Multiple Interactions between Polyphenols and a Salivary Proline-Rich Protein Repeat Result in Complexation and Precipitation†

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ABSTRACT: Polyphenols (tannins) in the diet not only precipitate oral proteins, producing an astringent sensation, but also interact with dietary proteins and digestive enzymes in the gut, resulting in a variety of antinutritive and toxic effects. Salivary proline-rich proteins (PRPs), which are secreted into the oral cavity, form complexes with and precipitate dietary polyphenols, and thus, they constitute the primary mammalian defense directed against ingested tannins. In order to characterize the interaction, NMR studies were performed which involved titrating a series of polyphenols into a synthetic 19-residue PRP fragment. The results show that the predominant mode of association is a hydrophobic stacking of the polyphenol ring against the *pro-S* face of proline and that the first proline residue of a Pro-Pro sequence is a particularly favored binding site. Measurement of dissociation constants indicates that the larger and more complex polyphenols interact more strongly with the PRP fragment; the order of binding affinity was determined as procyanidin dimer B-2 > pentagalloylglucose > trigalloylglucose >> proanthocyanidin monomer (−)-epicatechin ≈ propyl gallate. Smaller polyphenols can bind with one phenolic ring stacked against each proline residue, whereas larger polyphenols occupy two or three consecutive prolines. The more complex polyphenols interact with the PRP fragment in a multidentate fashion; moreover, they self-associate or stack when bound. Thus, a model is proposed in which multiple polyphenol/polyphenol and polyphenol/PRP interactions act cooperatively to achieve precipitation.

Polyphenols, otherwise known as tannins, are a distinct family of secondary metabolites present in the leaves, bark, and fruit of many higher plants. They are believed to participate in the defense of the plant by rendering the tissues unpalatable and non-nutritious and therefore unacceptable as food sources for herbivores and omnivores (Feeny, 1970; Mehansho et al., 1987b; Robbins et al., 1987a; Haslam, 1989; Nahrstedt, 1989). Tannins possess an abundance of phenolic groups, have molecular masses in the range of 500−5000 Da, and display a diversity of structures which forms the basis of their classification into two families: (1) the condensed proanthocyanidins which are oligomers of the flavan-3-ol skeleton and (2) the hydrolyzable tannins which are esters, usually with D-glucose, of gallic acid and its derivatives (Haslam, 1989). Polyphenols are described as astringent, and it is generally held that their most important characteristic is their propensity to form complexes with proteins, polysaccharides, and alkaloids (*e.g.* caffeine) (Hagerman & Butler, 1981; McManus et al., 1985; Spencer et al., 1988). It is believed that the efficacy of many herbal and folk medicines may be attributed to the astringent properties of the polyphenols that they contain (Haslam et al., 1989). The presence of polyphenols in foods and beverages (*e.g.* sorghum, millet, fruit, cocoa, coffee, tea, beer, and wine) produces a sensation of astringency, which is perceived as a diffuse feeling of extreme dryness and roughness that is not confined to a particular region of the mouth or tongue. This is thought to arise from the precipitation of oral proteins and mucopolysaccharides. Polyphenols in the digestive tract display many detrimental effects, including the inhibition of iron absorption (Mehansho et al., 1987b), esophageal cancer (Warner & Azen, 1988), and the irreversible complexation of gut enzymes and dietary proteins (Mehansho et al., 1987b; Robbins et al., 1991; Scalbert, 1991), the consequences of which result in polyphenol-rich foods being nutritionally poor.

Polyphenols have a significant affinity for extended proteins and peptides that contain a high proportion of proline residues in their sequences (Hagerman & Butler, 1981). Among the proteins that bind polyphenols most strongly are members of the family of proline-rich proteins (PRPs[1]) which comprise up to 70% of the protein in human parotid saliva (Kauffman & Keller, 1979; Bennick, 1987). Proline, glutamine, and glycine residues, arranged into 5−15 almost identical repeats, account for 70−80% of the amino acids (Bennick, 1982). It is thought that the major function of the proline-rich repeats is to bind and precipitate dietary polyphenols, thereby neutralizing their harmful actions (Mehansho et al., 1987b; Robbins et al., 1987b; Warner & Azen, 1988; Mole et al., 1990; McArthur et al., 1995). The

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[1] Abbreviations: PRPs, proline-rich proteins; TGG, β-1,3,6-tri-*O*-galloyl-D-glucopyranose; PGG, β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose; [$^2H_6$]DMSO, [$^2H_6$]dimethyl sulfoxide; NMR, nuclear magnetic resonance; TOCSY, two-dimensional total correlation spectroscopy; ROESY, two-dimensional rotating frame nuclear Overhauser effect spectroscopy; TSP, 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate; K_a, association constant; K_d, dissociation constant; $\Delta\delta_{max}$, maximum change in chemical shift; ROE, rotating frame nuclear Overhauser effect; NOE, nuclear Overhauser effect.

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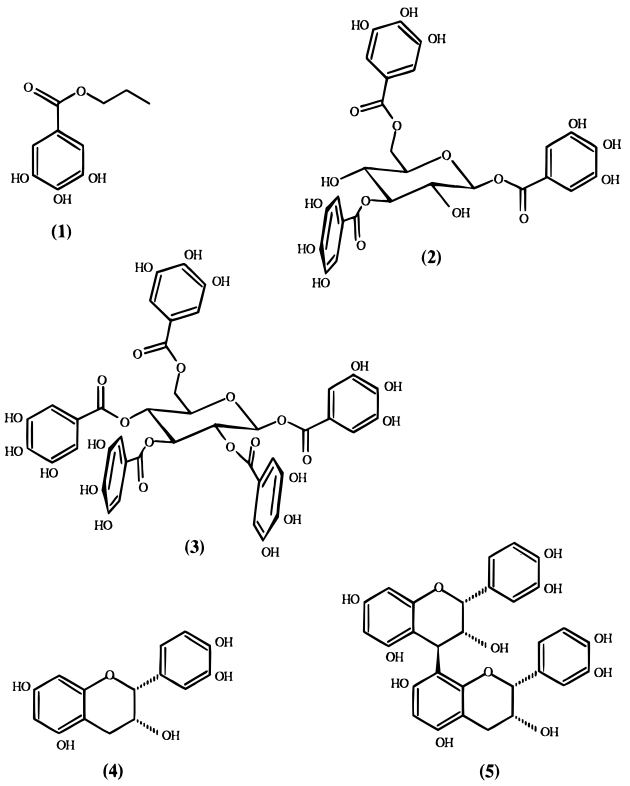

FIGURE 1: Polyphenols used in the study: (**1**) propyl gallate; (**2**) TGG, β-1,3,6-tri-*O*-galloyl-D-glucopyranose; (**3**) PGG, β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose; (**4**) (−)-epicatechin; and (**5**) procyanidin B-2.

strongest evidence that salivary PRPs act as defensive proteins directed against ingested polyphenols is provided by the diet-mediated induction of PRPs in rodents. PRPs are normally present at low concentrations in rodent saliva in order to perform their regulatory and dental functions (McArthur et al., 1995). However, they are induced to very high levels by dietary polyphenols in rats (Mehansho et al., 1983; Jansman et al., 1994) and mice (Mehansho et al., 1985) but not in hamsters (Mehansho et al., 1987a). The growth rates of rats, mice, and hamsters are initially retarded; however, within 3 days, the growth rates of rats and mice recover, but there is no change in the growth rate of hamsters. In the rats and mice, growth resumption coincides with the maximum secretion of salivary PRPs. There is no induction of PRPs in hamsters, and growth rates continue to be static over several months. However, hamster growth rates recover on supplementing the polyphenol-rich diet with gelatin (a proline-containing protein).

