The self-association of the black tea polyphenol theaflavin and its complexation with caffeine

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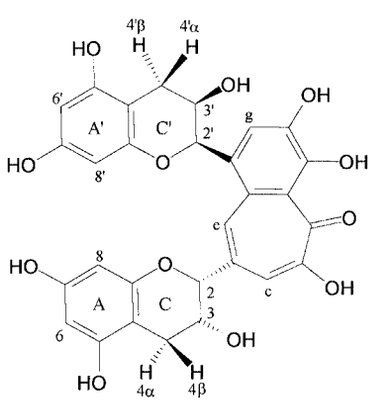
Caffeine is found in both coffee and tea whilst polyphenols are present in a wide variety of foods and beverages. Theaflavin and its gallate esters are polyphenolic molecules which can be isolated from black tea infusions. The theaflavin family of polyphenols contribute to the taste and colour of tea, and their complexation with caffeine is thought to be largely responsible for the formation of tea cream, a precipitate that forms as tea cools. The self-association of theaflavin and caffeine was studied using nuclear magnetic resonance methods (chemical shift changes and self-diffusion constants on dilution) and it is shown that caffeine forms stacks of molecules (K_s = 7.9 l mol^{-1} at 300 K), while theaflavin forms stable dimers (K_s = 230 l mol^{-1}). The theaflavin monomer consists of a planar benzotropolone ring system, with the two flavan rings approximately orthogonal to this plane, and stacked against each other. In the dimer, two benzotropolone rings align with an antiparallel geometry. Two molecules of caffeine bind to one molecule of theaflavin in a strictly sequential manner, with first and second association constants of 11.9 and 16.5 l mol^{-1}, respectively. It is proposed that the first caffeine inserts between the two flavan rings, and the second then binds to the newly liberated flavan surface.

Introduction

Polyphenols are a structurally diverse group of molecules found in a wide variety of higher plants. Polyphenolic molecules are believed to have a role in the defence of their host against herbivorous ingestion due to the unpalatable and anti-nutritional properties they possess.[1,2] The ubiquitous distribution of polyphenols accounts for their presence in a number of human foods and beverages such as cocoa, coffee, beer, black and green tea. In particular, much of the colour and taste of tea arises from the complex mixture of polyphenolic molecules it contains. These include groups known as the theaflavins and the thearubigins[2] which are both produced when the extract from leaves of the tea plant is fermented.[3] Whilst thearubigins are structurally ill-defined, the theaflavins have been purified and their covalent structure determined. The initially proposed benzotropolone structure of theaflavin was incomplete[4] and it was not until a mechanism for the formation of theaflavin from (−)-epicatechin and epigallocatechin was proposed that the structure was correctly determined as **1**.[5]



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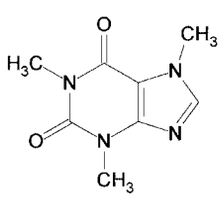
Another major constituent of green and black teas and coffee is caffeine (**2**), which is a stimulant of the central nervous



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system. It has long been known that caffeine interacts with polyphenolic molecules. Coffee contains the polyphenol potassium chlorogenate which was first extracted from green coffee beans as a 1 : 1 crystalline complex with caffeine.[6] The crystal structure of the potassium chlorogenate–caffeine complex shows that the aromatic nucleus of the potassium chlorogenate caffeoyl group stacks in alternate layers with the caffeine. The whole structure is coordinated around a potassium ion.[7] Caffeine has been shown to self-associate[8–10] and also to form heterogeneous stacks with the simple polyphenol methyl gallate[8,11] again in alternate layers, described as a "layer lattice". The caffeine in black tea forms insoluble complexes with most polyphenols but predominantly with theaflavin[12] in a process known as tea cream formation. The tea infusion becomes turbid as its temperature decreases (to <60 °C)[2] and the caffeine–polyphenol complexes become insoluble. This is not observed in coffee, probably due to the simple nature and high solubility of its polyphenolic constituents. Clearly this has implications for the availability of caffeine as a stimulant in tea when compared to coffee, with the caffeine in coffee being more readily available for absorption into the blood. Beverages with modified levels of caffeine and polyphenols are of commercial interest for example in the production of decaffeinated products, chilled tea with high clarity, and teas which remain stable under

a variety of conditions for extended periods of time. For this reason a greater understanding of the interaction between theaflavin and caffeine has been sought.

