CHAPTER 6

The Sweet Taste Receptor: A Single Receptor with Multiple Sites and Modes of Interaction

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Advances in Food and Nutrition Research, Volume 53
ISSN 1043-4526, DOI: 10.1016/S1043-4526(07)53006-8

Abstract

Elucidation of the molecular bases of sweet taste is very important not only for its intrinsic biological significance but also for the design of new artificial sweeteners. Up to few years ago design was complicated by the common belief that different classes of sweet compounds, notably sweet proteins, might interact with different receptors altogether. The recent identification and functional expression of the receptor for sweet taste have shown that there is but one receptor, drastically changing our approach to the development of new sweeteners. The explanation of how the sweet receptor can bind several different classes of molecules is that rather than multiple receptors there are, apparently, multiple sites on the single sweet taste receptor. In this chapter, the mechanisms of interaction of small and macromolecular sweet molecules will be examined, with particular emphasis on sweet proteins. Systematic homology modeling yields reliable models of all possible heterodimers of the human T1R2 and T1R3 sequences with the closed (A) and open (B) conformations of one of the metabotropic glutamate receptors (mGluR1), used as template. The most important result of these studies is the "wedge model," the first explanation of the taste of sweet proteins. In addition, it was shown that 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. This observation accounted for the first time for the peculiar phenomenon of synergy between some sweeteners.

I. INTRODUCTION

Taste plays a key role in the selection of food. The gustatory system of all animals is primarily involved in feeding behavior, allowing them to detect useful foods and avoid toxic substances. For instance, plant-feeding insects often rapidly reject foods containing toxic plant compounds (Glendinning, 1994, 1996; Wang *et al.*, 2004). Although there is not unanimous consensus on a sharp classification of tastes (Delwiche, 1996), the existence of five different tastes, that is sweet, bitter, salty, sour, and umami, is acknowledged by a vast majority of scientists (Lindemann, 2001). Sweet taste plays a central role for humans since most people respond positively to the sensation of sweetness, with a propensity for sweet foods that can be traced back to early life. Sweetness is also an important medical issue because there is an increasing number of people affected by diseases, like diabetes, hyperlipemia, caries, that are more or less directly linked to the secondary effects of sugar intake. New knowledge about the molecular basis of taste may suggest strategies to overcome diet-induced diseases (Mennella *et al.*, 2005). The design of safe low-calorie sweeteners is particularly important in this context.

Most people associate sweet taste with sugars, but it is not generally true that sweet molecules are sugars. Several hundreds of synthetic and natural sweeteners were found either by serendipity or by targeted research. In the past, organic chemists used to taste most of the compounds they synthesized and many substances turned out to be sweet (Moncrieff, 1967). The chemical constitution of sweet molecules varies widely from sugars to amino acids, peptides, proteins, olefinic alcohols, nitroanilines, saccharin, chloroform, and many other organic compounds. In addition to diversity in chemical constitution, sweet molecules can have drastically different dimensions, the extreme example being afforded by the existence of a few very sweet proteins (Morris, 1976). The molecular volume of a typical small molecular weight sweetener, like aspartame, can be estimated at 265 $Å^3$ whereas that of thaumatin, a well-characterized sweet protein, is of the order of 27,000 $Å^3$. Such a dramatic difference poses a big challenge if one tries to reconcile the sweetness–activity relationship of the two classes of molecules. We shall see that the interpretation of the mechanism of action of sweet proteins plays a crucial role for the understanding of structure–activity of sweet molecules in general. When nothing was known about taste receptors, the structure–activity relationship of sweet molecules was studied mainly using an indirect mapping of the active site of the receptor, by comparing the structures and the activities of the largest possible number of sweet compounds. This approach amounted to the identification of an ideal sweetener whose shape and electronic properties reflected the nature and topological arrangement of glucophores in the majority of sweet molecules. Indirect mapping led to the development of different models of active site (Goodman *et al.*, 1987; Iwamura, 1981; Kier, 1972; Shallenberger and Acree, 1967; Temussi *et al.*, 1978, 1984, 1991; Tinti and Nofre, 1991), consistent with the structure–activity of most small molecular weight sweeteners but hardly explaining the enormous sweetening power of sweet proteins.

The molecular biology of taste has been studied less than that of other stimuli, to the extent that Lindemann (1996) defined taste as ''the Cinderella of senses'' since its transduction mechanism was, at that time, the least well understood, but the cloning of several likely taste receptors rekindled the interest in this stimulus (Firestein, 2000) and opened brand new perspective also for the rational design of artificial sweeteners. In 2001, a major experimental effort led many groups to the identification of the sweet receptor (Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Li *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001). It was found that the sweet taste receptor is a special type of G-protein–coupled receptor (GPCR), one of the so-called metabotropic or class C GPCRs. This class of GPCRs, in addition to the seven-helix transmembrane domain (7TM), has a large extracellular domain, called Venus flytrap domain (VFTD), containing the active site

for typical ligands (Pin *et al.*, 2003). The knowledge of the receptor provided immediately a better understanding of the molecular bases of sweet taste but left many questions unanswered. Among these, one that arises when comparing sweet molecules is whether sweet compounds of size so different as sweet proteins can interact with the same receptors as small molecular weight compounds. Since it was shown that small and large molecular weight sweet molecules do interact with the same T1R2–T1R3 receptor (Li *et al.*, 2002), it was necessary to understand whether they use the same mechanism. We shall show how the answers to many unresolved issues on sweet taste came from modeling studies that show that the sweet taste receptor has multiple active sites. In the following sections, we shall give an overview of the history of sweet molecules with a particular emphasis on sweet proteins and the unique mechanism of their interaction with the T1R2–T1R3 receptor.

II. INDIRECT MAPPING OF ACTIVE SITES

A. Small molecular weight sweet molecules

Sugar, chemically better described as sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranose) is the natural compound generally associated with sweet taste. The sugar we use everyday, commercially extracted from sugarcane or sugar beet, is also the most commonly used substance for altering the flavor of food. In addition to sucrose, there are hundreds of other ''sugars,'' that is water-soluble crystalline carbohydrates or sugar alcohols characterized by a typical sweet taste. All these compounds are characterized by the presence on their skeleton of several hydroxyl groups, but it is not easy to find a special distribution of these substituents typical only of sweet saccharides. Figure 1 shows the molecular models of some typical natural sweet molecules, including sucrose. The two monosaccharides contained in sucrose, fructose and glucose, have the same number of hydroxyl groups but have a somewhat different sweetening power: 25% and 75% as sweet as sucrose, respectively.

Another class of polyol compounds that have been widely used as sugar substitutes is that of sugar alcohols. The most important of these sugar substitutes are erythritol, glycerol, mannitol (hexane-1,2,3,4,5,6-hexol), and sorbitol. Figure 1 shows the molecular models of glycerol and sorbitol. It can be appreciated that sorbitol has a chemical constitution very similar to that of monosaccharides, yet it has been used as sugar substitute since the body metabolizes it slowly.

Amino acids of the natural (*S*) configuration, that is the building blocks of proteins, are for the major part tasteless or bitter whereas glycine and some hydrophobic amino acids of *R* chirality are sweet (Solms *et al.*, 1965).

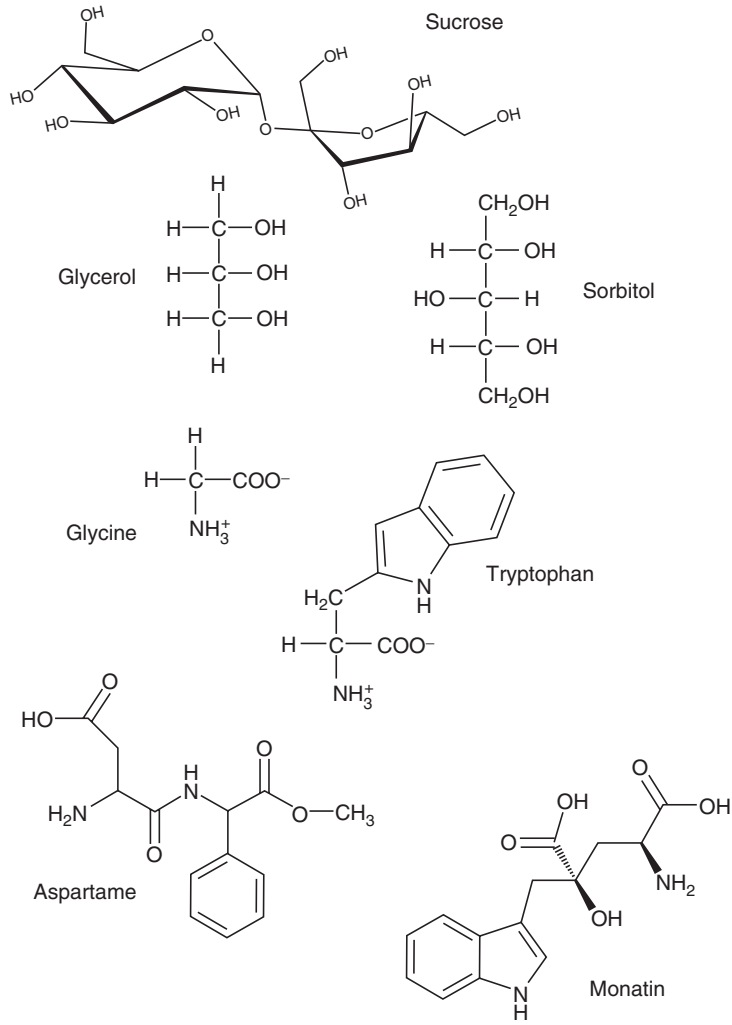

FIGURE 1 Molecular formulas of some sweet molecules, representative of the major natural classes: carbohydrates (sucrose), polyols (glycerol, sorbitol), amino acids (glycine, tryptophan), peptides (aspartame, monatin).

In fact, the very name of glycine comes from the greek word for sweet (γλυκοσ). Figure 1 shows the molecular models of glycine and of *R*-tryptophan, the sweetest of *R* amino acids. In addition to simple amino acids, there are several sweet peptides; most of them, although composed of natural amino acidic residues, are not natural and are related to aspartame, l-aspartylphenylalanine methyl ester, the first sweet dipeptide, discovered

by serendipity in the 1960s (Mazur *et al.*, 1969). A very interesting naturally occurring peptide, monatin, has been described and fully characterized (Bassoli *et al.*, 2005).

