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Isothermal Titration Calorimetry Study of the Interaction of Sweeteners with Fullerenols as an Artificial Sweet Taste Receptor Model

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A fullerenol-based synthetic sweetness receptor model, consisting of polyhydroxy groups for potential hydrogen bond donor along with a spherical hydrophobic center, was proposed according to the widely accepted sweetness hypothesis. An isothermal titration calorimetry (ITC) technique was used to study mimetic interaction of this sweet receptor model with a series of sweeteners having increasing sweetness intensity. The results showed that ITC is an effective method to provide thorough and precise characterization of the energies of molecular complex formation. Binding of all of the studied sweeteners with fullerenols was found through two sets of site models. More heat was released from sweeter synthetic compounds binding with fullerenols than from less sweet carbohydrates. The results imply that hydrogen bond formation is necessary for the sweeteners to bind to the fullerenol receptor in the first stage, whereas hydrophobic effect and conformation changes that lead to favorable entropy changes occur in most cases. The preliminary results of this study help to cover the lack of information about the thermodynamic basis of understanding of the initiation of the sweet sensation. It also adds complementary physicochemical measurements available for comparison with the sweetness hypothesis. On the other hand, a correlation between the thermodynamic parameters and sweetness intensity has been made as well, which exhibits potential as a useful tool in sensory analysis.

KEYWORDS: Fullerenols; thermodynamics; ITC; sweetener; sweetness intensity

INTRODUCTION

Sweet sensation is the result of a cascade of complex biological, physiological, and chemical events starting when the sweet molecule, which is carried by the saliva, reaches the sweet receptors inside the lingual epithelium. Although sweet sensation is exhibited by diverse classes of natural or chemical compounds, they all display the sweetness response. How these events occur is not yet entirely elucidated. Shallenberger and Acree (*1*) have proposed their pioneering hypothesis disclosing a correlation between sweet taste and different compounds regardless of their chemical class, which depends on the existence of an AH−B system capable of establishing two hydrogen bonds with a corresponding B−AH unit on the receptor along with the distance constraint between the donor and acceptor proton of 2.5−4.0 Å. Other attempts to improve the model have included adding a hydrophobic center (*2*) and determining the minimum, optimum, and maximum distance between the hydrophobic center and the hydrophilic AH−B moiety (*3*), which seems to be essential in defining the quality of taste in high-potency sweeteners. Tinti and Nofre (*4*) brought forward a culminating eight-sited model, attempting to add more sites to make it more applicable to all sweet compounds. As most workers accept that hydrogen bonding is the cornerstone of the sweetness mechanism, this three-body tenet has been used as a working hypothesis for structure−activity relationships and molecular modeling studies. Another substantial breakthrough is the emergence of a correlation between some aspect of the solute−water interaction and taste quality. Birch and Drew's group (*5*) has found very significant differences in the water-structuring properties of the various groups in carbohydrates by the calculation of radial distribution functions using computer modeling studies. The results showed that radial distribution functions for oxygen, carbon, and hydrogen atoms are able to reveal quite different and distinctive behavior of the solvent around the atom types and in particular can distinguish between hydrophilic and hydrophobic hydration effects, which implies that the water-structuring properties of carbohydrates are related to the sweetness response.

Despite the interest in elucidation of the sweetness mechanism and some structural relationship between the sweetness intensity and the structure of sweet compounds, there are no experimental data on the conformation of the isolated taste receptor or its complexes with sweeteners in the crystalline state. This shortcoming has been partially solved by a theoretical approach,

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which provided us with some details about the sweetener−receptor model interaction. Suami and Hough (*6*) have postulated a theoretical *o*-helical proteinaceous receptor model that has L-asparaginyl and L-prolyl residues at the N-terminus and adjacent sites, respectively, along with L-leucyl residues arranged in a right-handed *o*-helix. The host−guest relationship between kinds of sweeteners and the theoretical receptor model indicated that the α-helical protein receptor model may be very close to the dimensions of the active site of the native receptor (*7*). Morini et al. (*8*) have described the homology modeling of a sweet taste receptor, a heterodimeric G protein coupled receptor (GPCR) protein, formed by the T1R2 and T1R3 subunits, recognizing several sweet compounds including carbohydrates, amino acids, peptides, proteins, and synthetic sweeteners. This model accounts for many experimental observations on the tastes of sweeteners, including sweetness synergy, and can help in the design of new sweeteners. Such molecular precision seems to be more than coincidental. However, detailed interaction between the receptors and target sweeteners needs to be further elucidated experimentally.

As biomimetic chemistry provides a valuable tool for understanding biological processes and for the creation of functional synthetic systems, the introduction of a synthetic receptor to interact with the sweeteners can be an effective way to study the sweet sensation before the structure of the proteins of the taste receptor sites is determined exactly. A crucial issue among many possible questions is the comprehension of the structural and functional requirements for the effective and selective recognition of a specific sweetener. In nature, molecular recognition of sweetener relies on weak non-covalent interactions and the required affinity and selectivity achieved by polyvalency through a concerted array of H bonds involving multiple structural units. In synthetic receptors, such a combination is difficult to achieve by design, but by capitalizing on non-covalent interactions, encouraging results have been obtained in several cases. For example, peptide-functionalized nanoparticles have been constructed to function as artificial proteins and enzymes (*9*), glyconanoparticles have been used as useful models of cell adhesion (*10*), a variety of functionalized particles have been used for recognition in aqueous media (*11*, *12*), and gold nanoparticles functionalized with an L-amino acid-terminated monolayer have provided an effective platform for the recognition of protein surfaces (*13*).

Recently, we have carried out research on preference development of sweetness sensation (*14*). The ongoing interest in sweetness sense expression and the inspiring results of biomimetic research motivate us to investigate the molecular interactions between sweeteners and taste receptor model experimentally. As the initial work of constructing artificial sweetness receptors, we present herein an experimental taste receptor model based on polyhydroxylated fullerenes (fullerenols). In molecular recognition processes, structure underpins function and, in due course, the process is complemented with the thermodynamic certainty in which complex formation depends on the energies which make it a spontaneous event. As a consequence, the thorough and precise characterization of the energies of molecular complex formation, including its dependence on relevant environmental variables, is of great importance in molecular sciences research. To the best of our knowledge, comparatively little is known currently about the thermodynamic properties that govern the sweetener binding events. This deficiency limits our understanding of the molecular forces that dictate and control the affinity and specificity of binding. Fortunately, it has been reported that sensitive isothermal titration calorimetry (ITC) is the direct and usually preferred method to measure the heat released in the course of binding and interaction. The thermodynamic parameters attained by ITC can provide important insights into the underlying physical basis of sweetener−receptor interactions, which are essential for a better understanding of the kinetics and thermodynamics during sweetness chemoreception. The present study was initially undertaken with the aim of providing a thermodynamic basis of understanding the initiation of sweet sensation using a synthetic receptor model. On the other hand, as the sweetness intensity can be measured by panelist sensory evaluation (*15*) or by the development of smart taste sensors (*16*), we tried to correlate the thermodynamic parameters with sweetness intensity as well. Many thermodynamic observations can be rationalized in terms of the particular structural features of a complex, and it is hoped that, eventually, the correlation of thermodynamic data and sweetness intensity data from the systems will lead to an accurate quantitative model of binding thermodynamics in terms of sweetness intensity and can be used as a potential alternative tool for sweetness sensory analysis.