We have previously studied the interaction of β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (PGG) **(3)** (Figure 1) with the synthetic peptide GPQQRPPQPGNQQGPPPQGGPQ (Murray et al., 1994) representing a single repeat of mouse proline-rich protein MP5 (Layfield et al., 1992). We showed that the peptide adopts an extended and random conformation in solution (Murray & Williamson, 1994) and that the interaction is primarily a hydrophobic association between proline residues in the peptide and aromatic phenolic rings in PGG. The purpose of the work described here is to provide more insight into the molecular basis of polyphenol/PRP precipitation by conducting a detailed comparison of a range of different polyphenols. The polyphenols selected (Figure 1) were propyl gallate **(1)**, β-1,3,6-tri-*O*-galloyl-D-glucopyranose (TGG) **(2)**, and β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose **(3)**, which are members of the hydrolyzable tannin family of increasing complexity and molecular mass, and (−)-epicatechin **(4)** and procyanidin B-2 **(5)**, which are simple representatives of the proanthocyanidins. The PRP fragment QGPPPQGGPQQRPPQPGNQ used in this investigation has the same sequence as that studied previously but begins and ends at a different position within the repeat. This proline-rich peptide is preferred to the intact protein since it gives a simpler NMR spectrum.

EXPERIMENTAL PROCEDURES

Materials. The mouse salivary proline-rich peptide, QGPPPQGGPQQRPPQPGNQ, was synthesised as detailed previously (Murray & Williamson, 1994). The isolation and purification of the polyphenols from their natural sources are described in the references listed: (−)-epicatechin (Thompson et al., 1972), procyanidin B-2 (Thompson et al., 1972), TGG (Haddock et al., 1982), PGG (Armitage et al., 1961; Haddock et al., 1982), and propyl gallate (a gift from Foursquare Ltd).

Sample Preparation. Polyphenol solutions (0.50 mL) were prepared in the following solvent mixtures: B-2 (40 mM) in 90% H_2O/10% [$^2H_6$]DMSO, (−)-epicatechin (50 mM) in 90% H_2O/10% ^2H_2O, and PGG (40 mM), TGG (50 mM), and propyl gallate (50 mM) in both 90% H_2O/10% [$^2H_6$]-DMSO and 90% H_2O/10% ^2H_2O. Since polyphenols are not readily soluble in aqueous solvents, the polyphenol solutions were sonicated and heated briefly in a boiling water bath to ensure total solubility. The tannin solutions were adjusted to pH 3.8 ± 0.1 measured using a combination glass electrode (Russell pH meter) at room temperature. For the polyphenol titration experiments, 0.50 mL volumes of 4 mM proline-rich peptide solution (0.45 mL volume of 2 mM proline-rich peptide solution for the PGG titration in 90% H_2O/10% [$^2H_6$]DMSO) were prepared in 5 mm NMR tubes and were adjusted to pH 3.8 ± 0.1. For simplification and clarity, the solvent systems 90% H_2O/10% ^2H_2O and 90% H_2O/10% [$^2H_6$]DMSO will be subsequently abbreviated to water and 10% DMSO, respectively.

The self-association of the polyphenols was investigated in the solvent systems used during the proline-rich peptide/polyphenol titrations. Polyphenol solutions with a concentration of 10 mM (PGG) or 50 mM [propyl gallate, (−)-epicatechin, and TGG] were prepared in water and/or 10% DMSO, and 0.50 mL volumes were diluted with the appropriate solvent mixture.

NMR Experiments. The titration of the polyphenols into the proline-rich peptide was followed by the acquisition of two-dimensional total correlation spectroscopy (TOCSY) spectra, and occasionally additional two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectra, after each titration increment. All spectra were acquired at 276 K using a Bruker AMX-500 spectrometer connected to a liquid nitrogen dewar which provided a source of cold gas. The spectra were processed and displayed using FELIX (Biosym Technologies Inc.) operating on a Silicon Graphics workstation. The TOCSY and ROESY experiments were recorded with water presaturation using time-proportional phase incrementation into 600 4K complex files, with 32 scans per increment and spectral widths of 12.5 kHz in F_2 and 6.25 kHz in F_1. The TOCSY spin lock was achieved with a 100 ms DIPSI-2 pulse sequence at a field

strength of 10.9 kHz, and the ROESY spin lock was achieved with continuous irradiation for 100 ms using a field strength of 3.5 kHz. A Hahn echo with an echo delay of 2.54 ms was inserted in the pulse sequences prior to data acquisition for the application of a 32-point time domain Gaussian convolution filter to remove the solvent signal (Waltho & Cavanagh, 1993). The TOCSY and ROESY data were left-shifted by 32 complex points in t_2, and the first points were halved in both dimensions. Lorentz-to-Gaussian window functions were applied in t_2, and sine squared-bell window functions shifted by 90° were applied in t_1 prior to zero-filling the t_1 data to 4K. The data were Fourier transformed into 4K × 2K ($F_2 \times F_1$) real points, and the spectral widths and the number of points in F_2 were halved during processing, by the removal of 1K real points from both edges of the spectrum, resulting in a 2K × 2K real matrix with spectral widths of 6.25 kHz in both F_1 and F_2. Referencing of the spectra acquired in 10% DMSO was achieved by using the residual dimethyl sulfoxide resonance as a secondary reference at 2.73 ppm from external 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) (Murray & Williamson, 1994), whereas the spectra recorded in water were referenced to external TSP dissolved in the same solvent.

For the polyphenol concentration dependence studies, the dilutions were followed at 276 K by one-dimensional experiments acquired, using internal TSP as the reference, into 8K complex data points. Improvements in baseline flatness were achieved by using a spectral width of 12.5 kHz and by insertion of a 140 μs Hahn echo pulse sequence (Waltho & Cavanagh, 1993). The data were left-shifted by two complex points, and a Lorentzian window function was implemented before Fourier transformation.

Polyphenol Self-Association. The association constants for polyphenol self-association were determined from a least-squares fitting of eq 1 (Baxter et al., 1996) to the chemical shift data obtained from the one-dimensional dilution experiments:

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

where $\Delta\delta_i$ is the observed change in chemical shift, $\Delta\delta_{max}$ is the maximum change in chemical shift on saturation, K_a is the association contant, and $[A]_o$ is the total concentration of solute A. The equation assumes that solute molecules self-associate to form stacks (dimers, trimers, *etc*.) where the equilibrium constant K_a for each association step is the same.

Peptide/Polyphenol Association. Determination of the dissociation constant for the proline-rich peptide/polyphenol interaction was achieved by fitting eq 2 to the chemical shift data obtained from the peptide/polyphenol titrations. Equation 2 is a version of the 1/1 binding equation presented in Murray et al. (1994) modified to allow for binding of the polyphenol at multiple sites on the peptide.

$$\Delta\delta_i = \frac{\Delta\delta_{max}}{2}\left\{\left(1 + \frac{K_d}{n[P]_i} + \frac{[T]_i}{n[P]_i}\right) - \left[\left(1 + \frac{K_d}{n[P]_i} + \frac{[T]_i}{n[P]_i}\right)^2 - 4\frac{[T]_i}{n[P]_i}\right]^{1/2}\right\} \quad (2)$$

where $\Delta\delta_i$ and $\Delta\delta_{max}$ are defined as in eq 1, K_d is the dissociation constant, n is the number of binding sites present on the proline-rich peptide, $[P]_i$ is the total concentration of proline-rich peptide at each titration increment, and $[T]_i$ is the concentration of self-associated polyphenol species (monomers, dimers, trimers, *etc*.) at each titration increment (see below). $[P]_i$ is given by

$$[P]_i = \frac{[P]_o V_o}{V_o + V_i} \quad (3)$$

where $[P]_o$ is the initial concentration of peptide solution, V_o is the initial volume of peptide solution, and V_i is the total volume of polyphenol solution at each titration increment.

