The Major Birch Allergen, Bet v 1, Shows Affinity for a Broad Spectrum of Physiological Ligands*

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Bet v 1 is a 17-kDa protein abundantly present in the pollen of the White birch tree and is the primary cause of birch pollen allergy in humans. Its three-dimensional structure is remarkable in that a solvent-accessible cavity traverses the core of the molecule. The biological function of Bet v 1 is unknown, although it is homologous to a family of pathogenesis-related proteins in plants. In this study we first show that Bet v 1 in the native state is able to bind the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS). ANS binds to Bet v 1 with 1:1 stoichiometry, and NMR data indicate that binding takes place in the cavity. Using an ANS displacement assay, we then identify a range of physiologically relevant ligands, including fatty acids, flavonoids, and cytokinins, which generally bind with low micromolar affinity. The ability of these ligands to displace ANS suggests that they also bind in the cavity, although the exact binding sites seem to vary among different ligands. The cytokinins, for example, seem to bind at a separate site close to ANS, because they increase the fluorescence of the ANS-Bet v 1 complex. Also, the fluorescent sterol dehydroergosterol binds to Bet v 1 as demonstrated by direct titrations. This study provides the first qualitative and quantitative data on the ligand binding properties of this important pollen allergen. Our findings indicate that ligand binding is important for the biological function of Bet v 1.

Bet v 1 is a 17-kDa protein constituting 10% (w/w) of the protein fraction in aqueous extracts of mature pollen from the White birch tree (Betula verrucosa). It shows a considerable degree of heterogeneity when analyzed by two-dimensional gel electrophoresis and immunoblotting using rabbit antiserum raised against purified Bet v 1, because pollen from a single birch tree exhibits between 5 and 10 spots, whereas a pollen extract reveals 24 spots (1). Approximately 50 isoforms have been cloned and sequenced, displaying a primary amino acid sequence identity in the range of 99–72%. Both the solution and crystal structure of Bet v 1 have been determined (2). The main feature of the structure is a seven-stranded anti-parallel β-sheet that wraps around a 25-residue-long C-terminal α-helix. The β-sheet and the C-terminal part of the long helix are separated by two small consecutive helices. A most unusual feature of the structure is a large forked solvent-accessible cavity penetrating the core of the molecule (2). Moreover, the structure of Bet v 1 contains a P-loop motif, a structural element found in nucleotide binding proteins, where it is responsible for binding nucleotides (3).

Bet v 1 is a major cause of tree pollen allergy in humans (4) affecting an estimated 100 million people worldwide (5). Despite the immunological interest in Bet v 1, little is known about its biological function. It is homologous to a group of pathogenesis-related proteins, the PR-10 proteins, that are expressed during disease and in stress situations (6) and that seem to be ubiquitous in plants (7). The closest relatives are found in the pollens of the related trees alder (8), hornbeam (9), and hazel (10), and birch pollen-allergic individuals often cross-react to the pollens of these species. Bet v 1 homologues present in various fruits and vegetables, particularly apple (11), but also in cherry (12) and celery (13), cause the oral allergy syndrome upon oral ingestion in some birch pollen-allergic patients (14). The identity percentages to these homologues are in the range of 65–56%. Several more distantly related PR-10 proteins from a variety of plant species have been described. These proteins are not serologically cross-reactive, and homology percentages range between 54 and 33%. In addition, homology between the PR-10 proteins and a group of proteins termed the major latex proteins (15), including members from opium poppy (16) and bell pepper (17), has been described. Finally, Bet v 1 shows remarkable structural similarity to the 200-amino acid START domain of MLN64 (18), a protein involved in the mobilization of cholesterol in human placenta and brain (19), although there is no sequence homology.