MATERIALS AND METHODS

Materials. Ten sweeteners with increasing sweetness intensity used in the present research include D-galactose, maltose, D-trehalose, D-glucose, sucrose, D-fructose, sodium cyclamate, acesulfame-K (AK), saccharin, and sucralose. They were all purchased from Sigma-Aldrich or Supelco. C_{60} was from Henan Yongxin Co. of China. Polyhydroxylated fullerene was synthesized according to the reported method (*17*). Distilled and deionized water was used for the preparation of all solutions.

Isothermal Titration Calorimetry. An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure enthalpies associated with sweetener−receptor interactions at 298 K. ITC measurements were carried out through an inverse titration model (sweeteners in the cell). This titration sequence has been modified and will be discussed later. Fullerenols in the syringe were 0.6 mM. The concentration of sweetener has been screened until a reasonable ITC profile was achieved along with the smallest chi-square (χ^2) value of the fitting final data. In a typical experiment, a sweetener solution was placed in the 1430 μL sample cell of the calorimeter, and fullerenol solution was loaded into the injection syringe. Both solutions were prepared in deionized water and were degassed before use. The reference cell was filled with deionized water. The first drop was set to 2 μL, and then fullerenol solution was titrated into the sample cell as a sequence of 28 injections of 10 μL aliquots. The duration of each injection was 20 s, and there was an interval of 240 s between successive injections to achieve complete equilibration. The solution in the titration cell was stirred at a speed of 307 revolutions min^{-1} throughout the experiment. Control experiments included the titration of fullerenols into water, water into sweetness, and water into water. The last two controls resulted in small and equal enthalpy changes for each successive injection of water and, therefore, were not further considered in the data analysis. To maximize the internal consistency of the data, all data on all sweeteners were generated at the same conditions.

Raw data were obtained as a plot of heating rate (μcal s^{-1}) against time (min). The raw data were then integrated to obtain a plot of observed enthalpy change per mole of injected fullerenol (ΔH, kcal mol^{-1}) against molar ratio (fullerenols/sweetener). Binding stoichiometries, enthalpies, and equilibrium association constants were determined by fitting the data to a two-site binding model with MicroCal Origin 7 using a nonlinear least-squares approach (Levenberg−Marquardt algorithm). All data show the average and standard deviation of three independent titrations.

RESULTS AND DISCUSSION

It is well-known that fullerene C_{60} has a rigid hydrophobic ball with a dominant geometrical constraint which can act as a

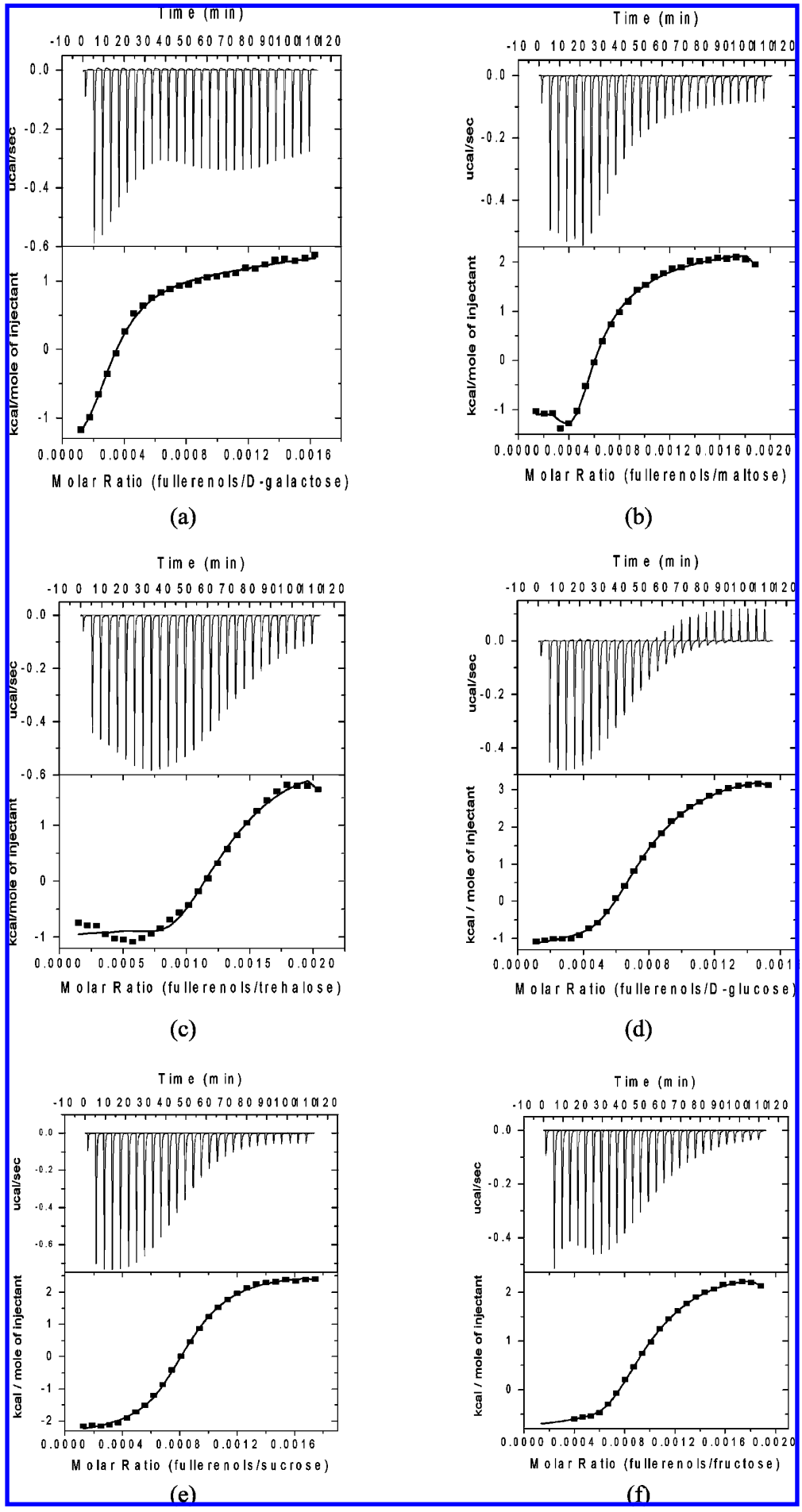
strong hydrophobic site. The polyhydroxylated C_{60} molecule provides enough three-dimensionally oriented hydroxyl groups for its interaction with sweeteners through hydrogen bond formation. Moreover, the solubility of polyhydroxylated fullerenes ensures that testing of the binding process can be carried out in water. This sweetness receptor consists of 18 or more hydroxyl groups for potential hydrogen bond donor along with a spherical hydrophobic center and was designed according to the widely accepted sweetness mechanism hypothesis. ITC can reveal the stoichiometry, enthalpy, free energy, and entropy changes that occur over the course of a reaction. ITC has been used mainly to quantify interactions in biochemical systems, but due to its high potentiality, it has been extended to different fields of molecular recognition (*18*). In the present research, the construction of this taste receptor is based on the accepted theory that all sweet compounds have in common a hydrophilic bipartite AH−B glycophore to form two hydrogen bonds to reciprocal AH and B units on the receptor and that the intensity of sweetness is determined by strategically placed hydrophobic areas of attraction on both the sweetener and the receptor. Therefore, the observed enthalpy changes that may arise as a result of changes in hydrogen bonding interactions can be determined by ITC.

