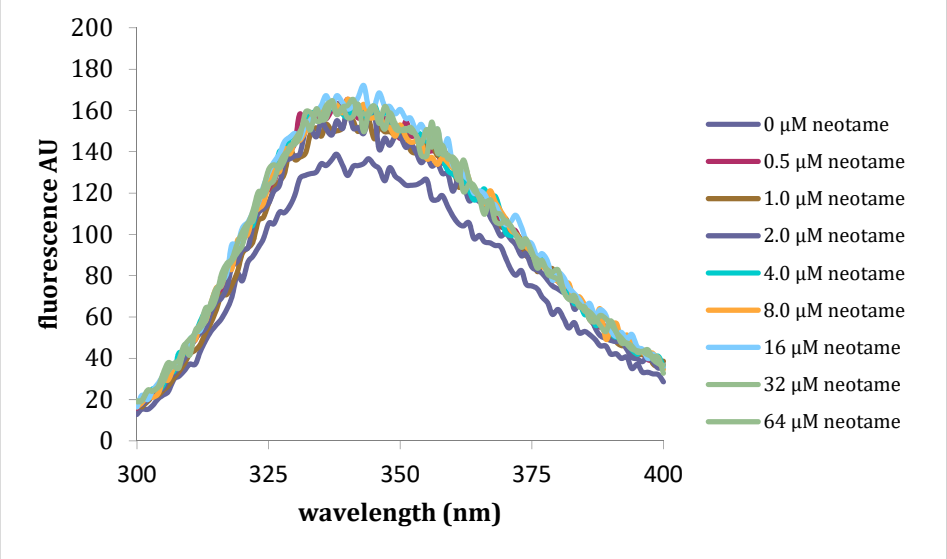
Ligand	K_D Values*
Acetasulfame	100 µM
Aspartame	> 300 µM
Cyclamate	ND
Neotame	50 µM
Saccharin	>1 mM
Sucrose	40 µM
Sucralose	40 µM
D-Tryptophan	500 µM
MSG	ND

Intrinsic fluorescence measurements indicates ligand binding affects tryptophans in T1R2 and T1R3 ATDs. Intrinsic fluorescence measurements were also performed in order to exploit the changing environment around the tryptophans as a marker for ligand binding. In figure 3A, neotame was added to the human HIS T1R2 ATD in increasing concentrations. Fluorescence intensity was found increase upon addition of neotame with a maximal value occurring at 10 µM. Interestingly, adding sucralose produced the opposite effect of a decrease in fluorescence intensity (figure 3B-D) and also tight binding (1/2 maximal value was less than 5 µM). MSG produced no change in intrinsic fluorescence intensity over the same concentration range of the other ligands. When the mouse protein was used (figures 3E-H), neotame was silent as was MSG

in changing intrinsic fluorescence intensity, while sucralose, as with the human protein produced a decrease in the fluorescence intensity with a half-maximal value of 10 µM. These results are in accordance with the fact that neotame does elicits a sweet-taste response in the human, but not the mouse.

Figure 3.

A)



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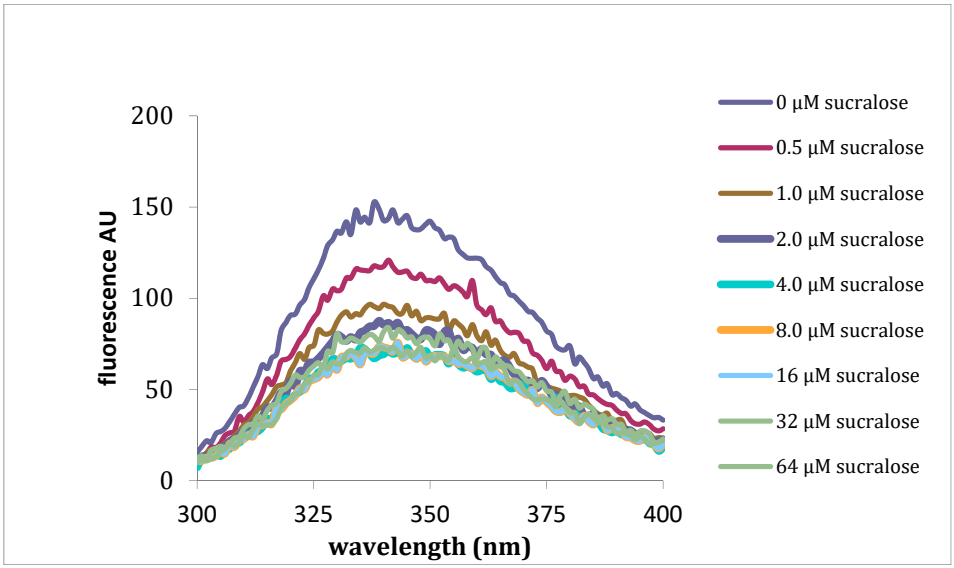