Extracts from plants led to the discovery of several intensely sweet natural glycosides. The best known of sweet glycosides is probably stevioside, a component of *Eupatorium rebaudianum* (Bridel and Lavieille, 1931) 150–300 times sweeter than sucrose. Another important natural glycoside is Glycyrrhizin, the flavoring agent of licorice (*Glycyrrhiza glabra*), ∼50 times as sweet as sucrose (Tahara *et al.*, 1971). However, the aglicone of Glycyrrhizin is a triterpene structurally similar to corticosteroids; hence, Glycyrrhizin at high doses induces hypertension. A much sweeter similar glycoside (3000 times sweeter than glucose) is Osladin, extracted from *Polypodium vulgare* (Tahara *et al.*, 1971).

Other small molecular weight sweeteners of natural origin are often classified as semisynthetic since the original natural substance, although not sweet, becomes sweet after minor chemical modification (Morris, 1976). The terpene perillaldehyde extracted from *Perilla frutescens* is not sweet but becomes 200 times sweeter than sucrose when the aldehyde functional group is changed into its syn-oxime, called perillartine (Acton and Stone, 1976). A similar relationship exists between narigin dihydrochalcone and neohesperidine dihydrochalcone and the corresponding bitter flavanone glycosides derived from citrus fruits (DuBois *et al.*, 1981). In turn, the aglicone of neohesperidine dihydrochalcone represents an entire class of natural compounds that can be called isovanillyl sweet compounds (Bassoli *et al.*, 2002b) since they contain the isovanillyl group (3-hydroxy-4-methoxyphenyl). The most representative natural isovanillyl molecules are phyllodulcin, dihydroquercetin 3-acetate, and hematoxylin, but the sweetest isovanillyl compounds are synthetic modifications discovered in the laboratory of Merlini (Bassoli *et al.*, 2002b). Figure 2 shows the molecular models of some larger typical natural sweet molecules (stevioside, Glycyrrhizin, neohesperidine dihydrochalcone, and phyllodulcin).

In addition to many natural products, there is a huge number of unrelated organic molecules that were found to be sweet in the course of many decades of research in synthetic organic chemistry. As mentioned in Section I , in the past it was a common practice among organic chemists to taste the new compounds they synthesized, and many substances turned out to be sweet. Accordingly, there are many sweeteners totally unrelated to the classes of Figures 1 and 2. Figure 3 shows the molecular models of some representative synthetic sweeteners. It can be appreciated that all of them are considerably more hydrophobic than most natural sweeteners. None of the mentioned compounds as well as many others discovered or synthesized 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.

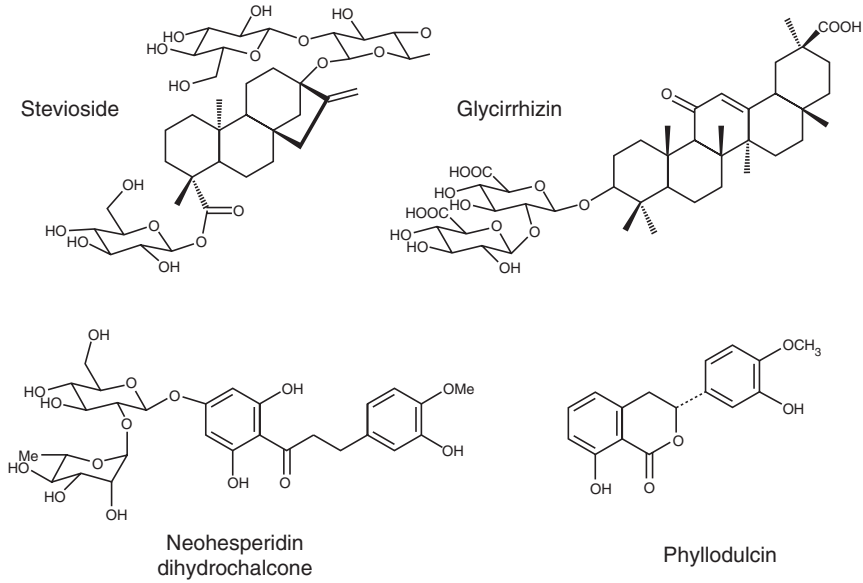

FIGURE 2 Molecular formulas of further typical natural sweet molecules, mainly related to terpenes: stevioside, glycyrrhizin, neohesperidine dihydrochalcone, and phyllodulcin.

B. Early structure–activity studies

Early attempts to understand structure–taste relationships of molecules were based on the search of specific atoms or groups of atoms (called ''sapophores'') that could impart a given taste to molecules (Cohn, 1914). In the case of sweet compounds, Cohn observed, for instance, that molecules containing several hydroxyl groups, or chlorine atoms, or the α-amino and carboxyl groups typical of amino acids are often sweet; accordingly, these groups of atoms were defined ''dulcigen'' groups (Cohn, 1914). This approach was further elaborated by Oertly and Myers (1919) who called these chemical groups ''glucophores'' and others, with the ability to increase the potency, ''auxogluc.'' It was soon clear that ''glucophores'' and ''auxogluc'' groups belonged to sweet and tasteless molecules with similar frequency, whereas other features, that is the steric disposition of groups, probably played an important role (Moncrieff, 1967).

The first successful generalization can be attributed to Shallenberger and Acree (1967) who hypothesized that the main signature for sweet molecules could reside on their skeleton in the presence of a hydrogen bond donor (AH) and a hydrogen bond acceptor (B) spaced 3–4 Å. These two groups, by interacting with a complementary pair of hydrogen bond donor and acceptor on the receptor, would act as the main

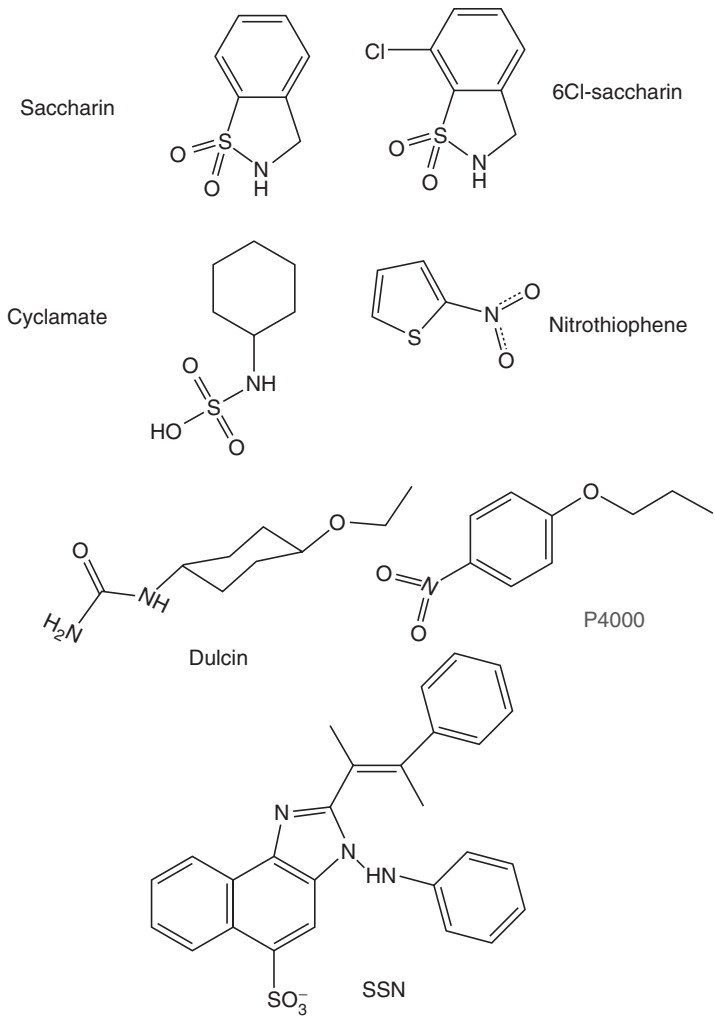

FIGURE 3 Molecular models of some representative synthetic sweeteners: saccharin, 6Cl-saccharin, cyclamate, nitrothiophene, dulcin, P4000 (*ortho*-propoxy-*meta*-nitro aniline), and SSN (3-anilino-2-styryl-3*H*-naphtho[1,2-d]imidazole-5-sulphonate).

anchoring points in binding. The model of Shallenberger and Acree can be regarded as a linear model. It was soon developed into a planar (triangular) geometry by Kier (1972) who introduced a third group, named the ''dispersion point,'' at a precise distance from the AH–B pair. Kier's model accounts for the experimental observation that many, albeit not all, synthetic sweeteners are flat rigid molecules. This model enjoyed great popularity among medicinal chemists, possibly because of its simplicity, but the identification

of a single dispersion point represented by virtually any uncharged atom or even a point on a chemical bond is an oversimplification. One of the weakest points of this model is that, in the absence of any stereochemical information, it could not allow selection between chemically similar, or even isomeric, sweet and tasteless or bitter compounds. For instance, of the three possible nitroanilines, the *meta*-isomer is sweet but the *ortho*- is not and the *para*- is almost tasteless (Moncrieff, 1967). The interest was soon redirected toward the development of more general models of the receptor active site derived from the shape of conformationally rigid sweet molecules, used as molecular molds. The most exhaustive approach was that of Temussi and coworkers (Kamphuis *et al.*, 1992; Temussi *et al.*, 1978, 1984, 1991). They suggested a more detailed model based on an accurate superposition of rigid sweet compounds, which should reflect the overall shape of the putative receptor cavity (Kamphuis *et al.*, 1992; Temussi *et al.*, 1978, 1984, 1991). The combination of several observations, using also flexible compounds, notably aspartame, whose solution structure had just been determined (Lelj *et al.*, 1976), led to a detailed quasi-planar outline of the active site. The main features of this model can be summarized as follows: (1) the active site of the receptor is a flat cavity with one side partially accessible even during the interaction with the agonist; (2) the lower part of the cavity hosts the AH–B entity complementary to that of the sweet molecule; (3) the upper part is hydrophobic and plays an important role in the case of very active sweeteners. This is often referred to as the ''Temussi model'' (Walters, 1995; Walters *et al.*, 1986). Figure 4A shows the main contour of the active site hosting a model of aspartame in an extended conformation.