Determination of the Titration Sequence and Fullerenol Concentration. Generally, when the ligand and macromolecule each have only one site for interaction with the other, then the system is symmetrical, and it does not matter which of the two is loaded into the cell or into the injection syringe in ITC measurement. The situation will be a little more complicated if the macromolecule has more than one site (even if there is only one set of sites). In the present research, the fullerenol is polyhydroxylated C_{60}, which bears 18 or more hydroxyl groups. It was expected to provide multiple sites necessary to bind the sweeteners according to the sweetness mechanism. The interaction of this biomimetic acceptor and sweeteners was proved to be binding in multiple sites by inverse titration sequence. When we put the macromolecule (fullerenols) into the cell and the ligand (sweeteners) in the syringe, the working line stays almost overlapped to the control titration line. After treatment with Origin 7.0 software, no clear and useful signals can be obtained (not shown). However, when we reverse the titration sequence, that is, the ligand is loaded into the cell and the macromolecule in the syringe, then the situation is different. The difference between the working line and control line is obvious, and after the subtraction of the control line, we can get clear ITC fitting curves. Moreover, in the reverse titration process, the heat releasing from the binding and interaction is more than that of regular titration, in which we can get useful information from the interaction of the fullerenols as a mimetic receptor with kinds of sweeteners. It is noteworthy that the different results obtained in a reverse titration process imply that multiple-site binding does exist during the interaction. Therefore, in the present research, all of the ITC measurements were conducted in this so-called reverse titration sequence.

Although easily performed, an ITC experiment requires great care in concentration determination and sample preparation. To further determine the appropriate concentration for the titration process, different concentrations of fullerenols were applied for the binding titration. The sucrose−fullerenol complex was used as a model system for fullerenol concentration determination. When 0.1 mM fullerenol was loaded in the cell, the heat flow curve rose continuously without getting to a flat line, which means the fullerenols injected were not enough for the sucrose binding. When the concentration of fullerenols was increased to 0.5 mM, the heat released per mole of injectant first displayed a drop trend and then it increased smoothly. With more concentrated fullerenols loaded in the syringe, the curve shows a similar figure and the saturation state was obtained. The results showed that 0.6 mM fullerenols was enough for sucrose binding. Therefore, in the following ITC fullerenols at this concentration were put into the syringe and used as the receptor model. For each sweet compound, different concentrations of sweetener were screened for the titration with 0.6 mM fullerenols until the smallest χ^2 value of the final data fitting was achieved along with a reasonable ITC profile.

ITC of Different Sweeteners with Fullerenols. It is well-known that different types of sweeteners display various times of onset, duration, decay, and extinction, and their sweetness intensities relative to sucrose are often evaluated by a human sensory panel (*19*). Sucrose is often used as the standard sweetener, and its relative sweetness intensity is set as 1; the relative sweetness intensity of other sweeteners was determined by panelist sensory evaluation. The developing electronic tongues (*16*) are considered to be quite accurate and reliable candidates after suitable training.

In the present work, four kinds of synthetic sweeteners [sodium cyclamate, acesulfame-K, saccharin, and sucralose with relative sweetness intensities of 50, 200, 500, and 600, respectively (*20*)] and six naturally occurring carbohydrates [D-galactose, maltose, D-trehalose, D-glucose, sucrose, and D-fructose with relative sweetness intensities of 0.32, 0.40, 0.45, 0.70, 1, and 1.7, respectively (*21*)] were chosen as typical sweeteners in our ITC experiments. These experiments on the binding of sweeteners were conducted with fullerenols at 298 K. **Figure** shows experimental data for a typical titration of fullerenols to 10 kinds of sweeteners with increasing sweetness intensity. The upper panel of each graph shows the trace recorded for each 10 μL injection made at 240 s intervals. The area of each peak was integrated and corrected for the heat of dilution, which was estimated by a separate experiment by injecting the titrant strand into the distilled water. By fitting the titration curve with a nonlinear least-squares method, the enthalpy change ΔH and the equilibrium binding constant K can be estimated with the assumption of a reasonable binding site model for fullerenol−sweetener formation. The lower panel of each graph shows the fit of each integrated heat value to a titration curve calculated on the basis of two sets of binding sites model. **Figure a** shows ITC measurement of D-galactose binding with fullerenols. It can be seen that the enthalpy change associated with each injection was exothermic for the first few injections, decreased appreciably after a certain number of injections, and then reached endothermic value for the following injections. In the case of maltose and trehalose shown in **Figure b**,**c**, decreasing profiles with exothermic heat flow were observed for the first few injections. Then the exothermic heat decreased smoothly until the saturation state was obtained. **Figure d** shows the ITC profiles of D-glucose binding with fullerenols. An upward pattern of the ITC titration peaks (**Figure d**, top) was observed. The heat flow of the first few drops is exothermic, after which it tends to be endothermic. Among sugars and polyols, erythritol, D-fructose, is reported to be differentiated by a relatively high hydrophobicity (*22*). The resultant positively integrated heat value demonstrates that the association between D-glucose and fullerenols is an endothermic reaction or an entropically driven process. This endothermic association has also been observed in peptide and lipid binding (*23*) and heavy metal ions binding by the phytochelatin (*24*). Sucrose (**Figure e**) and fructose (**Figure f**) show similar ITC profiles. Of all six


