Correlation of the Sweetness of Variants of the Protein Brazzein with Patterns of Hydrogen Bonds Detected by NMR Spectroscopy

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In sequence-function investigations, approaches are needed for rapidly screening protein variants for possible changes in conformation. Recent NMR methods permit direct detection of hydrogen bonds through measurements of scalar couplings that traverse hydrogen bonds (trans-hydrogen bond couplings). We have applied this approach to screen a series of five single site mutants of the sweet protein brazzein with altered sweetness for possible changes in backbone hydrogen bonding with respect to wild-type. Long range, three-dimensional data correlating connectivities among backbone $^1$HN, $^{15}$N, and $^{13}$C atoms were collected from the six brazzein proteins labeled uniformly with carbon-13 and nitrogen-15. In wild-type brazzein, this approach identified 17 backbone hydrogen bonds. In the mutants, altered magnitudes of the couplings identified hydrogen bonds that were strengthened or weakened; missing couplings identified hydrogen bonds that were broken, and new couplings indicated the presence of new hydrogen bonds. Within the series of brazzein mutants investigated, a pattern was observed between sweetness and the integrity of particular hydrogen bonds. All three "sweet" variants exhibited the same pattern of hydrogen bonds, whereas all three "non-sweet" variants lacked one hydrogen bond at the middle of the $\alpha$-helix, where it is kinked, and one hydrogen bond in the middle of $\beta$-strands II and III, where they are twisted. Two of the non-sweet variants lack the hydrogen bond connecting the N and C termini. These variants showed greater mobility in the N- and C-terminal regions than wild-type brazzein.

All known sweet proteins are derived from fruits native to Africa or South Asia. Of these, brazzein is the smallest and most stable (1, 2). Brazzein is found in nature in the fruit of the African plant Pentadiplandra brazzeana Baillon (1). Brazzein contains no carbohydrate, and its structure (3) bears no structural resemblance to sucrose, small molecule chemical sweeteners, or the two other sweet tasting proteins of known structure: monellin (4) and thaumatin (5, 6). Moreover, members of the class of sweet proteins contain no conserved stretches of amino acids.

Recently, the human sweet taste receptor has been identified (7, 8). It is a heterodimeric form of a typical seven-transmembrane helix $\gamma$-coupled receptor with an unusually large ectodomain (7, 8). Different sweet proteins presumably interact with and activate the same heterodimeric (T1R2/T1R3) receptor in somewhat different ways. Tentative models for such interactions have been presented (9). The characteristics that make a compound taste sweet to humans are not well understood, despite considerable interest and effort in the study of high potency and low calorie sweeteners.

Brazzein is an ideal system for investigating the chemical and structural requirements for extracellular triggering of a sweet response in humans and Old World monkeys, the only species known to perceive the protein as sweet (10–12). Brazzein is small, stable, and easily investigated in solution by NMR spectroscopy. We earlier designed a synthetic gene coding for brazzein and developed an efficient bacterial production system (a fusion protein expressed in Escherichia coli) (13) and have used this approach to discover mutants with altered sweetness (14). This system also has allowed us to produce rapidly stable isotope-labeled brazzein variants for structural evaluation by NMR spectroscopy. Although all members of the set of brazzein variants analyzed previously exhibited one-dimensional NMR spectra characteristic of folded protein, subtle conformational rearrangements could not be ruled out. The present investigation was undertaken to use direct NMR detection of hydrogen bonds to examine this question.

Although trans-hydrogen bond $^{13}$N-$^{13}$C NMR scalar coupling constants are very small ($|J_{NC}| < 1$ Hz), methods have been developed recently (15–18) which permit their detection in small proteins (<30 kDa). These methods employ constant time, long range, two- or three-dimensional NMR correlation experiment that identifies connectivities among backbone $^1$HN, $^{15}$N, and $^{13}$C atoms ($\text{HNCO}$) pulse sequences, either without or with the application of the transverse optimized spectroscopy (TROSY) technique (19) for increased sensitivity. Identification of hydrogen bonds by detection of trans-hydrogen bond couplings has been used to investigate protein secondary structure (20), protein stability (21), and ligand binding (22).