The total concentration of polyphenol molecules $[T]_o$ at each titration increment in the absence of any self-association is obtained from

$$[T]_o = \frac{[T]_a V_i}{V_o + V_i} \quad (4)$$

where $[T]_a$ is the concentration of the bulk polyphenol solution. However, as noted previously, polyphenol molecules self-associate in solution, and thus, $[T]_o$ must be re-expressed as the concentration of self-associated polyphenol species $[T]_i$. $[T]_o$ comprises the sum of the concentration of monomeric polyphenol [T], dimeric polyphenol $[T_2]$, *etc*. as

$$[T]_o = [T] + 2[T_2] + 3[T_3] + ... \quad (5)$$

which gives

$$[T]_o = [T]/(1 - K_a[T])^2 \quad (6)$$

and on rearrangement

$$[T] = [T]_o\{2/[1 + (4K_a[T]_o + 1)^{1/2}]\}^2 \quad (7)$$

Assuming that the self-association constant is independent of stack length, then the concentration of self-associated polyphenol species is given by

$$[T]_i = [T] + [T_2] + [T_3] + ...$$
$$= [T] + K_a[T]^2 + K_a^2[T]^3 + ... \quad (8)$$

which gives

$$[T]_i = [T]/(1 - K_a[T]) \quad (9)$$

Substitution of eq 7 into eq 9 yields the concentration of polyphenol species available to associate with the proline-rich peptide. The values of K_a used during the fitting of the peptide/polyphenol titrations are derived from the separate polyphenol self-association experiments.

This description of the association processes taking place carries a number of assumptions. (1) The proline-rich peptide contains a number of independent polyphenol binding sites which have an identical affinity for polyphenol species. (2) The presence of proline-rich peptide has no effect on the self-association of polyphenol molecules. (3) Any single polyphenol species may only bind to the peptide at one binding site at any one time. Of these, assumption (1)

requires justification, and assumptions (2) and (3) are valid in most cases, as discussed below.

It was found that, after least-squares fitting of eq 2 to the chemical shift data, the fitted parameters K_d and $\Delta\delta_{max}$ were highly correlated, which is a consequence of the complex precipitating well before saturation, resulting in truncation of the experimental saturation curve. In such cases, many different binding curves can be fitted to the data almost equally well, in which an increase in K_d is matched by an increase in $\Delta\delta_{max}$ (see Figure 4). In order to test the reliability of the fitting, a range of statistical tests were applied, including random variation of experimental data and Monte Carlo fitting. The most useful conclusions were obtained by fitting n (the number of equivalent binding sites) and $\Delta\delta_{max}$ for a range of fixed K_d values and quantifying the goodness of the fit by using the χ^2 statistic. The fitting was carried out on all available proton titration curves and provided a highly consistent value for n across all protons (for each polyphenol) and a reasonably consistent value for K_d.

This result provides a justification for assumption (1) above. If different binding sites have very different affinities, one would expect that very different values of n and K_d would emerge for protons within different binding sites. The consistent values of n obtained indicate that the affinities at different sites on the peptide are similar. Because the estimated errors in individual K_d values are relatively large, we present only a single consensus value for K_d of each polyphenol. The consensus values were obtained from the χ^2 *vs* $\log(K_d)$ curves, weighted by our confidence in the original data (determined largely by overlap in the cross-peaks). All the least-squares fitting was performed using a FORTRAN program operating on a Silicon Graphics workstation.

Analysis of the Polyphenol Association State when Bound to the Peptide. During the course of the peptide/polyphenol titration experiments, the polyphenol proton resonances shift upfield as a result of polyphenol/polyphenol aromatic stacking interactions (rather than polyphenol/peptide interactions which are expected to produce much smaller chemical shift changes). This may be modeled by assuming that the observed shift is a weighted average of the shifts of free and bound species:

$$\delta_{calc} = \delta_b f_b + \delta_f f_f \tag{10}$$

where δ_{calc} is the calculated chemical shift of a polyphenolic proton, δ_b is the chemical shift of the proton when bound to the peptide (*i.e.* $\delta_{monomer}$), δ_f is the chemical shift of the proton when unbound (*i.e.* free to self-associate), f_b is the fraction of polyphenol bound to the peptide, and f_f is the fraction of polyphenol free to self-associate. We have calculated two extreme cases: where the polyphenol is associated when bound to the peptide, to the same extent as it is when free (in which case, the calculated observed shift is identical to that seen in the absence of peptide), or alternatively where the polyphenol binds as a monomer. In this second case, the following equilibrium exists in a solution containing proline-rich peptide and polyphenol:

$$[B] + [T] \underset{K_d}{\rightleftharpoons} [BT]$$

where [B] is the concentration of unoccupied polyphenol binding sites on the peptide, [T] is the concentration of monomeric polyphenol, and [BT] is the concentration of the binding site/polyphenol complex.

$$[B]_i = [B] + [BT] \tag{11}$$

$$[T]_o = [T]_f + [BT] \tag{12}$$

and

$$[T] = [T]_f\{2/[1 + (4K_a[T]_f + 1)^{1/2}]\}^2 \tag{13}$$

where $[B]_i$ is the total concentration of polyphenol binding sites present on the peptide ($\equiv n[P]_i$), $[T]_o$ is the total concentration of polyphenol molecules, and $[T]_f$ is the concentration of free (unbound) polyphenol molecules. Note that eq 13 is analogous to eq 7.

By using the calculated number of polyphenol binding sites (see below), and the previously determined peptide/polyphenol dissociation constant and polyphenol self-association constant, the value of [BT] at each titration increment can be calculated by numerical methods. Hence, f_b and f_f can be obtained from $[BT]/[T]_o$ and $[T]_f/[T]_o$, respectively. The chemical shift values (δ_f) corresponding to the concentration of $[T]_f$ at each titration increment are determined from a polynomial curve fitting of the polyphenol self-association data. Thus, eq 10 can be solved to obtain the calculated chemical shift (δ_{calc}).

RESULTS

Initial Observations. All the proline-rich peptide/polyphenol titration experiments, apart from those of propyl gallate, were limited by the appearance of a cloudy precipitate which became denser on subsequent additions of polyphenol. The precipitate could be solubilized by warming, but on cooling, the precipitate reappeared. The polyphenol/peptide molar ratios at which a precipitate was first observed are as follows: TGG in water, 0.25; TGG in 10% DMSO, 1.5; PGG in water, <0.4 (first increment); PGG in 10% DMSO, 3.2 (2 mM peptide concentration); (−)-epicatechin in water, 4.0; and B-2 in 10% DMSO, 2.0.

Chemical Shift Effects. Resonance assignment of the proline-rich peptide was based on data published previously (Murray & Williamson, 1994). During the peptide/polyphenol titrations, many of the proton signals of the peptide, particularly those of proline, Gly2, Gly8, Arg12, and Gln15, were observed to shift upfield. Figure 2 presents the average observed change in chemical shift ($\delta_{initial} - \delta_{final}$) for the carbon-bound protons in each residue of the peptide on titration with the polyphenols.

Table 1 lists the observed chemical shift changes ($\delta_{initial} - \delta_{final}$) for the proline CαH signals on titration with the polyphenols. In all the peptide/polyphenol titration experiments, three proline CαH resonances, Pro3, Pro4, and Pro13, which occur N-terminal to another proline in the sequence, consistently shift upfield more than the other proline CαH signals. In addition, the stereospecifically assigned proline Cβ^{2}H peaks consistently display chemical shift changes of differing magnitudes; in all the titrations, the downfield Cβ^{3}H (*pro-S*) resonance shows larger upfield chemical shift changes than the upfield Cβ^{2}H (*pro-R*) signal. For example, the $\Delta\delta_{C\beta^3H}/\Delta\delta_{C\beta^2H}$ ratios for PGG in 10% DMSO were as