Time (min)
ucal/sec
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Molar Ratio (fullerenols/D-galactose)
(a)
Time (min)
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Molar Ratio (fullerenols/maltose)
(b)
Time (min)
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kcal/mole of injectant
Molar Ratio (fullerenols/trehalose)
(c)
Time (min)
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Molar Ratio (fullerenols/D-glucose)
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(e)
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kcal / mole of injectant
Molar Ratio (fullerenols/fructose)
(f)

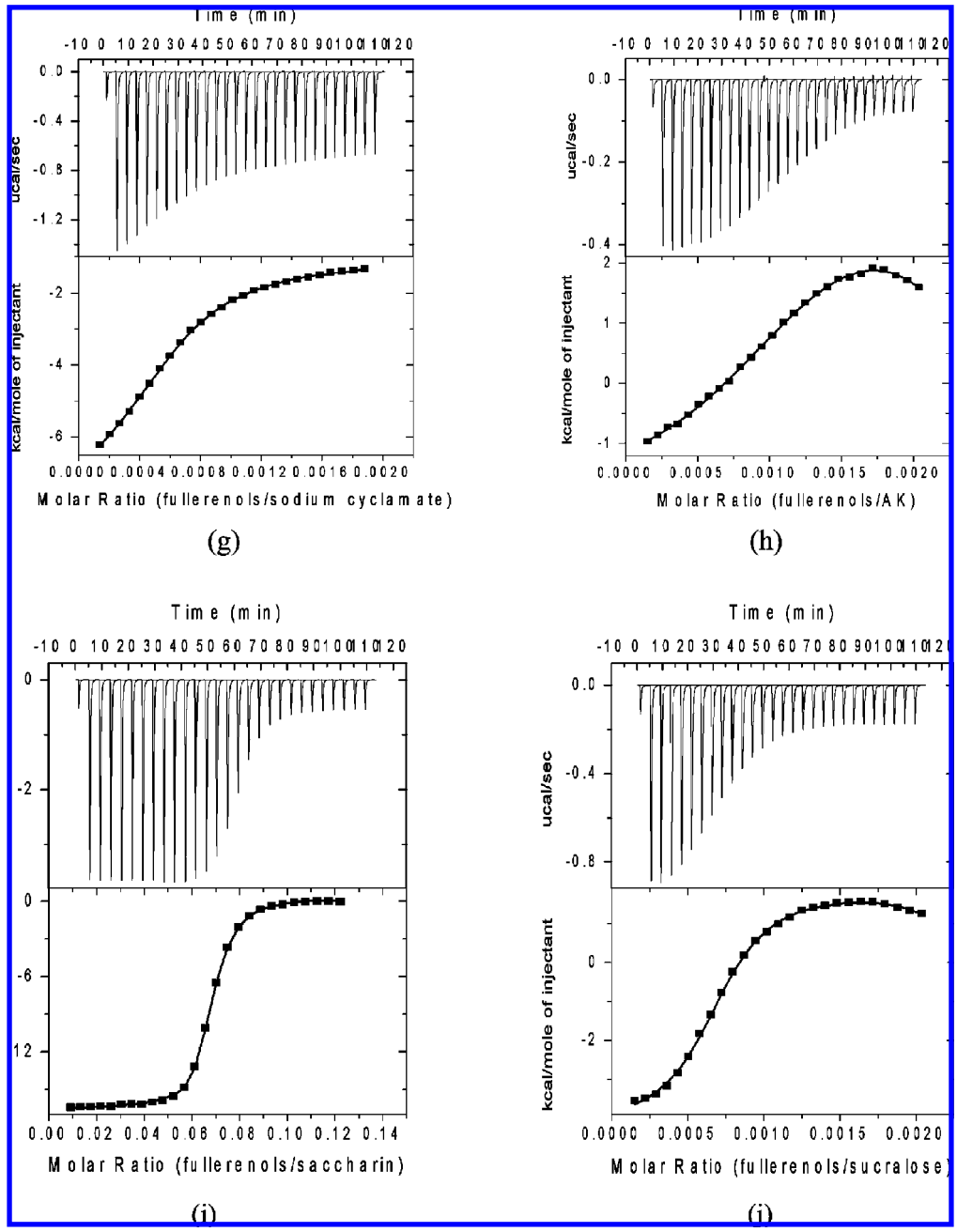

Figure 1. Isothermal titration calorimetry for sweetener binding to and interaction with fullernols at 298 K. The upper panelof each graph shows heat flow for each injection (microcalories per second) as a function of time (minutes); the lower panel shows the integration of each peak (kilocalories per mole of injectant) as a function of fullerenol to sweetener molar ratio by subtracting the heat of dilution. Fullerenol at the concentration of 0.6 mM was put in the syringe and sweetener was put in the cell. Sweeteners at different concentrations (in parentheses) are used as follows: (**a**) D-galactose (75 mM); (**b**) maltose (65 mM); (**c**) D-trehalose (60 mM); (**d**) D-glucose (80 mM); (**e**) sucrose (70 mM); (**f**) D-fructose (65 mM); (**g**) sodium cyclamate (65 mM); (**h**) acesulfame-K (AK) (60 mM); (**i**) saccharin (1 mM); (**j**) sucralose (60 mM).

natural carbohydrates, the heat flow of most of the injections tends to be endothermic; only the first few drops are exothermic.