Theory: Caffeine–theaflavin interaction

A model which assumes that two binding events occur in sequence was derived as equilibria (1a) and (1b), where C is

$$T + C \overset{K_a}{\rightleftharpoons} TC \quad (1a)$$

$$TC + C \overset{K_b}{\rightleftharpoons} TC_2 \quad (1b)$$

caffeine and T is theaflavin, TC is the caffeine–theaflavin complex after the first binding event and TC_2 the caffeine–theaflavin complex after the second binding event. However the concentration of caffeine available to bind to theaflavin will be affected by both the self-association of caffeine and the amount of caffeine which is already bound to theaflavin. The concentration of caffeine species ($[C]_{free}$) which are not bound to theaflavin is given by eqn. (2), where $[C]_i$ is the total concentration of caffeine oligomers. The number of free binding sites on theaflavin for caffeine is dependent both on the concentration of the bound species and on the total concentration of theaflavin $[T]_o$. This assumes that the self-association of theaflavin does not affect the way in which caffeine binds to it (see below). So, the number of free theaflavin binding sites is given by eqn. (3). Note that eqn. (3) describes the number of

$$[C]_{free} = [C]_i - [TC] - 2[TC_2] \quad (2)$$

$$[T]_{free} = 2[T]_o - [TC] - 2[TC_2] \quad (3)$$

binding sites rather than the number of theaflavin molecules, because the number of binding sites changes with the state of complexation of theaflavin.

The binding constants (K_a and K_b) are therefore given by eqns. (4) and (5).

$$K_a = ([C]_{free}[T]_{free})/[TC] \quad (4)$$

$$K_b = ([C]_{free}[TC])/[TC_2] \quad (5)$$

Substitution of eqn. (2) into eqn. (5) gives eqn. (6).

$$[TC_2] = ([TC][C]_i - [TC]^2)/(K_b + 2[TC]) \quad (6)$$

The concentration of caffeine species can be calculated from eqn. (7),[13] where [C] is the concentration of monomeric caffeine

$$[C]_i = [C]/(1 - K_s[C]) \quad (7)$$

and is given by eqn. (8), where $[C]_o$ is the total concentration

$$[C] = [C]_o\{2/[1 + (4K_s[C]_o + 1)^{1/2}]\}^2 \quad (8)$$

of caffeine and K_s is the self-association constant for caffeine. Note that eqn. (7), taken with eqn. (2), carries the implicit assumption that caffeine can bind to theaflavin as a self-associated species, in equilibrium with the free species. The concentration of caffeine species can therefore be calculated if the self-association constant and the concentration of caffeine are known. The resulting pair of equations can then be substituted into eqn. (4) to give an expression defining [TC] (and, from eqn. (6), also $[TC_2]$) in terms of $[T]_o$ and $[C]_i$, and the unknowns K_a and K_b. The values of K_a and K_b are determined by fitting the data for the observed change in chemical shift of the theaflavin resonances when caffeine is titrated into it, which depends on the mole fractions of the complexes [eqn. (9)] where $\Delta\delta_{calc}$ is the

$$\Delta\delta_{calc} = (\Delta\delta_{TC}[TC]/[T]_o) + (\Delta\delta_{TC2}[TC_2]/[T]_o) \quad (9)$$

predicted chemical shift change of theaflavin with caffeine and $\Delta\delta_{TC}$ and $\Delta\delta_{TC2}$ are the differences in chemical shift between unbound theaflavin and TC and TC_2 respectively. The chemical shift changes of all the theaflavin protons were fitted simultaneously to the same values of K_a and K_b using an EXCEL spreadsheet (Microsoft Corporation), allowing $\Delta\delta_{TC}$ and $\Delta\delta_{TC2}$ to vary freely for each proton fitted.