Aspartame was the starting point for another model, proposed by Iwamura (1981) on the basis of QSAR analyses of dipeptide analogues. This author claimed that his receptor model is different from that proposed by Temussi *et al.* (1978), but the results of the calculations reflected mainly the difficulty of using conformationally flexible compounds for a QSAR calculation. Another topological model, mainly based on the conformation of aspartame and other dipeptides, was developed by Goodman *et al.* (1997). This model (Figure 4B), while incorporating most of the features of the Temussi model, differed in some sterical aspects. According to their model, the overall topology of a sweet tasting molecule can be described as an ''L''-shaped structure with the aspartyl moiety forming the stem of the ''L'' and the hydrophobic group X forming the base of the ''L'' (Goodman *et al.*, 1997). The zwitterionic ring of the aspartyl residue is coplanar and essentially perpendicular to the X group. Figure 4B shows the superposition of the ''L''-shaped model and an ''L''-shaped structure for aspartame. Also the Temussi model was originally inspired by the solution conformation of aspartame (Lelj *et al.*, 1976), but it was soon realized that aspartame is too flexible to be used as a mold (Temussi *et al.*, 1984) and the final active site model, although consistent

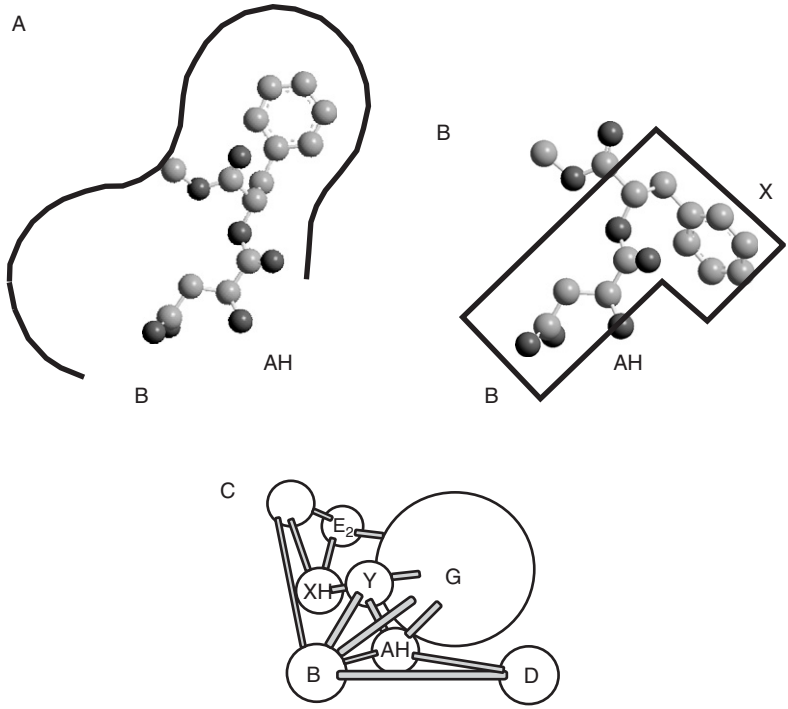

FIGURE 4 Three of the most popular indirect models of the active site of the sweet taste receptor. (A) Main contour of the active site proposed by Temussi and coworkers (Kamphuis *et al.*, 1992; Temussi *et al.*, 1978, 1984, 1991), hosting a molecular model of aspartame in an extended conformation. (B) A topological model, developed by Goodman *et al.* (1987). The "L"-shaped model and an "L"-shaped conformation of aspartame are superimposed. The hydrophobic side chain of Phe is denoted X, since it corresponds to the Kier's dispersion point. (C) 3D model of an idealized sweetener proposed by Tinti and Nofre (1991). Besides the AH–B entity, the model has six additional interaction points connected by a complex network of distances.

with the solution structure of aspartame, was built on more rigid molecules. A way to discriminate between the models of Figure 4A and B could have been to be able to predict the conformation of aspartame in the actual receptor. Experimental structural studies were not sufficient to give an unequivocal answer: the conformer found in the crystal structure of aspartame (Hatada *et al.*, 1985) is consistent with Goodman's model, whereas that of the more rigid and sweeter [(L-α-Me)Phe^2] aspartame (Polinelli *et al.*, 1992) is consistent with Temussi's.

The most popular model in the 1990s was that of Tinti and Nofre (1991). Following their discovery of very potent sweeteners containing a guanidinium ion (Nofre *et al.*, 1988; Tinti and Nofre, 1991), they proposed

a three-dimensional (3D) model for an ideal sweetener that besides the AH–B entity has six additional interaction points connected by a complex network of distances (Figure 4C). This model may suffer from little generality since it specifically tailors the architecture of only one type of compounds, but it has the great merit of being consistent with the most powerful known sweeteners. Just before the discovery of the sweet taste receptor, Bassoli *et al.* (2002a) proposed a unifying model able to explain and predict semiquantitatively the sweet taste of compounds belonging to different families. An entirely different approach has been proposed by Gokulan *et al.* (2005). By comparing the crystal structures of synthetic supersweetener and nonsweetener compounds complexed with murine monoclonal antibody (mAB) NC6.8, they found that receptor–ligand interactions imply a complex array of hydrogen bonds, electrostatic interactions, and several hydrophobic contacts. The main conclusion was that the difference between high-potency guanidine sweeteners and related zwitterionic low-potency tastants is determined by the nature and conformation of the hydrophobic group. Their results are very interesting but suffer from the same drawbacks of indirect modeling. Since both are not based on any knowledge of the actual receptor, they may bear little relationship with actual receptor binding. The long trial and error search for ideal sweeteners via indirect mapping or intuition did produce a number of high-potency sweeteners, notably those derived from aspartame, like neotame (Prakash *et al.*, 1999) or superaspartame (Tinti and Nofre, 1991), and the guanidinium compounds (Nofre *et al.*, 1988; Tinti and Nofre, 1991). However, none of the existing models of the active site could explain the enormous increase in activity in going from small molecular weight compounds to proteins: monellin, for example, one of the best characterized sweet proteins, is 100,000 times sweeter than sucrose on a molar basis (Hung *et al.*, 1999).

III. SWEET MACROMOLECULES

Although not very numerous, sweet macromolecules, both natural (Morris, 1976) and synthetic (Zaffaroni, 1975), are crucial for an understanding of the mechanism of the sweet receptor. The best known among proteins with a very strong sweet taste are brazzein (Ming and Hellekant, 1994), monellin, and thaumatin (Kurihara, 1992). Figure 5 shows molecular models of these three proteins. Other two known sweet proteins are mabinlin (Kurihara, 1992) and hen egg white (HEW) lysozyme (Maehashi and Udaka, 1998), whereas miraculin and curculin, which taste sweet when combined with sour substances, can be better described as taste-modifier proteins (Kurihara, 1992).

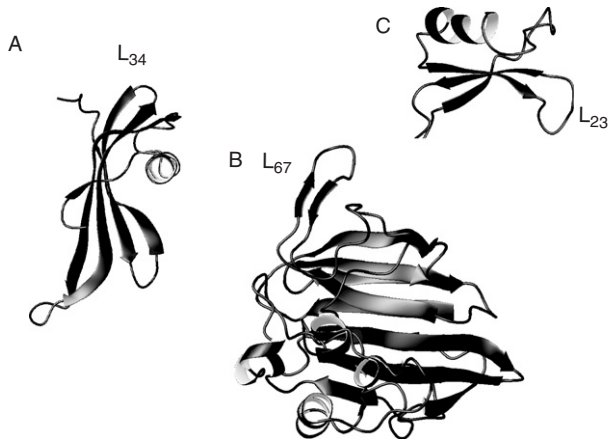

FIGURE 5 Ribbon representations of the three sweet proteins of known structure. (A) Structure of MNEI (pdb entry 1fa3): the most likely sweet finger is loop L_{34}. (B) Structure of thaumatin (pdb entry 1thw): the most likely sweet finger is loop L_{67}. (C) Structure of brazzein (pdb entry 2brz): hairpin L_{23} represents the only possible sweet finger.

A. Characterization of natural sweet proteins

1. Monellin

Until 1972, it was not known that a protein could taste sweet (Morris and Cagan, 1972). Monellin is one of the first two proteins with intense sweet taste unambiguously identified and characterized. Inglett and May (1969), who originally discovered it as the sweet principle of *Dioscoreophyllum cumminsii*, a plant taxonomically related to the sweet potato, believed it was a carbohydrate. Owing to the unexpected intensity of the sweet taste, Inglett and May called the plant "serendipity berries" (Morris, 1976). Later on, Morris and Cagan (1972) established that the sweet principle is a protein and named it monellin, after the Monell Chemical Senses Center where they worked. According to these authors, the sweetness of monellin relative to sucrose is 3000:1 on a weight basis, corresponding to a ratio of 90,000:1 on a molar basis. Monellin consists of two nonidentical subunits of 42- and 50-amino acid residues, called A and B respectively, that are not covalently linked but are held together only by secondary forces (Bohak and Li, 1976). The sequence of monellin bears no significant similarity to that of any of the other sweet proteins. In addition, when it was originally discovered, it was impossible to assign it to a known protein family, but Murzin (1993) on the basis of its solid state structure demonstrated that it belongs to the cystatin superfamily, albeit devoid of any activity as a protease inhibitor. The sweetness of monellin is exhibited only by the whole molecule, whereas the individual subunits are not sweet (Bohak and Li, 1976). Owing to the weak forces holding its two chains, when heated above 50° C monellin

dissociates into two chains and, as a consequence, it loses its sweetness altogether. Single-chain monellins in which the two chains are covalently linked retain all sweetening power but have greatly increased thermal stability (Kim *et al.*, 1989; Tancredi *et al.*, 1992). The first single-chain monellin (dubbed SCM) was designed by Kim *et al.* (1989) on the basis of the crystal structure of wild-type monellin. In SCM, the C-terminal residue of the B chain (B50E) is directly linked to the N-terminal residue of the A chain (A1R). SCM is as sweet as natural monellin, more stable on temperature or pH changes, and renatures easily even after heating to 100°C at low pH. A very similar behavior was shown by MNEI, a single-chain monellin obtained by inserting a Gly-Phe dipeptide between the B and A chains (Tancredi *et al.*, 1992). The structures of both two-chain and single-chain forms of monellin were thoroughly characterized by X-ray and nuclear magnetic resonance (NMR) studies (Hung *et al.*, 1998; Lee *et al.*, 1999; Somoza *et al.*, 1993; Spadaccini *et al.*, 2001). The solution structure of MNEI, shown in Figure 5A, can be described as an α-helix cradled into the concave side of a five-strand antiparallel β-sheet solution (Spadaccini *et al.*, 2001).

The huge difference in size between sweet proteins and all non-proteic sweeteners led several researchers to postulate the existence, on the surface of monellin, of some kind of ''sweet finger,'' that is a protruding structural element hosting one or more glucophores similar to those of small sweeteners. ELISA tests showed cross-reactivity between antibodies raised against monellin and those raised against thaumatin (Bodani *et al.*, 1993; Mandal *et al.*, 1991). On this basis, the sequence $TyrA^{13}$-$AspA^{16}$ of native monellin and that comprising residues Tyr^{57}-Asp^{59} of thaumatin were suggested as a potential sweet fingers (Kim *et al.*, 1991). However, point mutations on synthetic monellin (Ariyoshi and Kohmura, 1994) showed that even substantial changes of residues 13 and 16 of the A chain do not affect sweetness. Actually, according to Ariyoshi and Kohmura (1994), 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 both on SCM and wild-type monellin (Kohmura *et al.*, 1992; Somoza *et al.*, 1995) 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 Ile^{6}, Asp^{7}, Gly^{9} (Kohmura *et al.*, 1992), and Arg^{39} (Sung *et al.*, 2001) whereas mutations of Gln^{13}, Lys^{36}, Lys^{43}, Arg^{72}, Arg^{88} or deletion of Pro^{92}–Pro^{96} cause a decrease of one order of magnitude (Kohmura *et al.*, 1992; Somoza *et al.*, 1995). The distribution of key residues (for biological activity) on a large area was confirmed without recurring to any hypothesis on the mechanism of interaction, that is, in a completely objective way, by a surface survey based on novel NMR techniques (Niccolai *et al.*, 2001).