The three-dimensional ribbon diagram in Fig. 1 shows the...
Fig. 1. Backbone ribbon diagram of wild-type brazzein. Residues found to be critical for the sweetness of the protein (14) are indicated in color: red, enhanced sweetness; blue, decreased sweetness.

The backbone of wild-type\(^2\) brazzein and the positions of the five mutations investigated here. Four of the sites of mutation (Ala\(^2\) insertion, H31A, R33A, and D50A) are spatially close to one another. Two of the mutants (Ala\(^2\) insertion and H31A) have about twice the sweetness of wild-type brazzein; the other three mutants (R33A, R43A, and D50A) have greatly reduced sweetness (Table I) (14). R43A is essentially tasteless; it is only about twice as sweet as wild-type brazzein. The efficiency of labeling was indicated in color:

Analysis of the hydrogen bonds in these brazzein variants through measurements of trans-hydrogen bond couplings has shown that single site mutations can give rise to subtle structural changes. Wild-type brazzein and two variants with sweetness equal to or greater than wild-type had similar patterns of hydrogen bonds, whereas all three variants with reduced sweetness exhibited changes in hydrogen bonding and local mobility.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant brazzein and its variants were prepared as described previously (13). Brazzein samples were labeled with \(^{15}\)N or \(^{13}\)C by including in each liter of minimal growth medium 1 g of \(^{15}\)NH\(_4\)Cl (Isotec, Miamisburg, OH) and for double labeling 2 g of \(^{13}\)C\(^6\)glucose (Isotec). Cells were induced for 3–6 mg of pure lyophilized protein dissolved in 250–500 \(\mu\)l of 90% \(\text{H}_2\text{O}\) and 10% \(\text{D}_2\text{O}\); the pH was adjusted to 5.2.

NMR Data Collection—Unless otherwise noted, all NMR spectra were recorded at 37 °C. Data were collected on Bruker DMX500, DMX600, and DMX750 spectrometers, each equipped with a triple resonance \(^{1}H/^{13}C/^{15}N\) probe with three axis pulsed field gradient capability or on a DMX500 equipped with a triple resonance CryoProbe\(^\text{TM}\) with a z gradient. Quadrature detection in the indirectly detected dimensions was obtained with the States TPPI method (23). Water suppression was achieved with a Watergate sequence containing a 3-9-19 selective inversion pulse (24). Two-dimensional \(^{1}H/^{13}C/^{15}N\) HSQC (25) data sets were collected from \(^{15}\)N-\(^{13}\)C variants to screen for chemical shift changes relative to wild-type brazzein in the amide "fingerprint" region. Three-dimensional \(^{15}\)N TOCSY-HSQC-SE (26) and three-dimensional \(^{15}\)N NOESY-HSQC-SE (27) data sets were collected from the same samples and used to determine sequence-specific backbone resonance assignments.

Three of the brazzein variants investigated here (R33A, R43A, and D50A) showed chemical shift changes in regions distant from the site of mutation; these changes necessitated extensive peak assignments. For these variants and for wild-type recombinant brazzein as a control, the following data sets were acquired from \(^{1}H/^{15}\)N-\(^{13}\)C-labeled samples dissolved in 90% \(\text{H}_2\text{O}\) and 10% \(\text{D}_2\text{O}\) (for a total experimental time of 1 week/mutant): three-dimensional SE-HNCA (28), three-dimensional SE-NMR correlation experiment that identifies connectivities among backbone \(^{1}H/^{13}C\) of the same residue (29), three-dimensional SE-HNCO (28–29), three-dimensional \(^{15}\)NOESY-HSQC-SE (30), and three-dimensional HCC-HCOSY (31).

NMR data were processed with Felix98 (Accelrys Inc., San Diego). Time-domain data were apodized in each dimension with a squared sine bell function; then the data were zero filled to the final matrix size, Fourier transformed, and phase corrected. The initial value for the incremented delay was set in a manner that allowed predictable phasing in each dimension and minimized roll and offset of the base line (32). All \(^{1}H\) dimensions were referenced directly to internal DSS. The other nuclei (\(^{13}C\) and \(^{15}\)N) were referenced indirectly to DSS as described in the IUPAC recommendations (33). Processed NMR data were analyzed by using either Felix98 or XEASY (34) software packages.

