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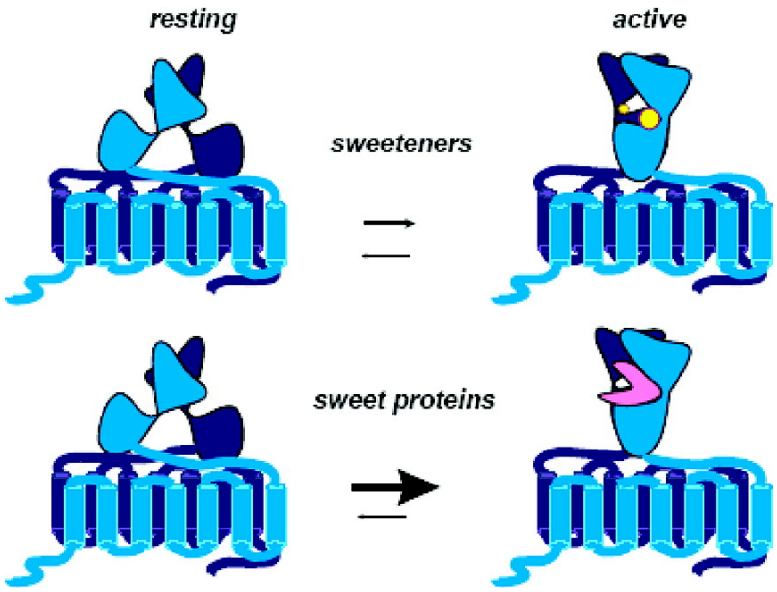

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From Small Sweeteners to Sweet Proteins: Anatomy of the Binding Sites of the Human T1R2_T1R3 Receptor

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The sweet taste receptor, a heterodimeric G protein coupled receptor (GPCR) protein, formed by the T1R2 and T1R3 subunits, recognizes several sweet compounds including carbohydrates, amino acids, peptides, proteins, and synthetic sweeteners. Its similarity with the metabotropic glutamate mGluR1 receptor allowed us to build homology models. All possible dimers formed by combinations of the human T1R2 and T1R3 subunits, modeled on the A (closed) or B (open) chains of the extracellular ligand binding domain of the mGluR1 template, yield four ligand binding sites for low-molecular-weight sweeteners. These sites were probed by docking a set of molecules representative of all classes of sweet compounds and calculating the free energy of ligand binding. These sites are not easily accessible to sweet proteins, but docking experiments *in silico* showed that sweet proteins can bind to a secondary site without entering the deep cleft. Our models account for many experimental observations on the tastes of sweeteners, including sweetness synergy, and can help to design new sweeteners.

Introduction

Coping with diseases linked to the consumption of carbohydrates benefits from the design of new sweeteners. The identification and functional expression of the receptor for sweet taste opens new perspectives, but owing to the difficulty of direct structural studies, modeling of sweeteners complexed to their receptor represents one of the best possible approaches.

The sweet taste receptor (SR) is a G protein coupled receptor (GPCR) similar to the dimeric mGluR1 receptor. Both belong to class C of GPCRs, which includes several metabotropic glutamate receptors, sweet and umami (monosodium glutamate) taste receptors, the Ca^{2+} sensing receptor, the γ-aminobutyric acid type B receptor, and pheromone receptors.[1] In addition to the seven helices transmembrane domain (7TMD), class C receptors have a large extracellular domain composed of a Venus fly trap domain (VFTD), containing the active site for ligands, and a cysteine-rich domain. The main structural difference between the SR and mGluR1 is that while the SR is heterodimeric, mGluR1 is homodimeric, albeit with two different conformations of its two chains (A and B). It is interesting to note that while several independent groups[2−6] hypothesized almost contemporaneously that T1R3 corresponded to the *Sac* gene, it was demonstrated only later[7] that to function as sweet taste receptor T1R3 has to combine with T1R2 to form a heterodimer. Moreover, T1R3 alone can recognize carbohydrates *in vivo*.[8]

A unique feature is that while the ligands of mGluR1 are either glutamate or closely related molecules, the ligands able to activate the sweet taste receptor vary widely in chemical constitution, ranging from sugars to amino acids, peptides, proteins, and several other classes of organic compounds. The very fact that sweeteners cover a particularly wide range of chemical constitution hints that at least some of them may interact with parts of the SR different from the two likely cavities corresponding to the Glu hosting cavities of mGluR1, either in the N-terminal domain or in the transmembrane helices. To date, this possibility has been confirmed by Xu et al.,[9] who found that the C-terminal transmembrane domain of T1R3 is required for recognizing cyclamate (an artificial sweetener as well as a compound known to work synergistically with some sweet tasting compounds[10]) and the sweet taste inhibitor lactisole. This finding is consistent with data reported for mGluR1 and some positive or negative allosteric regulators.[11−13]

Traditional structure−activity relationship (SAR) studies of sweet molecules used the shape and electronic properties of small-molecular-weight ligands to describe the nature and topological arrangement of glucophores of an ideal sweet compound and/or of the recognition active site of the sweet taste receptor. The main features of these artificial sites, common to all models, are the presence of complementary pairs (in the active site and the agonist) of hydrogen bond donors and acceptors (AH−B),[14] a flat shape, and a hydrophobic patch.[15−21] The main reservation on the use of artificial binding sites is that it is not certain whether the SR binds all the different compounds able to elicit sweetness in the same pocket. Recently, a model able to explain and predict, semiquantitatively, the sweet taste of compounds belonging to different families has been proposed and used[22] in support of the hypothesis that there is

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just one binding site, at least for small ligands, in the sweet taste receptor.

In addition to the many hundreds of small-molecular-weight sweeteners found over a century by serendipity or by targeted research, a few sweet proteins were also discovered in more recent times.[23] The size of sweet proteins is so different from that of most sweeteners that, for a long time, the prevailing belief was that they were likely to interact with a different receptor. Only recently, it has been demonstrated that small-molecular-weight sweeteners and sweet macromolecules interact with the same T1R2_T1R3 receptor,[7] supporting the notion that there is just one sweet taste receptor. However, it is not easy to understand how low-molecular-weight sweet compounds and sweet proteins can activate the same receptor. The mentioned indirect models of the active site based on the shape of small sweeteners could still be compatible with the interaction of proteins, provided one can identify, on the surface of the proteins, protruding features that can probe the active site, i.e., "sweet fingers" chemically similar to small sweeteners.[18] Among known sweet proteins, there is no sequence homology. There is also little similarity among the tertiary folds of brazzein, monellin, and thaumatin, the sweet proteins of a known 3D structure. The only common elements among the three proteins are small traits of secondary structure, β-sheet loops that might be consistent with sweet fingers since they host residues with glucophores already identified in small sweeteners. However, none of the cyclic peptides designed to mimic these potential sweet fingers, although assuming conformations consistent with the conformation of the same sequences in the parent proteins, was able to elicit a sweet taste.[24] This finding suggests that sweet proteins recognize a binding site different from the one that binds small-molecular-mass sweeteners and supports the alternative mechanism of interaction proposed by Temussi,[25] in which the sweet proteins stabilize a ligand-free active form of the SR through the binding to an external site. This hypothesis (henceforth referred to as the "wedge model") was based on *in silica* binding experiments of brazzein, monellin, and thaumatin to one of the possible forms of the sweet receptor modeled on *mouse* T1R2_T1R3 sequences that showed that all three proteins fit a large cavity of the receptor with the wedge-shaped surfaces of their structures.