The survey of the MNEI surface accessibility (Niccolai *et al.*, 2001) was performed by means of TEMPOL, a paramagnetic probe and a direct assessment of bound water by means of ePHOGSY, a pulse sequence that allows accurate detection of NOEs between bound water and protein hydrogens (Dalvit, 1996, 1998). The result of this integrated NMR study suggested that three MNEI regions are potentially suitable for interactions with other proteins: loop L_{34}, previously referred to as a potential sweet finger; the small N-terminal β-strand containing Ile^{6}, Asp^{7}, and Gly^{9}; and a basic patch containing Arg^{72} and Arg^{88}.

2. Thaumatin

The jelly-like exterior of the seeds of a West African plant, *Thaumatococcus danielli*, is intensely sweet. Inglett and May (1968) reported that it contains a sweet substance of ''unique chemical and physical nature'' but failed to identify it as a protein. The sweet substance that makes the seeds of *T. danielli* so sweet was later characterized by van der Wel and Loeve (1972) as a mixture of two proteins called thaumatin I and II endowed of a sweetening power about 1600 times higher than that of sucrose on a weight basis or 100,000 on a molar basis. So far, thaumatin is the only sweet protein that has been actually used as a sweetener: in the 1970s, Tate and Lyle began commercializing thaumatin, as extracted from *T. danielli*, under the trade name of Talin. Thaumatin is a single polypeptide chain of 207 residues (Iyengar *et al.*, 1979) and, according to the SCOP classification (Murzin *et al.*, 1995), belongs to the osmotin, thaumatin-like superfamily. The 3D structure of thaumatin, solved in the solid state by X-ray studies (de Vos *et al.*, 1985; Ogata *et al.*, 1992), contains three domains, mainly composed of β-sheets (Figure 5B).

The structure–activity relationship of thaumatin has been studied less than that of monellin. Comparing the amino acid sequence of thaumatin with that of monellin, the other sweet-tasting protein known at the time; Iyengar *et al.* (1979) 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. However, if one runs a comparison of the sequence of thaumatin with those of other sweet tasting proteins by means of modern bioinformatics means, for example ClustalX (Thompson *et al.*, 1997), the similarities are negligible (Tancredi *et al.*, 2004). Mandal *et al.* (1991) developed a library of monoclonal antibodies that react with different surface antigenic epitopes on thaumatin and, in a few instances, also cross-react with monellin. A similar study by Slootstra *et al.* (1995) identified two major overlapping conformational epitopes. This region contains an aspartame-like site which is formed by Asp^{21} and Phe^{80}, tips of the two extruding loops 19–29 and 77–84, which are spatially positioned next to each other. Since the aspartame-like Asp^{21}-Phe^{80} site is not present in nonsweet thaumatin-like proteins, they suggested that the

two loops contain important sweet taste determinants. Kaneko and Kitabatake (2001), by examining in detail the role of lysines in the structure–sweetness relationship of thaumatin, found that phosphopyridoxylation of Lys^{78}, Lys^{97}, Lys^{106}, Lys^{137}, or Lys^{187} reduced sweetness significantly. Combination of these results with those ensuing from modifications of other charged residues led them to suggest that there is a charged side of the protein that is important for sweetness. These studies are not conclusive but seem to point to a large surface of interaction also for thaumatin.

3. Brazzein

Brazzein, the smallest of sweet proteins, was discovered only in 1994 (Ming and Hellekant, 1994) in *Pentadiplandra brazzeana* B. This protein, whose sequence contains 54-amino acid residues, is ~2000 times sweeter than sucrose when compared to a 2% sucrose aqueous solution. Its taste was described as more similar to sucrose than that of thaumatin (Ming and Hellekant, 1994). As can be seen in Figure 5C, the 3D structure of brazzein, determined by ^{1}H NMR spectroscopy in solution at pH 5.2 (Caldwell *et al.*, 1998), is very simple. It contains one α-helix and three strands of antiparallel β-sheet. The structure is stabilized by four disulfide bonds, three connecting the helix to the β-sheet. It does not resemble either that of monellin or that of thaumatin; instead, it resembles those of plant γ-thionins and defensins and arthropod toxins. According to the SCOP classification (Murzin *et al.*, 1995), brazzein belongs to the Scorpion toxin-like superfamily.

All studies on the structure–activity relationship of Brazzein were performed by the same group that elucidated the 3D structure. Assadi-Porter *et al.* (2000) by introducing multiple mutations at several specific positions found that the mutations that affect most the sweetness of brazzein are localized within the tracts Asp^{29}LysHisAlaArg^{33} and Tyr^{39}AspGluLysArg^{43}, close to the C-terminus. These data were refined by Jin *et al.* (2003) who investigated more mutations. Three mutants, that is Ala2ins, Asp2Asn, and Gln17Ala, were found to be as sweet as wild-type brazzein. Four mutants, that is Asp29Ala, Asp29Lys, Asp29Asn, and Glu41Lys, were found to be significantly sweeter than wild-type brazzein. In other 8 mutants the sweetness decreased significantly although they were not tasteless, whereas in 10 mutants the sweetness did not differ significantly from that of water. At about the same time, Assadi-Porter *et al.* (2003) proposed a very innovative approach to identify the main structural determinants for the sweetness of brazzein. Assadi-Porter *et al.* (2003) applied NMR methods that permit direct detection of hydrogen bonds (Cordier and Grzesiek, 1999) to screen a series of five single site mutants of brazzein with altered sweetness, looking for possible changes in backbone hydrogen bonding with respect to wild-type.

Assadi-Porter *et al.* (2003) found that in the mutants, altered magnitudes of the couplings identified hydrogen bonds that were strengthened or weakened with respect to the wild type. Within the series of brazzein mutants investigated, a pattern was observed between sweetness and the integrity of particular hydrogen bonds. Assadi-Porter *et al.* (2003) concluded that their findings may be interpreted as supporting the hypothesis of an extensive receptor-binding surface in brazzein, involving loop 43 and the N- and C-terminal regions. The success of this approach probably reflects the fact that changes in the protein surface mirror changes of the underlying network of hydrogen bonds.

4. Mabinlin

Hu and Min (1983) have isolated two new sweet proteins from the seed of *Capparis masaikai* Levl., a plant that grows in the subtropical region of the Yunnan Province of China and named them mabinlin I and II, after the local name of the plant (mabinlang). The sweetness of mabinlin II with respect to sucrose was estimated as 375:1 on a molar basis; it remains unchanged by more than 48-hour incubation at boiling temperature (Liu *et al.*, 1993). Thus, although mabinlin is much sweeter than sucrose, it is considerably less sweet than monellin, thaumatin, and brazzein. Purified mabinlin II gave a single band having a molecular mass of 14kDa on SDS-PAGE, but two peptide chains (A and B) were separated from reduced and *S*-carboxamidomethylated mabinlin II by HPLC (Liu *et al.*, 1993). The amino acid sequences of the A chain and B chains consist of 33-amino acid and 72-amino acid residues, 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).

5. Lysozyme

As it is well known, lysozyme is a small enzyme that catalyzes the hydrolysis of polysaccharides comprising the cell walls of bacteria. It is exceptionally abundant in egg whites. Lysozyme is also one of the best characterized proteins from a structural point of view, both in solution (Schwalbe *et al.*, 2001) and in the crystal state (Strynadka and James, 1996). Figure 6A 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 structures of Figure 5, that are mainly rich in β-sheets, that the structure of lysozyme is prevalently α-helical. Its inclusion among sweet proteins is quite recent. The sweetening power of HEW lysozyme corresponds to a threshold value of around 7μM (Masuda *et al.*, 2005b), a value that is far from the nanomolar range of the three main sweet proteins but is higher than that of sucrose. Maehashi and Udaka (1998)

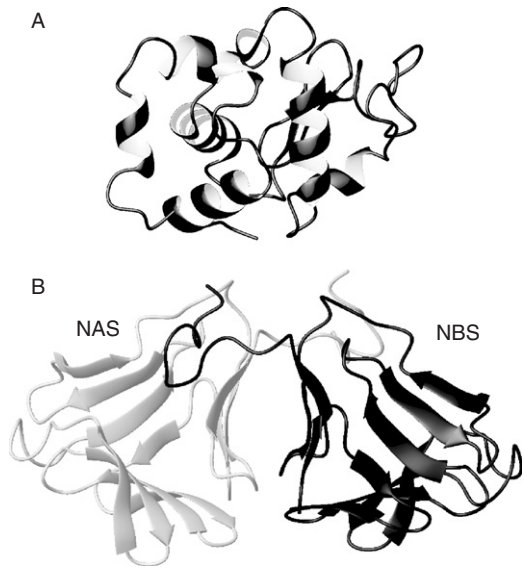

FIGURE 6 Ribbon representations of lysozyme and neoculin. (A) Tetragonal form of lysozyme solved at 1.3-Å resolution (pdb entry 193L). (B) One of the four crystallographically independent heterodimers of neoculin (pdb entry 2d04).

claimed that 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 (*vide infra*). Masuda *et al.* (2001, 2005a,b) have studied extensively the structure–activity relationship of lysozyme. The main results can be summarized as follows. Alanine substitution of lysine residues showed that two of six lysine residues, only Lys^{13} and Lys^{96}, are required for lysozyme sweetness, while the remaining four lysine residues do not affect significantly the sweetness. Similarly, single alanine substitutions of arginine residues showed that three arginine residues, Arg^{14}, Arg^{21}, and Arg^{73}, play significant roles in lysozyme sweetness, whereas mutation of Arg^{45}, Arg68, and Arg^{125} did not affect sweetness (Masuda *et al.*, 2005b).

6. Miraculin

The fruits of *Synsepalum 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 (Morris, 1976). Theerasilp and Kurihara (1988) isolated miraculin from alkaline extracts of the

miracle fruit and purified it with standard biochemical procedures. Miraculin is a single polypeptide chain with 191-amino acid residues (Theerasilp *et al.*, 1989). 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 disulfide bridges (Igeta *et al.*, 1991), 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 (Gibbs *et al.*, 1996). 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 (nonactive) occupancy of the receptor and it becomes sweet only after acidification (Kurihara and Beidler, 1969).