In the case of the synthetic compounds that have intensive sweetness, their binding with fullerenols gives quite different titration curves. Sodium cyclamate (**Figure g**) gives the larger endothermic heat during the initial binding with fullerenols. An obvious peak in the ITC profiles of acesulfame-K was observed in **Figure h**. Saccharin (**Figure i**) shows the largest endothermic heat compared with other sweeteners, whereas for sucralose an early binding plat figure is observed in **Figure j**. Obviously, synthetic compounds such as sodium cyclamate, acesulfame-K, and saccharin are sweeter than carbohydrates and have quite different chemical structures. Their anion moieties of the salts play key roles in their sweetness intensities. Compared to the carbohydrates, which remain in molecular state in water, the synthetic sweeteners tend to dissociate into ions. Desolvation is needed before its association with fullerenol molecules. The magnitude of the observed molar enthalpy changes and the general shape of the isotherms are completely different from those carbohydrates, which can be attributed to structural factors.

On the basis of earlier reports that hydrogen bonding is the cornerstone of the sweetness mechanism, we hypothesized that, at equal molar concentrations, the more intensive the perceived sweetness of a sweet compound, the more heat will release from the association of sweetener with the receptor. It seems that binding of artificial sweeteners such as sodium cyclamate,

Table 1. Thermodynamic Parameters Derived from ITC Profiles for Association of Sweeteners with Fullerenols at 298 K[a]

sweetener	N ($\times 10^{-3}$)	K (M^{-1})	ΔH (cal/mol)	ΔS (cal K mol^{-1})	ΔG (kcal mol^{-1})
D-galactose	$N_1 = 0.29 \pm 0.02$	$K_1 = 2.54 \times 10^{12} \pm 4.03 \times 10^{11}$	$\Delta H_1 = -3195 \pm 837$	$\Delta S_1 = 46.0$	$\Delta G_1 = -16.9$
	$N_2 = 1.34 \pm 0.23$	$K_2 = 2.78 \times 10^{11} \pm 3.42 \times 10^{10}$	$\Delta H_2 = 1477 \pm 104$	$\Delta S_2 = 57.3$	$\Delta G_2 = -15.6$
maltose	$N_1 = 0.75 \pm 0.01$	$K_1 = 1.04 \times 10^{13} \pm 1.16 \times 10^{12}$	$\Delta H_1 = -1201 \pm 46$	$\Delta S_1 = 55.5$	$\Delta G_1 = -17.7$
	$N_2 = 1.13 \pm 0.03$	$K_2 = 1.55 \times 10^{11} \pm 8.31 \times 10^{9}$	$\Delta H_2 = 2199 \pm 31$	$\Delta S_2 = 58.6$	$\Delta G_1 = -15.3$
D-trehalose	$N_1 = 1.27 \pm 0.03$	$K_1 = 4.13 \times 10^{12} \pm 9.98 \times 10^{11}$	$\Delta H_1 = -999 \pm 56$	$\Delta S_1 = 54.4$	$\Delta G_1 = -17.2$
	$N_2 = 0.76 \pm 0.04$	$K_2 = 1.13 \times 10^{11} \pm 1.54 \times 10^{10}$	$\Delta H_2 = 2003 \pm 115$	$\Delta S_2 = 57.3$	$\Delta G_2 = -15.1$
D-glucose	$N_1 = 0.74 \pm 0.005$	$K_1 = 3.22 \times 10^{12} \pm 2.47 \times 10^{11}$	$\Delta H_1 = -1353 \pm 33$	$\Delta S_1 = 52.7$	$\Delta G_1 = -17.1$
	$N_2 = 0.79 \pm 0.02$	$K_2 = 1.21 \times 10^{11} \pm 5.96 \times 10^{9}$	$\Delta H_2 = 3445 \pm 27$	$\Delta S_2 = 62.3$	$\Delta G_2 = -15.1$
sucrose	$N_1 = 0.80 \pm 0.005$	$K_1 = 2.12 \times 10^{12} \pm 3.19 \times 10^{11}$	$\Delta H_1 = -2503 \pm 36$	$\Delta S_1 = 48.0$	$\Delta G_1 = -16.8$
	$N_2 = 0.94 \pm 0.05$	$K_2 = 7.6 \times 10^{10} \pm 1.27 \times 10^{10}$	$\Delta H_2 = 2642 \pm 32$	$\Delta S_2 = 58.6$	$\Delta G_1 = -14.8$
D-fructose	$N_1 = 0.95 \pm 0.007$	$K_1 = 3.92 \times 10^{12} \pm 4.43 \times 10^{11}$	$\Delta H_1 = -806 \pm 38$	$\Delta S_1 = 54.9$	$\Delta G_1 = -17.2$
	$N_2 = 0.93 \pm 0.01$	$K_2 = 1.13 \times 10^{11} \pm 4.05 \times 10^{9}$	$\Delta H_2 = 2385 \pm 16$	$\Delta S_2 = 58.6$	$\Delta G_2 = -15.1$
sodium cyclamate	$N_1 = 0.50 \pm 0.013$	$K_1 = 1.14 \times 10^{12} \pm 1.52 \times 10^{11}$	$\Delta H_1 = -8390 \pm 325$	$\Delta S_1 = 27.0$	$\Delta G_1 = -16.4$
	$N_2 = 1.38 \pm 0.10$	$K_2 = 1.26 \times 10^{11} \pm 2.65 \times 10^{10}$	$\Delta H_2 = -1079 \pm 43$	$\Delta S_2 = 47.2$	$\Delta G_2 = -15.1$
acesulfame-K	$N_1 = 1.00 \pm 0.08$	$K_1 = 1.80 \times 10^{6} \pm 1.34 \times 10^{6}$	$\Delta H_1 = -2037 \pm 150$	$\Delta S_1 = 21.8$	$\Delta G_1 = -8.5$
	$N_2 = 1.16 \pm 0.1$	$K_2 = 2.99 \times 10^{5} \pm 1.98 \times 10^{5}$	$\Delta H_2 = 3904 \pm 827$	$\Delta S_2 = 38.1$	$\Delta G_2 = -7.5$
saccharin	$N_1 = 66.1 \pm 0.13$	$K_1 = 4.26 \times 10^{12} \pm 2.44 \times 10^{11}$	$\Delta H_1 = -16600 \pm 23$	$\Delta S_1 = 2.09$	$\Delta G_1 = -17.2$
	$N_2 = 36.1 \pm 4.85$	$K_2 = 4.57 \times 10^{10} \pm 3.68 \times 10^{9}$	$\Delta H_2 = 245 \pm 44$	$\Delta S_2 = 49.6$	$\Delta G_2 = -14.5$
sacralose	$N_1 = 0.67 \pm 0.005$	$K_1 = 3.40 \times 10^{7} \pm 1.85 \times 10^{7}$	$\Delta H_1 = -4257 \pm 62$	$\Delta S_1 = 20.2$	$\Delta G_1 = -10.3$
	$N_2 = 1.5 \pm 0.066$	$K_2 = 1.19 \times 10^{6} \pm 6.67 \times 10^{5}$	$\Delta H_2 = 1907 \pm 51$	$\Delta S_2 = 34.2$	$\Delta G_2 = -8.3$