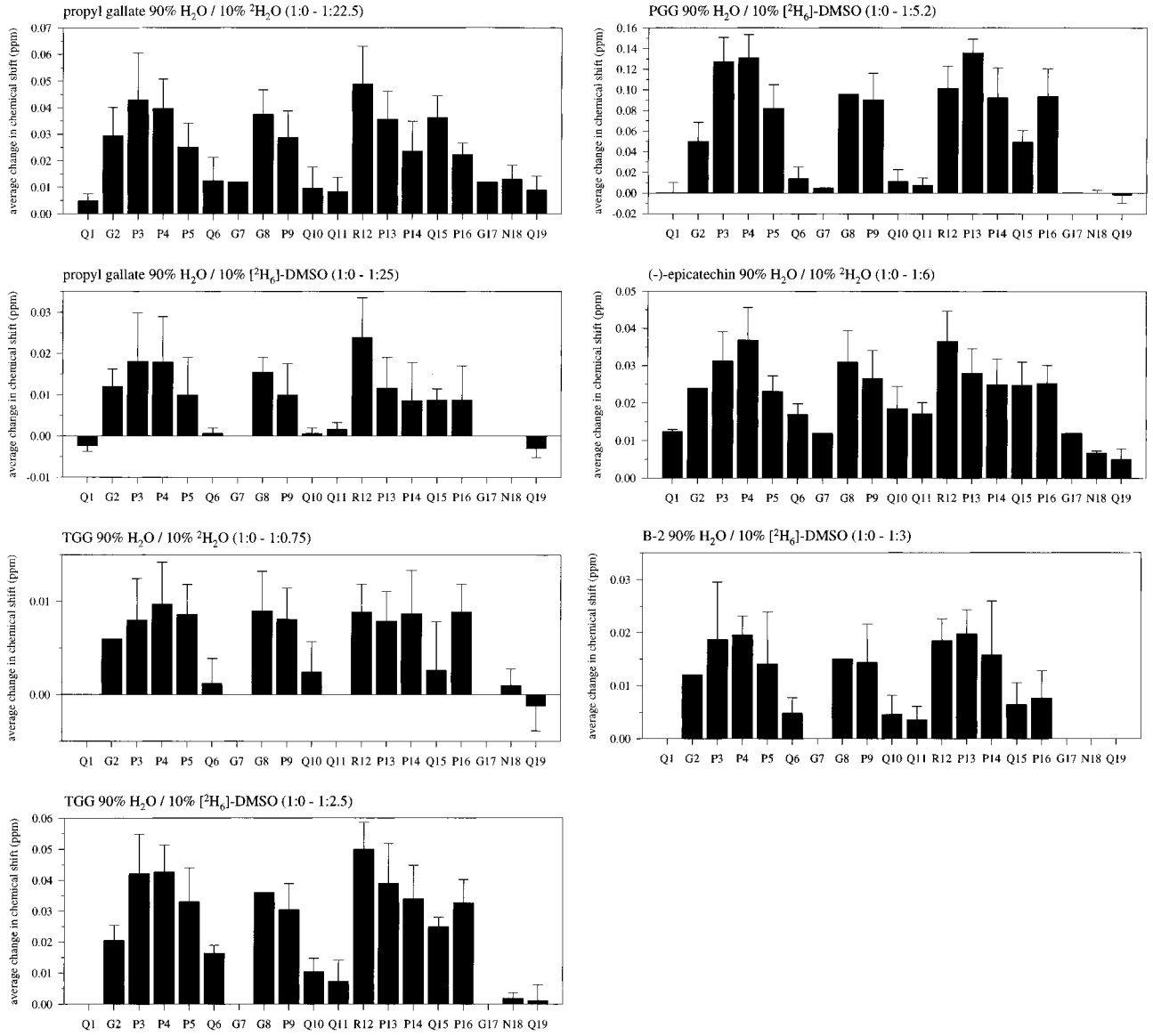

FIGURE 2: Average change in chemical shift (ppm), $\delta_{free} - \delta_{final}$, of the carbon-bound protons in each residue of the proline-rich peptide observed on titration with the polyphenols. The initial and final peptide/polyphenol molar ratios are given in parentheses. The error bars indicated in the figure represent the standard deviations of the chemical shift differences determined for protons within each residue. The experimental error in the chemical shift measurement is about 0.003 ppm.

Table 1: Comparison of the Observed Change in Chemical Shift (ppm), $\delta_{initial} - \delta_{final}$, of the Proline CαH Resonances on Titration with Polyphenol

	Pro3[a]	**Pro4**[a]	Pro5	Pro9	**Pro13**[a]	Pro14	Pro16
propyl gallate/water	**0.076**	**0.052**	0.017	0.023	**0.049**	0.018	0.021
propyl gallate/10% DMSO	**0.037**	**0.031**	0.000	0.000	**0.024**	0.000	0.000
TGG/water	**0.018**	**0.018**	0.006	0.007	**0.012**	0.006	0.007
TGG/10% DMSO	**0.064**	**0.049**	0.022	0.022	**0.061**	0.018	0.022
PGG/10% DMSO	**0.138**	**0.141**	0.050	0.050	**0.141**	0.050	0.055
(−)-epicatechin/water	**0.043**	**0.049**	0.018	0.021	**0.036**	0.016	0.021
B-2/10% DMSO	**0.036**	**0.025**	0.009	0.006	**0.024**	0.003	0.004

[a] Proline residues in the peptide sequence which are N-terminal to another proline residue (Pro3, Pro4, and Pro13) consistently have larger CαH chemical shift changes (bold text) than the remaining proline residues. Since the titrations were run to different final peptide/polyphenol molar ratios, chemical shift changes should only be compared within each system.

follows: Pro3, 1.44; Pro4, 1.48; Pro5, 1.26; Pro9, 1.22; Pro13, 1.19; Pro14, 1.43; and Pro16, 1.18.

NMR Parameters. In a previous NMR study (Murray & Williamson, 1994), it was established that the proline-rich peptide adopted an extended and unstructured conformation. During all the polyphenol titrations, the intensity of the interresidue rotating frame nuclear Overhauser effects (ROEs) and the size of the $J_{HN\alpha}$ coupling constants remained constant, suggesting that the conformation of the proline-rich peptide was not perturbed in the presence of polyphenol (data not shown). However, progressive exchange broadening of the peptide resonances was observed during most of the titrations. ROESY spectra acquired from the final molar ratio increments of the peptide/polyphenol titrations were analyzed

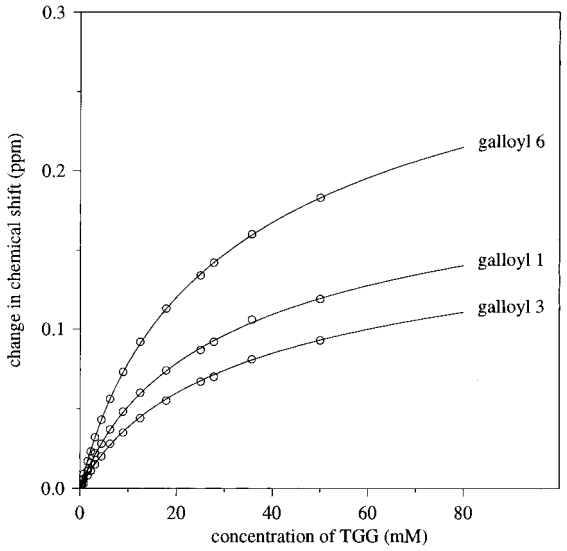

FIGURE 3: Comparison of the observed (○) and fitted (—) chemical shift differences (using eq 1) for the three galloyl proton resonances of TGG in 10% DMSO with increasing TGG concentrations.

for the presence of intermolecular ROEs between protons of the proline-rich peptide and protons of the tannin. Only the TGG and PGG systems (10% DMSO) resulted in intermolecular ROEs, which were observed between the galloyl protons and protons of proline, glycine (Gly2 and Gly8), and arginine residues. This result is consistent with our previous observations on the complexation of PGG with a similar salivary proline-rich peptide (Murray et al., 1994) and confirms that the chemical shift changes seen result directly from binding interactions.

Calculation of Association Constants for the Self-Association of Polyphenols. During the polyphenol dilution experiments, almost all the proton resonances displayed upfield changes in chemical shift, which is consistent with stacking interactions of the aromatic functions. Figure 3 compares the observed and fitted chemical shift changes for the three galloyl proton resonances (galloyl 1, galloyl 3, and galloyl 6) of TGG. The self-association constants (K_a) and $\Delta\delta_{max}$ values for the polyphenols are given in Table 2. The value of K_a for polyphenol self-association is required in the subsequent determination of K_d for the peptide/polyphenol interaction (see below).