7. Curculin

In 1990, a new taste-modifying protein named curculin was extracted from the fruits of *Curculigo latifolia* and purified by ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration (Yamashita *et al.*, 1990). Curculin consists of 114 residues, but the molecular weight suggests that native curculin is a homodimer of a 12,000-Da polypeptide. 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.

Until recently, however, it proved impossible to observe the taste-modifying properties in recombinantly expressed curculin. Almost simultaneously Shirasuka *et al.* (2004) and Suzuki *et al.* (2004) isolated a gene that encodes a novel protein highly homologous to curculin. The amino acid sequence of the novel gene has 77% identity to that of curculin but, in contrast to the previously reported isoform, the new protein is acidic, with an estimated isoelectric point of 4.7. Using cDNAs of the previously known curculin (dubbed curculin1) and the novel curculin isoform (curculin2), Suzuki *et al.* (2004) produced a panel of homodimeric and heterodimeric recombinant curculins by *Escherichia coli* expression systems. They found that sweet-tasting and taste-modifying activities were exhibited solely by the heterodimer of curculin1 and curculin2. Shirasuka *et al.* (2004) named neoculin this heterodimer and, on the basis of the isoelectric points, neoculin basic subunit (NBS) the polypeptide corresponding to curculin1 and neoculin acid subunit (NAS) curculin2. The crystal structure of neoculin, the first of taste-modifying proteins, showed a fold quite similar to that of monocot mannose-binding lectins. Figure 6B shows a ribbon representation of one of the four crystallographically independent heterodimers of neoculin.

B. Interaction of sweet proteins with the sweet receptor

The discovery of a few proteins with an intense sweet taste (Morris, 1976) was a great shock for all researchers studying sweetness–activity relationship. The dimensions of all sweet proteins are so different from those of typical sweeteners that it was difficult to hypothesize an interaction with the same active sites proposed for small molecular weight sweeteners. In addition, it is difficult to find commonalities among the sweet proteins. The most widely used approach for an understanding of the origin of a common function among proteins belonging to the same family is to compare their sequences, in search of corresponding parts. No sequence homology can be detected among monellin, thaumatin, brazzein, mabinlin, miraculin, and curculin. A pairwise alignment of these sequences performed by Clustal X (Thompson *et al.*, 1997) showed that the percentages of identical residues between monellin and the other proteins are 23% between monellin and miraculin and a bare 7% between monellin and curculin (Tancredi *et al.*, 2004). If HEW lysozyme is included in the sequence alignment, the result is a complete misalignment (Temussi, 2006).

Yet, the interaction of proteins with the receptor might be explained also on the basis of the quoted indirect models of active site based on the shape of small sweeteners, provided one could identify, on the surface of the proteins, protruding features that can probe the active site, that is ''sweet fingers'' chemically similar to small sweeteners. Therefore, many efforts have been devoted to the search of possible sweet fingers on proteins. As mentioned above, on the basis of early ELISA tests the sequence $TyrA^{13}$-$AspA^{16}$ of native monellin and that comprising residues Tyr^{57}-Asp^{59} of thaumatin were suggested as a potential sweet fingers (Kim *et al.*, 1991). Although mutagenesis studies on monellin (Somoza *et al.*, 1995) hinted at a much larger spread of key residues on the surface of the protein, it could not be excluded that sweet fingers play an important role in recognition. Accordingly, Tancredi *et al.* (2004) undertook a systematic investigation on brazzein, monellin, and thaumatin to identify possible sweet fingers. They examined in great detail the structures of brazzein, monellin, and thaumatin for the presence of common motifs. The similarity among the tertiary folds of these three proteins is very low. One of the best methods to search for structural similarities is by means of DALI (Holm and Sander, 1995): a 3D search of each of the three known structures (brazzein, monellin, and thaumatin) against the whole database by means of DALI did not even retrieve the other two proteins. However, there are structural elements common to the three proteins, in the form of single secondary traits, notably short β-sheet hairpins. Potential candidates for sweet fingers should be protruding structural features of sufficient length to enter the active site of the receptor but, in addition, they should host residues consistent with glucophores already identified in small sweeteners. Judging from all existing models of the receptor (*vide infra*), the

minimum length of a candidate substructure should be of the order of 20 Å, since the active site is located at the bottom of a deep cleft, 20–30 Å from the surface of the protein. Likely protruding elements should also have a sufficiently stable secondary structure; thus, we can restrict our search to β-hairpins present in all three proteins shown in Figure 5 as ribbon representations. Brazzein (shown in Figure 5C) is the simplest case since its very simple structure contains only one such hairpin, loop L_{23}. Its length and the presence of residues containing suitable glucophores are consistent with the requirements outlined above. In the case of MNEI (Figure 5A), a single-chain monellin, there is no choice as clear as the one for brazzein. Loop L_{23}, centered around Gly^{51}-Phe^{52}, can be excluded right away since it is not even present in native monellin; loop L_{45}, being an integral part of a rigid β-sheet, is not mobile enough to act as a flexible finger whereas loop L_{34}, although not completely free, is structurally similar to the loop of brazzein and, in addition, corresponds to the original sweet finger proposed by Kim *et al.* (1991). In the case of thaumatin, there are numerous loops with sufficient length to probe the receptor's cavity. However, also in this case, most of them are tightly bound to the body of β-sheet that forms the architecture of this protein and thus, cannot be freed for probing the receptor interior without disrupting the structure of the protein. The only loop that is not tightly bound to the body of β-sheet is loop L_{56}. In addition, this loop is also the one identified by DALI as the only structural element similar to corresponding ones in brazzein (L_{23}) and monellin (L_{34}). All three loops contain, among the side chains, an aromatic ring (e.g., belonging to either a Tyr or a Phe) in relative spatial orientation, with respect to a pair of hydrogen bond donors or acceptors, similar to that found in aspartame. Starting from the sequences of these loops, Tancredi *et al.* (2004) synthesized the corresponding cyclic peptides: $c[C^{56}YFDDSGSGIC^{66}]$, $c[C^{61}LYVYASDKLFRAC^{73}]$, and $c[C^{37}FYDEKRN-LQC^{47}]$, with cyclization assured by –S–S– bridges. The cyclic peptides do assume conformations consistent with the conformation of the same sequences in the parent proteins. However, none of them was able to elicit sweet taste (Tancredi *et al.*, 2004). If, as mentioned above, we add to this result the fact that mutants affecting sweetness of monellin are distributed over a large area, rather than being concentrated on a long protruding structural entity (Somoza *et al.*, 1995), it is fair to assume that the sweet fingers hypothesis can be abandoned.

IV. THE SWEET TASTE RECEPTOR

A. Molecular biology of taste receptors

Indirect studies that tried to map the sweet taste active site or to design idealized sweeteners *implied* the existence of specific sweet taste receptors, but it was only in the last few years that likely candidate receptors were

identified, expressed, and characterized. During the 1990s, the efforts devoted to the identification of the molecular components of taste transduction, using molecular biological methods, yielded a large number of proteins potentially involved sensory transduction mechanisms (Kinnamon, 2000). The first proteins identified in taste-receptor cells were components of the G-protein oligomers, that is, of the complex involved in signal transduction of many receptors, in particular α-gustducin (McLaughlin *et al.*, 1992) and α-transducin (McLaughlin *et al.*, 1994). From these studies, it was clear that the receptor most likely belonged to a class of GPCRs. However, although a number of candidate taste-GPCRs were proposed (Abe *et al.*, 1993; Hoon *et al.*, 1999; Matsuoka *et al.*, 1993), it was difficult to establish their functional significance unequivocally (Lindemann, 1999). The first taste-specific GPCR cloned and characterized with respect to its ligand was that of the umami taste, that is the taste receptor for monosodium glutamate, a key ingredient of oriental food (Chaudhari *et al.*, 2000). Soon after, Chandrashekar *et al.* (2000) reported the characterization of a large family of putative mammalian bitter taste receptors (T2Rs) and Matsunami *et al.* (2000) reported the identification of a family of candidate taste receptors (TRBs).

At the beginning of 2001, T1R3, the first putative sweet receptor, was finally identified (Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Li *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001). The approaches followed by these groups are well illustrated by Montmayeur *et al.* (2001). Several biochemical and electrophysiological studies had indicated that the detection of sweet, bitter, and umami taste transduction involved GPCRs (Gilbertson *et al.*, 2000). One of the genetic loci that control sensitivity to bitter or sweet in mouse or human compounds, the *Sac* locus, governs the sensitivity of mice to certain sweet tastants, including sucrose and saccharin (Bachmanov *et al.*, 1997; Blizard *et al.*, 1999; Capeless and Whitney, 1995; Fuller, 1974; Lush, 1989; Lush *et al.*, 1995). Montmayeur *et al.* (2001) followed an approach similar to that had located some T2r genes at or near genetic loci that control sensitivity to bitter taste (Adler *et al.*, 2000; Matsunami *et al.*, 2000). Searching the syntenic region of the human genome for genes encoding GPCRs, they identified T1R3, a gene encoding a GPCR that is expressed in a subset of taste cells in mouse, and found allelic differences in Sac taster versus nontaster strains that could result in differences in Sac phenotype. In addition, Montmayeur *et al.* (2001) found that *in situ* hybridization studies show that T1R3 is expressed in the same taste cells as T1R2, a related receptor, raising the possibility that the two receptors function as heterodimers or that these cells recognize more than one ligand. However, the common belief was that the sweet taste receptor was a (T1R3) homodimer like most metabotropic, or class C, GPCRs. Class C includes several glutamate receptors, sweet and umami (monosodium glutamate) taste receptors, the Ca^{2+}-sensing receptor, the γ-aminobutyric acid type B receptor, and pheromone receptors (Pin *et al.*, 2003). As all GCPRs, these

receptors have a 7TM, but in addition they have a large extracellular domain, called VFTD, containing the active site for typical ligands, and a cysteine-rich domain. As mentioned above, several groups (Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Li *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001) hypothesized almost at the same time that T1Rs (particularly T1R3 that corresponds to the *Sac* gene) were likely candidates for the sweet receptor. In analogy with other C receptors, it was assumed that each member of this family would form a homodimer in its active form. Only a few months later, Li *et al.* (2002) demonstrated that only heterodimer T1R2–T1R3 can function as a sweet receptor.

The very likely presence in the sweet taste receptor of cavities similar to those hosting Glu in mGluR1, a metabotropic glutamate receptor of known structure (Kunishima *et al.*, 2000), tells us that the sweet taste of small molecular weight sweeteners can certainly be accounted for, even if the details will remain in part obscure, at least till a receptor structure with better resolution than homology models will be available. Can the taste of sweet proteins be also explained by the knowledge of the receptor? There is no obvious answer. Let us first examine possible receptor models in detail.