Little experimental data exist on the functional properties of the numerous Bet v 1 homologues, yet some activities have been reported. The START domain of MLN64 is capable of binding one molecule of cholesterol (18). The PR-10 member from mung bean is a cytokinin-specific binding protein capable of binding cytokinins and cytokinin analogues with nanomolar affinity (20). Very recently, the major allergen from cherry, Pru av 1, whose backbone folding pattern is very similar to that of Bet v 1, was reported to bind the phytohormone homocastasterone (21). Furthermore, PR-10 members from ginseng (22) and white lupin (23) as well as Bet v 1 (24, 25) have been reported to show RNase activity. It thus appears that PR-10 proteins display enzymatic activity as well as ligand binding activity.

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¶The abbreviations used are: Bet v 1, major allergen from birch tree pollen; 2QF-COSY, double-quantum-filtered correlation spectroscopy; ANS, 8-anilino-1-naphthalenesulfonic acid; Cnc, critical mucic acid concentration; DHE, dehydroergosterol; IC50, apparent dissociation constant; IPA, N,N'-diisopentenyl)adamine; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; PR-10 protein, pathogenesis-related protein, subfamily 10; Pru av 1, major allergen from cherry; START domain, steroidalogenic acute regulatory-related lipid transfer domain; CD, circular dichroism; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; bis-ANS, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid.
which is also reflected in the classification of the START domain superfamily, including both START domain proteins and PR-10 proteins among others (26). We show here that Bet v 1 is able to bind a wide range of both synthetic and naturally occurring compounds with moderate to high affinity and discuss the implications of the identified ligands for a possible biological function of Bet v 1.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Lauric acid, stearic acid, and oleic acid were from Fluka (Busch, Germany); deuterium oxide, deuterated HCl, deuterated ethanol, and deuterated phosphate buffer were from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). All chemicals were of analytical or biological grade. Bet v 1.2801 was produced and purified as described previously (27). The protein concentration was determined spectrophotometrically using a molar extinction coefficient of 4990 M$^{-1}$ cm$^{-1}$ at 280 nm as determined by the method of Gill and von Hippel (28).

**Data Analysis and Inner Filter Effect Corrections**

Non-linear least squares regression analysis was carried out with the program Kaleidagraph, version 3.5 (Synergy Software, Reading, PA). Unless stated otherwise, the error mentioned throughout the text is the standard error of the mean. Where necessary, we corrected for inner filter effects using the equation (29),

$$\kappa = \frac{\ln(10) \cdot \alpha \cdot \delta}{10^{-\left(\frac{\lambda}{T_2}\right)} - 10^{-\left(\frac{\lambda}{T_1}\right)}}$$

(Eq. 1)

where $\kappa$ is the inner filter effect correction factor, $\alpha$ is the product of the molar extinction coefficient and the concentration, $\delta$ is the emission slit width, and $d$ is the cuvette path length. Cuvettes with low path lengths (3 mm) were used to reduce inner filter effects.

**ANS-Bet v 1 Interaction**

All fluorescence experiments were performed on an RTC2000 spectrometer from Photon Technology International (Lawrenceville, NJ) equipped with a 75-watt xenon arc lamp and a temperature control unit. Excitation and emission band paths were 5 nm. ANS was dissolved in 1 ml Me2SO and diluted to 100 ml with MilliQ water. The concentration was determined spectrophotometrically using a molar extinction coefficient of 4990 M$^{-1}$ cm$^{-1}$ at 280 nm as determined by the method of Gill and von Hippel (28).

**Identification of Naturally Occurring Ligands**

**ANS Displacement Assay**—Typically, 10 μM Bet v 1 and 10 μM ANS in 50 mM phosphate, pH 7, at 25 °C was used in the ligand titration experiments. The concentrations of the Bet v 1 and ANS stock solutions were determined spectrophotometrically. ANS was excited at 350 nm. The contribution of buffer, Bet v 1, and ligand to the measured fluorescence was subtracted. Ligands with limited solubility in water were dissolved in absolute ethanol. The final ethanol concentration did not exceed 10% (v/v) ensuring that the decreased polarity of the solvent did not change the quantum yield of ANS (36). Far-UV CD spectra showed that, below 30% (v/v), the presence of ethanol in the samples had no denaturing effect on Bet v 1 (data not shown). bis-ANS was dissolved in 40 μl of Me2SO and diluted to 1 ml with MilliQ water. The concentration was determined spectrophotometrically using a molar extinction coefficient of 16,790 M$^{-1}$ cm$^{-1}$ at 385 nm in water (37). Excitation was performed at 383 nm corresponding to the maximum absorption wavelength.