[a] ΔH and K were evaluated directly from the titration data using Origin software. Association constant K = [binding complex]/[fullerenols][sweetener]. ΔG and $T\Delta S$ were calculated using $\Delta G = -RT \ln K$ and $\Delta G = \Delta H - T\Delta S$. Mean and standard deviation values from the fitted data of three independent experiments are shown.

saccharin, and sucralose with fullerenols released more heat than other carbohydrates. However, there appears to be no direct correlation between the perceived sweetness and the heat flow measured for all of the sweeteners in this ITC study. On the other hand, by collecting ITC profiles of the 10 sweetener bindings with fullerenols, it can be seen that the heat flow from its binding with the fullerenols tends to switch from exothermic to endothermic during the binding process, except for sodium cyclamate and saccharin. It is well documented that, among several weak non-covalent interactions between host and guest molecules, the hydrophobic, hydrogen bond, $\pi-\pi$, C−H$\cdots\pi$, and van der Waals interactions are the main contributions to the enthalpic changes, whereas conformational changes and desolvation contribute to the entropic changes. Therefore, we can deduce from the ITC data that hydrogen bond formation is not always the key element during the binding process; otherwise, all of the heat flow should be exothermic. Other non-covalent interactions such as hydrophobic, van der Waals interactions and conformational changes, and desolvation contribution should be involved in the complicated process.

To further investigate the contribution of enthalpy or entropy to the association between the sweeteners and fullerenols, the ITC profiles were fit with two sets of binding site models to yield reasonable thermodynamic parameters of the experimental data. It is noteworthy to say that Bassoli et al. (*25*) have presented a model which is able to support the hypothesis that there is just one binding site, at least for small ligands, in the sweet taste receptor. In our study, the model with a single binding site or three independent binding sites did not fit the experimental data. These fittings (depicted as continuous lines in **Figure**) were derived using ΔH, the association constant (K), and the binding stoichiometry as free-floating parameters. The binding thermodynamic parameters that emerged from these fittings are summarized in **Table 1**.

ITC profiles of all of the sweeteners can be fitted by two sets of binding models. All of the free energy (ΔG) is negative, indicating binding of sweeteners with fullerenols is favorable energetically. Two kinds of equilibrium association constants existed in all of the sweeteners binding with fullerenols. If we suppose the sites on the fullerenol core, which display more negative free energy (larger equilibrium association constants), prefer to associate with sweeteners in the first step, those with less negative free energy will bind with the sweet molecules in the second step; thus, we can see that enthalpy changes contribute to the association process of all of the sweeteners with fullerenols in the first step because of negative ΔH_1. On the other hand, sweetener binding to the first high-affinity sites is exothermic, whereas the second set of sites bind sweetener endothermically except for sodium cyclamate. Although the detailed structural relationship behind the thermodynamic parameters needs to be further examined, we can get some information of the sweeteners' interaction with fullerenols on a molecular level. For example, both galactose and glucose are monosaccharide; the position of the −OH group on the fourth carbon is the only distinction between glucose and galactose. Glucose is defined as the −OH on the fourth carbon in a horizontal projection in the chair form, whereas in galactose the −OH on the same position is in an upward projection in the chair form. They also showed different ITC profiles (**Figure a**,**d**). The very different ΔG_1 values of the two monosaccharides may come from −OH position, which is important for the hydrogen bond formation. As they have the same carbon number and similar hydrophobic structures, they display similar amplitudes in ΔG_2. Maltose consists of two α-D-glucose molecules with the α bond at carbon 1 of one molecule attached to the oxygen at carbon 4 of the second molecule, which is called a 1α−4 glycosidic linkage. Trehalose has two α-D-glucose molecules connected through carbon 1 in a 1α−1 linkage. The

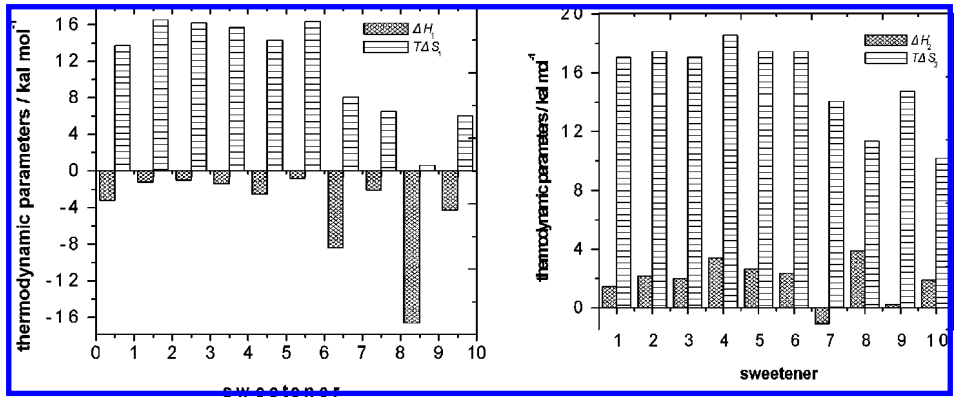

Figure 2. Standard enthalpy (ΔH) and entropy changes ($T\Delta S$) for the binding complex of sweeteners with fullerenols at 298 K. Sweeteners: 1, D-galactose; 2, maltose; 3, D-trehalose; 4, D-glucose; 5, sucrose; 6, D-fructose; 7, sodium cyclamate; 8, acesulfame-K (AK); 9, saccharin; 10, sucralose. The left bar graph shows the thermodynamic parameters of the first sets of site binding (ΔH_1 and $T\Delta S_1$). The right bar graph shows those of the second sets of site binding (ΔH_2 and $T\Delta S_2$).

difference is a little greater in K_1 than that in K_2, implying that the position of the glycosidic linkage mainly affects the hydrogen bond formation during the bonding process. In all cases, sweetener binding to the highest affinity set of sites is aided by both entropic and enthalpic contributions, whereas sweetener binding to the second and low-affinity set of sites is entropically driven. Similar results have also been found in the affinities of Ca^{2+} and the C2 domains of classical protein (*26*). The thermodynamic data herein clearly imply that H-bonded interactions contribute comparatively little for the sweetener binding with our artificial receptor; other forces drive the binding reaction and contribute the bulk of the favorable binding free energy. We assume that the overwhelming driving force for binding may stem from hydrophobic effects. Although hydrophobic force has an enthalpic component, about half of it originates from the entropy of the hydrogen-bonded three-dimensional network lattices of water molecules at room temperature.