B. Computer-generated models of the sweet taste receptor

How can the potencies of sweet molecules span a range of five orders of magnitude? How can molecules as large as proteins interact with the same receptor as small sweeteners? Precise answers to these difficult questions would require solving the structure of complexes of the sweet receptor with representative sweet molecules or, at least, solving the structure of the receptor alone. In the meantime, crucial information was gained from modeling studies of the T1R2–T1R3 receptor. In particular, the similarity of the sweet taste receptor to mGluR1, one of the metabotropic glutamate receptors, hinted at the possible coexistence of different mechanisms for the two classes of molecules and prompted Temussi (2002) to propose the so-called ''wedge model'' for proteins.

The first likely sweet taste receptor was a protein (dubbed T1R3) whose sequence has sufficient homology to several metabotropic GPCR. The sequence of T1R3 bears significant homology to several other metabotropic receptors, in particular it is 20% identical to that of mGluR1. It is a very happy coincidence that, at the time of the discovery of the sweet receptor, the structure of the N-terminal domain of mGluR1 had just been determined (Kunishima *et al.*, 2000). The knowledge of the structure of the N-terminal domain of mGluR1 allowed homology model building. Similarly to mGluR1, that is a homodimer of a single sequence, the first homology model of the sweet receptor was built as a homodimer of two

T1R3 chains (Max *et al.*, 2001). On the basis of the dimeric nature of their model, these authors postulated that taster to nontaster substitutions could affect N-linked glycosylation at N58, thus precluding correct dimerization. Almost simultaneously, another homodimeric model was used to show that the active site of T1R3 can consistently host three very sweet small molecular weight molecules (Walters, 2002). When Li *et al.* (2002) demonstrated that the actual taste receptor contains two similar but not identical proteins (dubbed T1R2 and T1R3) and that only the heterodimer T1R2–T1R3 can function as a sweet receptor for all classes of sweet molecules, it was necessary to revise homology models. The only possible template remains the structure of mGluR1, but it is necessary to build more than a single homology model since Kunishima *et al.* (2000) have shown that the extracellular N-terminal domain of mGluR1 exists in three different forms: one complexed with two molecules of glutamate and two ligand-free forms. Both the complexed receptor (Protein Data Base entry 1ewk.pdb) and the uncomplexed free form II (Protein Data Base entry 1ewv.pdb) can be called open–closed conformations and correspond to the active state of the receptor, whereas the other ligand-free form (Protein Data Base entry 1ewt.pdb) is in an open–open conformation and corresponds to a resting state of the receptor. Combining two sequences (T1R2 and T1R3) with two conformations amounts to four possible heterodimers. The first heterodimeric T1R2–T1R3 model, based on the mouse sequences and built using the complexed form as the template (1ewk.pdb), corresponded to only one of the two possible active models (Temussi, 2002). Morini *et al.* (2005) built all models of the human sequences and used them to identify all possible sites of interaction. Out of four T1R2–T1R3 heterodimers, two are inactive, ligand-free, open–open forms, and two are active, complexed, closed–open forms. If we model T1R2 on chain A and T1R3 on chain B of the 1ewt template, we get the two inactive dimers: Roo_AB (where R stands for resting, oo for open–open, and AB refer to the two chains of mGluR1), and Roo_BA when we model T1R3 on chain A and T1R2 on chain B of the 1ewt template. If we model T1R2 on chain A and T1R3 on chain B of 1ewk, we get Aoc_AB (where A stands for resting, oc for open–closed, and AB refer to the two chains of mGluR1), whereas when we model T1R3 on chain A and T1R2 on chain B of 1ewk, we get Aoc_BA, the two possible active dimers.

V. MECHANISMS OF INTERACTION

A. The "wedge model" mechanism for sweet proteins

The similarity between the sequences of the two chains of the T1R2–T1R3 receptor and that of the single chain of the homodimer of mGluR1 suggests that the two receptors might have the same general features,

particularly with respect to the mechanism of activation. If the T1R2–T1R3 receptor behaves like the mGluR1, it should also exist as a mixture of three forms: a complexed form, activated by low molecular weight sweeteners, a resting ligand-free form I, and ligand-free form II, with a structure nearly identical to that of the ''active,'' complexed form. As shown by Figure 7, the resting (Roo) and active (Aoc) forms are in equilibrium even in the absence of their ligands, that is glutamate for mGluR1 and sweeteners for the sweet taste receptor. In analogy to mGluR1, the ''normal'' way to activate the receptor, shown by Figure 7A, should be the binding of a small molecular weight sweetener that transforms resting free form I into the active complexed form.

However, the equilibrium between form I and form II can also be shifted if we can stabilize form II in another way. Figure 7B illustrates how stabilization can be achieved by external binding of a macromolecule on a secondary binding site on the surface of the receptor. This mechanism termed the ''wedge model'' was proposed on the basis of docking calculations of brazzein, monellin, and thaumatin to the Aoc conformation of a model receptor, built using the mouse sequences of T1R2–T1R3 (Temussi, 2002). This mechanism was soon supported by experimental and theoretical work. G16A-MNEI is a structural mutant that shows a reduction of one order of magnitude in sweetness with respect to its parent protein, MNEI, a single-chain monellin. This data was difficult to interpret since the mutation does not affect any part of the surface of MNEI but only, and slightly, its hydrophobic core. Comparison of the structures of wild-type monellin and its G16A mutant showed that

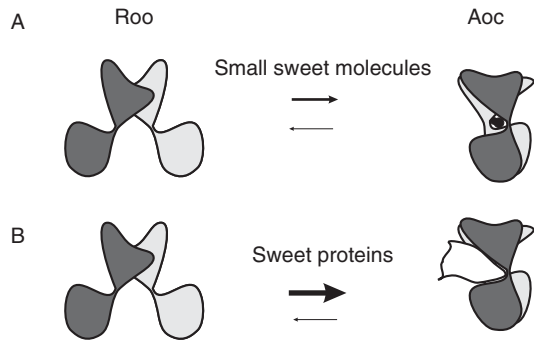

FIGURE 7 Modes of binding of small sweeteners and sweet proteins. (A) Binding of small molecular weight ligands transforms resting (open, open) free form I (Roo, left) into the complexed form (Aoc, right), identical to active (open, closed) free form II. Small ligands in the two cavities of Aoc are shown as black balls. (B) Free form II, stabilized by protein complexation (active form, right), activates long lasting signal transduction. The "wedge" protein is shown in white.

the mutation does not affect the structure of potential glucophores but produces a distortion of the surface owing to the partial relative displacement of elements of secondary structure. These results support the hypothesis that the mechanism of interaction of sweet tasting proteins involves a large part of the sweet protein surface, as proposed in the wedge model (Spadaccini *et al.*, 2003).

The original formulation of the wedge model was based on the homology model of only one of the possible conformations of the receptor, but it has been substantiated by exhaustive modeling using the human sequences (Morini *et al.*, 2005). After building all possible resting and active models, Morini *et al.* (2005) used them for docking calculations with experimental structures of brazzein, MNEI, and thaumatin, the sweet proteins of known 3D structure. The results for all three proteins are consistent with those found with the mouse receptor (Temussi, 2002). As a negative check Morini *et al.* (2005) calculated also the docking of sweet proteins to the inactive open–open Roo_AB and Roo_BA models. Figure 8 shows the interaction of MNEI with Aoc_AB, Aoc_BA, Roo_AB, and Roo_BA. All 10 molecules of MNEI are found, oriented in a similar albeit not identical way, in the same spot of the surface of the human Aoc_AB form, mainly belonging to the T1R3(B) chain. Efficient binding is assured mainly by shape and charge complementarity. As shown in the right-hand side of the upper 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. As shown in the lower panel, in the case of the inactive open–open Roo_AB and Roo_BA models, the molecules of MNEI bind to a very large area of the receptor, without any apparent regularity.

The wedge model received a further validation by the design of a single-chain monellin sweeter than wild-type monellin (Esposito *et al.*, 2006). According to the wedge model, the mechanism of interaction between a sweet protein and the sweet receptor hints at a largely positive nature of the surface of interaction of the protein with the receptor. Accordingly, three neutral residues, Met^{42}, Tyr^{63}, and Tyr^{65}, comprised in a critical area of interaction hosting key residues according to previous mutagenesis studies, were changed into either acidic or basic residues. The expectation that, in order to be consistent with the wedge model, all changes of neutral residues to acidic ones ought to be more detrimental for sweetness than the corresponding ones into basic residues was met by all mutants. In addition, careful selection of the best point mutation, that is Tyr^{65}, led to the discovery of a mutant, Y65R, that is even sweeter than MNEI itself. Tyr^{65} is at the center of the main interacting area predicted by the wedge model and close comparison of the surface electrostatic potential of MNEI and Y65R reveals that, indeed, the only change in going from MNEI to the mutant is an increase of the positive area of the interface at

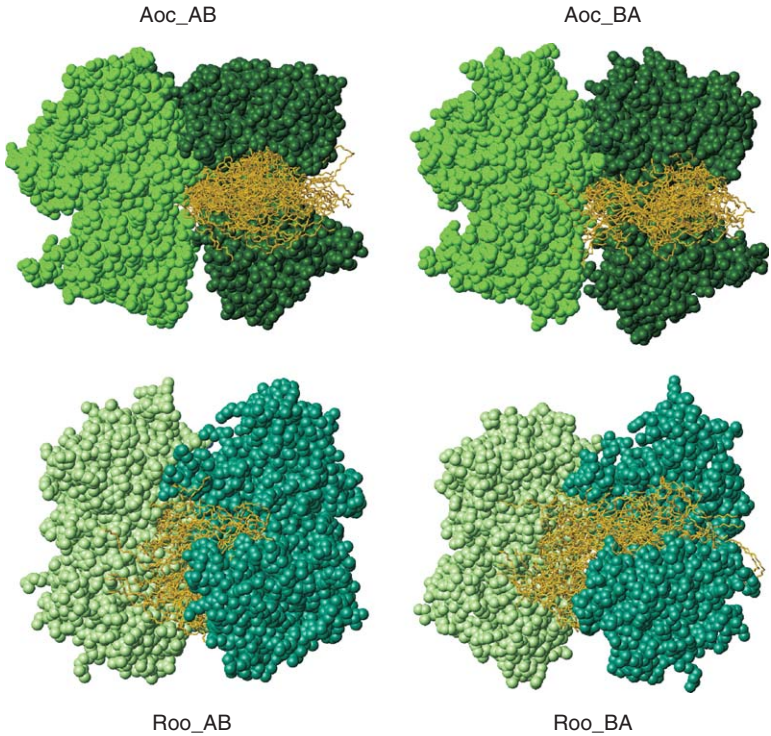

FIGURE 8 The four possible models of the human sweet receptor with bound MNEI molecules. Active models (Aoc_AB and Aoc_BA, upper panel): atoms of the T1R2 sequence are shown in green, whereas those of the T1R3 sequence are shown in dark green. Models of MNEI are represented as gold neon backbone bonds. Resting models (Roo_AB and Roo_BA, lower panel): atoms of the T1R2 sequence are shown in light green, whereas those of the T1R3 sequence are shown in blue-green. Models of MNEI are represented as gold neon backbone bonds. The models were generated by MOLMOL (Koradi *et al.*, 1996).

the expense of a neutral patch, without significant changes in the alternation of positive and negative areas on the crucial region of interaction (Esposito *et al.*, 2006).