In this paper, we report an exhaustive modeling of the *human* sweet taste receptor extracellular domain based on the structures of the resting and activated forms of the extracellular domain of mGluR1.[26,27] All the possible dimers formed by combinations of the two subunits T1R2 and T1R3 modeled on the A or B chains of the mGluR1 templates were examined, i.e., two models corresponding to the inactive open−open form and two models for the active closed−open form. All models were then used for docking calculations with models of brazzein, MNEI (a single chain monellin), and thaumatin, the sweet proteins of a known 3D structure. In each modeled subunit of the active closed−open form, the ligand binding site for low-molecular-weight sweeteners was defined. To validate the identified binding sites, a large number of sweet compounds belonging to different families, including sugars, peptides, and other intensive sweeteners, were docked into them, and the free energy of ligand binding was calculated and compared with the experimental free energy of ligand binding derived from the relative sweetness. With the help of these models, we were able to address several important questions. In particular, is the receptor configuration that of T1R2(A)_T1R3(B) or that of T1R2(B)_T1R3(A)? Which is the most likely active site for small-molecular-weight sweeteners? Are two ligands necessary to activate the receptor, or are both ligand binding sites occupied only in the presence of two synergistic sweet compounds?

Results

Sweet Taste Receptor Homology Modeling. We considered all possible receptor models based on the *human* sequence, modeling either T1R2 or T1R3 on open or closed chains of the relevant crystal structures of mGluR1: free form I, that contains two open conformers similar in the overall shapes but slightly different in detail (inactive open−open, PDB entry 1ewt), and the complexed form, identical to free form II, that hosts two molecules of glutamate in two cavities of chains A and B (complexed active closed−open, PDB entry 1ewk).[26] This amounts to two models for the inactive open−open form, henceforth dubbed Roo_AB (where AB means T1R2 modeled on chain A and T1R3 modeled on chain B of the 1ewt template) and Roo_BA (T1R3 modeled on chain A and T1R2 modeled on chain B of the 1ewt template), and two models for the complexed active closed−open form, henceforth dubbed Aoc_AB (T1R2 modeled on chain A and T1R3 modeled on chain B of 1ewk) and Aoc_BA (T1R3 modeled on chain A and T1R2 modeled on chain B of 1ewk).

Homology modeling of these four structures posed an interesting problem: the human T1R2 and T1R3 sequences should be aligned to the single glutamate sequence, but the corresponding template varies in all cases owing to the different conformations of its two chains. Accordingly, it was necessary to resort to ad hoc adjustments to yield an acceptable model for each of the four possibilities. The resulting slightly different alignments are characterized by different figures in the number of identities and homologies with the glutamate sequence that, to some extent, reflect the relative ease with which the corresponding models were built using the SwissModel web site.

***In Silico* Binding of Sweet Proteins to a Single Site: The "Wedge" Site.** If the SR had the same characteristics as mGluR1, it should exist as a mixture of ligand-free forms in equilibrium:[26,27] free form I, the "inactive" conformation with two open protomers (resting open−open, dubbed Roo), and free form II, nearly identical to the "active" complexed form (active open−closed, dubbed Aoc), that hosts two molecules of glutamate in two cavities of chains A and B. As shown by Figure 1, the "normal" way of shifting the equilibrium between the two forms toward the active form would be through the binding of the ligand (small-molecular-weight sweetener) in one or both cavities, analogous to cavities that bind glutamate in the two protomers of mGluR1. Alternatively, according to the wedge model, stabilization may result from the attachment of a sweet protein to a secondary binding site on the surface of free form II.[25]

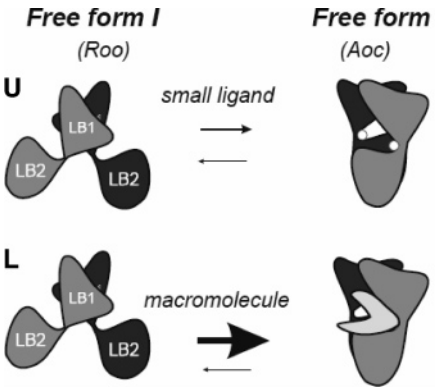

Figure 1. Scheme of the conformational equilibrium between free forms of the extracellular domain of the SR. Each protomer is represented as a bilobed entity composed of subdomains LB1 and LB2. On the right-hand side, the angle between LB1 and LB2 is smaller in the closed protomer (black). (U) Binding of a small-molecular-weight ligand transforms inactive free form I into the complexed form (Aoc), identical to free form II. (L) Free form II, stabilized by protein complexation, activates long lasting signal transmission. Small ligands in the two cavities of Aoc are shown as white balls of equal size. The wedge protein is colored gray.

To test the authenticity of the external binding site for sweet proteins, i.e., the single site previously found on the surface of a model of free form II of the mouse receptor,[25] we first checked whether the corresponding human Aoc_AB and/or Aoc_BA models could bind sweet proteins in the same part of the surface and, as a negative check, whether human Roo_AB and/or Roo_BA models, corresponding to free form I in equilibrium with free form II, could also bind sweet proteins in a single well-defined surface spot. In fact, were free form I also able to bind sweet proteins on a well-defined surface active site, the equilibrium depicted in the lower part of Figure 1 would not be shifted to the right.