Calculation of Dissociation Constants for the Peptide/Polyphenol Interaction. Dissociation constants for the peptide/polyphenol interactions and the number of binding sites present on the peptide for each polyphenol were obtained by least-squares fitting of eq 2 to the $\Delta\delta$ *vs* [polyphenol] binding curves and subsequent statistical analysis. Figure 4 presents $\Delta\delta$ *vs* [polyphenol] binding curves for a representative peptide proton in each of the peptide/polyphenol titrations. The best-fitted curves defined by eq 2 for a range of fixed K_d values are also illustrated. Curve fitting of each test value of K_d yielded a value for the χ^2 statistic, which describes the goodness of fit of the curve. Figure 5 shows the corresponding χ^2 *vs* log(K_d) plots for the data presented in Figure 4. The theoretical curve which gives the best agreement with the chemical shift data has a minimum χ^2 value. As an example, Table 3 presents the fitting results of the proline CαH chemical shift data derived during the peptide/TGG titration in 10% DMSO for various fixed K_d values. Best estimates of K_d were obtained for all

Table 2: K_a and $\Delta\delta_{max}$ for the Self-Association of the Polyphenols[a]

	K_a (M^{-1})	$\Delta\delta_{max}$ (ppm)
propyl gallate/water		
galloyl	12.1	0.85
$C\alpha H_2$	9.4	0.73
mean	10.7 ± 1.9	
propyl gallate/10% DMSO		
galloyl	7.6	0.49
$C\alpha H_2$	4.9	0.56
mean	6.2 ± 1.9	
TGG/10% DMSO		
galloyl 1	29.8	0.265
galloyl 3	25.8	0.219
galloyl 6	29.7	0.407
mean	28.4 ± 2.3	
PGG/10% DMSO		
galloyl 1	170.0	0.029
galloyl 4	256.4	0.049
galloyl 6	296.4	0.157
mean	241.2 ± 64.2	
(−)-epicatechin/water		
C2	21.9	0.774
C3	16.9	0.339
C4	27.3	0.173
C4	23.7	0.334
C6/C8[b]	39.3	0.156
C6/C8[b]	36.2	0.141
C2′	27.7	0.191
C5′	28.4	0.127
C6′	22.6	0.275
mean	27.1 ± 7.0	

[a] A self-association constant K_a (=300 M^{-1}) was estimated for B-2 on the basis of hydrophobicity consistent with the observed low solubility in DMSO/water mixtures, as well as aromaticity and size. [b] Individual assignment of the protons at positions C6 and C8 was not achieved.

protons in each of the polyphenol titrations, which were used to derive the consensus values of K_d and n given in Table 4. The peptide/polyphenol titrations for propyl gallate, TGG, and PGG were studied both in water and in 10% DMSO which allows a comparison of the effect of solvent on binding.

A few of the peptide $\Delta\delta$ *vs* [polyphenol] binding curves of the TGG, PGG, and B-2 titrations displayed sigmoidal behavior (*e.g.* Gly2 and Gly8 Cα protons in the peptide/TGG titration), whereas in the peptide/propyl gallate and peptide/(−)-epicatechin titrations, only normal binding curves were observed. The sigmoidal curves can be readily understood as a cooperative effect involving interactions of more than one aromatic ring of TGG, PGG, or B-2 with different regions of the peptide. For the larger polyphenols, these observations provide the first indication of multisite interactions, a point that is discussed later.

Self-Association of the Polyphenols when Bound. During the peptide/polyphenol titration experiments, the polyphenol resonances shift upfield with increasing polyphenol concentration, as shown for propyl gallate in Figure 6. These chemical shift changes give details about the association state of the polyphenols when bound to the peptide, because the observed chemical shifts are weighted averages of the shifts of the free and bound species, which in turn depend on the degree of association of these species. Figure 6 compares the observed galloyl proton chemical shift during the peptide/propyl gallate titration to the calculated shifts of the polyphenol binding to the peptide either as a monomer or as a polymeric stack (with a degree of association equal to that in free solution). It is clear that the observed shifts are

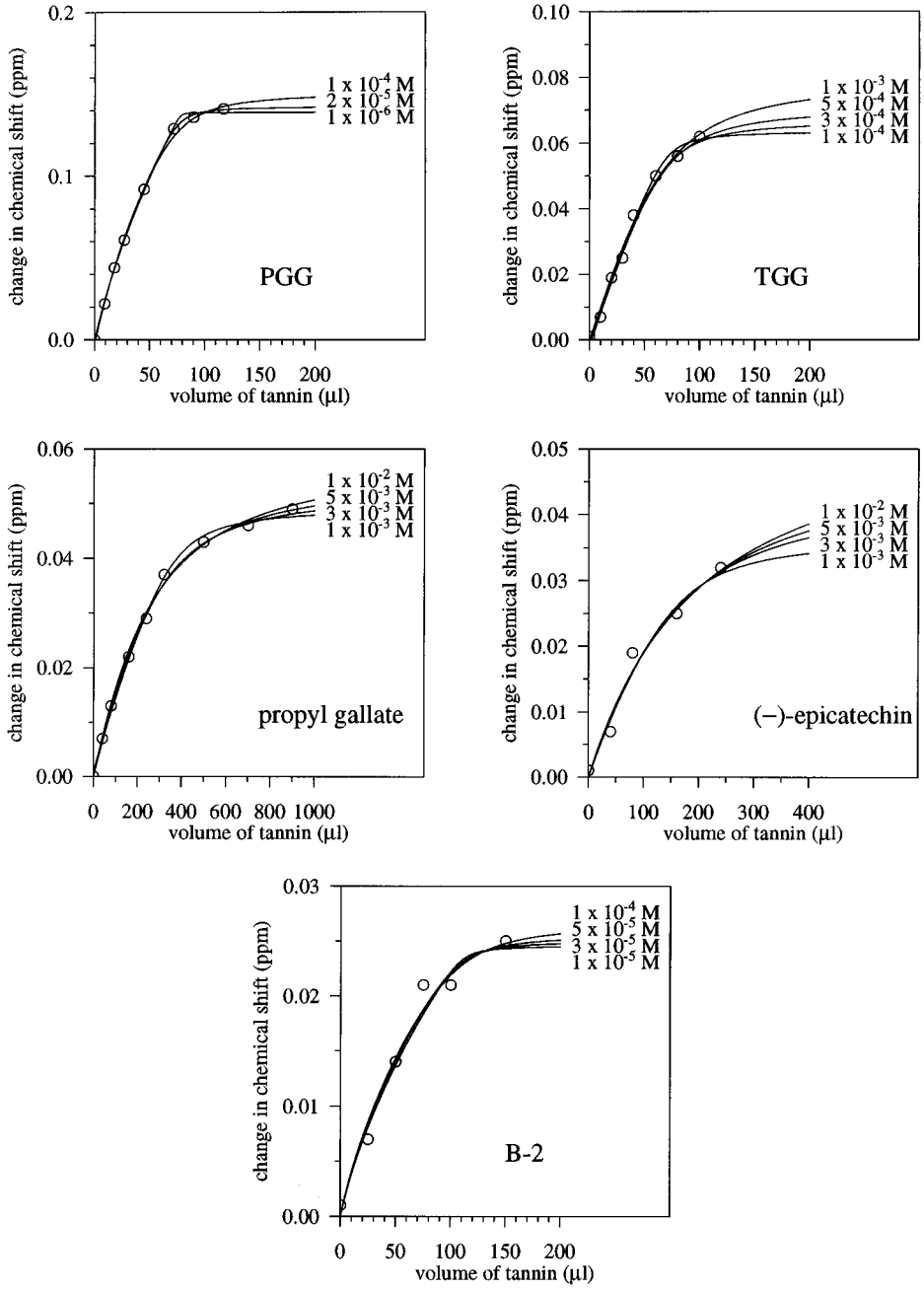

FIGURE 4: Observed changes in chemical shift (○) and the best-fit curves corresponding to a range of fixed K_d values (indicated in each panel) for the Pro13 CαH resonance in the PGG (10% DMSO), TGG (10% DMSO), and propyl gallate (water) titrations, the upfield Arg12 CβH resonance in the (−)-epicatechin (water) titration, and the upfield Pro13 CδH resonances in the B-2 (10% DMSO) titration.

much closer to those expected for binding as a stack, implying that the state of self-association of propyl gallate when bound to the peptide is almost as great as it is in free solution. Similar behavior was seen for other polyphenols [thereby justifying assumption (2) above]. As an example, Figure 7 presents the chemical shift changes seen for TGG.