Table 1 The self-association constants (K_s) and maximum changes in chemical shift ($\Delta\delta_{max}$) for caffeine in ^2H_2O–$(C^2H_3)_2SO$ and theaflavin in H_2O–$(C^2H_3)_2SO$ (9 : 1 v/v) at 300 K

	K_s/l mol^{-1}	$\Delta\delta_{max}$/ppm
Caffeine		
Methyl 1	7.1	−0.28 ± 0.06
Methyl 3	8.0	−0.28 ± 0.05
Methyl 7	7.9	−0.17 ± 0.05
H-8	8.8	−0.09 ± 0.05
Mean	7.9 ± 0.7	
Theaflavin		
g	124.2	−0.04 ± 0.02
e	296.6	−0.03 ± 0.01
c	266.1	−0.04 ± 0.01
Mean	229 ± 75	

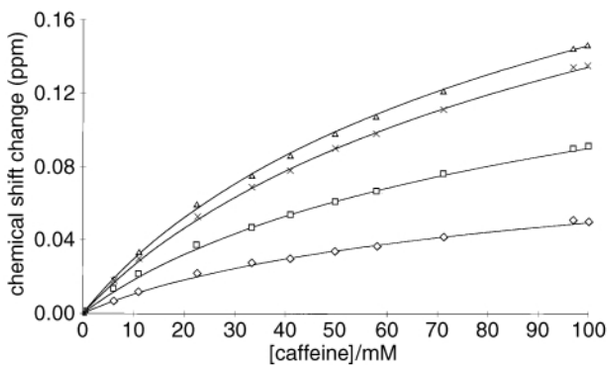

Fig. 1 Fitting of the caffeine resonance chemical shift changes with increasing caffeine concentration in ^2H_2O–[$^2H_6$]dimethyl sulfoxide to the isodesmic model for self-association. The observed data have been plotted using the following symbols; H-8 proton (◇), methyl 1 (×), methyl 3 (Δ), methyl 7 (□). The calculated data are represented in each case by the fitted line.

Results

Caffeine self-association

Chemical shift assignments for the proton resonances of caffeine, and a self-association constant (of 7.4 ± 0.9 l mol^{-1}) have previously been determined in H_2O/[$^2H_6$]dimethyl sulfoxide at 288 K.[8] The isodesmic model was used in ref. 8, which states that the association of solutes progressively into dimers, trimers, tetramers *etc.* happens with the same affinity constant.[9] Similar methods were applied here, using chemical shift measurements at a range of caffeine concentrations between 0.31 and 100 mM (Fig. 1) to derive a self-association constant of 7.9 ± 0.7 l mol^{-1} in the same solvent system but at 300 K, as listed in Table 1. Diffusion experiments showed a continuous decrease in the diffusion constant of caffeine with increasing concentration, in agreement with the expected increase in stack size (data not shown).

Theaflavin self-association

A complete assignment of the ^{1}H NMR signals of theaflavin in

Table 2 ^{1}H NMR chemical shifts for theaflavin in aqueous 10% [$^2H_6$]-dimethyl sulfoxide at pH 3.8[a]

Proton	δ_H(ppm)
g	7.732
e	7.415
c	6.892
8	6.079
8′	5.969
6	5.931
6′	5.811
2′	5.529
2	4.72–4.78[b]
3	4.247
3′	4.247
4β	2.921
4′β	2.842
4α	2.758
4′α	2.612

[a] Chemical shifts measured at 300 K and quoted relative to [$^2H_6$]-dimethyl sulfoxide solvent peak = 2.691 ppm (measured against internal DSS = 0.015 ppm). [b] Obscured by solvent peak.

