Cell. Mol. Life Sci. 63 (2006) 1876–1888
1420-682X/06/161876-13
DOI 10.1007/s00018-006-6077-8

Cellular and Molecular Life Sciences

Review

Natural sweet macromolecules: how sweet proteins work

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Received 11 February 2006; received after revision 31 March 2006; accepted 11 May 2006
Online First 4 July 2006

Abstract. A few proteins, discovered mainly in tropical fruits, have a distinct sweet taste. These proteins have played an important role towards a molecular understanding of the mechanisms of taste. Owing to the huge difference in size, between most sweeteners and sweet proteins, it was believed that they must interact with a different receptor from that of small molecular weight sweeteners. Recent modelling studies have shown that the single sweet taste receptor has multiple active sites and that the mechanism of interaction of sweet proteins is intrinsically different from that of small sweeteners. Small molecular weight sweeteners occupy small receptor cavities inside two subdomains of the receptor, whereas sweet proteins can interact with the sweet receptor according to a mechanism called the 'wedge model' in which they bind to a large external cavity. This review describes these mechanisms and outlines a history of sweet proteins.

Keywords. Sweeteners, taste receptor, modelling, sweet proteins, food.

Introduction

Although most foods contain several macromolecules, we do not normally think of macromolecules as food. Even less common, however, is to think of sweet macromolecules. Yet there are seven proteins that have a distinct sweet taste. These macromolecules have played an important role towards a molecular understanding of the mechanisms of taste. Unveiling the molecular bases of the sweet taste of proteins is important not only for our basic knowledge of biological systems, but also for its possible medical implications, since it can help in the design of new, safe sweeteners of natural origin.

We normally associate sweetness with sugars but sweetness is a taste imparted by molecules belonging to widely different chemical classes: sugars, amino acids, olefinic alcohols, nitroanilines, peptides, proteins and many other chemically distinct substances [1, 2]. Besides chemical diversity, we can divide the whole family of sweet compounds into small molecular weight molecules and high molecular weight compounds. Both synthetic [3] and natural macromolecules [4–6] have molecular weights and hence dimensions completely different from those of all other sweeteners. In addition, some of the sweet proteins have an enormous sweetening power and a marked persistence of the taste sensation. Monellin, the best characterised of these proteins is 100 000 times sweeter than sucrose on a molar basis [7].

Given the huge difference in size, when comparing small molecular weight and macromolecular sweet molecules, it is natural to wonder whether they interact with the same receptor. Soon after the discovery of the first taste receptors, it was indeed shown that small and large molecular weight sweet molecules interact with the same T1R2-T1R3 receptor [8]. However, it was still difficult to conceive a single mechanism by which low molecular weight sweeteners and sweet proteins could activate the same receptor.

Modelling studies now show clearly that the single sweet taste receptor has multiple active sites and that the mechanism of interaction of the sweet proteins is intrinsically different from that of small sweeteners [9]. In this review,

we give an overview of the field from the mode of action of small molecular weight sweeteners up to the unique mechanism of interaction of sweet proteins with the T1R2-T1R3 receptor.

Natural sweeteners

Small molecular weight sweeteners

The natural compound generally associated with sweet taste is sugar, a synonym of saccharose (D-glucopyranosyl–D-fructofuranose), *i.e*. the disaccharide containing one unit of glucose and one of fructose. It is also the most commonly used sugar for altering the flavour and other taste properties of food. The sugar we use everyday, also called table sugar, is commercially extracted from sugar cane or sugar beet. In addition to sucrose there are hundreds of other compounds generally referred to as sugars. They are water-soluble crystalline carbohydrates or sugar alcohols, including several monosaccharides, disaccharides, and a few trisaccharides, having a typical sweet taste. Two of the most important of these sugar substitutes are probably erythritol and fructose. Fructose is a monosaccharide found in many foods and one of the three most important sugars found in blood. Fructose is often recommended for people with diabetes but its benefits are tempered by concern that it may have an adverse effect on plasma lipids. Erythritol is a natural sugar alcohol, which has been approved for use in many countries. It is less sweet than table sugar, has good taste and is virtually non-caloric. Erythritol is generally free of side effects, but it is important to note that if one consumes large quantities of erythritol, it may cause a laxative effect.

Amino acids of S configuration, *i.e*. those that are used in Nature to build proteins, are for the major part tasteless or bitter, whereas glycine and several hydrophobic amino acids of R chirality are sweet [10].

Several glycosides extracted from plants are intensely sweet. The best known of them is stevioside, that is a component of *Eupatorium rebaudianum* [11]. It is 150–300 times sweeter than sucrose but it has been reported to be highly toxic [12]. Another glycoside found in nature is glycyrrhizin, the flavouring agent of licorice (*Glycyrrhiza glabra*), that is approximately 50 times as sweet as sucrose [13]. Owing to the nature of the aglicone, a triterpene with structural similarity to corticosteroids, this sweetener induces hypertension at high doses. A much sweeter similar glycoside (3000 times sweeter than glucose) is Osladin, extracted from *Polypodium vulgare* [13].

Other small molecular weight sweeteners of natural origin have been classified as semi-synthetic since the natural substance had to be slightly modified to taste sweet [14]. The essential oil from *Perilla frutescens* contains the terpene perillaldehyde that is not sweet but becomes 200 times sweeter than sucrose when changed into its syn-oxime, called perillartine [15]. A similar case is that of naringin dihydrochalcone and neohesperidine dihydrochalcone, derived from bitter flavanone glycosides of citrus fruits [16].

Among the semi-synthetic natural sweeteners Morris [14] includes also aspartame, L-aspartyl-phenylalanine methyl ester [17], that is the most successful among 'natural sweeteners' employed so far. Recently, a very interesting naturally occurring peptide, monatin, has been described and fully characterised [18].

However, none of the mentioned compounds nor many others discovered or synthesised in the last 100 years, when taken as sweetener, is free from drawbacks. Therefore, much hope has been attached to the most unusual class of natural sweet compounds, namely sweet proteins.

Sweet proteins

The best known and characterised among proteins with a very strong sweet taste are monellin, tahumatin and brazzein [19, 20]. In addition, there are two less sweet proteins, mabinlin [19] and hen egg white lysozyme [21], and two taste modifier proteins [19], miraculin and curculin, which taste sweet when combined with sour substances. Among these proteins, lysozyme is the only one whose function, besides sweetness, is well defined. Monellin and brazzein seem related to protease inhibitors but monellin lacks the loop necessary for recognition by the proteolytic enzyme and no activity as a protease inhibitor was found for brazzein so far.