DISCUSSION

Polyphenol Self-Association. Association constants for the self-association of the polyphenols were determined from the $\Delta\delta$ *vs* [polyphenol] data and may be used to rank the polyphenols in order of their propensity to self-associate: propyl gallate < (−)-epicatechin < TGG ≪ PGG. This trend corresponds with the increasing number of aromatic rings and with the increasing size and hydrophobicity of the polyphenol molecules. For (−)-epicatechin, TGG, and PGG, a measure of relative hydrophobicity is given by the distribution coefficients between octan-1-ol and water at 20 °C (Spencer et al., 1990): (−)-epicatechin, 1.6; TGG, 1.5; and PGG, 31.7.

Nature of the Binding Site. The appearance of a cloudy precipitate during the titrations (except for those involving propyl gallate) demonstrates that the polyphenols bind and form complexes with the proline-rich peptide. Polyphenol aggregation during the peptide/polyphenol titrations can be excluded as the cause of the precipitate, since precipitates were not observed in solutions of the polyphenols alone at concentrations in excess of those used in the titration experiments. The residue-specific chemical shift changes for protons of the peptide on addition of polyphenol were shown in a previous study to arise as a direct result of a binding interaction which was confirmed by the presence of

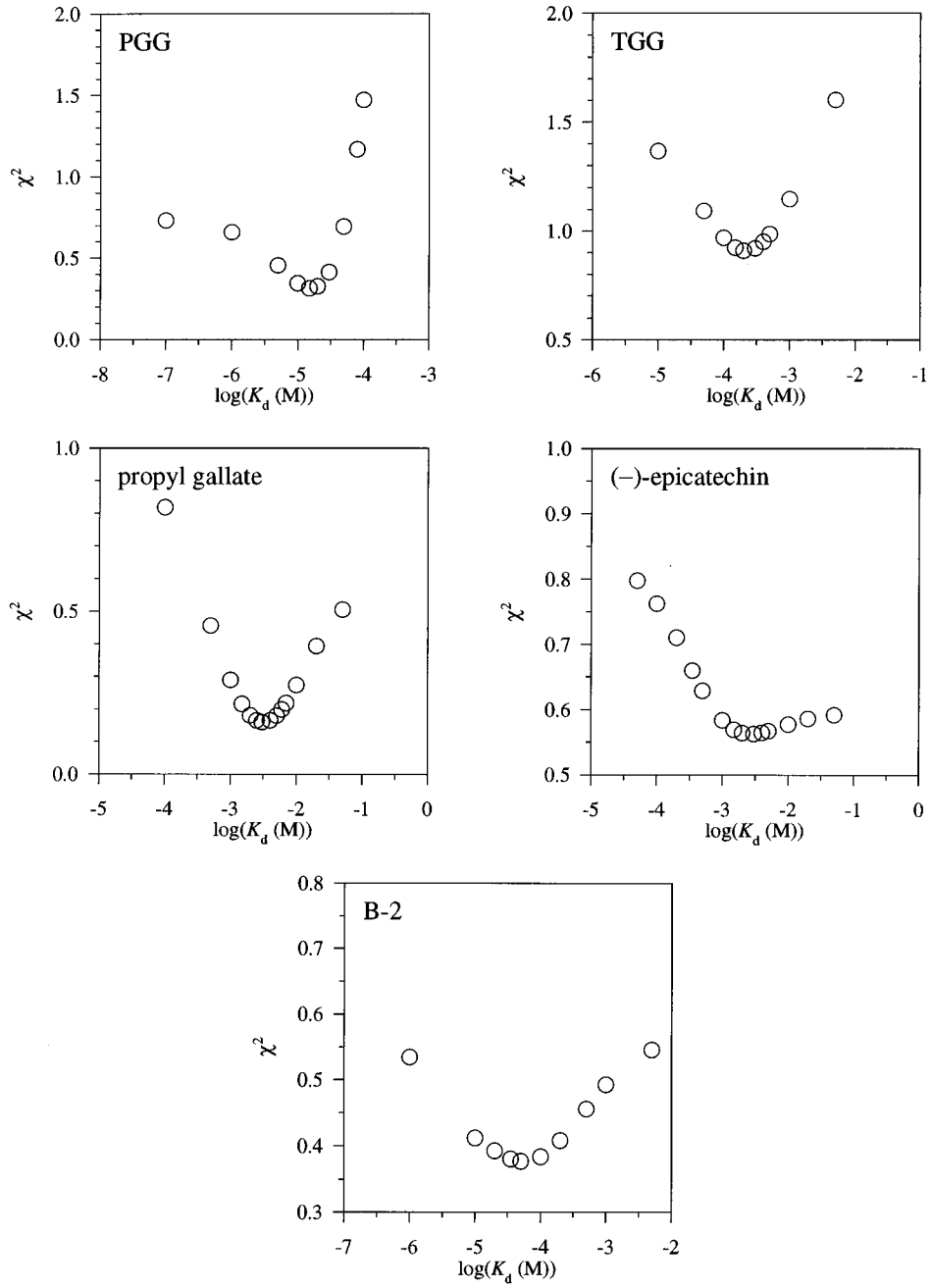

FIGURE 5: Relationship between log(K_d) and the χ^2 statistic obtained in the least-squares curve fitting of the respective peptide protons presented in Figure 4. For the various peptide/polyphenol titrations, the minimum value of χ^2 corresponds to the best-fit value of K_d.

intermolecular nuclear Overhauser effects (NOEs) (Murray et al., 1994). In the present study, intermolecular ROEs were observed for the TGG and PGG titrations between the galloyl proton resonances and proton signals of the proline-rich peptide. Exchange broadening of the peptide resonances was observed, which also substantiates intermolecular association.

In all the peptide/polyphenol titration experiments, upfield changes in chemical shift were observed for the proton resonances of proline residues and for the residues preceding proline, namely Gly2, Gly8, Arg12, and Gln15 (Figure 2). Such chemical shift changes may have arisen either as a result of a binding interaction between peptide and polyphenol or indirectly from a change in the conformation of the peptide associated with binding. However, it was observed for all the titrations that there were no changes in coupling constant values and that the intensities of the sequential $C\alpha H_i - NH_{i+1}$ ROEs of the free peptide and of the peptide in the presence of polyphenol were comparable, suggesting that the conformation of the peptide does not alter appreciably on titration with polyphenol; *i.e.* the structure of the proline-rich peptide remains extended (Murray & Williamson, 1994). Moreover, the peptide signals that shift upfield on titration with polyphenol are precisely those observed to have ROEs to the polyphenol resonances. The upfield chemical shift changes observed for peptide protons during the various titrations are therefore most likely to be induced by the aromatic functions of the polyphenol being in close proximity to the residues of the peptide.