Backbone assignments were determined for R33A, R43A, and D50A on the basis of the following three-dimensional triple resonance experiments: HNCA, H/CACO, HNOCC, \(^{15}\)N TOCSY-HSQC, and \(^{15}\)N NOESY-HSQC. The two-dimensional \(^{1}H/^{15}\)N HSQC spectrum was used as a reference to correlate each cross-peak to its corresponding amide \(^{1}H\) and \(^{15}\)N in each three-dimensional spectrum. The sequential connectivities obtained mainly from HNCA and H/CACO experiments were confirmed by the analysis of NOE patterns from \(^{15}\)N NOESY-HSQC spectra. The HCC-HCOSY and side chain H/CACO experiments provided assignments for aliphatic \(^{13}\)C resonances. Aliphatic side chain \(^{13}\)C assignments were obtained from \(^{15}\)N TOCSY-HSQC-SE and HCC-HCOSY experiments.

The quantitative J correlation scheme was used to detect trans-hydrogen bond \(^{1}H/^{13}C/^{15}N\) scalar couplings (\(J_{\text{HCN}}\)) (18), which served to identify hydrogen bonds in the protein. The constant time, long range HNCO pulse sequence of Cordier and Grzesiek (15) was used on the Bruker 750 MHz spectrometer with a conventional probe and on the Bruker 500-MHz spectrometer with a CryoProbe\(^\text{TM}\). In each case, two data sets were collected. The first was a long range HNCO experiment with the evolution time (66.6 ms) set for the detection of trans-hydrogen bond couplings (15). The long range HNCO data set consisted of 2048 \(\times\) 80 \(\times\) 64 complex points each with 32 transients. The second was a reference HNCO experiment, with delay time between the two \(^{13}\)C inversion pulses set for 16.6 ms to maximize the one bond coupling \(J_{\text{HCN}}\) evolution (15). For the reference data, 4 transients were coadded for each of the 2048 \(\times\) 80 \(\times\) 64 complex points. The reference spectrum was used as a control to distinguish couplings through hydrogen bonds from those through covalent bonds and to determine whether the signal from a potential hydrogen bond might be missing as the result of relaxation effects. For the detection and analysis of hydrogen bonds involving backbone NH groups, the long range HNCO data were collected at spectral widths of 20, 15, and 30 ppm for the \(^{1}H\), \(^{13}C\), and \(^{15}\)N dimensions, respectively. The carrier frequencies for \(^{1}H/^{13}C\) and \(^{15}\)N were placed at 4.7, 175, and 119 ppm, respectively. Selective carbon \(^{13}\)C pulses were given with G3 and G4 shapes (35). Linear prediction was used to achieve a total number of 2048 \(\times\) 120 \(\times\) 128 points prior to final data processing and analysis.
To evaluate hydrogen exchange rates, a series of $^1$H–$^{15}$N HSQC spectra of wild-type and mutant brazzeins were collected as a function of the time after dissolving a sample previously lyophilized from H$_2$O in D$_2$O.

$^1$H–$^{15}$N steady-state heteronuclear NOE (NOE) data (36) were collected on a 600-MHz NMR spectrometer for wild-type brazzein and the mutants R33A, R43A, and D50A. Spectra were collected with and without a 4 s $^1$H saturation delay (37).

Data Processing and Analysis—The software package Felix98 running on a 10-processor SGI Onyx (SGI, Mountain View, CA) was used to process the data. The XEASY program was used for peak picking and spectral analysis. The program CHIPIT (38) was used to quantify the chemical shifts and intensities of peaks accurately. Measurements from this program were used for hydrogen bond calculations as described earlier (16, 17, 39).

Sequence-specific Assignments—Extensive $^1$H, $^{15}$N, and $^{13}$C resonance assignments were determined for the wild-type, R33A, R43A, and D50A proteins. Only $^1$H and $^{15}$N assignments were obtained for the Ala$_2$ insertion and H31A mutants. These assignments have been deposited in BioMagResBank.