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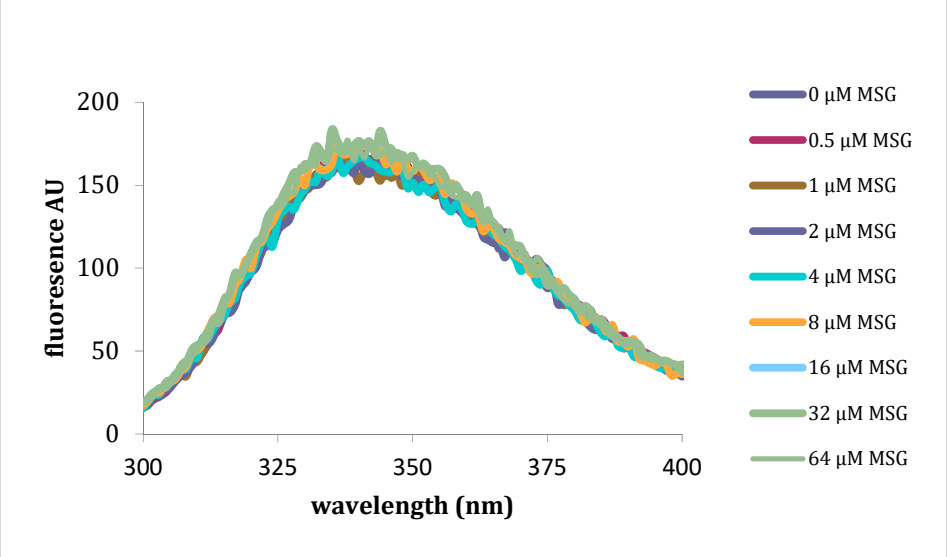

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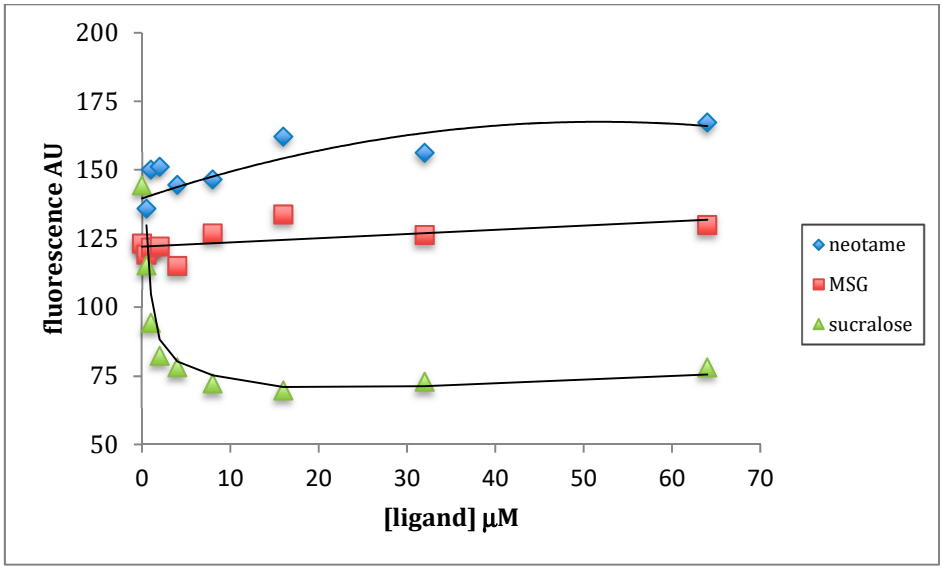