B. Interaction of small sweeteners with the sweet receptor

Like their template, the active conformations of the sweet taste receptor contain two cavities that can host ligands: a smaller one in the closed protomer and a larger one in the open protomer. It is natural to assume that most sweeteners interact with the sweet receptor via optimal fitting of one or both these cavities, but it is difficult to ascertain whether the

precise shape of the closed cavity is influenced by the binding. In fact, Gokulan *et al.* (2005) warned that the considerable conformational changes implied by the binding mechanism of VFTD domains might prevent accurate modeling of the active sites of the receptor. However, Morini *et al.* (2005) by exploiting the conservation of key residues and the similarity of some sweetener with glutamate were able to show that all active sites of the two active protomers can actually be used to account for the sweetening power of a very large number of sweet molecules. The sweet receptor can be activated by simple hydrophobic amino acids, notably D-tryptophan and synthetic dipeptides, generally derived from aspartame. These molecules have the same amino acidic moiety typical of all α-amino acids, including glutamate, that is an amino group adjacent to a carboxyl group. It is fair to assume that reliable active sites in T1R2–T1R3 receptor models should retain all the features necessary to bind this moiety. In other words, residues lining the wall of the part of the cavity that binds amino acidic moieties should be highly conserved in going from mGluR1 to T1R2–T1R3. In fact, as pointed out by Morini *et al.* (2005) for all their models, residues directly interacting with the α-amino acid moiety in mGluR1 are well conserved not only in T1R2–T1R3 and in other mGluRs but also in the sequences of other families of metabotropic GPCRs (Pin *et al.*, 2003). On the other hand, residues of the other part of the cavity are expected to be more variable, since in the sweet taste receptor this part of the active site ought to accommodate molecular fragments of different size and chemical constitution. In addition, residues corresponding to those that bind the side chain of glutamate in mGluR1 should possibly turn from polar to hydrophobic in T1R2–T1R3 to accommodate molecular moieties more similar to that of tryptophan rather than that of glutamate. In agreement with these ideas, in the alignments corresponding to the four models, Morini *et al.* (2005) found that residues binding the glutamate side chain in mGluR1 are invariably changed to less polar or uncharged residues (Morini and Temussi, 2005).

mGluR1 can bind two glutamate molecules: both closed (MOL1) and open (MOL2) protomers bind glutamate at active sites lined by the interfaces of subdomains LB1 and LB2 (Kunishima *et al.*, 2000; Tsuchiya *et al.*, 2002), with the only difference that, in the open protomer, the LB2 interface is not used for binding. Figure 9 illustrates these two modes of binding. Constitution and size of sweeteners can be so diverse that we cannot be sure *a priori* that in the sweet taste receptor both ligand-binding sites are available for sweet ligands. Even simple visual inspection of the models reveals that active sites of closed protomers, that is T1R2(A) and T1R3(A), are so small that they cannot possibly host some of the larger synthetic sweeteners (Morini *et al.*, 2005). 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. Figure 9B illustrates the

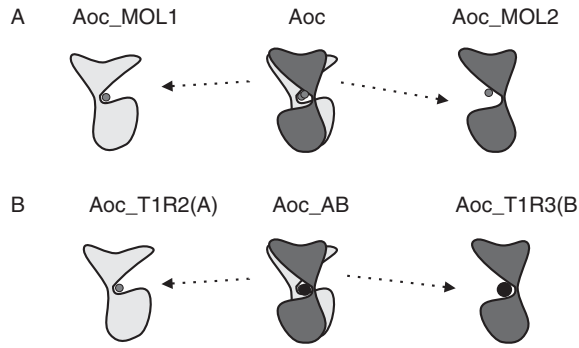

FIGURE 9 Mode of binding of ligands in the active sites of the protomers of metabotropic receptors. (A) Binding of two molecules of glutamate in the two protomers of mGluR1. The glutamate that binds to the closed protomer (MOL1) of the active form Aoc uses residues from both LB1 and LB2; the one that binds to the open protomer (MOL2) uses only residues from LB2. The two molecules of glutamate are represented as black balls of equal size. (B) Binding of two sweeteners of different dimensions to the active form Aoc_AB of T1R2–T1R3. The smaller sweetener, represented as a small gray ball, binds to the closed T1R2(A) site; the larger one, represented as a larger black ball, binds to the open T1R3(B) site using both LB1 and LB2 lobes.

binding of a small sweetener in site T1R2(A) and that of a larger (non-proteic) one in site T1R3(B). In order to probe semiquantitatively the fit of sweeteners in the active sites of the models, Morini *et al.* (2005) chose a large number of sweet molecules belonging to different families, including sugars, peptides, and supersweeteners. Their fit was evaluated by means of PrGen (Vedani *et al.*, 1995), a program that allows the comparison of calculated binding affinity for ligands with the experimental sweetness. In the binding sites of the open protomers, it is possible to fit a large number of representative sweet compounds. In T1R2(B) and T1R3(B), 16 and 22 molecules, respectively, were used as training sets to derive the model and then to predict binding energy of other sets. Interestingly, although PrGen allows changes in the relative positions of the residues defining the site, the final active sites showed only minor changes with respect to those of the original homology models.

Figure 10 shows the agreement between predicted and experimental free energies of binding for the open sites of both possible active conformations: T1R2(B) and T1R3(B). Open symbols refer to compounds used in training sets, whereas filled symbols refer to compounds of test sets. The compounds are those reported in the corresponding tables of Morini *et al.* (2005). Their relative sweetening power, referred to sucrose, are comprised between 200,000 (corresponding to a ΔG of -13.8 kcal/mol) of sucrononic acid and 0.26 (corresponding to a ΔG of -5.9 kcal/mol) of D-glucose. On the other hand, it proved very difficult or impossible to

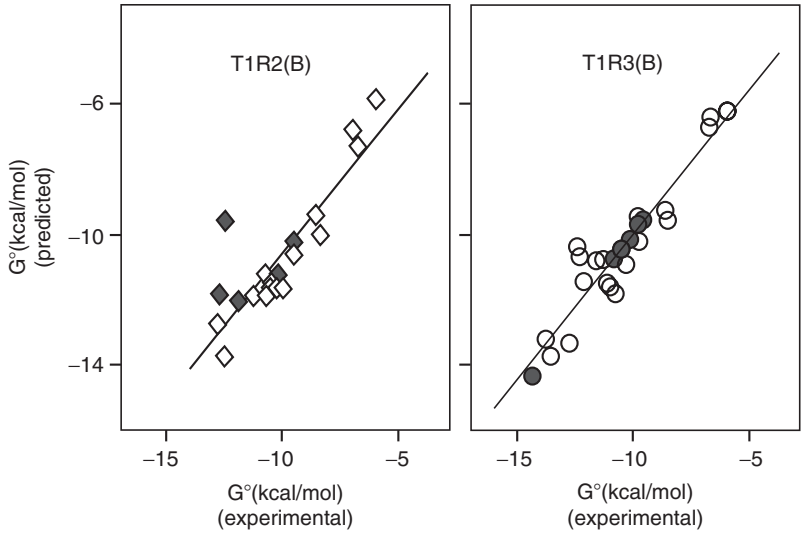

FIGURE 10 Correlation between calculated experimental and experimental binding affinities of sweet compounds inserted in the open sites of active conformations: T1R2 (B) and T1R3(B). Open diamonds, in the left-hand side panel, represent compounds used in the training set, whereas black diamonds represent compounds of the test set. Open circles, in the right-hand side panel, represent compounds used in the training set, whereas black circles represent compounds of the test set.

dock most of the larger ligands in the binding sites of closed protomers of the active closed–open form. In fact, it was possible to fit only four compounds in T1R2(A) with good correlation between experimental and calculated binding affinity; while in T1R3(A), although it was possible to dock the same four compounds, the correlation between experimental and calculated binding affinity was poor. Figure 11 shows the contact surface representation of the two sites of Aoc_AB with typical sweet molecules inside. The fit of saccharine in the T1R2(A) site is shown in the right-hand side of the figure and the fit of sucrononic acid (one of the guanidinium supersweeteners, 200,000 times sweeter than sucrose) in the T1R3(B) site in the left-hand side. It is easy to appreciate that large sweeteners, like sucrononic acid, can only enter the wider B sites.

A special case is represented by flexible compounds, such as aspartame, which can exist in several conformations. For consistency with the PrGen calculations of Bassoli *et al.* (2002a), the first choice for the conformation of aspartame used by Morini *et al.* (2005) to derive the models was the folded conformation found in the crystal structure (Hatada *et al.*, 1985) and used as paradigmatic in one of the indirect models (Yamazaki *et al.*, 1994). However, our indirect model (Kamphuis *et al.*, 1992) is consistent

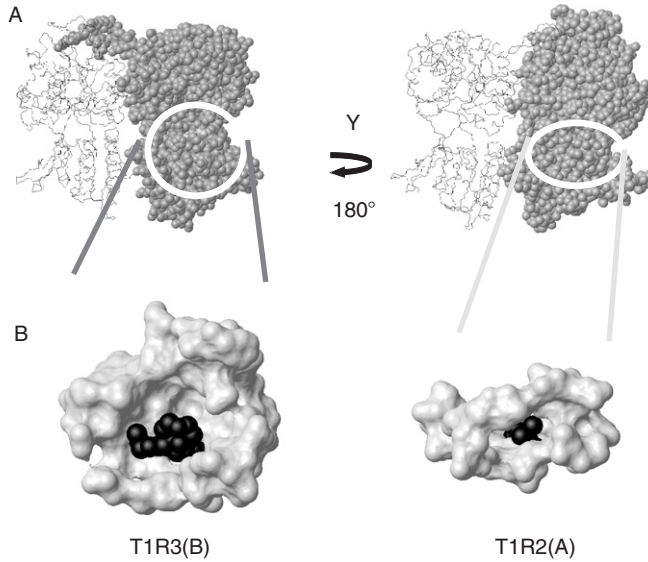

FIGURE 11 Contact surface representation of the two sites of Aoc_AB with typical sweetener molecules inside. (A) Individual protomers, T1R2(A) and T1R3(B), are represented with all heavy atoms colored in gray. The other protomer is represented by only by a line along the backbone. (B) The fit of saccharine in the T1R2(A) site is shown on the right-hand side and the fit of sucrononic acid (one of the guanidinium supersweeteners, 200,000 times sweeter than sucrose) on the T1R3(B) site is shown in the left-hand side. The two protomers, T1R2(A) and T1R3(B), are as gray contact surfaces; the sweet molecules are represented with black atoms. All models were generated by Molmol (Koradi *et al.*, 1996).

with an extended form of aspartame, corresponding to the crystal structure of [(L-α-Me)Phe^2] aspartame (Polinelli *et al.*, 1992). Accordingly, Morini *et al.* (2005) checked the possibility of using [(L-α-Me)Phe^2] aspartame among the test compounds in an alternative training set in lieu of folded aspartame. Both calculations showed that the folded conformation can fit both open and closed sites whereas the extended conformation of aspartame can be accommodated only in the open ''B'' cavities.