RESULTS

Chemical Shift Comparisons—To characterize the effect of mutations on the structure, $^{15}$N-labeled samples of each of the five proteins were investigated by NMR. $^1$H–$^{15}$N HSQC data were collected for each under identical conditions of pH and temperature. Discounting changes near the site of mutation, only two of the five proteins showed $^1$H–$^{15}$N HSQC patterns closely similar to that of wild-type recombinant brazzein; these were the two sweeter proteins, Ala$_2$ insertion and H31A (Fig. 2).

The three proteins with reduced sweetness exhibited large backbone chemical shift differences from wild-type. These shift differences are projected onto the structure of brazzein in Fig. 3. For R33A (Fig. 3A), the largest chemical shift changes are in the loop that contains the mutated residue and in the C terminus. Because the side chains of Arg$_{33}$ and Asp$_{50}$ in wild-type brazzein are less than 5 Å apart (Fig. 4), these dual changes are not unexpected. Regions of R33A which showed smaller chemical shift differences include the middle of the $\alpha$-helix and the N-terminal end of $\beta$-strand II.

R43A, with a mutation in the $\beta$-turn (loop 43) between strands II and III, exhibited large chemical shift changes in residues 13–14, in residues 18–21 of the disordered loop, at the N-terminal end of the $\alpha$-helix and smaller chemical shift changes in the N- and C-terminal residues and in loop 33 (Fig. 3B).

D50A exhibited more global chemical shift differences from wild-type than the other mutants (Fig. 3C). These included large chemical shift changes in the loop 33 region and in the residues near the N terminus and smaller chemical shift changes in the three $\beta$-strands, $\alpha$-helix, and in residues 12–19 of the disordered loop.

Trans-hydrogen Bond Couplings—A representative long range HNCO spectrum used in measuring $^{3}$H$_{\text{NC}}$ couplings is shown in Fig. 5. Examples of intraresidue, sequential, and through-hydrogen bond connectivities are shown. Table II lists the experimental $^{3}$H$_{\text{NC}}$ couplings determined for wild-type brazzein. The table also lists apparent hydrogen bond distances derived from these couplings on the basis of the empirically established relationship (17),

$$R_{\text{NO}} = 2.75 - 0.25 \ln(|^{3}J_{\text{NC}}|)$$  \hspace{1cm} (Eq. 1)

where $R_{\text{NO}}$ is the distance (in Å) between the two heavy atoms of the hydrogen bond. Standard deviations of the derived $R_{\text{NO}}$ distances were calculated by error propagation (39).

Hydrogen Bond Patterns—The same pattern of hydrogen bonding was observed in wild-type brazzein and in the two sweeter variants (Ala$_2$ insertion and H31A). This “wild-type pattern” is shown schematically in Fig. 6A. The conservation of hydrogen bonding is consistent with the minor chemical shift differences observed with these mutants (Fig. 2). The hydrogen bonding patterns deduced for the three “non-sweet” brazzein variants (R33A, R43A and D50A) are shown in Fig. 6, B–D. These differ from the wild-type pattern, as do the chemical shifts for these mutants.

The R33A mutant lacks three of the hydrogen bonds observed in wild-type brazzein (Fig. 6B, missing hydrogen bonds are shown in orange): Lys$_{85}$–Asn$_{85}$O (in the middle of the...
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The middle of the brazzein. The fifth missing hydrogen bond comes from the bonds are the ones that link stabilize the disordered loop. Four of the five missing hydrogen bonds, by replacing one that is lost, serves to shorten the loop 43 by one residue. The other two new hydrogen bonds stabilize the disordered loop. Four of the five missing hydrogen bonds are the ones that link β-strands II and III in wild-type brazzein. The fifth missing hydrogen bond comes from the middle of the α-helix.