All four models were used for *in silico* binding experiments with models of brazzein, MNEI, and thaumatin, the sweet proteins of a known 3D structure. All docking calculations were performed by means of the program GRAMM in the low-resolution mode (see the Methods section). This docking method is best suited to circumvent the multiple minima problem and is very useful in cases in which likely binding sites are not known in advance. The results for all three proteins are consistent with those described previously for the mouse receptor. Only the results for MNEI with both the Aoc models will be discussed in detail. Among the first 50 preferred solutions found by GRAMM, none is on the side of the receptor model facing the membrane. All MNEI models are centered on a large cavity on the concave side of the B chain of the dimeric receptor. Figure 2 shows the interaction of MNEI with each of the two possible activated forms of the receptor. Figure 2U (upper panel) shows the human Aoc_AB form together with 10 of the molecules of MNEI calculated by the docking procedure. All 10 molecules are found in the same spot on the surface, mainly belonging to the T1R3(B) chain. They are oriented in a similar, albeit not identical, way. Efficient binding is assured both by shape and charge complementarity, since the cavity is predominantly negative and the interacting surfaces of the proteins are mainly positive. The other two views of the complex pictured in the U panel, obtained via rotation of the central model around the *x* axis (left) or around the *y* axis (right), show that the remaining parts of the surface of the Aoc_AB model do not bind any MNEI molecules. As shown in Figure 2L (lower panel), the results obtained by docking MNEI on Aoc_BA are similar from a structural point of view, since the MNEI molecules, also in this case, bind to a cavity on the T1R2(B) chain. Figure 3 shows the potential surface of the sides of Aoc_AB and Aoc_BA that hosts the binding cavities. Both the T1R3(B) and T1R2(B) protomers, shown in the U and L panels, respectively, host prevalently negatively charged cavities, but whereas that of the T1R3(B) protomer is completely accessible from outside, that of the T1R2(B) protomer is partially obstructed. Besides, as shown by the rotated images on the right-hand side of Figure 3, the cavity on T1R2(B) is not as wide as that on T1R3(B). It is difficult to ascertain whether these differences are authentic, since the modeling of the two protomers is based on low sequence similarities, using a template whose resolution is 2.2 Å. Owing to the inevitable limitations inherent to modeling, it is possible that the obstruction observed in T1R2(B) is an artifact. Nonetheless, the preference of sweet proteins for a binding site located prevalently on the B chain of the models is clearly shown by the docking calculation.

As a negative check, we calculated the docking of MNEI to the inactive open−open Roo_AB and Roo_BA models. Figure 4 shows that in both cases the molecules of MNEI bind to a very large area of the receptor without any apparent regularity. Therefore, a specific binding of MNEI to this form of the receptor seems unlikely.

Identification of Active Sites Binding Small Ligands. The residues that directly interact with the α-amino acid moiety in mGluR1 are well conserved, not only in other mGluRs but also in other families of class C GPCRs;[1] therefore, they can be used to locate the ligand binding site. Moreover, we can rely on the chemical similarity of some sweeteners to glutamate to assess the relative likelihood of possible active sites. In fact, the T1R2_T1R3 receptor can bind, among many other molecules, simple hydrophobic amino acids and synthetic dipeptides, e.g., aspartame. Both types of molecules are characterized by the amino acidic moiety typical of all α-amino acids, including glutamate. We can hypothesize that the cavity of the T1R2_T1R3 receptor should retain most of the features necessary to build this moiety, i.e., the residues lining the wall of the moiety of the cavity that binds the amino and carboxyl groups of amino acids. On the other hand, the residues of the remaining part of the cavity are expected to be more variable, albeit on average hydrophobic, since in the sweet taste receptor they accommodate several molecular fragments of different size and chemical constitution. In the alignments used to derive the models, as expected, the residues corresponding to those binding the amino acidic moiety of glutamate are well conserved in all the modeled protomers, while the residues lining the wall of the active site in correspondence to the glutamate side chain are changed, on average, to less polar or uncharged residues.[28] We would like to note that T1R3 presents more conserved residues and conservative mutations in the active site,

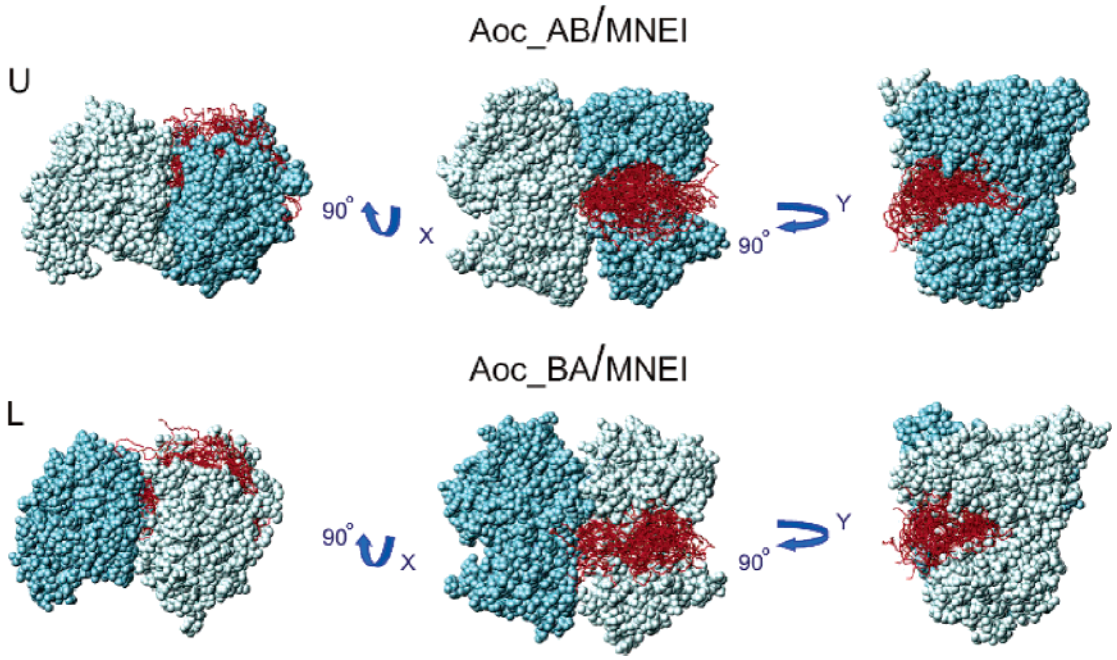

Figure 2. Models of the active form of the human sweet receptor with bound MNEI molecules. (U) Model Aoc_AB together with ten bound molecules of MNEI calculated by the docking procedure. Atoms of the T1R2 sequence are shown in lighter blue, whereas those of the T1R3 sequence are shown in darker blue. Models of MNEI are represented as red neon backbone bonds. On the left-hand side of panel U, the Aoc_AB complex is shown after a rotation of 90° around the *x* axis; on the right-hand side, the same complex is shown rotated 90° around the *y* axis. (L) Model Aoc_BA together with ten bound molecules of MNEI calculated by the docking procedure. Atoms of the T1R2 sequence are shown in lighter blue, whereas those of the T1R3 sequence are shown in darker blue. Models of MNEI are represented as red neon backbone bonds. On the left-hand side of panel L, the Aoc_BA complex is shown after a rotation of 90° around the *x* axis; on the right-hand side, the same complex is shown rotated 90° around the *y* axis. The models were generated by MOLMOL.[42]