**Analysis of Displacement Data**—A simple rectangular hyperbolic binding model was employed to express the affinity of the ligand,

$$F_{\text{obs}} = D \times \frac{1 - \frac{IC_{50}}{I_{50} + [L]}}{I_{50} + [L]} + F_{\text{baseline}}$$

(Eq. 3)

where $F_{\text{obs}}$ is the observed fluorescence, $F_{\text{Baseline}}$ is the fluorescence at saturation, and $L$ denotes ligand. This model yields the parameter $IC_{50}$, which is a crude measure of the $K_d$ for the displacing ligand (38). $IC_{50}$, $\Delta F$, and $F_{\text{baseline}}$ are fitted as free parameters by non-linear least squares regression analysis. In a few instances direct titrations were also performed by monitoring the intrinsic fluorescence of the protein with excitation at 278 nm.

**DHE-Bet v 1 Interaction**—Dehydroepiandrosterone (DHE) was dissolved in absolute ethanol. The concentration of DHE was determined spectrophotometrically using an extinction coefficient of 10.550 M$^{-1}$ cm$^{-1}$ at 325 nm (39). 10 μM DHE was titrated with Bet v 1, and excitation was performed at 325 nm. The contribution of buffer to the measured ellipticity were subtracted. The use of “Good” buffers (29) had no effect on ANS binding to Bet v 1 (data not shown).
RESULTS

Bet v 1 Binds ANS in the Native State

The fluorescent probe ANS is traditionally used to detect molten globules, i.e., partly folded proteins that accumulate under mildly denaturing conditions. ANS is believed to bind to molten globules due to the presence of solvent-exposed hydrophobic patches, which are a particular characteristic of this protein state (40). Certain proteins also bind ANS in the native state, however, provided this conformation displays exposed hydrophobic sites (41–43). Because Bet v 1 contains a hydrophobic solvent-exposed cavity, we tested whether Bet v 1 in the native conformation is able to bind ANS specifically. Fluorescence emission spectra of ANS in the presence and absence of Bet v 1 are shown in Fig. 1. In water, ANS is essentially non-fluorescent and exhibits a maximum emission wavelength ($\lambda_{\text{max}}$) of ~515 nm. In the presence of Bet v 1 under native conditions (pH 7), ANS fluorescence displays a pronounced increase in intensity with a concomitant shift of $\lambda_{\text{max}}$ to 474 nm, both of which indicate transfer of ANS to a less polar environment. These data show that Bet v 1 binds ANS in the native state.