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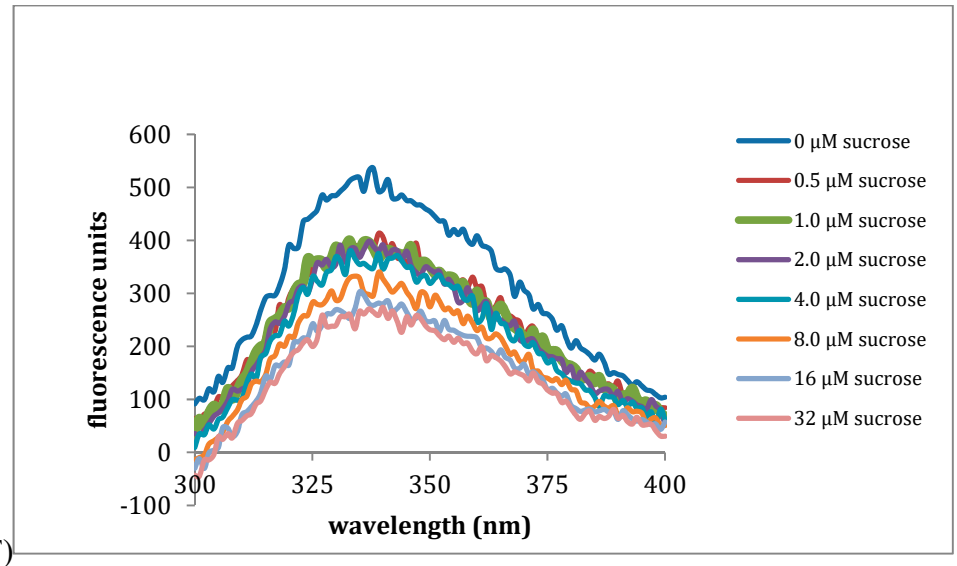

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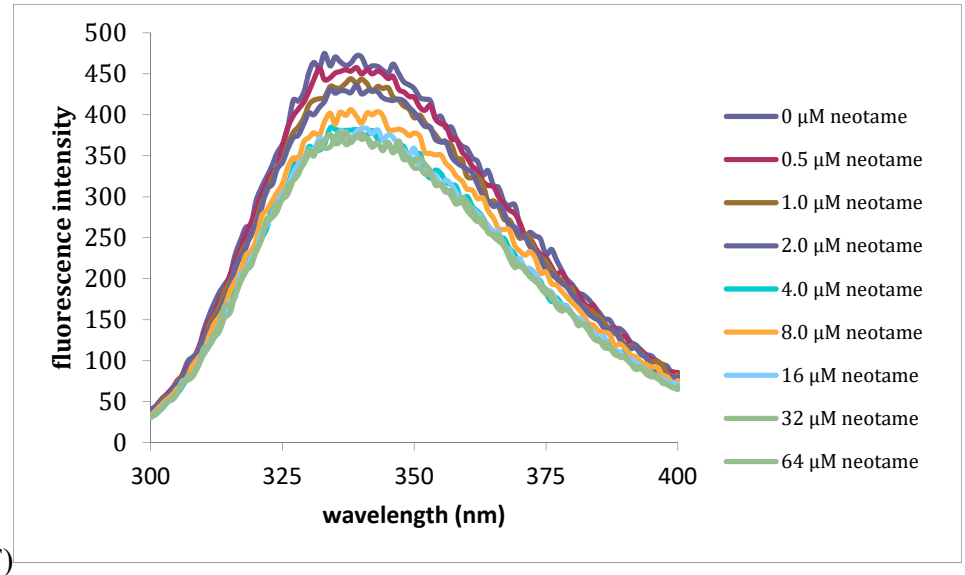