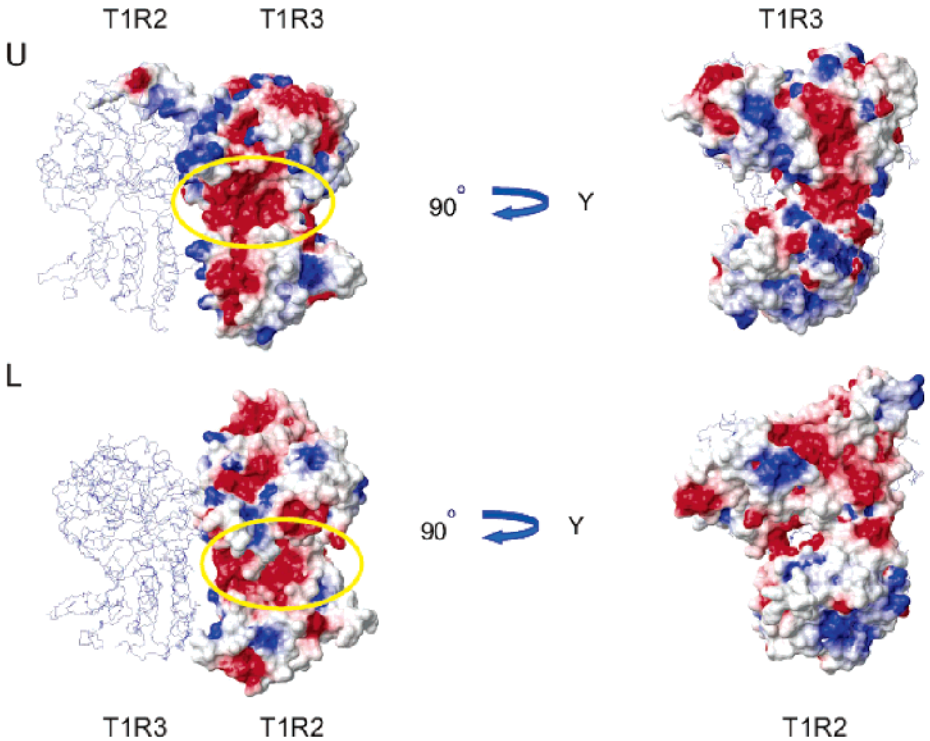

Figure 3. Electrostatic potential surface of the B chains of the Aoc_AB and Aoc_BA forms of the T1R2_T1R3 receptor. (U) The T1R2(A) protomer of Aoc_AB is represented as line backbone bonds, and the T1R3(B) protomer is represented as an electrostatic potential surface. On the right-hand side, the same model is shown rotated 90° around the *y* axis. (L) The T1R3(A) protomer of Aoc_BA is represented as line backbone bonds, and the T1R2(B) protomer is represented as an electrostatic potential surface. On the right-hand side, the same model is shown rotated 90° around the *y* axis. The models were generated by MOLMOL.[42]

as compared to those presented by T1R2. Another consideration concerns the fact that we do not know whether in the sweet taste receptor both ligand binding sites are available for sweet ligands or if—and how—they are involved in a synergistic mechanism. Therefore, we decided to locate both ligand binding sites (one in

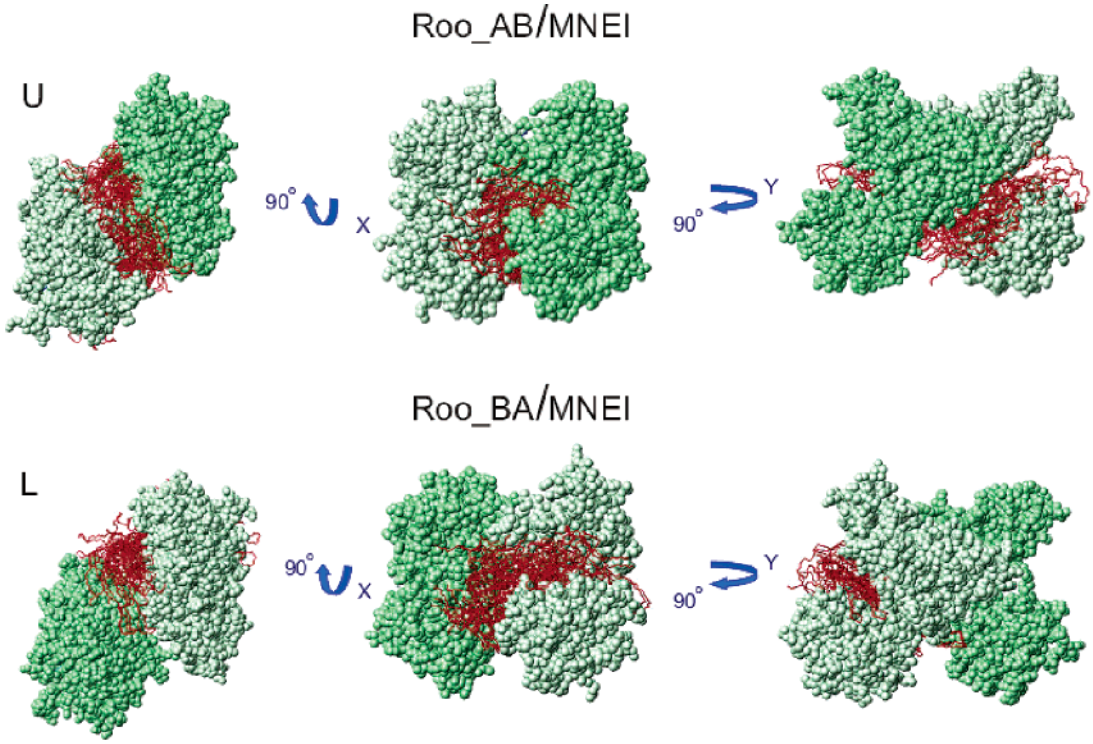

Figure 4. Models of the resting form of the human sweet receptor with bound MNEI molecules. (U) Model Roo_AB together with ten bound molecules of MNEI calculated by the docking procedure. Atoms of the T1R2 sequence are shown in lighter green, whereas those of the T1R3 sequence are shown in darker green. Models of MNEI are represented as red neon backbone bonds. On the left-hand side of panel U, the Roo_AB complex is shown after a rotation of 90° around the *x* axis; on the right-hand side, the same complex is shown rotated 90° around the *y* axis. (L) Model Roo_BA together with ten bound molecules of MNEI calculated by the docking procedure. Atoms of the T1R2 sequence are shown in lighter green, whereas those of the T1R3 sequence are shown in darker green. Models of MNEI are represented as red neon backbone bonds. On the left-hand side of panel L, the Roo_BA complex is shown after a rotation of 90° around the *x* axis; on the right-hand side, the same complex is shown rotated 90° around the *y* axis. The models were generated by MOLMOL.[42]

each protomer) on the two models derived from the two forms Aoc_AB and Aoc_BA.