The R43A mutation, located in the β-turn between the anti-parallel β-strands II and III (the opposite end of β-strand III away from Arg), results in changes to eight hydrogen bonds (Fig. 6C). Five hydrogen bonds in the wild-type pattern are missing: Arg HN–Asp O, Asp HN–Ser O, Glu HN–Ile O, Asp HN–Asn O, and Lys HN–Asn O. Three new hydrogen bonds appear (shown in gray): Cys HN–Glu O, Tyr HN–Gln O, Asn HN–Glu O. One of the new hydrogen bonds, by replacing one that is lost, serves to shorten the loop 43 by one residue. The other two new hydrogen bonds stabilize the disordered loop. Four of the five missing hydrogen bonds are the ones that link β-strands II and III in wild-type brazzein. The fifth missing hydrogen bond comes from the middle of the α-helix.

The D50A mutation results in the loss of five hydrogen bonds and the gain of one new hydrogen bond (Fig. 6D). The missing hydrogen bonds are Tyr HN–Cys O, Asp HN–Ser O, Glu HN–Ile O, Glu HN–Phe O, and Lys HN–Asn O. The new hydrogen bond is Asn HN–Leu O.

The three non-sweet mutants have two features in common: loss of hydrogen bond Lys HN–Asn O, which appears to indicate a kink in the α-helix, and loss of hydrogen bond Glu HN–Ile O in the center of β-strands II–III. 

Quantitative Analysis of J Couplings—Apart from the qualitative changes in hydrogen bonding patterns summarized in Fig. 6, changes in trans-hydrogen bond couplings can be evaluated in terms of changes in hydrogen bond distances (17). Fig. 7 compares the magnitudes of JNC couplings measured for hydrogen bonds that are conserved in wild-type brazzein and the three non-sweet brazzein mutants. The mutations resulted in an increase in the magnitude of most trans-hydrogen bond coupling constants (strengthening of the hydrogen bonds). Of the three mutants, R33A (Fig. 7A) showed the smallest changes from wild-type brazzein with four hydrogen bonds strengthened (Tyr HN–Asp O, Arg HN–Asp O, Phe HN–Gln O, and Asp HN–Asn O) and three hydrogen bonds weakened (Asp HN–Asp O, Gln HN–Phe O, and Cys HN–Lys O).

The R43A mutation (Fig. 7B) resulted in four stronger hydrogen bonds, four slightly stronger hydrogen bonds, and two weaker hydrogen bonds. The stronger ones are Leu HN–Tyr O, Asp HN–Asp O (α-helix), Phe HN–Gln O (strands II–III), and Tyr HN–Cys O (strands I and II connecting the N terminus to the C terminus). The slightly stronger hydrogen bonds are Tyr HN–Cys O (strands I and II), Cys HN–Cys O (α-helix), Gln HN–Phe O, and Ile HN–Glu O (strands II–III) (Fig. 7B). The weaker hydrogen bonds are Tyr HN–Asn O (in the beginning of the α-helix) and Cys HN–Lys O (in the middle of strands I–II).

In D50A (Fig. 7C) the mutation led to six stronger hydrogen bonds (increased magnitude of JNC couplings): Tyr HN–Cys O (strands I and II), Leu HN–Tyr O, Asp HN–Asp O (α-helix), Arg HN–Asp O (loop 33 to the C terminus), Phe HN–Gln O (strands III–II), and Asp HN–Asn O (loop 43) and two hydrogen bonds weakened (Cys HN–Cys O, and Cys HN–Lys O).

Backbone Dynamics—To evaluate how changes in hydrogen bonding affect backbone dynamics, heteronuclear (1H)–15N NOE data were collected for wild-type brazzein and the three non-sweet brazzein variants (those with the most extensive changes to the hydrogen bond patterns). The results are shown in Fig. 8.

For all four variants, the secondary structure of the protein

![Image of brazzein structure](http://www.jbc.org/Downloadedfrom)
The initial stretch of β-strand (strand I), the α-helix, the short loop 33, and the remaining two stretches of β-sheet (strands II and III), are regions where NOEs with the highest values of about 0.6 are found, indicative of well structured residues with low mobility. Some residues in the disordered loop (residues 8–11, 14–16) and in the C terminus (residues 51 and 52) display low mobility as well.
Surprisingly, the mobilities on the ps–ns time scale as reported by the NOE data of residues 14–16 in the central region of the disordered loop were lower than nearby residues. This suggests that the conformational variability for this loop is caused by dynamic processes on a slower time scale (μs–ms) not accessible for laboratory frame nuclear spin relaxation measurements.