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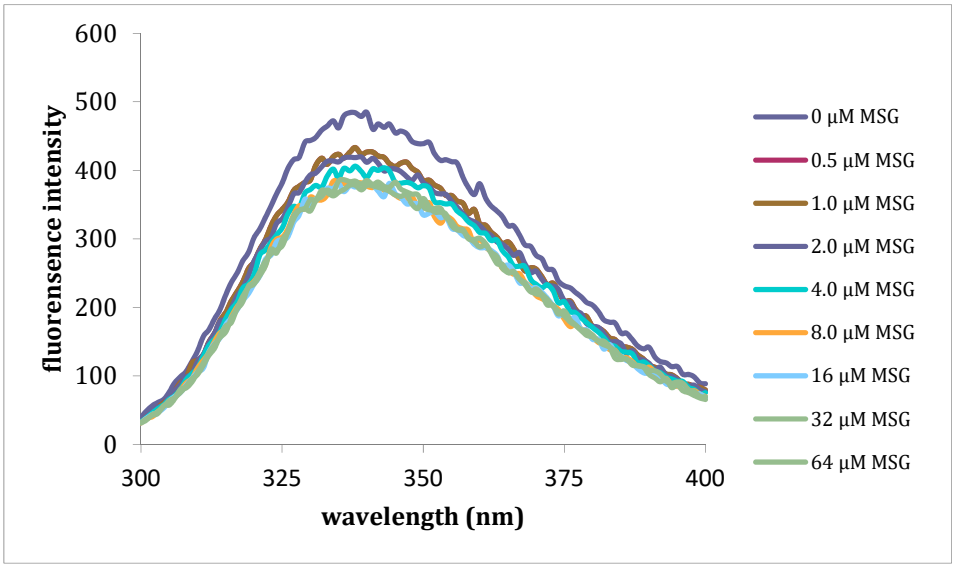

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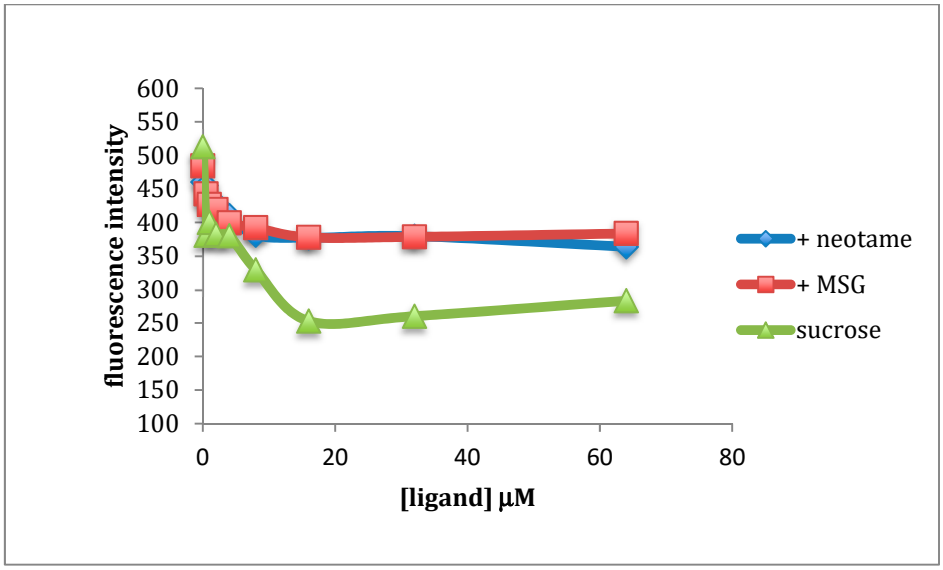

Figure 3. Intrinsic fluorescene emission spectra (excitation at 280 nm) of His tagged human and mouse T1R2 ATD. Panel A) human T1R2 ATD ± neotame; B) human T1R2 ATD ± sucralose; C) human T1R2 ATD ± MSG; D) changes in intrinsic fluorescence signal at 336 nm in the presence of human T1R2 ATD and increasing concentrations of either neotame, sucralose or MSG; panel E) mouse T1R2 ATD ± sucrose; F) mouse T1R2 ATD ± neotame; panel G: mouse T1R2 ATD ± MSG; H) changes in intrinsic fluorescence signal at 336 nm in the presence of mouse T1R2 ATD and increasing concentrations of either sucrose, neotame, or MSG.