Proline residues and residues preceding proline, *i.e.* Gly2, Gly8, Arg12, and Gln15, have the largest chemical shift changes on titration of the peptide with polyphenols. Since the analysis above has concluded that all proline sites have very similar binding affinities for polyphenol, the different magnitudes of the chemical shift changes cannot arise from

Table 3: Results of the Least-Squares fitting of eq 2 to the Proline CαH Chemical Shift Data of the Peptide/TGG Titration in 10% DMSO

	K_d (M)[a]	$\Delta\delta_{max}$ (ppm)	n	χ^2
Pro3 CαH				
	1×10^{-6}	−0.064	1.8	0.533
	1×10^{-5}	−0.064	1.8	0.385
	5×10^{-5}	−0.067	1.8	0.314
	1×10^{-4}	−0.069	1.8	0.348
	5×10^{-4}	−0.082	1.9	0.625
	1×10^{-3}	−0.095	2.1	0.792
Pro4 CαH				
	1×10^{-6}	−0.048	1.6	1.271
	1×10^{-5}	−0.049	1.6	1.209
	5×10^{-5}	−0.050	1.6	1.101
	1×10^{-4}	−0.052	1.6	1.063
	5×10^{-4}	−0.061	1.7	1.159
	1×10^{-3}	−0.069	1.7	1.292
Pro5 CαH				
	1×10^{-6}	−0.022	1.9	0.209
	1×10^{-5}	−0.022	1.9	0.188
	5×10^{-5}	−0.022	1.8	0.141
	1×10^{-4}	−0.023	1.8	0.119
	5×10^{-4}	−0.028	1.9	0.089
	1×10^{-3}	−0.032	2.0	0.090
Pro9 CαH				
	1×10^{-6}	−0.022	1.9	0.044
	1×10^{-5}	−0.022	1.9	0.043
	5×10^{-5}	−0.023	1.9	0.052
	1×10^{-4}	−0.024	1.9	0.062
	5×10^{-4}	−0.029	2.0	0.097
	1×10^{-3}	−0.033	2.2	0.113
Pro13 CαH				
	1×10^{-6}	−0.059	1.6	1.490
	1×10^{-5}	−0.060	1.6	1.367
	5×10^{-5}	−0.061	1.6	1.092
	1×10^{-4}	−0.064	1.6	0.969
	5×10^{-4}	−0.075	1.6	0.986
	1×10^{-3}	−0.084	1.6	1.147
Pro14 CαH				
	1×10^{-6}	−0.018	1.6	0.145
	1×10^{-5}	−0.018	1.6	0.138
	5×10^{-5}	−0.019	1.7	0.125
	1×10^{-4}	−0.019	1.7	0.125
	5×10^{-4}	−0.023	1.9	0.157
	1×10^{-3}	−0.027	2.0	0.178
Pro 16 CαH				
	1×10^{-6}	−0.022	1.8	0.036
	1×10^{-5}	−0.022	1.8	0.030
	5×10^{-5}	−0.023	1.8	0.032
	1×10^{-4}	−0.024	1.8	0.036
	5×10^{-4}	−0.028	2.0	0.057
	1×10^{-3}	−0.032	2.0	0.070

[a] For each fixed K_d value, the best-fitted $\Delta\delta_{max}$ value, the number of binding sites (n), and χ^2 are listed.

Table 4: Summary of the Fitting Results for the Peptide/Polyphenol Systems

	K_d (M)[a]	n
propyl gallate/water	5×10^{-3}	8.2 ± 1.8
TGG/10% DMSO	1×10^{-4}	1.8 ± 0.1
PGG/10% DMSO	5×10^{-5}	2.2 ± 0.7
(−)-epicatechin/water	5×10^{-3}	3.7 ± 0.5
B-2/10% DMSO	5×10^{-5}	1.3 ± 0.2

[a] The data for propyl gallate in 10% DMSO, TGG in water, and PGG in water could not be fitted. The estimated errors in fitted K_d values are a factor of 4 for propyl gallate, 5 for TGG, and 3 for the other polyphenols listed.

different affinities. They most likely reflect different binding geometries, in that the protons showing the largest chemical shift changes have the most well-defined binding sites and

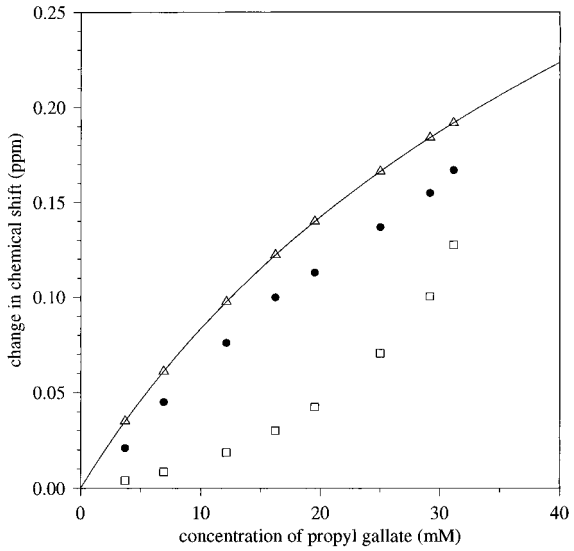

FIGURE 6: Comparison between the observed change in chemical shift of the galloyl proton of propyl gallate during the peptide/polyphenol titration (●), its change in chemical shift during the self-association experiments (—), and its calculated changes in chemical shift if it binds to the peptide as a monomer (□) or as a stack with the same degree of self-association as the free polyphenol (△).

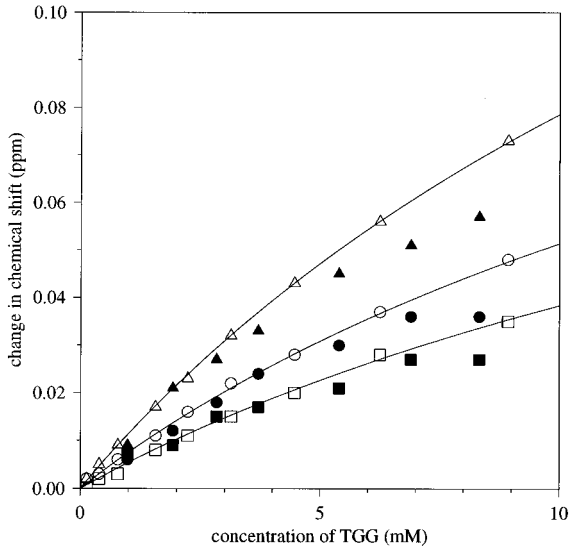

FIGURE 7: Changes in chemical shift of the galloyl 1 (circle), galloyl 3 (square), and galloyl 6 (triangle) proton resonances of TGG observed in the peptide/TGG titration in 10% DMSO (filled symbols) compared with those obtained during the TGG self-association titrations (open symbols) in the same solvent system. For clarity, the best-fit curves (using eq 1) of the self-association data are included.

are therefore least subject to conformational averaging of the ring-current shift.

Proline residues that are followed by prolines in the peptide sequence have CαH resonances that display larger chemical shift changes than the CαH signals of the other proline residues (Table 1). Thus, not only do the chemical shift data indicate that proline residues constitute polyphenol binding sites, but also the differential CαH chemical shifts imply that proline residues followed by prolines are geometrically preferred polyphenol binding sites. This is consistent with the special nature of the X-Pro sequence, in which proline imposes a β-sheet conformation on the preceding residue (MacArthur & Thornton, 1991).

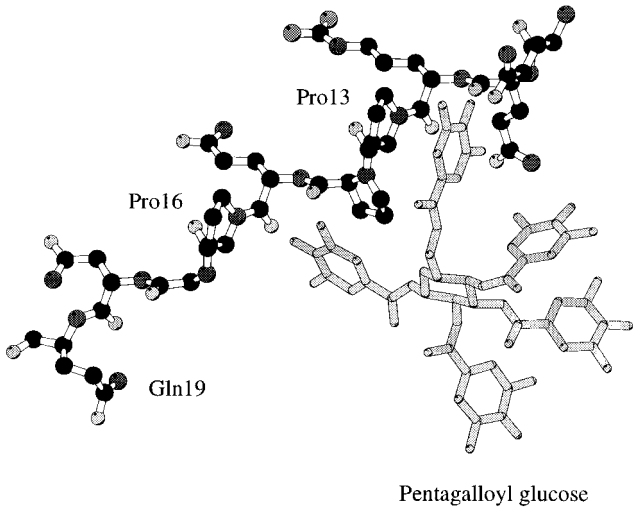

FIGURE 8: Proposed stacking interaction between the galloyl rings of PGG and the proline residues of the proline-rich peptide. The galloyl rings associate in a face-to-face manner with the binding site comprising a pyrrolidine ring and the preceding peptide bond, which is consistent with the upfield direction of the chemical shift changes observed. The larger and more complex polyphenols can occupy more than one adjacent proline residue. The primary interaction in this example is with the pyrrolidine ring of Pro13. The figure was prepared using MOLSCRIPT (Kraulis, 1991).