Monellin. One of the first two proteins unambiguously identified for being endowed with intense sweet taste was monellin. It was discovered as the sweet principle of *Dioscoreophyllum cumminsii,* a plant taxonomically related to the sweet potato by Inglett and May [22]. Owing to the unexpected intensity of the sweet taste, these authors called the plant 'serendipity berries' [14]. Morris and Cagan [5] established that the sweet principle was a protein and named it monellin, after the Monell Chemical Senses Center where they worked. They reported a sweetness, relative to sucrose, of 3000 : 1 on a weight basis, corresponding to 90 000 : 1 on a molar basis. Hung et al. [7] later reported a figure of 100 000 : 1 on a molar basis. Bohak and Li [23] showed that monellin consists of two non-identical subunits of 42 and 50 amino acid residues, called A and B, respectively, that are not covalently linked but are held together by secondary forces. They also established that the sweetness of monellin is exhibited only by the undissociated whole molecule, whereas the individual subunits are not sweet.

The sequence of monellin displays no significant similarity to that of any of other sweet proteins and, when origi-

nally discovered, it was not immediately obvious whether it belonged to a known protein family. On the basis of a comparison of the solid state structure of monellin with those of chicken cystatin [24] and of human stefin B [25], Murzin [26] showed that, despite a low sequence similarity, monellin belongs to the cystatin superfamily. However, monellin and cystatins do not have the same sequence in the regions corresponding to the cystatin active site, therefore monellin is completely unable to function as a cysteine protease inhibitor.

Owing to the weak forces holding its two chains, monellin dissociates into two chains and loses sweetness when heated above 50 °C. Single-chain constructs, *i.e*. monellins in which the two chains are covalently linked, can retain all sweetening power but have greatly increased thermal stability, [27, 28]. On the basis of the crystal structure of monellin, Kim et al. [27] designed a single-chain monellin (dubbed SCM) in which the C-terminal residue of the B chain (B50E) is directly joined to the N-terminal residue of the A chain (A1R). SCM is as potently sweet as natural monellin, more stable to temperature or pH changes and renatures easily even after heating to 100 °C at low pH. A very similar behaviour was shown by MNEI, a single chain monellin obtained by linking B and A chains *via* a Gly-Phe dipeptide [28]. Native two-chain and single-chain forms have been thoroughly characterised by X-ray and NMR studies [29–32]. The solution structure of MNEI [32] (Fig. 1a) is quite similar to that of native monellin and of SCM [29, 30]; it can be described as an α-helix cradled into the concave side of a five-strand anti-parallel β-sheet.

Owing to the huge difference in size between sweet proteins and conventional sweet molecules, it seemed natural, to explain proteins' activity, to assume the existence on the surface of monellin of some kind of 'sweet finger', *i.e*. a protruding structural element hosting one or more glucophores similar to those of small sweeteners. This view was explicitly embedded in the Temussi's model of the active site of the receptor [33].

On the basis of ELISA tests, which showed cross-reactivity between antibodies raised against monellin with thaumatin [34, 35], the sequence TyrA13-AspA16 of native monellin (corresponding to residues Tyr57-Asp59 of thaumatin in the assays) was suggested as a potential sweet finger [36]. Preliminary structural work on the solution conformation of MNEI showed that the relative spatial arrangement in solution of the side chains of Tyr65 and Asp68 (corresponding to TyrA13-AspA16 of monellin) is consistent with the corresponding arrangement of the side chains of Phe and Asp in aspartame [28]. However, point mutations on synthetic monellin [37] showed that substantial changes of residues 13 and 16 of the A chain do not affect sweetness. Actually, TyrA13Gly and TyrA13Phe have a sweetening power slightly higher than wild-type monellin, whereas the AspA16Abu and AspA16D-Asp have activities nearly twice as high. In addition, extensive mutagenesis studies on SCM and monellin [38, 39] hinted at an area of interaction with the receptor much larger than that of a sweet finger. The residues whose mutation causes a decrease of sweetness of two or more orders of magnitude are Ile6, Asp7, Gly9 [39] and Arg39 [40], whereas mutations of Gln13, Lys36, Lys43, Arg72, Arg88 or deletion of Pro92-Pro96 cause a decrease of one order of magnitude [38, 39].

Thaumatin. In 1968, Inglett and May [22] reported that an intensely sweet substance was present in the jelly-like exterior covering of the seeds of a West African plant, *Thaumatococcus danielli*, but although they described it as of 'unique chemical and physical nature', the sweet principle was characterised only later by van der Wel and Loeve [41] as a mixture of two proteins called thaumatin I and II. According to van der Wel and Loeve [41], thaumatins (I and II) have an intense sweet taste, with a sweetening power about 1600 times higher than that of sucrose on a weight basis or 100 000 on a molar basis. It is practically the only sweet protein that has been actually used as a sweetener. In the 1970s Tate and Lyle began commercialising it under the trade name of Talin.

The primary structure of the sweet-tasting protein thaumatin was elucidated by Ivengar et al. [42]. The protein contains a single polypeptide chain of 207 residues. Comparing the amino acid sequence of thaumatin with that of the other sweet-tasting protein known at the time, monellin, they located five sets of identical tripeptides that might be part of a common antibody recombination site and possibly be involved in the interaction with the sweet-taste receptor. According to the SCOP classification [43], thaumatin I belongs to the osmotin, thaumatin-like superfamily. Thaumatin-like proteins are synthesised by plants in response to fungal infection. They are also reported to be endowed with endo-beta-1,3-glucanase activity and alpha-amylase inhibiting properties [44]. The 3-D structure of thaumatin, solved in the crystal state by X-ray studies [45], contains three domains. As shown in Figure 1b, the central part of the molecule is built as an 11-stranded beta-sandwich folded into two Greek key motifs. All beta-strands of the sandwich are antiparallel except the parallel N-terminal and C-terminal ones. The second domain is made of two beta-strands forming a beta-ribbon that folds back against the main sandwich to form a smaller sandwich-like structure. The third domain, a disulphide-rich region stretching away from the sandwich portion of the molecule, contains one alpha-helix and three short helical fragments.