Monitoring direct ligand binding by saturation transfer difference spectroscopy (STD). We used STD to monitor ligand protons binding to functionally relevant ligands to the human and mouse T1R2 ATD (figure 4A). Neotame showed increasing signal in STD spectra, whereas, MSG molecule produced no change in the STD profiles (no STD peaks) (figure 4B) similar to CD results in figure 4A over the same range of concentrations. MSG was chosen as a negative control as it is known to interact with only the umami receptor (T1R1) only. Changing temperature from 7-37 ºC did not affect binding of MSG to the receptor.

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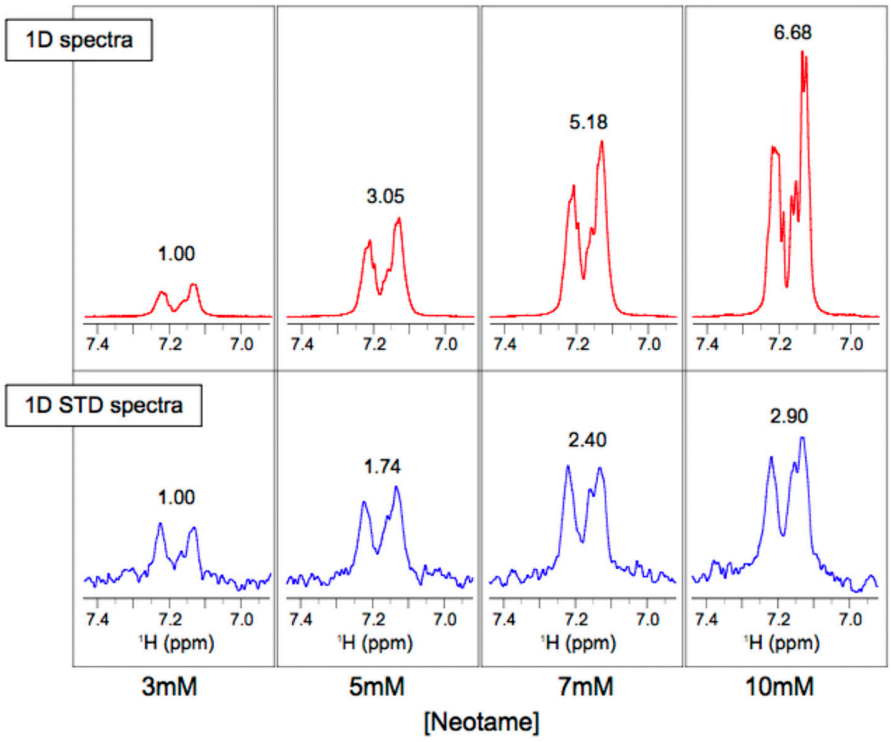
Figure 4

A)