[$^2H_6$]dimethyl sulfoxide has been published.[3] Most of the signals in aqueous 10% [$^2H_6$]dimethyl sulfoxide could be assigned by solvent titration. However, the H-4 and H-4′ protons have overlapping multiplets and were distinguished using two-dimensional total correlation spectroscopy (TOCSY) and two-dimensional rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) spectra. Also, a complete assignment of the A-ring resonances by the solvent titration method alone was not possible. During the titration, signal overlap became severe and the direction of the chemical shift changes reversed for some of the resonances making unequivocal assignment unachievable. On addition of acidic ^2H_2O to the [$^2H_6$]dimethyl sulfoxide, the A-ring resonances were observed to exchange with deuterium in the solvent leading to a loss of signal intensity: in particular it was noted that two of the A-ring resonances (H-8 and H-8′) underwent deuterium substitution significantly more rapidly than the other two resonances (H-6 and H-6′). This phenomenon allowed some of the signal ambiguities to be resolved. The remaining ambiguity was resolved using TOCSY on a sample containing all the A-ring proton resonances, where weak correlations from H-6 and H-8 to H-4β and from H-6′ and H-8′ to H-4′β allowed H-6/H-8 to be distinguished from H-6′/H-8′, completing the assignment. ^{1}H assignments are given in Table 2.

The chemical shift changes of theaflavin on dilution demonstrate that self-association is occurring. In order to fit the self-association of theaflavin, it is necessary to have a model for the association, of which the two most obvious are simple dimerisation and formation of infinite stacks in an isodesmic manner, as for caffeine. It is not possible to distinguish these models using the chemical shift changes alone, because the two equations describing chemical shift changes on dimerisation or stacking have identical forms.[8] Therefore, we used pulsed-field gradient experiments to measure the self-diffusion constants for theaflavin on dilution (Fig. 2). This experiment works by phase encoding the signals linearly from the top to the bottom of a sample by applying a magnetic field gradient. Diffusion of the molecules in the sample will destroy the phase gradient so that when a second opposite pulsed-field gradient is applied there is a loss in phase coherence resulting in reduced signal intensity. The rate at which the signal intensity falls with an increase in the strength of the gradient applied can be used to calculate how fast a molecule diffuses. The diffusion rate of theaflavin decreases as its concentration is increased, indicating that it self-associates. Fig. 2 shows that the way in which the diffusion rate of theaflavin changes matches the predicted change in diffusion rate upon theaflavin dimer formation and

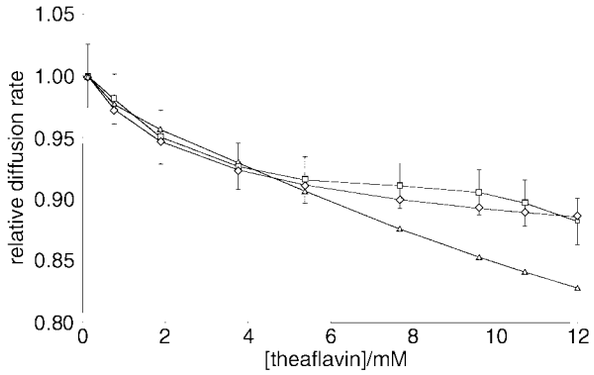

Fig. 2 Self-diffusion rate of theaflavin as a function of theaflavin concentration. The diffusion rate decreases as the theaflavin concentration is raised. This change in diffusion rate closely matches that calculated when theaflavin is considered to exist as only monomers and dimers, whereas the average rate of diffusion for the first five oligomeric forms of theaflavin (monomer, dimer, trimer, tetramer and pentamer) at the ratio calculated from the self-association constant is much lower than the observed data. Observed data (□), dimer model (◇), isodesmic model (Δ). The experimental errors, denoted by the error bars, represent the 95% confidence limits obtained from non-linear fitting of the experimental decay rates of intensity with gradient strength.