Apart from the mentioned ELISA study that pointed to residues Tyr57-Asp59 of thaumatin as the tip of a possible sweet finger [36], the first systematic study on the struc-

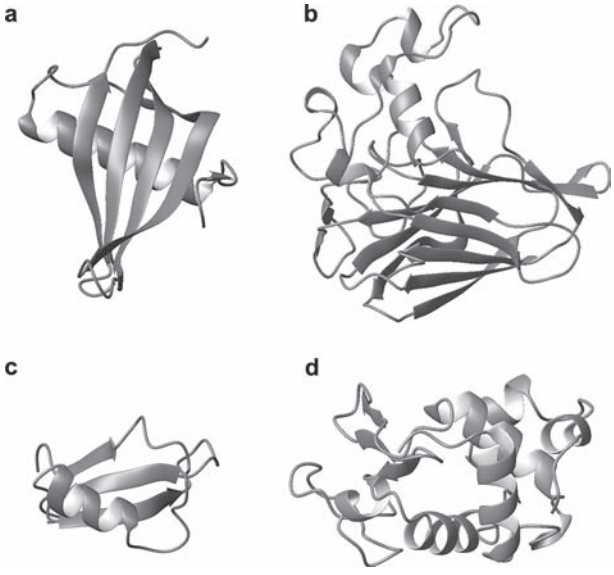

Figure 1. Ribbon representations of the structures of four sweet proteins. (*a*) Solution structure of MNEI [32]. The main elements of secondary structure are a five-strand anti-parallel β-sheet wrapped around an α-helix. (*b*) Crystal structure of thaumatin [45]. The structure can be described in terms of three domains: a central 11-stranded β-sandwich, a β ribbon containing 2 β strands and 1 α helix. (*c*) Solution structure of brazzein [48]. The main elements of secondary structure are a 3-stranded anti-parallel β-sheet and an α-helix. (*d*) Crystal structure of the tetragonal form of lysozyme solved at 1.3-Å resolution (pdb entry 193L). The structure of lysozyme is mainly helical but contains also short 3-stranded β-sheet. Models were generated with Molmol [92].

ture-activity relationship of thaumatin was reported by Mandal et al. [34]. Using a competitive inhibition binding immunoassay, these authors developed a library of monoclonal antibodies, which react with different surface antigenic epitopes on thaumatin. Some of these monoclonal antibodies also cross-react with monellin. A similar study by Sloostra et al. [46] identified two major overlapping conformational epitopes. This region contains an aspartame-like site, which is formed by Asp21 and Phe80, tips of the two extruding loops KGDAALDAGGR19-29 and CKRFGRPP77-84, which are spatially positioned next to each other. Since the aspartame-like Asp21-Phe80 site is not present in non-sweet thaumatin-like proteins, they suggested that the KGDAALDAGGR19-29- and CKRFGRPP77-84 loop contain important sweet-taste determinants.

More recently, Kaneko and Kitabatake [47] examined in detail the role of lysines in the structure-sweetness relationship of thaumatin. They found that phosphopyridoxylation of Lys78, Lys97, Lys106, Lys137or Lys187 reduced sweetness significantly. Combination of these results with those ensuing from modifications of other charged residues led them to suggest that there is a cleft-containing side of the protein that is important for sweetness.

Brazzein. The last of the intensely sweet proteins found in tropical plants was discovered by Ming and Hellekant [20] in *Pentadiplandra brazzeana* B. Brazzein is a single-chain polypeptide of 54 amino acid residues; it is 2000 times sweeter than sucrose in comparison to 2% sucrose aqueous solution, and 500 times in comparison to 10% of the sugar. In addition, according to Ming and Hellekant [20], its taste is more similar to sucrose than that of thaumatin.

Figure 1c shows that the 3-D structure, stabilised by four disulphide bonds, contains one alpha-helix and three strands of antiparallel beta-sheet [48]. It does not resemble either that of monellin or of thaumatin. Instead, it resembles those of plant gamma-thionins defensins and arthropod toxins. The Structural Classification Of Proteins (SCOP) [43] assigns brazzein to the Scorpion toxin-like superfamily. No function of brazzein has been reported, but the structural similarity with plant defensins hints at a possible role as a serine protease inhibitor.

All studies on the structure-activity relationship of brazzein were performed by the same group that elucidated the 3-D structure. Assadi-Porter et al. [49] looked for sweetness determinant sites of brazzein by introducing multiple mutations at several positions. The mutations that dramatically changed the sweetness of brazzein are localised within two regions ($Asp^{29}LysHisAlaArg^{33}$ and$Tyr^{39}AspGluLysArg^{43}$), suggesting that these are the critical regions of the molecule for eliciting sweetness. In the 3-D structure of brazzein, the region containing Arg33 is close to residues Tyr54 and Tyr51, thus in close contact with the C terminus. Overall, their data indicate that the C terminus is a necessary component for sweetness in brazzein.

These data were refined by Jin et al. [50] who investigated more mutations. Four mutants, Asp29Ala, Asp29Lys, Asp29Asn, andGlu41Lys, were found to be significantly sweeter than wild-type brazzein. Three mutants, Ala2ins, Asp2Asn, and Gln17Ala, were found to be as sweet as wild-type brazzein. In eight other mutants the sweetness decreased significantly but they were not tasteless, whereas in ten mutants the sweetness did not differ significantly from that of water.

Mabinlin. In 1983 Hu and Min [51] isolated two sweet proteins from the seed of *Capparis masaikai* Levl. (local name mabinlang), a plant that grows in the subtropical region of the Yunnan province of China and named them mabinlin I and II. The sweetness of mabinlin II with respect to sucrose was estimated as 375:1 on a molar basis and it was unchanged by at least 48-h incubation at nearly boiling temperature [52]. Accordingly, although it is much sweeter than sucrose, it is not as intensely sweet as monellin, thaumatin and brazzein. Liu et al. [52] purified mabinlin II by ammonium sulphate fractionation, ion-exchange chromatography and gel filtration. Puri-

fied mabinlin II thus obtained gave a single band having a molecular mass of 14 kDa on SDS-PAGE. Two peptides (A chain and B chain) were separated from reduced and S-carboxamidomethylated mabinlin II by high performance liquid chromatography (HPLC). The amino acid sequences of the A and B chains consist of 33 and 72 amino acids, respectively. The A chain is mostly composed of hydrophilic amino acid residues, and the B chain also contains many hydrophilic residues. High similarity was found between the amino acid sequences of mabinlin II and 2S seed storage proteins, especially 2S albumin AT2S3 in *Arabidopsis thaliana* (mouse-ear cress).

Lysozyme. Lysozyme is a relatively small enzyme that catalyses the hydrolysis of polysaccharides comprising the cell walls of bacteria. It is exceptionally abundant in egg whites. The effect of lysozyme is similar to that of penicillin, which also weakens the cell walls of bacteria, only by irreversibly inhibiting an enzyme required for cross-linking macromolecules formed in the biosynthesis of the cell wall.