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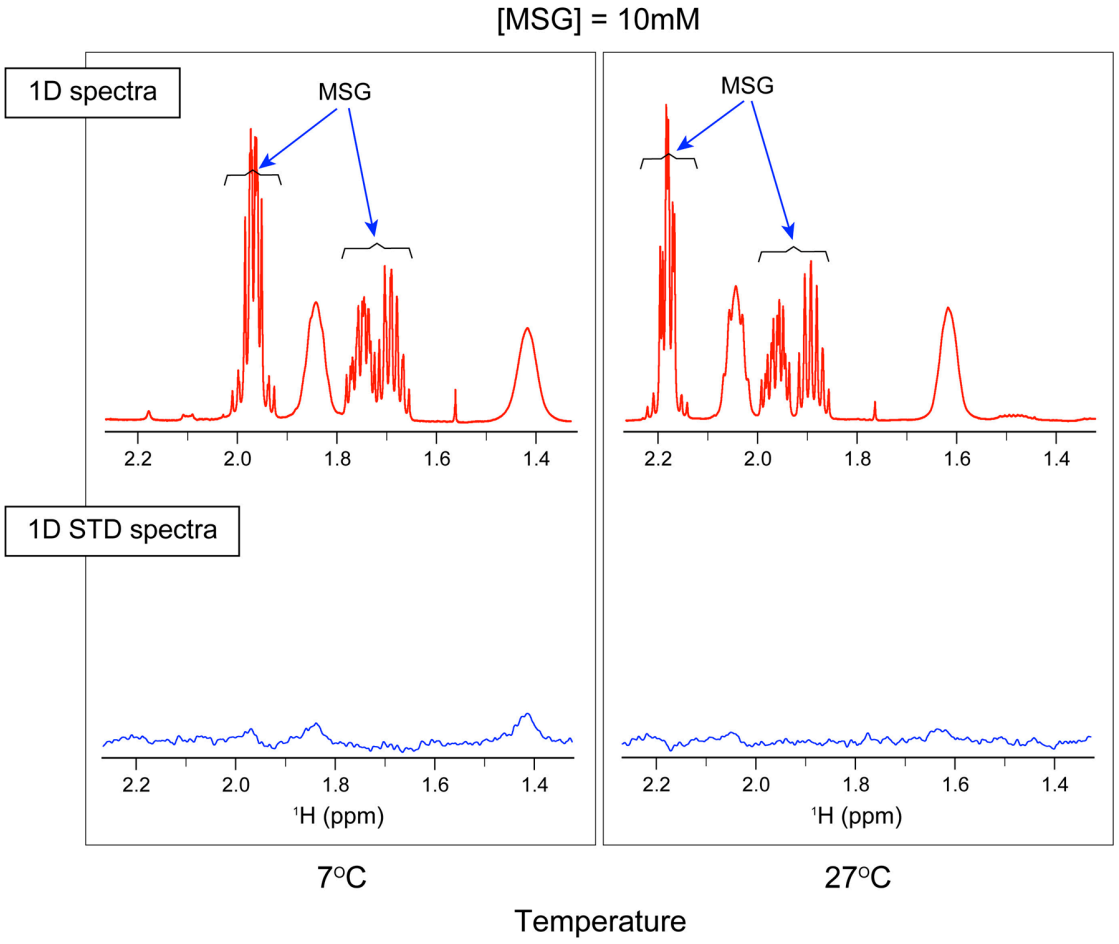

Figure 4. Saturation transfer difference spectra of His tagged human T1R2 ATD. A) human T1R2 ATD ± neotame at 27^{0}C; B) human T1R2 ATD ± MSG measured at 7 and 27^{0}C.

Monitoring the thermal stability of the tertiary structure of the human and mouse HIS-T1R2 ATD in the ligand-bound and unbound state. DSC was used to study the thermal stability of the tertiary structure of the human and mouse His-T1R2 ATD in the ligand-bound and unbound state. Table 2 shows summary of values determined from DSC for the thermo profiles of apo and neotame bound form of human and mouse ATDs. Thermo profile for the unbound protein best fit deconvoluted into two two-state transitions. Addition of the ligand neotame resulted in a thermal destabilization of both transitions of 2.5 and 8.5 °C each and a

small loss of overall enthalpy (ΔH). These results suggest that the loss of α-helix upon binding may be correlated to an overall thermal destabilization of the protein-ligand complex. In contrast, when the mouse counterpart of the T1R2 ATD was used and interacted with neotame, only small decreases in T_m were observed (< 1 °C for each transition), and the overall ΔH increased for the protein in the presence of ligand (Table 2). These results demonstrate a useful method for discerning the thermal stability of the species-specific T1R2 ATD protein in the presence and absence of ligand.

Table 2. Summary of DSC measurements for the apo and neotame bound states of human and mouse ATDs.