Each of the obtained protomer models was superimposed on the corresponding template (containing the ligand glutamate) in such a way that it was possible to obtain the modeled protomer containing the ligand glutamate in a position corresponding to the binding site. To define the amino acids lining the ligand binding site, a subset of SR residues was created, using the ligand glutamate as the center and considering the amino acids within a distance of 15 Å from the center as part of the subset. We also visually checked that all the amino acids surrounding the ligand were included. Even at a glance, it is clear that active sites built on closed protomers, i.e., T1R2(A) and T1R3(A), are so small that they cannot possibly host some of the large sweeteners (different from proteins). Once again, this finding reflects the fundamental difference between the template (mGluR1), that can host only small molecules of well-defined chemical constitution and volume (i.e. glutamate and congeners), and the SR, which can host sweeteners covering a particularly wide range of chemical constitution and molecular volume.

In mGluR1, both closed (MOL1) and open (MOL2) protomers bind glutamate at similar sites defined by the interfaces of two subdomains: LB1 and LB2.[26,27] The structures of the LB1 interface lining the glutamate binding cavities in closed and open protomers are essentially identical, but the LB2 interface of the open protomer is not used for glutamate binding. As a consequence, the buried surface area is larger in the closed protomer than it is in the open protomer. On the contrary, owing to the dimensions of many sweeteners, the active sites of Aoc_AB and Aoc_BA of the SR can use both LB1 and LB2 interfaces (*vide infra*) and, thus, the volume of the active sites of open protomers is larger than that of the corresponding closed protomers.

Although both sites of the active receptor (A_oc) of mGluR1 are occupied by a glutamate molecule, it has not yet been ascertained whether the mechanism of the conformational switch between R_oo and A_oc is driven by only one of the sites or by both. Also, in the case of the SR, it is possible that occupancy of the two sites by different modalities and/or molecules of different size plays a central role in receptor activation. Accordingly, we investigated ligand binding in all four identified active sites, namely, T1R2(A), T1R3(A), T1R2(B), and T1R3(B).

Docking and Binding Affinity of Small-Molecular-Weight Sweeteners. A large number of sweet molecules (Figure 5) belonging to different families, including sugars, peptides, and other intensive sweeteners (chosen in such a way that they represent most classes of chemical constitution and a fair range of dimensions), were inserted in the identified active sites. Their fit was optimized by means of PrGen,[29] a program that allows a semiquantitative prediction of binding affinity for ligands and its comparison with the experimental biological activity, i.e., sweetness. We would like to point out that PrGen has only been used as a tool to

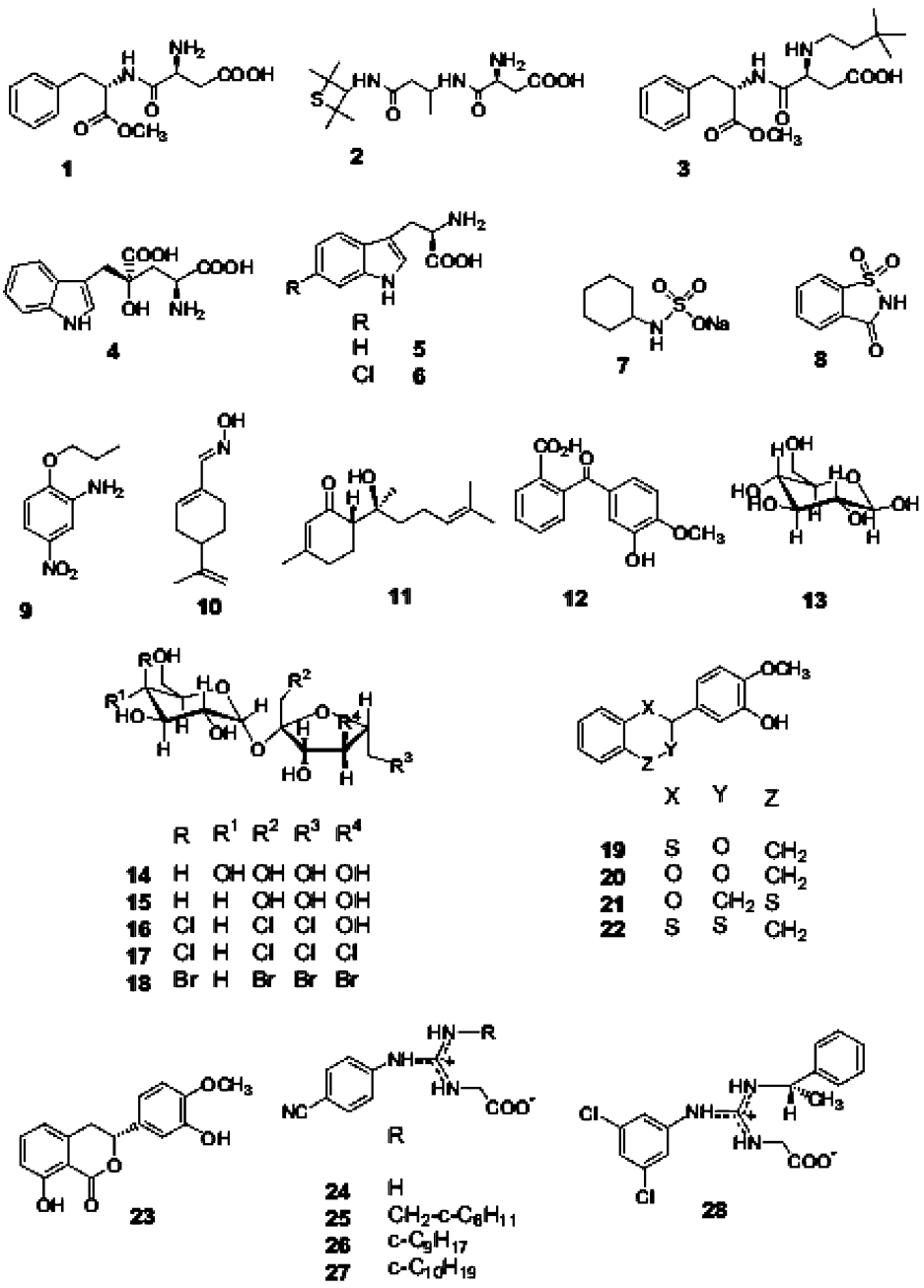

Figure 5. Molecular structures of sweet compounds which were inserted in the ligand binding sites and evaluated with PrGen, either in the training set or in the test set.

evaluate the reliability of the identified ligand binding sites without essentially altering the architecture of the sites yielded by homology modeling.