ANS Binding Is Characterized by Moderate Affinity and 1:1 Stoichiometry

The affinity of the interaction at pH 7 as well as pH 4 was examined by titration of ANS with Bet v 1 while following the ANS emission at 477 nm. The resulting binding curves, displayed in Fig. 2, show that ANS binds to Bet v 1 in a saturable manner. Fitting the raw data to Equation 2 yields $K_d$ values of $18.5 \pm 5.0$ $\mu$M and $3.8 \pm 0.3$ $\mu$M at pH 7 and pH 4, respectively. Thus, binding is characterized by moderate affinity and is more favorable at low pH than at neutral pH, which is expected because ANS is negatively charged in the entire pH scale (29). A pH profile of 20 $\mu$M ANS mixed with 2.5 $\mu$M Bet v 1 (Fig. 3) corroborates the result of the titration experiments as ANS fluorescence peaks at pH 3.6 while still being significant at pH 7. Higher ANS concentrations were found to stabilize Bet v 1 by shifting the acid pH denaturation to lower values (data not shown). Thermal scans monitored by far-UV CD spectroscopy confirmed the stabilizing effect of ANS as $T_m$ is raised from 45.3 ± 1.0 °C to 55.2 ± 0.5 °C when melting 10 $\mu$M Bet v 1 mixed with 100 $\mu$M ANS at pH 4 (data not shown). Fig. 3 also shows the unfolding profile of Bet v 1 as monitored by the intrinsic tyrosine fluorescence and far-UV CD spectroscopy. The two unfolding curves show that secondary and tertiary structure unfolds in parallel, which further supports the finding that ANS indeed binds to the native state of Bet v 1 and not to a partially unfolded state. Fig. 4 shows the titration of ANS with Bet v 1 at pH 7 under stoichiometric conditions. This experiment yields a binding stoichiometry of 0.9 ± 0.1, indicating that Bet v 1 possesses a specific binding site for ANS. The same experiment at pH 4 gives a stoichiometry of 1.1 ± 0.2 (data not shown). This finding supports the conclusion that higher affinity rather than an increase in the number of binding sites causes the increased ANS fluorescence at pH 4 compared with pH 7.
Two-dimensional NMR Data Show That Distinct Regions of the Protein Are Affected by ANS Binding

To identify the ANS binding site on the protein we compared two-dimensional NMR spectra of free and bound Bet v 1. Although the overall appearance of the Bet v 1 NOESY spectrum did not change after addition of ANS, numerous protons with a change in chemical shift of more than 0.02 ppm could be identified. These are summarized in Table I, whereas in Fig. 5 they are plotted on a molecular model of Bet v 1. One NOE could be found from a presumed ANS atom (8.33 ppm) to a protein atom at 0.75 ppm, presumably a methyl signal, but no assignment could be obtained. From a 2QF-COSY spectrum recorded in D$_2$O, only three additional resonances were detected after addition of ANS (at 8.33, 7.32, and 7.82 ppm, where the proton at 7.32 ppm exhibits a cross peak to the two other protons). Looking at the distribution of the perturbed protein protons, they can be seen to form one large patch along the β-sheet and several distinct regions in the α-helices (see Fig. 5). Very few perturbed protons can be detected on the outer surface of the protein. Although these data do not unequivocally pinpoint an
ANS binding site, they do suggest that ANS binds in the cavity of Bet v 1. This is further corroborated by the two NOEs vanishing between Tyr-83 Hε and Ile-102 Hγ1 and Hγ3, respectively. Fig. 5 shows the position of the side chains of these residues in the cavity. The broad swathes of perturbed atoms indicates either that there is no specific binding site for ANS within the cavity or that ANS binding causes some minor structural rearrangements in the protein molecule.

**Binding of bis-ANS and SDS to Native Bet v 1**

To test whether Bet v 1 can bind amphiphilic molecules in general, we also examined binding of bis-ANS and SDS. bis-ANS, which shows similar fluorescence characteristics as ANS, bind to Bet v 1 with high affinity, yielding a $K_d$ of 53.6 ± 15.0 nM (data not shown). This is consistent with the observation that bis-ANS is superior to ANS as a probe of non-polar cavities in proteins, often binding with an affinity orders of magnitude higher (44). The titration experiment under stoichiometric conditions reveals a binding stoichiometry of 1:1 (data not shown), bis-ANS typically induces conformational changes on the secondary and tertiary level upon interaction with proteins (37, 45). This is also observed upon interaction with Bet v 1, because far-UV CD spectra reveal a slightly different content of secondary structure (data not shown). Bet v 1 was also found to bind SDS specifically. By following the intrinsic tyrosine fluorescence of the protein upon titration with SDS, the apparent dissociation constant ($IC_{50}$ value determined from Equation 3) yields 28.2 ± 8.5 μM (Fig. 6). An indirect assay was also performed where a preformed complex between Bet v 1 and ANS was titrated with SDS. The decrease in ANS fluorescence as a function of SDS concentration shows that SDS is able to displace ANS (Fig. 6). The $IC_{50}$ value obtained from Equation 3 yields 7.7 ± 0.6 μM, which suggests that there is a high affinity SDS binding site overlapping the ANS binding site in addition to the one or more weaker binding sites revealed by the tyrosine fluorescence experiment.