	HIS-hT1R2 ATD - neotame	HIS-hT1R2 ATD + neotame
T_m1	52.19± 0.22 °C	49.64 ± 0.14 °C
ΔH1	14980 ± 2170 cal/mole	17770 ± 2510 cal/mole
T_m2	62.60 ± 0.91 °C	54.09 ± 0.41 °C
ΔH2	22030 ± 2300 cal/mole	18500 ± 2620 cal/mole
	HIS-mT1R2 ATD - neotame	HIS-mT1R2 ATD + neotame
T_m1	54.88 ± 0.11•C	54.20 ± 0.15 •C
ΔH1	20390 ± 1260 cal/mole	50010 ± 2910 cal/mole
T_m2	62.49± 0.32 •C	61.78 ± 0.89 •C
ΔH2	15130 ± 1170 cal/mole	25300 ± 3280 cal/mole

DISCUSSION AND CONCLUSIONS

In order to better understand the mechanism by which the sweet taste response is generated, we have used a set of methodologies to describe binding events that appear to correlate to sweet

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taste. Earlier work [8] described the ATD region of T1R3 as a soluble, folded and functional protein. We extend the work now to include the equivalent portion of human T1R2 and compared with mouse T1R2 which have species-specific binding properties. We have used the recombinant protein that contains the ATD region of human T1R2 as a model system to target small molecule ligands. It is important to note that the material we are working with appears as a homodimer as monitored by gel filtration chromatography. This suggests that the ATD region of human T1R2 is capable of dimerization with an identical partner that is in a form that resembles its heterodimeric counterpart.

The CD and STD and DSC experiments, taken together, appear to provide a roadmap to binding of the small molecules effectors of the sweet taste response. Small molecules (MSG) that do not affect a positive response and regions of the protein (SUMO) thought not to be involved the binding process proved to be negative controls when tested. These experiments provide both a more local molecular and a more global picture of the ATD region of T1R2 and what transpires in the initial event of the binding of a sweet taste response.

Recently, Dong and colleagues [13] have used fullerenol as a model for the sweet-taste receptor to investigate the binding affinities of structural enantiomers of sweet-taste ligands. Their basic findings demonstrated a correlation between sweet intensity and binding energy. Our methodologies would be well suited to confirm these results using the actual protein-binding interface. In addition, these results would give a more detailed description of the events that lead to either a productive or destructive binding event.

In addition, others [12] have used taste receptor molecules to study the binding of various small sweet molecules. That study utilized transiently transfected cells and cellular responses to sweet taste stimulus by monitoring Ca^{++} flux. This is a downstream event that yields very little

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information on the molecular actions that involve a sweet-taste response in the receptor itself. Our model system for studying these interactions, in contrast, can lead to uncovering the seminal details that occur in a productive sweet taste response.

We have described a system by which we can both produce to purity the ATD region of the human and mouse T1R2 and quantify by a series of biophysical techniques the crucial events leading to a successful sweet taste response. We suggest that these methods can be applied to a wide range of expansions of the research. Just to name a few, we can use our expression and purification system on the equivalent ATD regions of T1R3 and T1R1, and use site-directed mutagenesis to elucidate important amino acids in these binding events. We can also study a much wider range of sweet tasting ligands as well as those that are inhibitors as well as enhancers of sweet taste response.

In conclusion, we describe a novel methodological system to purify and study biophysical the interactions involved in the sweet taste response.

MATERIALS AND METHODS

Production and Purification of T1R2 ATD constructs from mouse and human.

The ATD region of the protein was cloned into a 8xHis tag vector with either a TEV cleavage site or in a SUMO vector [10]. The human constructs ranged from residues Asn24−Met494 for human (ATD-hT1R2) in the SUMO fusion, or Ser25-Thr489 in the 8xHis-Tag. The mouse 8xHis-Tev-ATD-mT1R2 construct ranged from residues Gly2−Pro466. DNA coding for the amino terminal ligand binding domain of the sweet taste receptor from ATDs was expressed as inclusion bodies in *Escherichia coli* BL21-CodonPlus(DE3)-RIPL cells grown at 37 °C in 1L of LB medium. The purification procedure follows with modifications as previously described [9, 10]. The inclusion bodies were solubilized in 6 M guanidinium chloride and refolded by dialysis

against a buffer containing 50 mM Tris.acetate (pH 8.0), 50 mM KCl, 2 mM Zw3-14 and 2 mM DTT. The amino terminal domain (ATD) was purified to homogeneity on a Superdex 200 prep grade FPLC column and analyzed by 12% SDS-PAGE. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard [14]. The yield from the 1 liter of culture media was 5-10 mg of final protein.