Lysozyme is also one of the best characterised proteins from a structural point of view, both in solution and in the crystal state. It has been defined 'a model enzyme in protein crystallography' [53]. The refined high-resolution structure solved by NMR techniques has been recently published by Schwalbe et al. [54]. Figure 1d shows a ribbon representation of the tetragonal form of lysozyme solved at 1.3-Å resolution (pdb entry 193L). It is interesting to note, with respect to the other structures of Figure 1 that are mainly beta, that it is prevalently alpha helical.

Its inclusion among sweet proteins is quite recent. In 1988, Maehashi and Udaka [21] claimed that hen egg white (HEW) lysozyme has a distinct sweet taste, whereas lysozymes from other sources such as turkey and soft-shelled turtle also showed sweetness but with different tastes, heavy or light. In contrast, human lysozyme is tasteless. The amino acid sequences of the various lysozymes are similar to that of HEW lysozyme, but no lysozyme sequence shows significant homology to other sweet proteins (see below). The sweetening power of HEW lysozyme corresponds to a threshold value of around 7 μM [55], a value that is far from the nanomolar range of the three main sweet proteins but is higher than that of sucrose. Masuda and coworkers have recently extensively studied the structure-activity relationship of lysozyme [55–57].

The main results can be summarised as follows. Alanine substitution of lysine residues showed that out of six lysine residues, only two, Lys13 and Lys96, are required for lysozyme sweetness, while the remaining four lysine residues do not significantly affect the sweetness. Similarly, single alanine substitutions of arginine residues showed that three arginine residues, Arg14, Arg21, and Arg73, play significant roles in lysozyme sweetness; mutation of Arg45, Arg68, Arg125 did not affect sweetness [55].

Miraculin. The fruits of *Synepalum dulcificum* have been known for more than a century to cause sour substances to taste sweet. This very unusual property earned the berries the name of miracle fruit [14]. It was only in 1988 that Theerasilp and Kurihara [58] isolated miraculin from alkaline extracts of the miracle fruit. Miraculin was purified from the extracted solution by ammonium sulphate fractionation, ion-exchange chromatography, and affinity chromatography. The purified miraculin thus obtained gave a single sharp peak in reverse-phase HPLC, with a single band with a molecular weight 28,000 in SDS-PAGE.

The complete amino acid sequence of miraculin was determined soon after by Theerasilp et al. [59]. Miraculin is a single polypeptide with 191 amino acid residues. The calculated molecular weight based on the amino acid sequence and the carbohydrate content (13.9%) was 24,600. High homology was found between the amino acid sequences of miraculin and soybean trypsin inhibitor. The primary structure of miraculin was completed with the determination of the location of disulphide bridges [60], but no tertiary structure is yet available.

It has been claimed that miraculin can have a maximum value of sweetness 400 000 times that of sucrose [6]. However, it is difficult to compare this figure to those of the sweet-tasting proteins since the mechanism of action of miraculin apparently requires preventive (non-active) occupancy of the receptor, and it becomes sweet only after acidification [61].

Curculin. In 1990, a new taste-modifying protein named curculin was extracted from the fruits of *Curculigo latifolia* and purified by ammonium sulphate fractionation, ion-exchange chromatography, and gel filtration [62]. Purified curculin thus obtained gave a single band of 12 000 on SDS-PAGE. The molecular weight, determined by low-angle laser light scattering (27 800) suggested that native curculin is a dimer of a 12 000-Da polypeptide. Curculin consists of 114 residues. Curculin itself elicits a sweet taste, albeit not very strong (equivalent to the sweetness of 0.35 M sucrose). After curculin, water elicits a sweet taste, and sour substances induce a stronger sense of sweetness. No protein with both sweet-tasting and taste-modifying activities had previously been found.

Recently, Suzuki et al. [63] isolated a gene that encodes a novel protein that is highly homologous to curculin. Using cDNAs of the previously known curculin (designated as curculin1) and the novel curculin isoform (curculin2), they produced a panel of homodimeric and heterodimeric recombinant curculins by *Escherichia coli* expression systems. It was revealed that sweet-tasting and taste-modifying activities were exhibited solely by the heterodimer of curculin1 and curculin2.

Early models of the active site of the sweet taste receptor

To put the structure-activity relationship of sweet proteins in the right perspective, it is necessary to review what is known about the structure-activity relationship of small molecular weight sweeteners.

Early attempts to interpret structure-activity relationships of sweet molecules were based on the search for specific functional groups of the molecules that could impart sweetness. These studies tried to attribute this feature to chemical groups such as OH, carboxyl or amino moieties, called 'glucophores', but it was soon clear that the same groups could be found in sweet and tasteless molecules with similar frequency [1]. The interest was redirected towards the development of more general models of the receptor active site derived from the shape of conformationally rigid sweet molecules, used as molecular moulds [2, 33, 64–69].

The first model suggested the importance of a hydrogen bond donor (AH) and a hydrogen bond acceptor (B) spaced at 3–4 Å that would interact with complementary sites of the receptor [2]. This linear model was developed into a triangular geometry by Kier [64], who introduced a third group, a 'dispersion point', at a precise distance from the AB pair. Both models were oversimplifications since, in the absence of any stereochemical information, they could not allow selection between sweet and tasteless isomers. To take into account the chemical nature of the compounds, Temussi and coworkers suggested a more detailed model based on an accurate superposition of rigid sweet compounds [33, 65–67]. This is often referred to as the 'Temussi model' [70, 71]. Contemporaneously, Tinti and Nofre discovered a new class of very potent sweeteners containing a guanidinium ion [72, 73] and accordingly proposed a 3-D model for an ideal sweetener that besides the AH-B entity has six additional interaction points connected by a complex network of distances. With respect to the previous ones, this model has the obvious advantage of being consistent with the most powerful known sweeteners, but it has little generality since it specifically tailors the architecture of only the guanidinium compounds. Recently, a unifying model has been proposed, based on an artificial active site, which is able to explain and predict semiquantitatively the sweet taste of compounds belonging to different families [74]. However, none of the existing models could explain the enormous increase in activity in going from small molecular weight compounds to proteins.

The discovery of the sweet receptor

While indirect studies of active sites had to imply the existence of sweet taste receptors, in the last few years these receptors have actually been identified, expressed and characterised. The sweet taste receptor is a class C G protein-coupled receptor (GPCR) similar to glutamate receptors, the Ca^{2+} sensing receptor, the γ-aminobutyric acid type B receptor and pheromone receptors [75]. These receptors, as all GPCRs, have a seven-helix transmembrane domain (7TM) but also a large extracellular domain containing active sites for typical ligands, and a cysteine-rich domain. A distinctive feature of the sweet taste receptor, with respect to other class C GPCRs is that it is a heterodimer. Several groups [76–82] hypothesised that T1Rs (particularly T1R3) were likely candidates for the sweet receptor and, in analogy with other class C GPCRs, assumed that they would form homodimers. However, it was only a few months later that Li et al. [8] demonstrated that only the heterodimer T1R2-T1R3 can function as a sweet receptor.