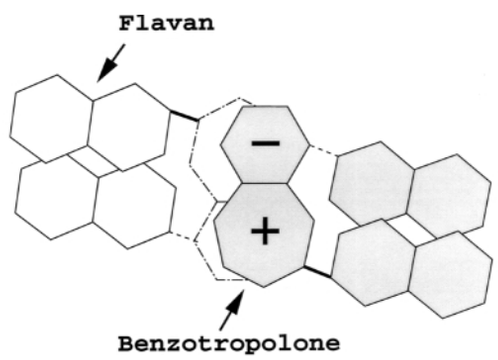

Fig. 3 A low-energy conformation of the theaflavin dimer that satisfies the observed NOEs and chemical shift changes. The electron distribution of the benzotropolone system promotes the formation of stable dimers in an antiparallel manner.

not that predicted by the isodesmic association model of theaflavin self-association. Fitting of a dimerisation model to the dilution shifts of H-c, H-e and H-g (the three protons that shift the most) yields the self-association constant given in Table 1.

ROESY spectra were obtained at different theaflavin concentrations. Similar nuclear Overhauser enhancements (NOEs) were measured in all spectra, implying that the conformation of theaflavin does not change greatly on dimerisation. In particular, NOEs were observed in all cases between H-8′ and H-2, H-8 and H-4′β, and H-8 and H-2′. These NOEs were used as constraints in a molecular modelling study. All three NOEs can be simultaneously satisfied by stacking the aromatic A rings over each other, without increasing the conformational energy of the molecule (Fig. 3). However, there are a large number of conformations of similar energy, all of which have these three pairs of protons close enough to give the observed NOEs, particularly if fast conformational exchange between conformations is allowed.[14] Therefore it is most likely that theaflavin adopts a range of conformations in which the A-rings are stacked, both in the monomer and in the dimer, which are in fast exchange with each other on the chemical shift timescale. The two faces of the flavan systems are different: one face (β) is significantly more hydrophobic and less hindered than the other. The NOEs listed above, as well as molecular modelling studies, suggest that the predominant conformers have the two hydrophobic faces stacked against each other.

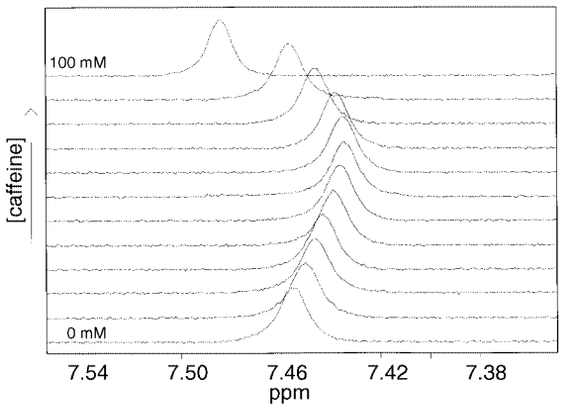

Fig. 4 A stack plot of one-dimensional proton spectra illustrating the change in resonance frequency of H-e from theaflavin upon addition of caffeine.

In ROESY spectra acquired from samples containing high proportions of dimer, an NOE between H-g and H-2 was observed. This NOE cannot be satisfied by any conformation of a single theaflavin molecule without destroying the planarity of the conjugated benzotropolone functionality. However, it can be satisfied by a dimer in which the two benzotropolone rings are stacked in an antiparallel manner, as shown in Fig. 3. This dimer structure is also strongly suggested by the chemical shift changes on dilution, in which the largest chemical shift changes are observed for the three protons directly attached to the benzotropolone system. On dilution of theaflavin the proportion of dimer is reduced, and it is observed that the H-g to H-2 NOE decreases in intensity, again consistent with antiparallel stacking.