**Identification of Physiologically Relevant Ligands**

The ability of native Bet v 1 to form a well-defined fluorescent complex with ANS was utilized to establish an ANS displacement assay, suitable for screening physiologically relevant ligands for binding to Bet v 1. Thus, the binding of non-fluorescent ligands can be indirectly measured as a reduction in ANS fluorescence. Table II provides a summary of the ligands identified and their structures and affinities of interaction with Bet v 1.

**Interaction with Fatty Acids—**Several studies have suggested a role for fatty acids in pollen-stigma interactions in plants (46, 47). We tested fatty acids with even-numbered chain lengths of 8–22 carbon atoms for binding to Bet v 1. To assess the influence of fatty acid chain conformation, oleic acid was also examined. $IC_{50}$ values of all fatty acids tested are shown in Fig. 7. The data show that the affinity between fatty acids and Bet v 1 exhibits a parabolic dependence on chain length and is maximal for chain lengths of 14–18 carbon atoms. A lower chain length significantly reduces affinity in a more or less linear fashion. Likewise, chain lengths of more than 20 carbon atoms reduce the affinity dramatically. Surprisingly, oleic acid binds with identical affinity compared with stearic acid, i.e., the presence of a cis double bond at position 9 in the chain has no effect on binding.

**Interaction with Flavonoids—**Two plant pigments belonging to the flavonoid group, flavone and 4’,5,7-trihydroxyflavone (naringenin), were tested for binding toward Bet v 1. Naringenin has been demonstrated to occur in birch trees (48). ANS displacement curves for flavone and naringenin yield $IC_{50}$ values of 33.2 ± 4.8 and 28.6 ± 2.3 μM, respectively (data not shown), implying that Bet v 1 binds these compounds with moderate affinity.

**Interaction with Cytokinins—**Cytokinins are plant growth hormones that regulate differentiation and proliferation of plant cells. The PR-10 protein from mung bean has been shown to bind cytokinins with high affinity (20); we tested if this is also the case for Bet v 1. Fig. 8 shows the results of a titration of a Bet v 1-ANS complex with N6-(Δ2-isopentenyl)adenine (IPA), a very potent naturally occurring cytokinin, and N6- furfuryladenine (kinetin), an extremely potent cytokinin. IPA

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2 We can disregard complications with micelle formation for fatty acids with chain lengths up to 20 carbon atoms, because their Cmc values are well above the apparent $K_d$ (71). C22 fatty acid has a Cmc value of around 10 μM, which may, however, interfere with our measurements. If so, the effect will be a slight underestimation of the apparent $K_d$. 

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**Fig. 5. Bet v 1 protons affected by ANS binding.** Stereo view of Bet v 1 showing the backbone folding pattern with secondary structural elements in gray. The pink spheres represent protons whose chemical shift is changed by more than 0.02 ppm when comparing NOESY spectra of Bet v 1 alone and in complex with ANS. The side chains of Tyr-83 and Ile-102 are represented by sticks in green. The NOEs disappearing upon addition of ANS are in blue. The image was prepared with MolMol, version 2.6 based on Protein Data Bank file 1bv1.
Fig. 6. SDS binding to Bet v 1. Titration of 10 μM Bet v 1 and 10 μM ANS with SDS in 50 mM phosphate, pH 7, 25°C. Fitting the data to Equation 3, IC50 yields 7.7 ± 0.6 μM. Far-UV CD spectra of Bet v 1 and SDS showed that denaturation commences at ~500 μM SDS (data not shown) changing the mixed α/β spectrum of Bet v 1 to the characteristic all-α spectrum. ANS was excited at 350 nm; the emission at 477 nm is displayed. Inset: direct titration of 10 μM Bet v 1 with SDS in 50 mM phosphate, pH 7, 25°C. Fitting the data to Equation 3, IC50 yields 28.2 ± 8.5 μM. Tyrosine was excited at 287 nm; the emission at 307 nm is displayed.