For all the peptide/polyphenol titrations, the proline Cβ^3H resonance shows larger upfield chemical shift changes on addition of polyphenol than the Cβ^2H signal, which implies that the polyphenols associate preferentially with the proline ring face containing Cβ^3H (this face also contains CαH). From model building studies, this face of the pyrrolidine ring adopts a more accessible geometry favorable for polyphenol association.

Size of the Binding Site. Curve fitting of the experimental chemical shift data yielded values for the number of polyphenol binding sites on the peptide for each polyphenol studied (Table 4). The results show that for the smallest polyphenol, propyl gallate, there is roughly one binding site per proline residue. The observed chemical shift changes (Figure 2 and Supporting Information) suggest that the binding site may extend across the peptide bond to the preceding residue, *i.e.* Gly2, Gly8, Arg12, and Gln15, and in particular that arginine can constitute part of a binding site. As the polyphenol size increases, the number of binding sites is reduced, until for the larger polyphenols there are only one or two binding sites on the peptide. Thus, although in principle each proline can bind a polyphenolic ring, in practice, steric hindrance limits the number of available binding sites, and for a large polyphenol such as PGG, three successive proline residues may be covered on interaction. This result serves to illustrate that proline residues perform dual functions; not only do they act as binding sites, but they also help to keep the peptide extended and thus maximize the available binding surface (Williamson, 1994). Figure 8 shows the proposed geometry of interaction between a molecule of PGG and the proline-rich peptide. As one galloyl function of PGG interacts with one binding site (proline residue and preceding peptide bond), the other galloyl functions are brought into close proximity of the peptide, allowing secondary interactions to take place.

Interpretation of the Peptide/Polyphenol Dissociation Constants. Consensus values of the dissociation constants have been obtained for the various peptide/polyphenol interactions. The data may be used to rank the polyphenols in order of their effectiveness in binding to the peptide: B-2 > PGG > TGG $\gg$ (−)-epicatechin $\approx$ propyl gallate. The hydrolyzable polyphenols (propyl gallate, TGG, and PGG) all contain the same galloyl binding function; however, the dissociation constant data indicate that the polyphenols form complexes with the proline-rich peptide with different affinities. The data therefore suggest that the galloyl function is not the only factor responsible for the strength of the association observed but that the polyphenol molecule as a whole confers the specific proline-rich peptide binding properties. In particular, it is clear that larger polyphenols, possessing a greater number of phenolic rings with a concomitant increase in hydrophobicity, form complexes with the proline-rich peptide more effectively.

Binding Interaction. It has previously been suggested that polyphenol/peptide binding is driven either by hydrophobic association or by hydrogen bonding via the many phenolic hydroxyl functions present on the polyphenols. The upfield nature of the chemical shift changes seen here, and the marked reduction of the binding on addition of DMSO, imply that the dominant force is hydrophobic (Hamaguchi, 1964), although hydrogen bonding may well play a secondary role (Lilley, 1993; Luck et al., 1994). Further evidence that hydrophobic association is the primary mode of interaction between the proline-rich peptide and polyphenol molecules is provided by non-specific chemical shift changes. For all the peptide/polyphenol titrations performed, small changes in chemical shift were observed for protons of Gln1, Gln6, Gly7, Gln10, Gln11, Gly17, Asn18, and Gln19. As discussed above, the essential polyphenol binding site consists of a proline residue, together with the preceding amide bond and amino acid residue. Hence, the chemical shift changes observed for protons of Gln1, Gln6, Gly7, Gln10, Gln11, Gly17, Asn18, and Gln19 arise from indirect or nonspecific polyphenol association and will be termed nonspecific chemical shift changes. In every case where titrations were carried out in both water and 10% DMSO, the nonspecific chemical shift changes were proportionately larger for the titration performed in water (*e.g.* Figure 2). This implies that in 10% DMSO the peptide/polyphenol association is weaker but more specific (*i.e.* binding occurs primarily at proline residues and residues preceding proline). Nonspecific chemical shift changes are suggested to arise from nonspecific peptide/polyphenol interactions which are promoted by hydrophobic association.

Multidentate Binding of Polyphenols. The peptide/TGG titration in water was limited to a final molar ratio of 1/0.75 due to the onset of precipitation, which was already evident at a stoichiometry of 1/0.25. Analysis of the resulting chemical shift changes for protons of proline, arginine, and glycine (Gly2 and Gly8) indicated that the peptide/TGG system had achieved saturation prior to attaining a 1/1 stoichiometry. This is feasible since TGG possesses three well-exposed galloyl functions which are capable of binding up to three polyphenol binding sites present on either the same peptide or different peptide molecules. Thus, as far as the peptide is concerned, saturation can be reached at less than an equimolar ratio. These results indicate that more

than one galloyl function of a polyphenol species may associate with the proline-rich peptide molecules, suggesting that precipitation of soluble peptide/polyphenol complexes can be accomplished by noncovalent cross-linking mediated by the multidentate nature of the polyphenol molecules. Thus, the sigmoidal $\Delta\delta$ *vs* [polyphenol] curves observed for a few resonances during titrations with TGG, PGG, and B-2 probably arise from cooperative binding between the aromatic functions present on the polyphenol molecules and neighboring polyphenol binding sites contained on the proline-rich peptides. We note that this cooperativity contradicts assumption (3) made in Experimental Procedures, but since the cooperativity is only seen in a small number of cases, it is not sufficient to invalidate the general treatment above.

These studies have shown that the larger and more hydrophobic polyphenols bind more strongly to the proline-rich peptide, and in addition, they have suggested that some of the binding interactions for the larger polyphenols may arise from multidentate association. The results presented agree with previous studies carried out on the noncompetitive inhibition of β-glucosidase activity by polyphenols (Haslam, 1974) and the complexation of tannins with globular proteins such as bovine serum albumin (Hagerman & Butler, 1981; McManus et al., 1985) and hemoglobin (Bate-Smith, 1973) and with the alkaloid caffeine (Cai et al., 1990). These latter studies demonstrated that larger and more complex hydrolyzable tannins are more effective in their activity. Although these investigations and the present study reach similar conclusions concerning the relationship between the efficacy of association and functional complexity, the mode of interaction of polyphenols with salivary PRPs and with globular proteins is certainly different. Whereas tannin/PRP association is a face-to-face stacking of aromatic groups onto proline residues, the interaction with globular proteins probably involves surface-exposed aromatic residues (Baxter et al., 1996; Murray, 1994). In this context, it is of interest to note that there is a family of histidine-rich proteins known as histatins that are present in saliva, which have also been proposed to bind strongly to polyphenols (Yan & Bennick, 1995).

Self-Association of the Polyphenols when Bound. The discussions so far have been concerned with the interpretation of the data from the point of view of the peptide. In contrast, by comparing the chemical shift changes observed for protons of the *polyphenols* during the peptide/polyphenol titration experiments with those seen during the polyphenol concentration dependence studies, we have shown that the polyphenols are self-associated when bound to almost the same extent as they are in free solution. Thus, polyphenols can be considered potential multivalent adapters, able to cross-link both peptide and other polyphenol molecules, resulting in precipitation. This is significant since the most striking physiological effect of polyphenols is their astringent taste, which arises from polyphenol/protein precipitation in the oral cavity (Haslam, 1989); this point is currently under investigation in our laboratory. Ongoing studies suggest that one function of the multiple tandem repeats often found in salivary PRPs is to markedly increase the binding affinity of complex multidentate polyphenols for these proteins by promoting multiple interactions (Charlton et al., 1996). The cooperative effect of the large number of interactions is production of a high-molecular mass aggregate, which precipitates and generates the astringency and antinutritive properties characteristic of polyphenols.

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SUPPORTING INFORMATION AVAILABLE

Tables of the chemical shift data obtained during the peptide/polyphenol titrations and the polyphenol self-association experiments (30 pages). Ordering information is given on any current masthead page.

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