Caffeine–theaflavin binding

When the concentration of caffeine is increased in the presence of a fixed concentration of theaflavin, the proton resonances of theaflavin undergo a change in chemical shift, indicating an interaction between caffeine and theaflavin. Almost identical patterns of chemical shift changes were seen at three different theaflavin concentrations (from 0.2 mM at which theaflavin is largely monomeric to 1.5 mM at which it is largely dimeric), implying that the interaction between caffeine and theaflavin is independent of the dimerisation state of theaflavin, and is therefore most likely occurring at the flavan ring(s) of theaflavin. This site is also implied by the associated chemical shift changes, which are largest for the protons on the flavan rings. Many theaflavin protons have an unusual chemical shift behaviour on titration with caffeine, initially moving upfield, but as the caffeine concentration is increased they then begin to move downfield (Fig. 4). This chemical shift behaviour cannot be fitted to a single binding event or to a model involving two independent binding events. However, it can be fitted well by a model requiring two sequential binding steps. The theaflavin chemical shift data from all the protons which exhibited measurable changes could be fitted simultaneously to eqn. (9) to generate values for the two binding constants K_a and K_b (Fig. 5). The values of K_a and K_b are tabulated in Table 3 for the three different concentrations of theaflavin used.

Using the affinity constants for caffeine binding to theaflavin (K_a and K_b) the concentration of each caffeine species can be calculated. Because the interaction between caffeine and theaflavin is relatively weak the population of the two bound forms under most conditions is lower than that for which NOEs can be detected using NMR, so intermolecular NOEs between caffeine and theaflavin cannot be measured. At higher concentrations of caffeine and/or theaflavin, such that intermolecular NOEs might be measurable, precipitation of the complex starts to occur during the NMR measurement and leads to poor quality spectra.

Table 3 The association constants (K_a and K_b) for the ordered binding of caffeine to theaflavin in ^2H_2O–$(C^2H_3)_2SO$ (9 : 1 v/v) at 300 K

[Theaflavin]/mM	K_a/l mol^{-1}	K_b/l mol^{-1}
1.5	11.7	15.4
0.4	14.3	21.6
0.2	9.7	12.4
Mean	11.9 ± 2.3	16.5 ± 4.7

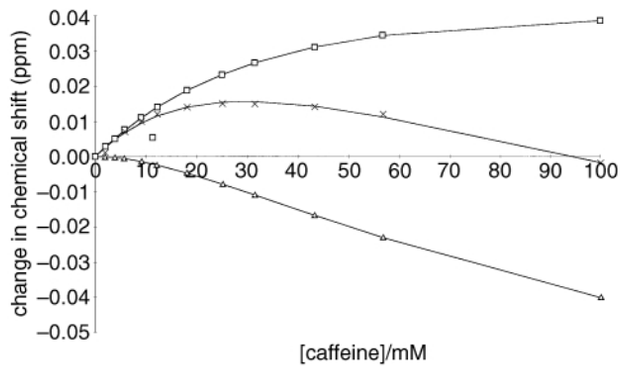

Fig. 5 A least-squares fitting of the observed chemical shift changes of the theaflavin H-8 proton when caffeine is titrated into theaflavin. Observed data (×), calculated shift change due to formation of TC (□), calculated shift change due to the formation of TC_2 (Δ).

Discussion

Caffeine and the theaflavins contribute to the organoleptic properties of black tea. The stimulatory effects of caffeine are well documented. Caffeine also has a bitter taste. Theaflavin (together with its mono- and di-galloyl derivatives) are constituents of the polyphenol content of tea not only influencing its colour but also contributing to the characteristic taste. In particular, the astringency of tea (defined as a feeling of dryness in the mouth) is due to the complexation of proteins by polyphenols.[2] Therefore, if caffeine and theaflavin are depleted, either chemically or by complexation and precipitation, the taste of tea will be affected.

The results presented here show clearly that caffeine forms aggregates in solution in which the molecules are stacked.[8,9] Theaflavin on the other hand forms dimers. The self-diffusion coefficients (Fig. 2) for theaflavin as a function of increasing concentration initially decrease but then level off, in agreement with the predicted behaviour of dimers. However, just before the theaflavin concentration reaches the point of saturation, the diffusion coefficient again decreases, suggesting that the dimers are themselves starting to associate together. Presumably it is this aggregation that leads to the precipitation of theaflavin.