Saturation Transfer Difference Spectroscopy (NMR-STD). The ligand binding activity of ATD-T1R2 for all ligands was confirmed with a NMR-STD binding assay as previously described [11, 15]. Aliquots of the pure labeled SUMO-T1R2 protein were incubated with desired titrating ligands at 0.5-20 fold molar excess and concentrated to a final concentration of ~0.05 mM in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 2.7 mM KCl, 5 mM DTT, 5X protease inhibitor (Roche) and 0.05% NaN_3. Mono sodium glutamate (MSG, non-sweet molecule) was used as a negative control. STD NMR data collection and analysis was as previously described [15].

Circular Dichroism Spectroscopy (CD)

CD spectra were recorded at 25 °C in an Aviv circular dichroism spectrometer Model 202SF equipped with a Peltier temperature control. Samples were added to a 0.01 cm path length quartz cuvette with a concentration of the ATD (from human and mouse) of about 0.3 mg/mL (4.5 mM) in 10 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 7.4. Increasing concentrations of ligands (neotame and sucrose) were included with the protein (0-64 mM) for the binding studies and MSG was used as a negative control. Data was collected every 1 nm with an averaging time of 5 seconds. The spectral bandwidth was 1 nm. Spectra were corrected for buffer and ligands contributions and converted to mean ellipticity in deg cm^2 $dmol^{-1}$. The % α-helix was computed using the deconvolution program from Chen et al [16].

Intrinsic Fluorescence Spectroscopy (Fl)

Samples were prepared in the same way as with the CD experiments. Experiments were performed at 25 °C on Carey Eclipse instrument (Agilent, 5301 Stevens Creek Blvd, USA). Spectra were substracted from the buffer ± ligand backgrounds before presentation. Curves at 340 nm were a best fit to the data by a regression/trend line.

Differential Scanning Calorimetry (DSC)

DSC was performed using a Microcal VP-DSC microcalorimeter. Samples of His-T1R2 ATD at about 20 mM were dialyzed against 4 x 1 liter of 10 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 7.4 in the absence or presence of 0.2 mM ligand for 16 hours at 4° C prior to use. Buffer without protein was hermetically sealed in the reference and sample compartments and repeated thermograms were generated from 10-95 °C and at 1 °C/min. After confirming that the repeated buffer/buffer thermoprofiles were identical, the protein sample was exchanged in the sample compartment and scanned against the reference buffer. Data was analyzed using the Microcal software package. The thermogram were background subtracted, normalized for concentration to ΔC_p (mcal deg^{-1}) and baselines established on the pre- and post-transitional data. The T_m (°C) and ΔH (kcal mol^{-1}) were determined from the corrected curves using non-2-state transitional curve fitting.

Conclusions.

We have described complementary experimental strategies for studying the conformational and binding variations that occur in the ATD domain of T1R2 of the human and mouse sweet receptor. This domain contains majority of the proposed binding pockets for small molecule sweeteners that upon binding leads to the productive execution of downstream sweet taste response. Our protein production system along with STD-NMR and the other biophysical

methods could provide important tools for mapping binding sites of natural and synthetic sweeteners for this complex GPCR.

Acknowledgements

This work was supported grants from the U.S. National Institutes of Health to (NIH) grant R01 DC009018 (to F.M.AP) and the National Magnetic Resonance Facility at Madison, which is supported by NIH grants from the National Center for Research Resources (5P41RR002301-27 and RR02301-26S1) and the National Institute for General Medical Sciences (8 P41 GM103399-27). The FL, CD and DSC were collected using instruments in the Biophysical core laboratory at UW-Madison.

Authors Contributions

FAP designed experiments, STD data collection and analysis, and wrote manuscript. HR prepared ATD constructs and protein purification, JR collected CD, DSC, and FI and wrote the manuscript. MT helped with STD-NMR data collection. All authors involved in writing the manuscript and preparing figures.

Conflict of Interests

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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