Regions of high mobility include loop 43 along with the C-terminal end of strand III and the N-terminal end of strand II and also regions of the disordered loop (residues 13, 17–18) including the start of the α-helix (residue 19). In the terminal regions, residues 4 and 54 exhibit high mobility, as well as residue 53 in the R33A and D50A variants. Notably, the D50A variant, which has lost the Asp50 HN–Ser34 O hydrogen bond, is the sole variant to show high mobility for residue 34. Similarly, the R33A variant, which has lost the Glu36 HN–Ile48 O hydrogen bond, shows high mobility at residue 36.

The mobility of the loop 43 region is increased greatly by mutating residue 43. As the only studied variant with a lost hydrogen bond between Asp40 HN–Asn44 O, it displays higher mobility for residues in the loop region than is seen for any other residues in any of the variants, except for the three N- and C-terminal residues (residues 4, 53, and 54) in some variants.

Two of the variants, R33A and D50A, both have lost the Tyr51 HN–Cys4 O hydrogen bond, which ties down the terminal regions, whereas this hydrogen bond is retained in the most pronouncedly non-sweet variant, R43A. In wild-type brazzein and the three variants, the presence or absence of the Tyr51 HN–Cys4 O hydrogen bond is closely correlated with the dynamic behavior of the N and C termini based on the NOE values. In wild-type brazzein and the R43A variant, the N-terminal residue 4 shows high mobility as expected; however,
comparatively low values of the generalized order parameters at 37°C values. The fast overall tumbling of brazzein, corresponding to blue (R33A (green), R43A (blue), and D50A (magenta)). Error bars have been omitted for clarity. The errors in the reported NOE values are between 0.01 and 0.03.

The seemingly low [1H]-15N NOE values observed for this protein are quantitatively in agreement with the observed T1 and T2 values. A full, preliminary dynamics analysis yields comparatively low values of the generalized order parameters for the backbone amide nitrogens, consistent with the NOE values. The fast overall tumbling of brazzein, corresponding to a τc value of 3.1 ns, and resulting from the small size of this protein, yields a theoretical maximum (Sθ = 1.0) NOE value of 0.74 at 600 MHz (40). Even moderately high generalized order parameters representing the most rigid parts of the structure thus result in apparently low NOE values. The even lower NOE values (resulting from fairly high amplitude local internal motions) are likely a result of the almost complete lack of side chain-side chain core packing interactions in this small protein.

With the exception of the cysteines, Cys22-Cys47 and Cys26-Cys33, which are completely buried (less than 20% solvent accessible), only six residues are less than 40% accessible to solvent (41). However, one residue has been identified which runs contrary to this general trend. In the wild-type protein, it was demonstrated that the side chain of Tyr11 is in intermediate exchange at 37°C (42). The aromatic ring flip is slowed down by extensive packing interactions. In wild-type brazzein, Tyr11 has substantially higher NOE values than most other residues, in agreement with the model of core packing. Tyr11 also has the highest average NOE value in all variants.

**Effect of Temperature on Hydrogen Bonding**—To evaluate the effect of temperature on the hydrogen bonding pattern, we measured 3bJSNC values for both wild-type and R43A brazzein proteins at 10°C. These values were compared with those determined at 37°C (Table II). Although the pattern of hydrogen bonds for R43A was the same at the two temperatures, that for wild-type brazzein showed differences (the loss of two hydrogen bonds and the gain of one hydrogen bond at 10°C). The hydrogen bonds lost at 10°C were Glu36 HN–Ile46 O and Asp10 HN–Asp44 O (both between β-strands II and III). The hydrogen bond gained at 10°C was Asn44 HN–Glu41 O. For the hydrogen bonds conserved at both temperatures, the magnitude of the 3bJSNC values increased at low temperature by 0.1–0.5 Hz, indicative of stronger hydrogen bonding.