The self-association constant for theaflavin is roughly 30 times stronger than that for caffeine. From the structure of theaflavin there is a clear rationale for why this might be so. The theaflavin dimer, as depicted in Fig. 3, has a compact and complementary interface between the two monomers with the electron deficient tropolone ring lying directly over the electron rich catechol ring resulting in favourable π–π interactions and a molecular environment in which intermolecular hydrogen bonds can occur.

The simplest model for the binding of caffeine to theaflavin that is consistent with the experimental data is an ordered binding of two caffeine molecules. The interactions occur at the flavan rings, and the model requires that the second caffeine cannot bind until the first caffeine is bound. In view of the structure of theaflavin (Fig. 3), in which the two flavan rings are stacked in a parallel fashion, we propose a model for the binding, shown in Fig. 6, which agrees with all the data presented above. In the model, the two faces of the flavan system are distinguished by the hydroxy group on the α-face, which is

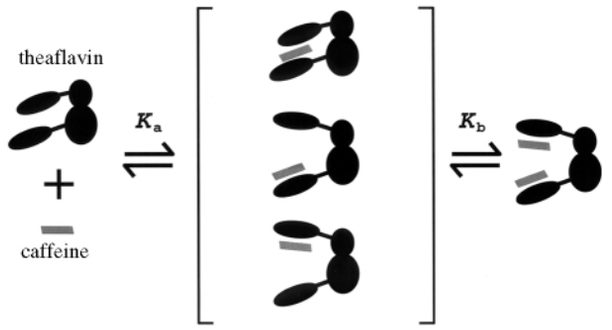

Fig. 6 Proposed model for the binding interaction between caffeine and theaflavin. For simplicity only the theaflavin monomer is shown. In the structure of uncomplexed theaflavin the two flavan rings are stacked together. The first caffeine binds in between the two flavan rings, forcing them apart. This liberates a second binding site, to which a second caffeine can bind. The model implies that the two faces of the flavan ring have different binding affinities.

assumed to be the less favourable face both for self-association and for caffeine binding. It is possible to construct more complicated models in which the α-face also binds, but the experimental data fit well to the simpler model and do not justify the more elaborate models.

The results listed in Table 3 show that the second binding event (K_b) is slightly more favourable than the first (K_a), despite the conformational change required to theaflavin. The most obvious rationale for this is that the intermediate structures contain either an exposed hydrophobic surface (bottom two structures of Fig. 6) or conformational strain (top structure of Fig. 6), neither of which are present in the final complex.

The concentrations of caffeine and theaflavin in tea (as theaflavin itself plus its galloyl derivatives) are roughly 0.5 and 0.05 mM, respectively. In addition, tea contains many other polyphenols of similar structure which could play a similar role to theaflavin. This study was carried out in 10% dimethyl sulfoxide, in which binding constants are weaker than in water.[13] A comparison with previous studies (*e.g.* ref. 9) suggests that the binding constants will be roughly 30% stronger in water. This would imply that in tea caffeine is extensively self-associated and that theaflavin is almost entirely dimeric. However the weak binding of single caffeine molecules to dimeric theaflavin indicates that the concentration of both caffeine and theaflavin present in heterogeneous complexes will be low compared to their total concentration. If this is the case then it is likely that the thearubigins and/or other polyphenolic molecules (including the gallated theaflavins) present in black tea play an important role in the formation of tea cream. If the structure of the complex has any similarity to the model proposed in Fig. 6, at least one of the two bound caffeines is free to self-associate with other caffeine molecules, thereby encouraging the formation of larger molecular assemblies. As the temperature is reduced, the binding constants become stronger, favouring supramolecular assembly. Such assemblies are typically insoluble. This observation therefore provides an explanation of the turbidity which forms as tea cools, known as tea creaming. The formation of tea creams reduces the concentration of soluble caffeine and polyphenols in tea, therefore changing the taste and mouthfeel of the tea. The formation of tea cream, and the change in organoleptic properties associated with tea creaming, are predicted to be reduced if the concentration of either caffeine or polyphenols in tea is reduced.