For the sake of the direct visualization of all series of data, standard enthalpic (ΔH) and entropic changes ($T\Delta S$) are illustrated by the bar graphs shown in **Figure 2**. From the viewpoint of thermodynamics, all of the binding between sweeteners and fullerenols is driven by favorable entropic changes ($T\Delta S > 0$), accompanied by either favorable ($\Delta H < 0$) or unfavorable ($\Delta H > 0$) enthalpic changes. The favorable free energy of all six carbohydrates' binding to the first sites of fullerenols is derived from a large positive entropic contribution along with a relatively minor enthalpic contribution in this spontaneous event. It can also be seen that the ratio of ΔH_1 to $T\Delta S_1$ in the first binding step of the synthetic sweeteners is larger than that of natural carbohydrates, which means enthalpy changes contribute more to free energy changes during the binding process for synthetic sweetener compounds. In the second step, all of the enthalpy changes (ΔH_2) were positive except for sodium cyclamate, which indicated that the binding enthalpy contributes unfavorably to the free energy of binding and the process is totally driven by entropic changes. The relatively complicated entropic changes can be analyzed with less certainty from the standpoints of both fullerenol acceptor and sweetener guest. Generally, the host−guest association process, which leads to the loss of conformational freedom, is inherently accompanied by a decrease in entropy. On the other hand, before association, both the receptor fullerenols and the guest molecule sweeteners are highly solvated due to the existing hydroxyl groups or other polarized groups, and the solvent molecules around the host and the guest are highly ordered. During the association, the solvation shells of both fullerenols and sweetener undergo reorganization accompanied by the loss of some solvent molecules. This process creates disorder in the system and thus leads to a favorable entropic gain, which compensates for the entropic change, to various degrees, arising from the loss of conformational freedom upon association. At the first binding step, the van der Waals and hydrogen bond interaction play crucial roles in the enthalpy contributions. At the second step, the binding of most of the sweeteners with fullerenols is endothermic; consequently, the unfavorable positive enthalpy is compensated by the large positive entropy. These data seem to prove that hydrated sweeteners and fullerenols need to be decomposed before the sweetener−fullerenol complex forms. Thus, the release of both the sweetener and the hydrated fullerenols into the solution yields increasing entropy, whereas the positive enthalpy is caused by the energy spent to decompose strongly hydrated fullerenols and sweeteners.

In biosystem processes, much attention has been focused on hydrogen bonding as a key factor in the stability of DNA minor groove ligands (*27*). Because of its relative strength compared with van der Waals interactions, being longer range and more angularly dependent, hydrogen bonding is often a powerful structuring force. This is certainly true for carbohydrates, which have hydroxyl groups that can simultaneously donate and accept hydrogen bonds. However, it has also been found that DNA binding with some simple intercalators is driven almost equally by hydrophobic effects and van der Waals contacts within the intercalation site (*28*). As mentioned above, the involvement of hydrogen bonding in sweetness was first hypothesized for sugars, after which it was extended to add hydrophobic centers. One assumption that underlies these models is that the conformation of sugar is important and that water is one of the partners in sweet taste chemoreception should also be considered. Here, our results demonstrate that hydrogen bond is involved in carbohydrates sweet chemoreception. The contribution of entropies changes derived from ITC profiles absolutely plays a role in sweet chemoreception of all of sweeteners. Binding of sweeteners to fullerenols may be governed by a broad range of factors, including solution conditions, electrostatic interactions, hydrogen bonding interactions and hydrophobic interaction.

Correlation of the ITC Thermodynamic Parameters to the Sweetness Intensity. Early researchers have already made some physicochemical approaches to the structural relationship to the intensity of sweeteners. For example, Hansch (*29*) has found a good correlation for nitroanilines between relative sweetness, Hammett constant, and hydrophobicity. Daniel (*30*)