The sequence of T1R3 is 20% identical to that of mGluRs, the only class C GPCR whose structure, at the time of the discovery of the sweet receptor, was at least partially known. The knowledge of the structure of the N-terminal domain of mGluR1 [83] prompted the building of the first homology model of a sweet receptor that, similarly to mGluR1, was a homodimer of two T1R3 chains [79]. Soon afterwards, a similar model showed that the active site of T1R3 could consistently host three very sweet small molecular weight molecules [84].

The demonstration that the heterodimer T1R2-T1R3 is the authentic sweet receptor [8] opened the way to more realistic homology models. Even if the only possible template is the structure of mGluR1, it is appropriate to refer to 'models' rather than to a single model since Kunishima et al. [83] have shown that the extracellular N-terminal domain of mGluR1 exists in two different conformations: an active open-closed one, corresponding to the crystal forms complexed with the ligand (1ewk.pdb) and to the uncomplexed free form II (1ewv.pdb), and another inactive open-open free form I (1ewt.pdb). Combining the two sequences of the sweet receptor (T1R2 and T1R3) with the two conformations of mGluR1 leads to four possible heterodimers. The first T1R2-T1R3 model based on the mouse sequences and on the complexed form of the mGluR1 template is one of these four models [85].

The four T1R2-T1R3 heterodimers are two models for the open-open form, and two models for the complexed closed-open form. All these models were examined systematically by Morini et al. [9] and used to identify all possible sites of interaction. The model with T1R2 on chain A and T1R3 on chain B of the 1ewt template was called Roo_AB, that with T1R3 on chain A and T1R2 on chain B of the 1ewt template Roo_BA. The model with T1R2 on chain A and T1R3 on chain B of 1ewk, was called Aoc_AB and that with T1R3 on chain A and T1R2 on chain B of 1ewk is Aoc_BA.

Active sites for small sweeteners

The sweet receptor can be activated by simple hydrophobic D-amino acids and synthetic dipeptides, e.g. aspartame. All of these molecules, like glutamate, have the same amino acidic moiety composed of an amino group adjacent to a carboxyl group. Morini et al. [9] hypothesised that the active sites of the T1R2-T1R3 receptor should retain all the features necessary to bind this moiety. In other words, the polar residues lining the wall of part of the cavity that binds amino acidic moieties should be highly conserved in going from mGluR1 to T1R2-T1R3. It turns out that the residues directly interacting with the α-amino acid moiety in mGluR1 are indeed well conserved. In contrast, the residues of the other part of the cavity, i.e. those binding the side chain of glutamate in mGluR1, are expected to possibly turn from polar to apolar in T1R2-T1R3 since in the sweet taste receptor they ought to accommodate on average rather hydrophobic molecular fragments. In the alignments corresponding to the four models, Morini et al. [9] found that the residues binding the amino acidic moiety of glutamate are well conserved in all protomers, while residues binding the glutamate side chain in mGluR1 are changed to less polar or uncharged residues.

In the active (open-closed) form of mGluR1 both active sites host a glutamate molecule, but owing to the diversity in chemical constitution and size of sweeteners, we cannot be sure whether in the sweet taste receptor both ligand-binding sites are available for sweet ligands in the active forms Aoc_AB, and Aoc_BA. According to Morini et al. [9], who examined the binding of a large number of sweeteners in each cavity of these models, the active sites of closed protomers, T1R2(A) and T1R3(A), are so small that they cannot possibly host most of the large synthetic sweeteners. In mGluR1 both closed (MOL1) and open (MOL2) protomers bind glutamate at active sites delimited by the interfaces of subdomains LB1 and LB2 [83, 86], with the only difference that, in the open protomer, the LB2 interface is not used for binding. On the contrary, owing to the large dimensions of some sweeteners, the active sites of open protomers in Aoc_AB and Aoc_BA can use both LB1 and LB2 interfaces. The sweeteners chosen to probe the binding by docking are representative of different families including sugars, peptides and supersweeteners. In the binding sites of the open protomers, it was possible to fit a large number of representative sweet compounds. In contrast, it proved very difficult to dock many of the larger ligands in the binding sites of closed protomers of the active closed-open form.

The models described by Temussi and coworkers [9, 85, 87] clarify the role of the two protomers of the sweet receptor. Since T1R3 is common to both sweet and umami receptors [8], it seemed natural to attribute specificity and even the main role in activation to T1R2 and T1R1 protomers of the two receptors, respectively. The wedge hypothesis for proteins (see below) had already shown that T1R3 plays the major role in the external binding site for proteins [85]. Subsequently, the exhaustive modelling of Morini et al. [9] demonstrated that both protomers can host non-protein ligands in the active state of the sweet receptor. Recently, this view received experimental support by the finding that T1R2 and T1R3 give distinct contributions to the detection of sweet stimuli [88]. This finding is particularly remarkable since the authors seem unaware of the modelling work performed by Temussi and coworkers [9, 85, 87].

The glucophores of proteins

The early models of active sites based on the shape of small sweeteners could still have been compatible with the interaction of proteins provided the surface of the proteins hosted protruding features that can probe the active site, i.e. 'sweet fingers' chemically similar to small sweeteners. Therefore, many of the early studies on sweet proteins were devoted to the search of possible sweet fingers on proteins.

When trying to understand the origin of a common function in a family of proteins, it is customary to look for corresponding parts of members of the family by comparing their sequences and/or their 3-D structures. No sequence homology could be detected among monellin, thaumatin, brazzein, mabinlin, miraculin and curculin. A pairwise alignment of these sequences performed by Clustal X [89] showed that the percentages of identical residues, between monellin and the other proteins comprised 23% between monellin and miraculin and a bare 7% between monellin and curculin [90]. If we repeat the sequence alignment including also HEW lysozyme, the result, shown in Figure 2a, is a complete mis-alignment: only 3 sites bear some homology. Even if the alignment is restricted to the three most sweet proteins (Fig. 2b), there are only 2 sites with identity and about 15 sites with homology.