Interaction with Dehydroergosterol—Based on the ability of Pru av 1 and the START domain to bind homocastasterone and cholesterol, respectively, a sterol was tested for binding to Bet v 1. We used DHE, a naturally occurring compound, because it is fluorescent and therefore can be utilized in direct binding experiments. Furthermore, it has similar properties to cholesterol (49). Fig. 9 shows the binding curve obtained from a titration of 10 μM DHE with Bet v 1. The fluorescence of DHE is seen to increase upon interaction with Bet v 1 giving rise to a saturable binding profile. Applying Equation 3 to assess the binding affinity yields an IC50 value of 20.9 ± 3.0 μM. To elucidate whether the binding site for DHE overlaps that of cytokinin, we titrated a saturated complex between Bet v 1 and DHE with Bet v 1 and cytokinin (data not shown). The intrinsic fluorescence of Bet v 1 was found to decrease upon interaction with cytokinin, and a direct binding experiment yielded an IC50 value of 92.4 ± 12.3 μM for cytokinin (data not shown). This value is very similar to the one obtained from the indirect assay (84.1 ± 13.8 μM), showing that the presence of ANS does not interfere with binding of cytokinin. Thus, the data from the indirect and direct experiments suggest the presence of two separate binding sites, which are non-cooperative. This conclusion was further tested by titrating a saturated Bet v 1-kinetin complex with ANS to investigate whether the affinity of ANS for Bet v 1 is affected by bound kinetin (data not shown). This yields an IC50 value of 37.0 ± 3.7 μM, which is to be compared with a value of 28.3 ± 5.4 μM for ANS. Thus, kinetin has only a marginal effect on the affinity of ANS for Bet v 1 and so corroborates the results of the direct titration.

Interaction with Indole-3-acetic Acid, Gibberellic Acid, and Abscisic Acid—A number of molecules did not show any affinity for Bet v 1, namely the plant hormones indole-3-acetic acid, gibberellic acid, and abscisic acid, as judged from their inability to displace ANS from Bet v 1 or influence the fluorescence of ANS bound to Bet v 1 (data not shown).

DISCUSSION

The major allergen from birch pollen, Bet v 1, is homologous to the group of PR-10 proteins. PR-10 proteins were originally characterized at the transcriptional level, demonstrating increased gene expression in stress situations, such as during microorganism infection (50). However, only limited functional studies have been performed on the corresponding proteins. It is conceivable, though, that the unusual feature of the Bet v 1 tertiary structure, the internal cavity, plays a central role in the biological function of the molecule and probably does so through specific interaction with an unknown ligand. In this study we have shown that Bet v 1 binds ANS in the native state and have exploited this property of Bet v 1 in an ANS displacement assay to identify a range of physiologically relevant amphiphilic ligands, most of which bind with low micromolar affinity.