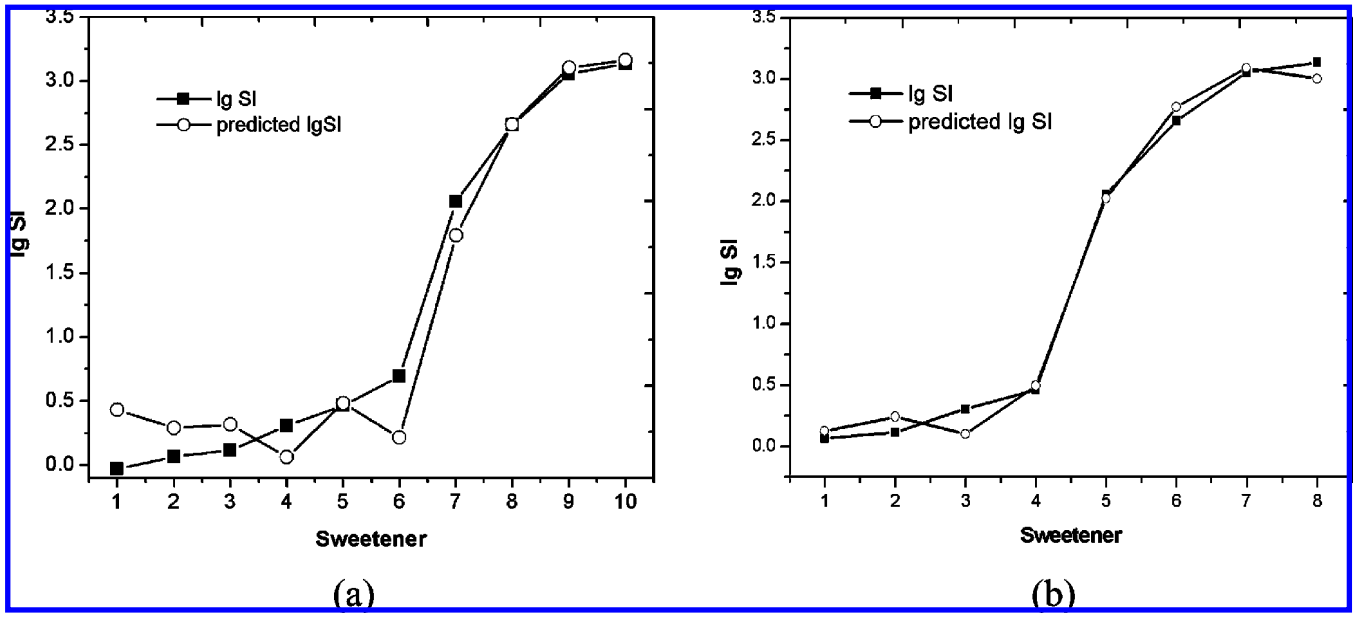

Figure 3. Correlation of sweetness intensity to the thermodynamic parameters of sweeteners binding to fullerenols. SI is the molar relative sweetness intensity. (**a**) Sweeteners: 1, D-galactose; 2, maltose; 3, D-trehalose; 4, D-glucose; 5, sucrose; 6, D-fructose; 7, sodium cyclamate; 8, acesulfame-K (AK); 9, saccharin; 10, sucralose. Multiple $R = 0.978257$. (**b**) Sweeteners: 1, maltose; 2, D-trehalose; 3, D-glucose; 4, sucrose; 5, sodium cyclamate; 6, acesulfame-K (AK); 7, saccharin; 8, sucralose. Multiple $R = 0.996329$.

determined a molecular connectivity coefficient, which takes into account the chiral center and other physical properties. A comparable procedure was followed by Laffort (*31*) and Patte (*32*) to link the olfactory activity of some molecules and their physicochemical properties such as solubility, polarizability, and molar volumes. Mathlouthi (*22*) has even determined a hydrophobicity scale for some sweeteners. Very recently, Goraieb' group (*21*) presented correlations of conventional energy-dispersive X-ray fluorescence spectra of common sugars with degrees of sweetness using chemometric tools. The present study was initially undertaken with the aim of providing a thermodynamic basis of understanding of the initiation of sweet sensation using a synthetic receptor model. We also hope to find a quantitative model of binding thermodynamics in terms of sweetness intensity. This is of utmost importance compared to sensory analysis in determining sweetness degree, which was reported to be a very laborious and time-consuming task (*15*). According to the proposed hypothesis, at equal molar concentrations, the higher the perceived sweetness of a sugar, the more heat will flow when the receptor associates with the sweeteners. Although our preliminary results showed that more heat released from most of synthetic compounds (acesulfame-K, saccharin, sucralose) compared with less sweet compounds (carbohydrates) during their bindings to fullerenols, it is hard to find a direct correlation between the perceived sweetness and enthalpy changes during the binding. Perhaps the lack of direct correlation between the perceived sweetness and the heat flow is due to the complex nature of sweet taste perception and the challenges associated with correlating molecular level parameters with macroscopic sensory data. However, our results imply that conformation changes and hydrophobic interaction related to entropy changes should be involved in the biomimetic recognition. Considering the contribution of both enthalpy and entropy changes to the binding process, further examination of the thermodynamic parameters indicates that combination of the enthalpy changes and free energy can be correlated well with sweetness intensity through multivariate correlation analysis. The results are shown in **Figure 3a**.

Figure 3a is the correlation of molar relative sweetness intensity (SI) to the thermodynamic parameters of 10 sweeteners in their binding process. The following correlation equation with the multivariate correlation coefficient $R = 0.978$ was obtained:

$$\lg \mathrm{SI} = -0.14107\Delta H_1 - 0.1837\Delta H_2 - 0.12968\Delta G_1 + 0.4813\Delta G_2 + 5.56907$$

$$\text{multiple } R = 0.978257$$

Larger multivariate correlation coefficients R ($R = 0.996329$) can be achieved if we use just eight sweeteners for the correlation as shown in **Figure 3b**. From this correlation equation, it can be seen that the more negative the enthalpies are, the larger is the SI value. This implies that heat release contributes to sweetness chemoreception in all cases. As ΔG represents combinational results of change in both enthalpy and entropy changes and it is negative in all of the present cases, we can conclude that larger positive entropies will help sweeter compound chemoreception in the first step binding. Carbohydrate sweeteners, which are usually less sweet than artificial intensive sweeteners, prefer to bind strongly with the receptor in the first step (with more negative ΔG_1). As our fullerenol receptor has so many hydroxyl groups on the sphere core, hydrogen bond formation is preferential, which leads to favorable enthalpy changes. In the second step, the fullerenol core is more hindered after being occupied by some sweetener molecule and the conformation change is needed for the following sweetener molecules' binding; therefore, this process is closely related to entropy changes. Intensive sweeteners tend to bind strongly with the receptor by predominately hydrophobic interaction because they have fewer hydrogen donors or acceptors. Although the correlation presents the potential model as a useful tool in sensory analysis, caution needs to be exercised when fewer variables are linked or connected with numerous unknowns.

In summary, the thermodynamic results showed that binding of all of the studied sweeteners with fullerenols was through two sets of site models. The heat released from most of the

synthetic sweet compounds binding to fullerenols was greater than that of less sweet sugars. Our results show that hydrogen bond formation is necessary for the sweetener binding with the fullerenol receptor, whereas hydrophobic effects and conformation changes that lead to favorable entropy changes exist dominantly during most of the association cases. Moreover, as many thermodynamic observations can be rationalized in terms of the particular structural features of a complex, correlation has been made of the thermodynamic parameters to sweetness intensity as well. The preliminary results of this study help to address the lack of information about the thermodynamic basis of understanding of the initiation of sweet sensation. They also add complementary physicochemical measurements available for comparison of the sweetness hypothesis. It is hoped that, eventually, the correlation of thermodynamic data and sweetness intensity data from sensory evaluation will lead to an accurate quantitative model of binding thermodynamics in terms of sweetness intensity and will be an attractive alternative for sensory analysis. We also found that at the same experimental conditions, typical titration drops of the ITC profile shape characterize the different sweeteners (not shown). Hence, it is reasonable to suggest that the vertical and horizontal parameters of the temporal profile such as the peak area, peak height, peak width, and peak angle contains a number of factors of sweeteners, including onset or appearance time, time to maximum sweetness intensity, linger time, and extinction time. Compared with time−intensity profiles drawn by the sensory evaluation panelists, these ITC titration profiles yield more impersonal and accurate information regarding fundamental aspects of sweetener−receptor interactions. It must be stressed, however, that a crucial issue among many questions is the comprehension of the structural and functional requirements for the effective and selective recognition of a specific sweetener. The preliminary results of the present study showed that only some of the sweetener intensity has been correlated to the thermodynamic parameters. Further modification of the artificial sweet receptor structure and thermodynamic research on more sweeteners with different structures including enantiomers binding synthetic receptor are in progress.

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