**DISCUSSION**

In sequence-structure-function investigations, it is important to determine whether the overall conformation of a protein is retained when individual residues are mutated. Without this information, sequence-dependent changes in function are difficult to interpret. The NMR technique is a powerful tool for screening protein variants for structural differences. A frequently used approach is to compare chemical shifts in [1H]-15N correlated spectra as a mean of mapping backbone regions of the protein which are altered. The present study shows how this information can be extended through measurements of 3bJSNC values to evaluate possible changes in hydrogen bonds. This approach is faster and more economical than the determination of full three-dimensional structures and is amenable to high throughput sequence-function investigations.

Direct measurements of through-bond 3bJSNC connectivities allowed us to identify unambiguously pairs of atoms involved in hydrogen bonds in wild-type brazzein (Table II). The pattern of observed trans-hydrogen bond scalar couplings defines the secondary structural motifs in proteins and provides definitive information about hydrogen bond donors and acceptors. For example, 3bJSNC from the amide hydrogen of residue i to the carbonyl oxygen of residue i-3 or i-4 is expected for an α-helix, and interstrand hydrogen bonds are expected for β-sheets. Wild-type brazzein contains four hydrogen bonds that connect flexible regions in various parts of the protein. One hydrogen bond (Asp40 HN–Glu41 O) closes loop 43 (residues 40–44), and two hydrogen bonds (Asp50 HN–Ser34 O and Arg33 HN–Asp50 O) connect one end of loop 33 (residues 30–33) to the C terminus. An additional hydrogen bond (Tyr51 HN–Cys4 O) connects the N and C termini. The remaining hydrogen bonds are involved in secondary structural motifs, in the α-helix and antiparallel β-strands.

The hydrogen bond connectivities determined here for wild-type brazzein and the variants are consistent with the overall protein secondary structure determined from unlabeled protein isolated directly from fruit (3): three antiparallel β-strands and a small α-helix. Table II compares the hydrogen bonds determined by the method of trans-hydrogen bond coupling with those reported in the solution structure of brazzein; also shown are H/D exchange rates determined in this study. A distinct advantage of trans-hydrogen bond scalar couplings over NOEs for detection of hydrogen bonding is that the J couplings are observed even if the Hβ exchanges rapidly with solvent (15), a situation that could obscure the NOE. Two hydrogen bonds were observed through trans-hydrogen bond couplings that were not detected previously (Table II); both involve backbone amides that exchange readily with solvent. These results reveal that β-strand I and the α-helix are each extended by one residue. Moreover, the trans-hydrogen bond couplings indicate changes in two additional hydrogen bonds: Leu29 HN–Asp25 O and Ala32 HN–Cys26 O in the solution structure are seen to be actual (Leu29 HN–Tyr24 O and Asp29 HN–Asp25 O, respectively (Table II). We intend to utilize these hydrogen bonds as tight restraints in determining a refined solution structure of wild-type brazzein. It is unclear at present whether the differences in hydrogen bonding represent errors in the earlier structure or a rearrangement resulting from the absence of pGlu1.

Brazzein adopts a cysteine-stabilized αβ (CSαβ) fold in which the α-helix and β-strands are stabilized by the presence of four disulfide bridges (3). Several families of proteins having diverse functions (toxins, insect defensins (43), and the rape-seed family of serine proteinase inhibitors (44)) share the CSαβ fold with brazzein. Apart from the conserved cysteines, little sequence identity is found among members of the different families. Brazzein is the only CSαβ protein known to be sweet. CSαβ proteins provide an excellent example of how a single fold class can be associated with a wide range of functions and how detailed structural information is needed to explain the functional diversity and specificity of these proteins.
The two “sweet” brazzein variants maintained the same hydrogen bonding pattern as the wild-type, whereas the three non-sweet variants showed numerous changes in hydrogen bonding (Fig. 6). The feature common to all three non-sweet variants was the loss of hydrogen bond Lys31 H3–Asn48 O, which appears to indicate a kink in the α-helix, and the loss of hydrogen bond Glu70 H2–Ile81 O in the center of β-strands II and III, where the strands are twisted.