In both binding sites obtained from the protomers whose structure is based on the open chain (B) of the template of the active closed−open form 1ewk ("type B sites"), it has been possible to dock a large number of representative sweet compounds, used as a training set to derive the model: 16 have been docked in the T1R2-(B) ligand binding site, and 22 have been docked in the T1R3(B) ligand binding site with good correlation between experimental and calculated binding affinities (Tables 1 and 2). It should be noted that only an approximate positioning of the ligands within the binding site is required at the beginning of the calculation, because the positions (as well as the conformations) of all the molecules are adjusted individually during the optimization of the training set to provide the best fit with the experimental data. Moreover, both site models refined by PrGen were also able to semiquantitatively predict free energies of binding for an independent set of sweet compounds used as a test set (5 in the T1R2-(B) and 6 in the T1R3(B) ligand binding site, as shown in Tables 1 and 2).

It is interesting to note that, despite its extreme flexibility, aspartame was also included in the compound set for consistency with the set previously used in the pseudoreceptor based on amino acid residues not related to the SR sequence.[22] For the same reason, the starting conformation of aspartame in the present work was, again, a folded conformation found in the crystal structure[30] and subsequently used in one of the indirect models.[31] Since a previous model[32] had used an extended form of aspartame, corresponding to the crystal structure of [(L-α-Me)Phe^2] aspartame,[33] we also put [(L-α-Me)Phe^2] aspartame as a test compound in an alter-

Table 1. Compounds Inserted in the T1R2(B) Ligand Binding Site and Modeled with PrGen: Common Name (When Available), MRS (Molar Relative Sweetness), and Experimental and Predicted Free Energies of Ligand Binding for Training and Test Sets[a]

cmpd	common name	exp MRS	exp MRS (as $\Delta G°$, kcal mol^{-1})	pred MRS (as $\Delta G°$, kcal mol^{-1})
		Training Set		
1	aspartame	172	−9.700	−9.491
2	alitame	1937	−11.110	−10.894
3	neotame	11057	−12.120	−12.362
4	monatin	1025	−10.740	−10.646
5	D-tryptophan	21	−8.480	−9.289
6	6-Cl-D-tryptophan	906	−10.670	−10.777
7	cyclamate	26	−8.600	−8.665
8	saccharin	161	−9.660	−9.421
9	P4000	2293	−11.210	−10.881
10	perillartine	966	−10.710	−10.599
12		198	−9.780	−9.628
13	D-glucose	0.26	−5.920	−5.791
14	sucrose	1	−6.713	−6.665
15		0.95	−6.670	−6.812
20		4527	−11.600	−11.139
23	phyllodulcin	502	−10.320	−10.944
		Test Set		
16	sucralose	755	−10.560	−10.470
18		13012	−12.220	−8.905
19		14426	−12.280	−11.235
21		401	−10.190	−10.811
22		16968	−12.370	−11.814

[a] Correlation coefficient for the training set (16 compounds) = 0.984, rms deviation = 0.323 kcal mol^{-1}; rms deviation for the test set (5 compounds) = 1.599 kcal mol^{-1}.

native training set in lieu of aspartame. Both calculations showed that, consistent with its conformational flexibility, both the folded and the extended conformations of aspartame can be accommodated in the "B" cavities.

Figure 6 shows the fit of [(L-α-Me)Phe^2] aspartame[33] in the cavity of T1R3(B); it is interesting to note that this extended conformation can span nearly the entire distance between LB1 and LB2.

A detailed analysis of the optimized model, by means of one of the tools of PrGen itself, pinpoints the residues contributing most to the ligand binding. In the case of T1R3(B), the first four amino acids indicated are Glu301, Ser170, Tyr218, and Asp 190, corresponding, respectively, to Asp318, Thr188, Tyr 236, and Asp 208 in mGluR1; for T1R2(B), the first three amino acids indicated by PrGen are Ser165, Glu 302, and Tyr215, corresponding, respectively, to Ser186, Asp318, and Tyr236 in mGluR1: in both cases, amino acids were among those interacting with the α-amino acid group,[28] and both LB1 and LB2 domains were involved.

As expected, it proved very difficult to dock a large variety of ligands in the binding sites hosted by structures built using chain A (closed) of the complexed active closed−open form as the template 1ewk ("type A sites"). In our models, it was possible to dock only four compounds in T1R2(A) with good correlation between experimental and calculated binding affinities, while, in T1R3(A), although it was possible to dock the same four compounds inserted into T1R2(A), it was not possible to obtain an acceptable correlation between experimental and calculated binding affinities (data not shown).

Table 2. Compounds Inserted in the T1R3(B) Ligand Binding Site and Modeled with PrGen: Common Name (When Available), MRS (Molar Relative Sweetness), and Experimental and Predicted Free Energies of Ligand Binding for Training and Test Sets[a]

cmpd	common name	exp MRS	exp MRS (as $\Delta G°$, kcal mol^{-1})	pred MRS (as $\Delta G°$, kcal mol^{-1})
		Training Set		
1	aspartame	172	−9.700	−10.248
2	alitame	1937	−11.110	−11.580
3	neotame	11057	−12.120	−11.439
4	monatin	1025	−10.740	−11.820
5	D-tryptophan	21	−8.480	−9.602
6	6-Cl-D-tryptophan	906	−10.670	−10.722
7	cyclamate	26	−8.600	−9.340
8	saccharin	161	−9.660	−9.587
9	P4000	2293	−11.210	−10.782
11	hernandulcin	691	−10.510	−10.646
12		198	−9.780	−9.514
13	D-glucose	0.26	−5.920	−6.269
14	sucrose	1	−6.713	−6.715
15		0.95	−6.670	−6.500
19		14426	−12.280	−10.716
20		4527	−11.600	−10.829
22		16968	−12.370	−10.414
23	phyllodulcin	502	−10.320	−10.934
24		1728	−11.040	−11.605
25		32200	−12.750	−13.370
26	sucrononic acid	200000	−13.810	−13.235
28		128400	−13.550	−13.736
		Test Set		
10	perillartine	966	−10.710	−9.761
16	sucralose	755	−10.560	−10.811
17		2674	−11.300	−10.180
18		13012	−12.220	−9.612
21		401	−10.190	−10.510
27		156210	−13.660	−14.337

[a] Correlation coefficient for the training set (22 compounds) = 0.932, rms deviation = 0.760 kcal mol^{-1}; rms deviation for the test set (6 compounds) = 1.264 kcal mol^{-1}.