There is also little similarity among the available 3-D structures. In a 3-D search of each of the structures of monellin, thaumatin and brazzein against the whole data base by means of DALI [91], the other two proteins are not even found. The only common elements among the 3-D structures of brazzein, monellin and thaumatin (Fig. 1) are small tracts of secondary structure, *i.e.* β-sheet hairpins, which could correspond to sweet fingers since they host residues with glucophores already identified in small sweeteners. In all three hairpins, there are residues that can form hydrogen bonds with a complementary AH-B entity of the receptor and another with apolar side chain at a precise spatial location with respect to the hydrogen bonding groups [33]. Following this lead, Tancredi et al.

[90] synthesised three cyclic peptides corresponding to these loops and stabilised by S-S bonds. 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 sweet taste [90].
Failure to produce small peptides corresponding to possible sweet fingers speaks against this possibility as a likely mechanism for sweet proteins. Another evidence against the sweet fingers hypothesis is the fact that mutants affecting sweetness of monellin are distributed over a large area, rather than being concentrated on a long protruding structural entity [38]. The same is true also for brazzein [50]. The only alternative mechanism proposed so far to interpret the interaction of sweet proteins with the sweet receptor is the so-called 'wedge model' [85].

Active site for proteins: The wedge model

Given that the sequences of the chains of the T1R2-T1R3 receptor and that of mGluR1 are sufficiently similar to allow model building, it seems fair to assume that the sweet receptor has also the same general features of mGluR1. Kunishima et al. [83] showed that the extracellular N-terminal domain of mGluR1 exists in three different crystal forms, one complexed with the ligand (1ewk.pdb) and two, free form I (1ewt.pdb) and free form II (1ewv.pdb), without ligand. Free form I, an 'inactive' conformation rather different from the conformation of the complex, is in equilibrium with free form II whose conformation is nearly identical to that of the 'active', complexed form. If the T1R2-T1R3 receptor behaves like mGluR1, it should also exist as a mixture of three forms: a complexed form containing a low molecular weight sweetener (corresponding to a molecule of glutamate), free form I, the 'inactive' conformation and free form II, whose structure is nearly identical to that of the 'active', complexed form.
As shown by the cartoon of Figure 3a, the equilibrium between form I and form II can be shifted in favour of the active form by the binding of a small molecular weight sweetener that transforms free form I into the complexed form, structurally identical to free form II. However, the equilibrium between form I and form II can also be shifted if we can stabilise form II it in another way. Figure 3b illustrates how stabilization can be achieved by external

a

```
mnei        ---------------------------------GEWEIIDI-G-----------PFTQNLG-KFAVD--------EENKI          26
brazzein    ------------------------------------------------------------QD--------KCKKV           7
thaumatin   ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWARTDCYFDDSGSGICKTGDCGGLLRCKRF        80
mabinlin    ------EPLCRRQFQQHQHLRACQRYIRRRAQRGGLVDE------------------QRGPALRLCCN-------QLRQV        49
lysozyme    ---KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFN-----------TQATNRNTDGSTDYG-------ILQIN        59
curculin    -----MAAKFLLTILVTFAAVASLGMADNVLLSGQTLHADH---------------SLQAGAYTLTIQN------KCNLV        54
miraculin   --MKELTMLSLSFFFVSALLAAAANPLLSAADSAPNPVLDIDGEKLRTGTNYYIVPVLRDHGGGLTVS-------ATTPN        71

                                                :
mnei        G---------------QYGR-LT-------FN------KVIR-----------PC--MKKTIY------ENEG--F-R          53
brazzein    -----------------YEN----------YP-------VSK-------------CQLANQCNY---------------          24
thaumatin   GR---PPTTLAEFSLNQYGKDYIDISNIKGFNVPMNFSPTTR-----G-------CRGVRCAADIVG---QCPAKLK-A        140
mabinlin    N-----------------KP----------CVC-----PVLR--------------QAAHQQLYQG----QIE------          72
lysozyme    ------------------SR----------WWCNDGRTPGSR----------N--LCNIPCSALLS----SDIT------          89
curculin    K----------------YQNGRQ------IWAS-----NTDRRGS----------GCRLTLLSDGNLVIYDHNNNDVWGS          97
miraculin   GTFVCPPRVVQTRKEVDHDRPLA-------FFPENPKEDVVRVSTDLNINFSAFMPCRWTSSTVWRLDKYDESTGQYF-V        143

          :                .
mnei        EIKGYE-----------YQLYVYASDKLFR---------------ADISEDYK---TRGRKLLRFNG-PVPPP-------          96
brazzein    ---DCK-----------LDKHARSGECFY-----------------D----------EKRNLQCICD-YCEY-----------          54
thaumatin   PGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKR--------LCPDAFSYVL---DKPTTVTCPGSSNYRVTFCPTA-----        207
mabinlin    --GPRQ-----------VRQLFRAARNLP----------------------------NICKIPAVGR--CQFTRW---          104
lysozyme    --ASVN----------CAKKIVSDGNGMN-----------------AWVAWRN--RCKGTDVQAWIR-GCRL--------        129
curculin    ACWGDNGKYALVLQKD--GRFVIYGPVLWS-------------LGPNGCRRVNGGITVAKDSTEPQHEDIKMVINN---        158
miraculin   TIGGVKGNPGPETISSWFKIEEFCGSGFYKLVFCPTVCGSCKVKCGDVGIYID---QKGRRRLALSDKPFAFEFNKTVYF        220
```

b

```
                                                                                  * :
mnei        -----------GEWEIIDIGPFTQNLGKFAVDEEN------KIGQYGRLTFNK---VIRP---------------CMKK          26
brazzein    ------------------------------------------------------------QD--------------KCKV           7
thaumatin   ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWARTDCYFDDSGSGICKTGDCGGLLRCKRF        80

                          * ::                                  :   ::       :    :    .
mnei        ----------T--IY-ENE-----------------------------GFR---EIKG----YEYQLYVYASDK--LF          53
brazzein    -------------Y-ENY----------------P-------------VSKC-QLAN----QCNYDCKLDKHAR----          24
thaumatin   GRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMNFSPTTRGCRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCT        140

             ..       :             .   .:
mnei        RADIS----EDYK-----------TRGRKLLR------FNGPVPPP            96
brazzein    SGEC-------FY-----------DEKRNLQC------ICDYCEY-            54
thaumatin   TGKCGPTEYSRFFKRLCPDAFSYVLDKPTTVTCPGSSNYRVTFCPTA           207
```

Figure 2. Sequence alignments of sweet proteins. (*a*) Alignment of monellin, thaumatin, brazzein, mabinlin, miraculin, curculin and HEW lysozyme. (*b*) Alignment of monellin, thaumatin and brazzein.