The loss of the hydrogen bond Tyr51 N2–Cys4 O in the R33A and D50A variants along with the observed significantly increased local backbone mobility in the N and C termini suggest that these mutations result in a widespread loss of nonbonding and electrostatic interactions in this region of brazzein. The fruit brazzein structure initially seemed to indicate a highly disordered C-terminal region, whereas the NOE results show that at least on the ps–ns time scale, the wild-type and the R43A variant have well structured N and C termini. The disruption of nonbonding and electrostatic interactions, in addition to the breakage of the Tyr51 N2–Cys4 O hydrogen bond, resulting from the R33A and D50A mutations, is further supported by the substantial chemical shift changes observed for the residues in this region of the two variants. In contrast, the R43A variant shows the same hydrogen bond pattern (Tyr51 N2–Cys4 O), local mobility, and chemical shifts as the wild-type. This suggests that the local structure of the terminal regions for this mutant is essentially identical to that of wild-type brazzein.

Quantitative measurements of trans-hydrogen bond couplings reveal detailed information about the hydrogen bond lengths and hence strengths of hydrogen bonds, which may be correlated with structural stability and rigidity. A surprising feature of the results for the non-sweet brazzein variants is that the mutations resulted in strengthening of many hydrogen bonds (Fig. 7). This may be a consequence of replacing larger, charged, side chains (Arg or Asp), which may encounter repulsive electrostatic and steric interactions, with the methyl group charged, side chains (Arg or Asp), which may encounter repulsive electrostatic and steric interactions, with the methyl group of alanine. In addition, the loss of certain hydrogen bonds may relieve conformational strain, allowing for structural adjustments more favorable to preserving the remaining hydrogen bonds and overall fold.

The observed NOE values led to the important realization that the structures of brazzein and its variants are stabilized mainly by the observed hydrogen bonds and the cysteine cross-links. Hence, the (small) variations in the NOE values among brazzein variants report on the structural consequences of the hydrogen bonds lost and gained. In general, the observed NOE values are slightly lower for the mutants. This is in agreement with the net loss of at least two core hydrogen bonds in all mutants. Only minor differences in the NOE values are observed (with the exception of a few terminal residues) showing that the overall fold and stability in the mutants are highly preserved.

Sweetness generally is thought to involve the formation of hydrogen bonds between the sweet molecule and sweet receptor (45). Our previous mutagenesis studies suggested that two surface areas are involved in the sweetness of brazzein (14). Similar results had been observed with the other sweet proteins, thaumatin and monellin (46–48). For example, mutagenesis studies of monellin implicated two distinct sites (residues 6–9 and 69) in interaction with the receptor (49). Recent computer models of complexes between TIR2–TIR3 receptors and each of the three sweet proteins (brazzein, thaumatin, and monellin) provided a structural justification for extensive binding surfaces in each case (9).

The results presented here show that the overall fold is preserved in all five variants. However, subtle changes in the local structure, hydrogen bond pattern, and in the backbone dynamics greatly affect the perceived sweetness of the variants. In two of the non-sweet variants (R33A and D50A), the mutations result in dramatic local variations in the N- and C-terminal regions of the molecule. In the third non-sweet variant (R43A), the N and C termini appear identical to the wild-type, whereas the loop 43 and regions of the disordered C-terminal residues (residues 7–18) experience substantial changes in structure and backbone dynamics. Surprisingly, the loss of the two hydrogen bonds between loop 33 and the C terminus in the R43A mutant has no measurable effect on the local chemical shifts and local backbone mobility. This suggests that this pair of hydrogen bonds has negligible consequences for the stability of the structure. In the sweet variants (Ala2 insertion and H31A), the hydrogen bond pattern is unaltered, and only minor changes are observed in the chemical shift of residues close to the site of mutation, suggesting that the overall and the local structure are preserved.

These 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. On the other hand, as is evident from the data presented here, the studied mutations introduce small, yet significant structural changes in regions remote from the mutation site. Consequently, the results from this study may equally well be interpreted as supporting a model involving a spatially limited protein-receptor interaction surface. The observed modifications in sweetness may thus be rationalized as a conformational alteration at the receptor binding site in brazzein, arising from the propagated effect of the structural modifications introduced at the remote mutation site.

The present correlations between sweet protein structure, dynamics, and sweetness will guide future experiments aimed at understanding the molecular interactions at the heterodimeric sweet taste receptor that lead to signal transduction and eventual perception of sweetness.

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