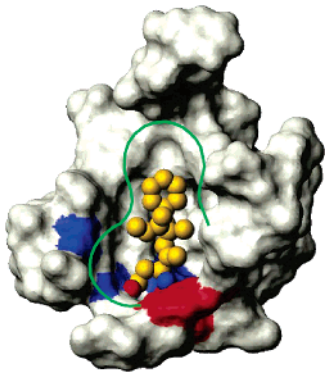

Figure 6. Fit of [(L-α-Me)Phe^2] aspartame in the active site of the T1R3(B) protomer of Aoc_AB. The residues lining the walls of the active site are represented as a contact area. The residues acting as the complementary AH unit in the receptor are colored red, and those acting as B are colored blue. The molecule of [(L-α-Me)Phe^2] aspartame is represented with atoms colored in gold except for the carboxylate and ammonium groups that are colored in red and blue, respectively. The green outline indicates the boundary of the old indirect model.[18] Models were generated using MOLMOL.[42]

Discussion

The Function of the Binding Sites. Homology models of the extracellular domain of the sweet receptor published to date are homodimers[2,34] or a heterodimer based on the *mouse* sequence.[25] The present paper presents an exhaustive modeling of the heterodimeric

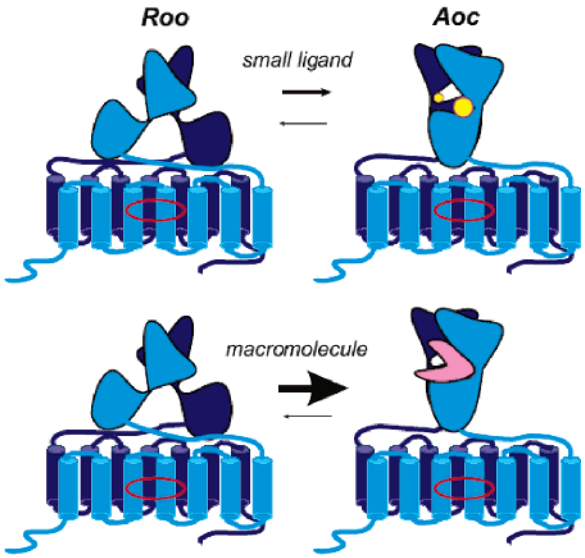

Figure 7. Scheme of the possible mechanisms of interaction of sweet molecules with the sweet taste receptor. (U) Binding of a small-molecular-weight ligand transforms inactive free form I (Roo) into the complexed form (Aoc). Two small ligands of unequal size are shown in the two cavities of Aoc as a large ball and a smaller ball (colored in yellow) in the open and closed protomers, respectively. (L) Free form II (Aoc), stabilized by protein complexation, activates long lasting signal transmission. The wedge protein is colored in pink. The further possibility of direct interaction in the 7TM domain by cyclamate and/or lactisole is highlighted in both panels with a red ellipse.

human sweet taste receptor based on the crystal structure of the extracellular domain of mGluR1 and docking experiments of both sweet proteins and small sweet ligands with the obtained models. Our results, together with the previously reported data, show that at least four binding sites could be present on such heterodimers: two sites for small sweet compounds (one in each VFTD), one *wedge site* for sweet proteins, and one site for allosteric modulators in the 7TMD (Figure 7).

We have previously suggested that sweet proteins can interact with the SR according to a mechanism, entirely different from that of small sweeteners, in which proteins bind to a large external cavity (the *wedge* model).[25] We exploited this mechanism to probe the different possible combinations of T1R2 and T1R3 on chains A and B, since the global shape of the SR models should be less influenced by sequence differences and by the unavoidable low resolution of homology modeling. By modeling each of the human protomer sequences on either the A chain or the B chain of the mGluR1 template, we obtained two models for the inactive open−open form, Roo_AB and Roo_BA, and two models for the complexed active closed−open form, Aoc_AB and Aoc_BA. Docking calculations with sweet proteins show convincingly that both Aoc_AB and Aoc_BA can interact with sweet proteins using sites with a very similar shape on the surface of the receptor, located mainly on the B chain, that is, either T1R2(B) or T1R3(B), whereas the corresponding resting models, Roo_AB and Roo_BA, cannot bind sweet proteins in a specific binding site. The small differences in the mode of binding are not sufficient to discriminate between the T1R2(A)_T1R3(B) and T1R2(B)_T1R3(A) models, but it is fair to say that the exhaustive modeling on the human sequences validates the wedge model, originally derived from only one of the possible combinations of protomers and based on the mouse sequences.

The docking of small sweeteners to the different possible combinations of T1R2 and T1R3 allows us to draw the conclusion that both T1R2 and T1R3 may be, in principle, able to bind sweet ligands in binding sites corresponding to those of mGluR1. Using a large set of sweeteners, chosen on the basis of our previous study on the completely artificial pseudoreceptor,[22] it was immediately clear that both type A sites were definitely too small to host the biggest sweeteners, but they were able to accommodate at least four compounds, namely, saccharin, alitame, aspartame, and 6-Cl-D-tryptophan. On the other hand, both T1R2(B) and T1R3(B) can host a very large number of small-molecular-weight sweeteners with a good correlation between experimental and calculated binding affinities. Also in this case, it is not possible to establish beyond a doubt whether the sweet taste receptor will more likely be T1R2(A)_T1R3(B) or T1R2(B)_T1R3(A), although T1R2(B) cannot host the large guanidine sweeteners and some other compounds successfully inserted in T1R3(B).

It is important to emphasize that homology models are necessarily static models that reflect only in part the dynamic situation of the receptor *in vivo*. Thus, it is not surprising that the type A sites, modeled after the closed protomer, can only host some of the smallest ligands. It is fair to hypothesize that these sweeteners can occupy both sites of the active closed−open forms, Aoc_AB and Aoc_BA, in a fashion very similar to that of glutamate in mGluR1. On the other hand, binding of larger sweeteners to the cavity of the open protomer would prevent the conformational transition of *that protomer* to a closed form, since these molecules will act as spacers between the subdomains LB1 and LB2 involved in the conformational transition (Figure 7). However, binding of larger sweeteners to the cavity of the open protomer cannot hinder an open−closed conformational transition of the *other protomer*, either with its active site occupied by a smaller sweetener or with an empty cavity. Such a mechanism would be very similar to the shift of the equilibrium between free form I and free form II caused by external binding of a protein in the *wedge model* (Figure 7). In fact, we can regard large sweeteners as potential wedges *inside* the cavity of the open protomer. Obviously, simultaneous binding of two large molecules at the two protomers would stabilize Roo conformations, thus inactivating the receptor.