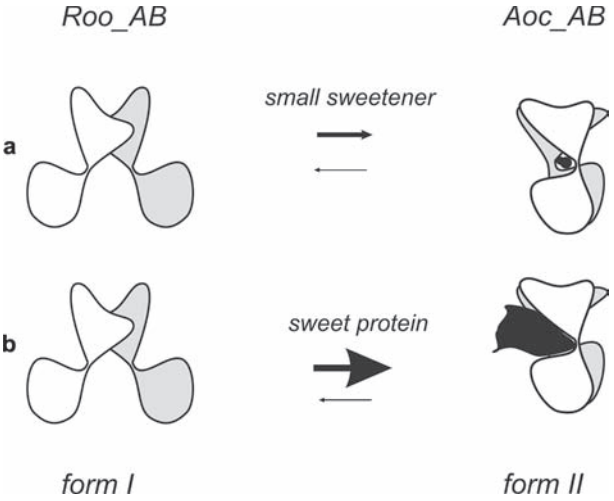

Figure 3. Modes of binding of small sweeteners and sweet proteins. (*a*) Binding of small molecular weight ligands transforms inactive free form I (Roo) into the complexed form (Aoc), identical to free form II. Small ligands in the two cavities of Aoc are shown as black balls. (*b*) Free form II, stabilised by protein complexation, activates long lasting signal transmission. The 'wedge' protein is shown in black.

binding of a macromolecule on an external large cavity of the surface of the receptor, belonging mainly to the open protomer. This cavity constitutes a secondary binding site, distinct from the two active sites that host small sweeteners. The mechanism of interaction of sweet proteins, termed the 'wedge model' was originally proposed on the basis of docking calculations of brazzein, monellin and tahumatin to a homology model of the receptor built using the mouse sequences of T1R2 and T1R3 for the closed (chain A) and open (chain B) conformations of mGluR1, respectively [85]. Recently, the mechanism has been confirmed by exhaustive modelling based on the human sequences [9]. All three sweet proteins fit a large cavity of the human receptor with wedge-shaped surfaces of their structures. To test whether this cavity is the only possible binding site for sweet proteins, Morini et al. [9] first performed docking calculations on the surface the active Aoc_AB and/or Aoc_BA receptor and then, as a negative check, repeated the docking on Roo_AB and/or Roo_BA models, corresponding to free form I, in equilibrium with free form II.

The results for all three proteins are consistent with those described previously for the mouse receptor. All protein models are centred on a large cavity of the concave side of the open chain of the dimeric receptor. Figure 4 shows the interaction of brazzein and thaumatin with one of the two possible activated forms of the receptor (the corresponding figure for MNEI can be seen in [9]). Figure 4a shows the human Aoc_AB form together with 15 of the molecules of brazzein calculated by the docking procedure. All 15 molecules are found in the same spot of 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 view of the complex of the A panel, obtained via rotation of the central model around the Y axis (right), shows that the remaining parts of the surface of the Aoc_AB model do not bind any MNEI molecules. Figure 4b shows the corresponding complex of human Aoc_AB form together with 10 of the molecules of thaumatin calculated by the docking procedure. The molecules of the three sweet proteins with Roo_AB and/or Roo_BA models bind to a very large area of the receptor, without any apparent regularity.

We have previously seen that sweet proteins have almost no sequence similarity and very little structural similarity, yet they interact with the receptor with the same mechanism. So, what do they have in common?

They have a similar wedge shape on the part of the surface that interacts with the external cavity of the receptor, but most of all they have similar electrostatic properties in the interacting surface, which complements the electrostatic potential of the active site.

Figure 5 shows the comparison of the internal (in contact with the receptor) and external (exposed to solvent) surface of MNEI and brazzein. This comparison is particularly significant since it is a more acidic protein than monellin: the isoelectric points of MNEI and brazzein are 9.0 and 6.7, respectively. From inspection of Figure 5, it

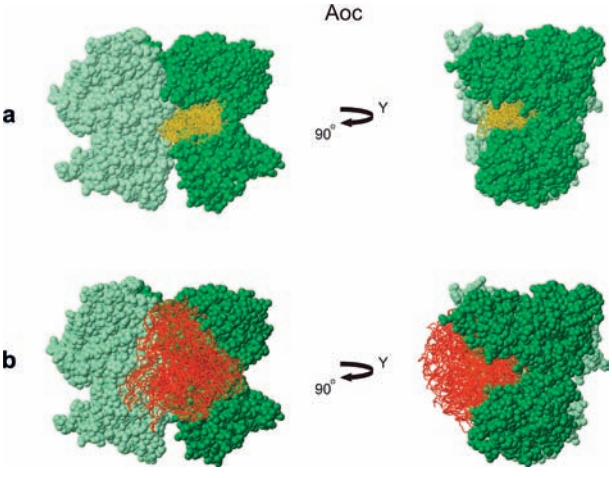

Figure 4. Comparison between the docking of brazzein and thaumatin on Aoc_AB, the active form of the receptor. The protomers are shown as full atom representations. (*a*) Two views of Aoc_AB together with 15 of the molecules of brazzein calculated by the docking procedure. The T1R2 is coloured in pale green, T1R3 is coloured in dark green. The 15 representative brazzein molecules are shown in gold neon representation. (*b*) Two views of Aoc_AB together with 10 of the molecules of thaumatin calculated by the docking procedure. The T1R2 is coloured in pale green, T1R3 is coloured in dark green. The 10 representative thaumatin molecules are shown in red neon representation. Models were generated with Molmol [92].