Experimental

Materials

Theaflavin was purified as previously described.[3] Caffeine was purchased from Sigma and deuterated solvents for NMR from Fluorochem.

NMR experiments

NMR spectra were acquired on Bruker AMX400 and DRX500 spectrometers using a 5 mm inverse probe equipped with z gradients. Experiments were performed at 300 K, pH 3.8 in ^2H_2O–[$^2H_6$]dimethyl sulfoxide [$(C^2H_3)_2SO$] or H_2O–$(C^2H_3)_2SO$ (9 : 1 by vol.). All spectra were referenced on the dimethyl sulfoxide solvent peak at 2.691 ppm (equivalent to internal DSS [2,2-dimethylsilapentane 5-sulfonic acid] = 0.015 ppm). TOCSY experiments[15] were acquired using the MLEV-17 sequence flanked by two 2.5 ms trim pulses. The spin-lock field strength was 9.4 kHz, the mixing time was 127 ms and a relaxation delay of 1 s was employed. 155 increments of 2K data points were recorded covering a spectral width of 2809 Hz in each dimension. ROESY experiments[16] were recorded using a continuous spin-lock pulse of 300 ms duration at a field strength of 2.2 kHz. 512 increments of 2 K data points were recorded covering a spectral width of 3067 Hz in each dimension, with a relaxation delay of 1.2 s. 2D experiments were acquired using time-proportional phase incrementation (TPPI)[17] and with on-resonance solvent presaturation. Data matrices were zero filled and multiplied by a cosine-squared window in both dimensions prior to Fourier transformation.

Diffusion experiments were measured using the bipolar pulse pairs longitudinal eddy-current delay (BPP-LED) sequence.[18] The dephasing and rephasing gradient pulses both consisted of a bipolar pair of rectangular shaped gradients, each of 4 ms separated by a delay of 350 μs. The time between dephasing and rephasing gradients was 18.8 ms, and the gradient strength was varied from 1 to 38 G cm^{-1}.

Chemical shift titrations

These were performed using a 5 mm NMR tube capped with a septum and using a Hamilton syringe for solution transfer. A measured volume of a theaflavin solution of known concentration [0.2, 0.4 or 1.5 mM] was replaced with a known volume of a solution having the same theaflavin concentration plus 100 mM caffeine giving a caffeine concentration span of 0 to 100 mM. Self-association constants were determined by diluting caffeine from 100 mM down to 0.31 mM, and theaflavin from 12 mM to 0.3 mM using 13 steps.

Caffeine and theaflavin self-association

The self-association constants for caffeine and theaflavin were determined from a least-squares fitting of eqn. (10) to the

$$\Delta\delta_i = \Delta\delta_{max} K_s [A]_o \{2/[1 + (4K_s[A]_o + 1)^{1/2}]\}^2 \quad (10)$$

chemical shift changes upon dilution of the two molecules,[8] where $\Delta\delta_i$ is the observed change in chemical shift, $\Delta\delta_{max}$ is the maximum change in chemical shift upon saturation, K_s is the self-association constant and $[A]_o$ is the total concentration of solute molecule A. Eqn. (10) defines the isodesmic model.[9] $\Delta\delta_i$ can be solved for each increment of the solute dilution by least squares fitting of eqn. (10) to the observed chemical shift data, to obtain a value for K_s and $\Delta\delta_{max}$.

Molecular modelling

Molecular modelling was carried out by constructing the molecular skeleton of theaflavin using Insight/Discover (Molecular Simulations, Inc.) and applying NOE constraints in a simulated annealing structure calculation.

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