Sweetness Synergy. Our observations provide, for the first time, a possible interpretation of the phenomenon of sweetness synergy, that has so far escaped an interpretation at the molecular level. The starting point is that at least three of the four compounds that, in our calculations, were able to bind to type A sites, aspartame, saccharin, and cyclamate, are known to be synergistic with other sweet compounds, suggesting that, although the binding in a single subunit is sufficient for receptor activation, the binding of a ligand in the second subunit increases the response.

To date,[9] it has been reported that the sweet taste receptor has separate sites for aspartame (on the T1R2

extracellular domain) and cyclamate (on the T1R3 7TM domain), but nothing was said about the synergistic effect of other compounds such as saccharin. Since a saccharin−cyclamate combination itself is synergistic, DuBois[35] advanced the hypothesis that, "if binding cooperativity is the mechanism mediating sweetness synergy, the sweet receptor must have at least three binding sites to explain the synergism of just three compounds" (aspartame, saccharin, and cyclamate). In favor of this hypothesis is the fact that in our modeling experiments it was possible to insert saccharin (together with a few other compounds) in the type A closed protomer; therefore, the three binding sites for small-molecular-weight sweeteners will be one on the T1R2 extracellular domain, one on the T1R3 extracellular domain, and one in the T1R3 7TM domain. A fourth binding site is represented by the external cavity that hosts proteins in the wedge model. Also, the reported synergy between sweet proteins and several sweet compounds is in accordance with our results, since sweet proteins interact with the sweet receptor according to the wedge model, not occupying any of the above-mentioned ligand binding sites for small-molecular-weight sweeteners.

Summary and Conclusions

The present work describes the homology modeling of all possible dimers formed by combinations of the human T1R2 and T1R3 sequences of the sweet receptor using the A and B chains of the ligand binding domain of the mGluR1 glutamate receptor. The resulting ligand binding sites for low-molecular-weight sweeteners were probed by docking a set of the most representative sweet compounds and calculating their free energies of binding. We conclude that only type B sites, either T1R2(B) or T1R3(B), can host a very large number of small-molecular-weight sweeteners with a good correlation between experimental and calculated binding affinities. Simultaneous binding to the A and B sites is not possible with two large sweeteners but is possible with a small molecule in site A and a large one in site B. In addition to these two sites on the VTFD, our models showed an external binding site that can host sweet proteins.

Our work accounts for many experimental observations on the taste and synergy of sweeteners, validating the derived models and active sites identified. Nevertheless, the existence of further binding sites or alternative activation mechanisms cannot be excluded. For instance, stevioside is reported to be synergistic with aspartame, cyclamate, acesulfame-K, and thaumatin, which in our model occupy all the four available binding sites; if this were true, another site for stevioside might be present in the sweet taste receptor. Last but not least, the semipredictive models of the sweet taste receptor presented here are more reliable than those obtained so far, since they are based on the amino acids present in the "true" binding sites, and their use could provide specific hints for the design of new sweeteners.

Methods

Homology Modeling and Protein Docking. Sequence alignments were first generated with ClustalW;[36] for some sequences, an interactive improvement of the alignment has been necessary to yield an acceptable model.

All the derived sweet taste receptor models were built using the facility of the SWISS MODEL tool of the EXPASY program in the oligomeric mode.[37−39]

All docking calculations of sweet proteins with the obtained sweet taste receptor models were performed by means of the program GRAMM in the low-resolution mode.[40,41] A major obstacle to the docking of protein structures obtained with modeling is significant errors in these structures. Unfortunately, most docking methodologies are sensitive to structural inaccuracies, leading, for instance, to unwanted conformational changes upon the formation of the complex. GRAMM allows docking at variable "resolutions", depending on the accuracy of the structural components to be docked. The low-resolution docking is fast and may tolerate structural inaccuracies on the order of 7 Å, which is a precision characteristic of many protein models. GRAMM is based on the following algorithm: the two molecules are projected onto a three-dimensional grid, the degree of surface overlap as a consequence of relative movements of the molecules in three dimensions is then calculated as a correlation function by means of a Fourier transformation, and finally, the relative orientations of the molecules in three dimensions are systematically scanned. The procedure is equivalent to a six-dimensional search but is much faster. The sweet proteins used are brazzein (PDB entry 1brz), MNEI (a single chain monellin, PDB entry 1fa3), and thaumatin (PDB entry 1thw).

To visualize the obtained models and locate the ligand binding site, we used MolMol[42] running on several computers and InsightII/Discover, 97.0 (Biosym Technologies, San Diego, CA), running on a Silicon Graphics IRIS 4D-35GT.

Docking of Small Molecules and Free Energy Calculations. Once the models of the protomers were obtained, each one was overlapped (through InsightII) to its template containing the ligand glutamate, superimposing the amino acid residues that directly interact with the α-amino acid moiety in mGluR1 and that are conserved in the considered protomer. Then, the template protein was removed, while the ligand glutamate was left: in this way, it has been possible to obtain the modeled protomer containing the ligand glutamate in a position corresponding to the binding site. To define the amino acids of the ligand binding site, a subset (through InsightII) was created, using the ligand glutamate as center and considering the amino acids at a distance up to 15 Å from the center as part of the subset. We also visually checked, using MolMol,[42] that all the amino acids surrounding the ligand were included.

To dock and evaluate semiquantitatively free energies of binding of the sweet ligands, the program PrGen 2.1[29] was used.

Three-dimensional molecular models of the ligands to be inserted in the active site were built on a Silicon Graphics IRIS 4D-35GT, using the program InsightII/Discover, 97.0 (Molecular Simulations Inc., San Diego, CA). The initial models were energy refined by molecular mechanics techniques with conjugate gradients until a maximum energy derivative value of 0.008 kcal mol^{-1} $Å^{-1}$ was obtained using the CVFF force field, one of the force fields currently available as part of the Discover program from within the InsightII software suite.[43] Conformational analysis was performed wherever necessary by molecular dynamics. For atomic partial charges of the ligand atoms, we used Mulliken charges calculated on the minimized structures using the MOPAC program with the MNDO Hamiltonian. Molar relative sweetness values (MRS) for the compounds and free energies of ligand binding were calculated as previously described.[22]

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