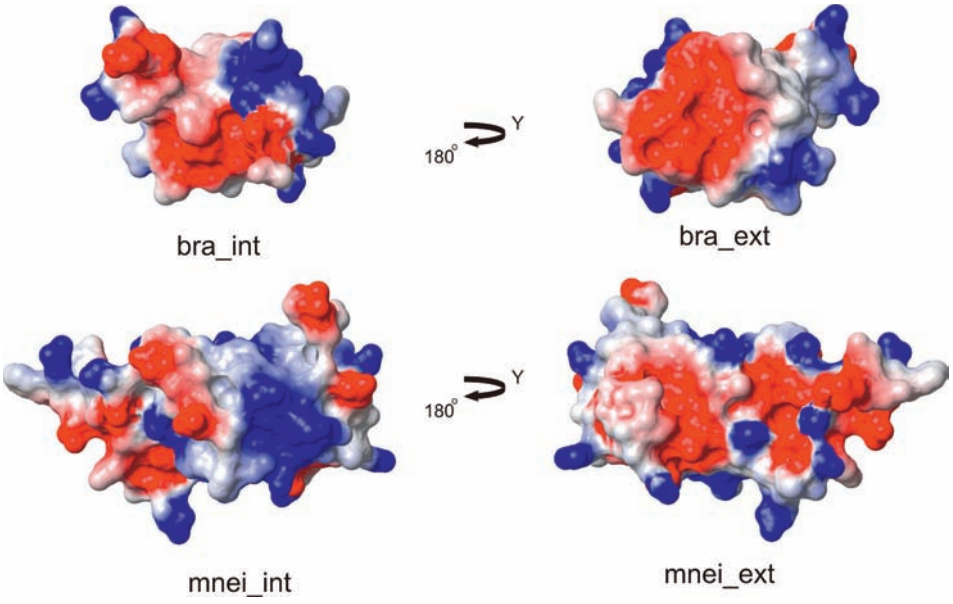

Figure 5. Comparison of the electrostatic potentials of the internal surfaces (in contact with the receptor) and external surfaces (exposed to solvent) of MNEI and brazzein. Models were generated with Molmol [92].

appears that much of the negative electrostatic potential of brazzein is concentrated on the external surface.

How many binding sites?

Homology modelling and docking show convincingly that there are two active sites for small sweet molecules and one site for sweet proteins [9]. Owing to the complexity and variety of sweet compounds, it was natural to suspect the existence of additional binding sites. Indeed, during the last few years, a large number of elegant molecular biology experiments, particularly those using receptor chimeras, have contributed to the interpretation of the interactions of taste receptors with different ligands, giving strong support to the concept of multiple sites. The two major candidates for additional sites are one for cyclamate and lactisole, a sweet taste inhibitor [93–96] and another for sweet proteins [97].

The idea of producing chimeras between different receptors to clarify the role of T1R protomers was originally suggested by Zhao et al. [98]. By a detailed characterization of the behavioural and physiological features of T1R1, T1R2, and T1R3 knockout mice, these authors first demonstrated that sweet and umami tastes are exclusively mediated by T1R receptors and that removal of individual T1R subunits selectively affects these two tastes. In turn, the identification of cells and receptors for sweet and umami tastes allowed them to design a strategy to separate the role of receptor activation from cell stimulation in encoding taste responses. Mice engineered to express a modified κ-opioid receptor in T1R2-3-expressing cells became specifically attracted to a κ-opioid agonist, thus proving that activation of sweet-receptor-expressing cells is the key determinant of behavioural attraction to sweet tastants [98].

Although the need for coexpression had already hinted at a heterodimer [8], the direct evidence that T1R2 and T1R3 function as a heteromeric complex came from a study that exploited differences in sweet taste between human and rat, particularly in terms of ligand specificity and sensitivity to inhibitors [93].

Following the lead of Zhao et al. [98], Xu et al. [93] used chimeras between human and rat T1Rs genes (with junctions at residues just before estimated TM domains) to map binding sites on the T1R2-T1R3 receptor. When the N-terminal domain of T1R2 of the human receptor was replaced with the corresponding rat sequence, the responses to aspartame and neotame were abolished, showing that the N-terminal domain of human T1R2 is necessary to recognise aspartame and neotame, and thus gives to the heterodimeric complex the necessary specificity. However, to their surprise, they also found that the C-terminal TM domain of T1R3 is required to recognise cyclamate, a well-known sweetener, and the sweet taste inhibitor lactisole [93]. When they replaced either the N-terminal or the C-terminal half of human T1R2 with the corresponding rat sequence, the response to cyclamate was not affected, but the TM domain of human T1R3 is sufficient, when coexpressed with T1R2, to recognise cyclamate. Similar to that seen for cyclamate, lactisole, a human-specific sweet taste inhibitor requires the human T1R3 C-terminal domain to inhibit the receptor's response to typical sweet agonists.

Using similar methods, Jiang et al. [94] confirmed these observations but also, most importantly, determined the

molecular basis for sensitivity to cyclamate and lactisole by identifying a few residues within the TM region of human T1R3 specifically involved in recognition of these two molecules [94, 95].

Initially, from studies with mouse/human chimeras, these authors determined that the molecular basis for sensitivity to lactisole is based on only a few residues within the TM region of the human T1R3 protomer. Alanine substitution of residues in the TM region of human T1R3 revealed four key residues required for sensitivity to lactisole. Molecular modelling of the TM region of human T1R3 and automatic docking of lactisole to the predicted binding pocket confirmed that this inhibitor docks to a binding pocket within the transmembrane region that includes these four key residues [95].

Subsequently, extending the study to cyclamate, the same authors showed that activation of the sweet receptor by cyclamate requires the human form of T1R3. Then, from additional chimeras and mutants, and systematic alanine-scanning mutagenesis, they identified several residues within the TM domain of human T1R3 involved in sweet receptor responses to cyclamate [94].

The molecular bases of lactisole recognition were further refined by Winnig et al. [99]. By functional analysis of specific receptor mutants they found that mutation V738A in the fifth TM domain of rat T1R3 is sufficient to confer lactisole sensitivity to the rat sweet taste receptor, although the sensitivity of this receptor mutant is ca. twofold lower than that of the human sweet taste receptor. In addition, mutation K735F in rat T1R3 results in a rat sweet taste receptor that is as sensitive to lactisole as its human counterpart [99]. All previous data suggest that the TM domain of the T1R3 protomer of the sweet receptor hosts a genuine fourth site.

An additional site for sweet proteins, different from that of the wedge model, was proposed by Jiang et al. [97]. Since human T1R2-T1R3 responds to brazzein, whereas the chimera of human T1R2-mouse T1R3 does not, these authors reasoned that the cause could be located in the cysteine-rich region of T1R3 and, using human/mouse chimeras of T1R3 paired with human T1R2, determined that residues 536–545 of the cysteine-rich region of human T1R3 were necessary for responsiveness to brazzein. It is not easy to tell whether this is a genuine additional fifth active site or just a critical region that cannot be easily perturbed without affecting the global response of the receptor. Data from other class C receptors suggest a predominantly structural role for the cysteine-rich region of all metabotropic receptors. In the case of the human Ca^{2+} receptor, Hu et al. [100] showed that the hCaR cysteine-rich domain plays a critical role in signal transfer from Venus's-flytrap domain to the 7TM of the hCaR and for sequence specificity in communication. Any mutation in this region may undermine the structural integrity of the sweet receptor. On the other hand, the wedge mechanism [9, 85, 87] does provide a simple explanation for the critical role played by T1R3 in the interaction with sweet proteins without invoking this additional site.

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