C. Multiple binding sites

Early indirect models of the active site of the sweet taste receptor tried to account for the largest possible number of sweet compounds, but it was generally believed that some classes of sweet compounds, notably sweet proteins, might interact with different receptors altogether. Can we reconcile old views with the picture emerging from molecular biology and

homology modeling? The answer is that rather than multiple receptors there are, apparently, multiple sites on the single sweet taste receptor.

The consensus feature of all indirect models was the presence of AH–B groups, in which the AH group is a hydrogen bond donor and the B group is a hydrogen bond acceptor. This feature is indeed present in both sites of the active forms of the T1R2–T1R3 receptor, as derived from homology modeling, using mGluR1 as template (Morini *et al.*, 2005). The cavity that accepts sweet proteins (Morini and Temussi, 2005; Morini *et al.*, 2005; Temussi, 2002) can be considered as a third independent active site. These three sites account for most observations on sweet molecules, including elusive concepts like synergy (*vide infra*). However, owing to the complexity of sweet compounds, it cannot be excluded that additional binding sites exist elsewhere in the heterodimeric sweet taste receptor. Two major candidates for additional sites are those proposed by Xu *et al.* (2004) for a site accepting both agonist cyclamate and the sweet taste inhibitor lactisole and by Jiang *et al.* (2004) for sweet proteins.

Agonist specificity between human and rat allows rational design of specific chimeras of the receptor. Using this technique, Xu *et al.* (2004) mapped binding sites on the T1R2–T1R3 receptor by generating chimeras between human and rat T1Rs genes, with junctions at residues positioned at the borders of estimated transmembrane domains. These authors found that when the N-terminal domain of human T1R2 is replaced with the corresponding sequence of rat T1R2, the responses of the receptor to aspartame and neotame are abolished, showing that the N-terminal domain of human T1R2 is necessary to recognize typical sweeteners such as aspartame and neotame. However, when they replaced either the N-terminal or the C-terminal domain of human T1R2 with rat sequence, the response to cyclamate was not affected. Apparently, the transmembrane domain of human T1R3, when coexpressed with T1R2, is sufficient to recognize cyclamate. Similarly, they showed that lactisole, a human-specific sweet taste inhibitor, like cyclamate requires the human T1R3 C-terminal domain to inhibit the receptor's response to typical sweet agonists. These observations were confirmed by Jiang *et al.* (2005). In addition, these authors, by means of alanine-scanning mutagenesis, identified six residues of the transmembrane domain specifically involved in the recognition of cyclamate. Contemporaneously, Winnig *et al.* (2005) found that a single residue, that is valine 738 on the fifth helix of the transmembrane domain of T1R3, is responsible for the lactisole insensitivity of rat sweet taste receptor. These findings do not rule out the possibility that cyclamate is recognized also by one of the two cavities of the VFT domain but suggest convincingly that the transmembrane domain of the T1R3 protomer of the sweet receptor hosts a genuine fourth site. Figure 12 summarizes all the sites described so far for T1R2–T1R3.

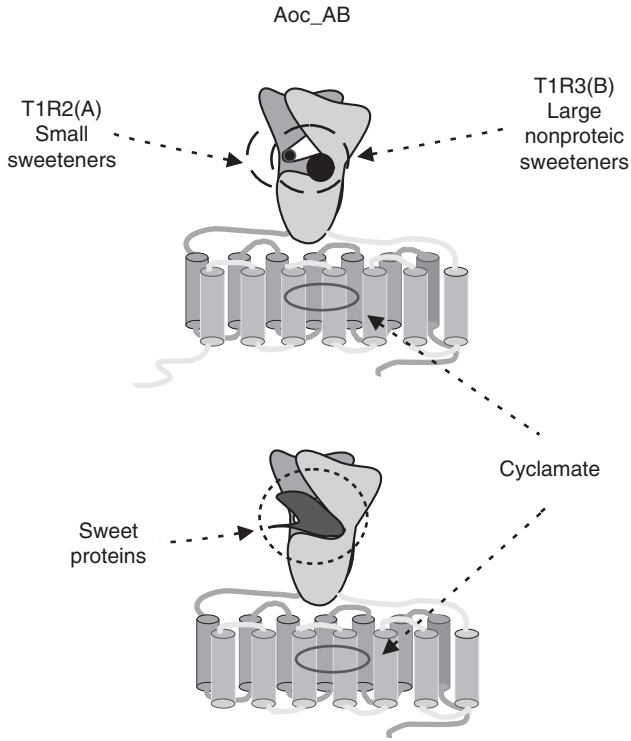

FIGURE 12 Binding sites for the T1R2–T1R3 receptor: two active sites of different dimensions for smaller and larger (non-proteic) sweeteners, the cyclamate site in the TM helices domain, and the external "wedge" site for proteins.

Chimera studies led Jiang *et al.* (2004) to propose yet another site for sweet proteins. Starting from the observation that human T1R2–T1R3 responds to brazzein whereas the chimera of human T1R2–mouse T1R3 does not, these authors argued that critical residues for this difference could be located in the cysteine-rich region of T1R3. Using human/mouse chimeras of T1R3 paired with hT1R2, they determined that, in particular, residues 536–545 of the cysteine-rich region of human T1R3 were required for responsiveness to brazzein. It is difficult at the moment to ascertain whether this is a genuine additional fifth active site since the cysteine-rich domain is a critical region that cannot be easily changed without affecting the global response of the receptor. It is fair to hypothesize that the cysteine-rich region has a crucial structural role in the conformational transitions of the sweet receptor as shown for other metabotropic receptors. For instance, in the case of the human Ca^{2+} receptor, Hu *et al.* (2000) have shown that the hCaR cysteine-rich domain plays a critical role in signal transfer from VFT to 7TM of the hCaR and for sequence specificity

in communication. Any mutation in this region may simply undermine the structural integrity of the sweet receptor. On the other hand, the wedge mechanism (Morini and Temussi, 2005; Morini *et al.*, 2005; Temussi, 2002) would provide a simple explanation for the critical role played by T1R3 in the interaction with sweet proteins without invoking an additional site.

Synergy between different sweeteners is a peculiar phenomenon of sweetness that has for a long time escaped a detailed interpretation at molecular level (DuBois, 2004). Sweetness synergy has been observed in several combinations of sweeteners. It had long been known that aspartame and cyclamate are synergistic in sensory experiments (Schiffman *et al.*, 1995). The modeling of all possible conformations based on the human T1R2 and T1R3 sequences (Morini *et al.*, 2005) suggested, among other aspects of the sweet taste, the first possible interpretation of this phenomenon. When exploring the four active sites of the heterodimers formed by human T1R2 and T1R3 sequences, using the A (closed) and B (open) chains of the ligand-binding domain of the mGluR1 glutamate receptor, it was immediately clear that both ''type A sites'' are definitely too small to host the bigger non-proteic sweeteners, but they can 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 affinity. The starting point to understand synergy is that at least three of the four compounds that, in the docking study of Morini *et al.* (2005), were able to bind to ''type A sites,'' aspartame, saccharin, alitame, and cyclamate, are known to be synergistic with other sweet compounds (DuBois, 2004), suggesting that, although the binding in a single subunit is sufficient for receptor activation, the additional binding of a ligand in the second subunit increases the response. The crucial point is that synergy can only be observed either with two small sweeteners or with a small and a large one, but never with two large non-proteic sweeteners. It is easy to see from Figure 9B that only the combinations small–small and small–large are consistent with the Aoc conformation of the receptor whereas two large sweeteners could stabilize the inactive Roo conformation.

VI. BEYOND THE SWEET RECEPTOR

Our understanding of the structure–activity relationship of sweet molecules increases enormously with the discovery of the sweet taste receptor and with the subsequent availability of reliable homology models. Some points need a more detailed explanation, but we should probably wait for

detailed 3D structures of the receptor and its complexes with different classes of sweeteners before we get an answer.

For instance, it is not fully understood whether the T1R3 protomer has an active role in accepting large synthetic sweeteners and proteins or just a role in transmitting information to the transmembrane domain. Specificity of the T1R2–T1R3 heterodimer is assured by T1R2, since the companion T1R1–T1R3 receptor is specific for umami compounds. The docking calculations of Morini *et al.* (2005) are in favor of the Aoc_AB active form of the receptor, that is, with T1R2(A) and T1R3(B), but their data are not conclusive. Another interesting issue that has not yet received an explanation is the mechanism of action of substances that can suppress sweet taste, in particular gymnemic acid. However, it is fair to say that the main aspects of the interaction of sweet molecules with their receptor have been elucidated. The most unexpected findings are probably the explanations of phenomena such as sweeteners synergy and the taste of sweet proteins.

As mentioned in Section I, an important motivation to study structure–activity relationships of sweet molecules is the possibility to design new sweeteners. In principle, detailed homology models of the two active sites of the active form of the human sweet receptor could indeed suggest key modifications of existing sweeteners or even entirely new scaffolds. In practice, however, the resolution of the models is not sufficient for accurate design; thus, also this problem must wait for a detailed solid state structure of the receptor. Besides, the actual use of new synthetic molecules is problematic since it requires long and costly tests before they can be introduced in the market. So far, sweet proteins have not been used as sweeteners, but they are very promising. The elucidation of their mode of action may open the way to modifications of existing proteins and even to the *de novo* design of new sweet proteins.

ACKNOWLEDGMENTS

I wish to thank Annalisa Pastore (NIMR) for many critical readings of the chapter and sweet suggestions. Financial support from MIUR (FIRB 2003) is gratefully acknowledged.

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