Bet v 1-ANS Interaction

Three observations show that Bet v 1 in the native conformation binds ANS: 1) the fluorescence intensity of ANS increases 48-fold at 462 nm upon addition of Bet v 1, 2) λmax of ANS is concomitantly blue-shifted from ~515 to 474 nm, and 3) the conformational stability of Bet v 1 is augmented in the presence of ANS. Moreover, the binding isotherm is rectangular hyperbolic and reaches saturation suggesting that binding is specific, i.e. a well-defined number of binding sites exist. Indeed, titrating ANS with Bet v 1 at concentrations well above Kd strongly indicates a 1:1 binding stoichiometry. NMR data indicate that binding occurs in the cavity, because Bet v 1 protons perturbed in chemical shift upon addition of ANS are almost exclusively confined to this region. Some degree of dispersion within the cavity is observed, however, which prohibits a more detailed analysis of particular amino acid residues participating in binding. The dispersion of perturbed protons could indicate that ANS binds in different positions in the cavity, creating different Bet v 1-ANS conformers. Such conformers must be overlapping, however, to fulfill a stoichiometry
of 1:1 binding. Also, ANS fails to induce a CD signal when in complex with Bet v 1 (unlike the dye Congo red, which also binds Bet v 1), suggesting that the environment of ANS in the cavity is not strictly asymmetric (data not shown). A similar distribution of perturbed protons was found from NMR analysis of the homologous protein Pru av 1, the major cherry allergen, mixed with the phytosteroid homocastasterone (21). In this case the perturbed protons surrounded the lower part of the cavity.

Mode of Ligand Binding

Promiscuity in Binding—Bet v 1 seems promiscuous with respect to ligand binding activity, because it binds compounds differing considerably in size, shape, and hydrophobicity with comparable affinity. For example, Bet v 1 binds myristic, palmitic, and stearic acid with very similar affinities, although these compounds differ by up to four carbon atoms in chain length. Also, the cis double bond in oleic acid, which induces a kink in position nine in the alkyl chain, does not affect the affinity relative to stearic acid. Such broad specificity, however, particularly toward fatty acids, has been found in a number of transport-like proteins, for instance, elicitors, which bind sterols and fatty acids (51), lipid transfer proteins, which bind fatty acids and phospholipids (52), and serum albumins (53). In particular, human serum albumin binds stearic and oleic acid with similar affinities, and crystallographic analysis showed that the cis double bond in oleic acid poses negligible steric hindrance on binding to the different sites on the protein (53).

Cytokinins Bind to an Alternative Site—The interaction of cytokinins with Bet v 1 is different compared with fatty acids and flavonoids in that the ANS fluorescence increases with increasing cytokinin concentration. In principle, two phenomena can explain this effect: 1) a change in the free energy of association of ANS (i.e., higher affinity so that more ANS molecules are in complex) and 2) a change in probe response (i.e., the quantum yield of ANS is enhanced). The data show that 1) the IC50 values determined from a direct and indirect titration with kinetin are equal within experimental error and 2) the affinity of ANS for Bet v 1 is not significantly affected by bound kinetin. This suggests that Bet v 1 contains two ligand binding sites: one site able to bind ANS but not kinetin and a second site able to bind kinetin but not ANS. The two sites are non-overlapping and non-cooperative, however, kinetin binds in close proximity to ANS and by doing so influences the quantum yield of ANS. Upon titration with kinetin, λmax of ANS shifts from 477 to 470 nm (data not shown) clearly indicating a decreased polarity of the ANS binding site. Further evidence for the existence of two separate sites comes from the independent binding of DHE and kinetin. Where is the second site then located? It is tempting to suggest that kinetin binds to the P-loop for three reasons: 1) cytokinins are adenine derivatives, and the P-loop is found in many nucleotide binding proteins (3); 2) the affinities of IPA and kinetin are similar suggesting that the adenine moiety is the interacting group; and 3) in Bet v 1 the P-loop forms part of the largest entrance to the cavity allowing the two ligands to bind close to each other. With the present study, two members of the PR-10 family have been shown to bind cytokinins. Primary sequence alignment shows that they share 29% identity and that the P-loop motif also is
present in the mung bean protein (20). Unfortunately, no data are available concerning the cytokinin binding site in the mung bean protein. We are currently investigating the binding of various ligands using x-ray crystallography to obtain a more detailed picture of the Bet v 1(H18528) ligand interactions.

**Relation to Pru av 1 and START**—Direct titration experiments of DHE with Bet v 1 show that Bet v 1 is able to bind sterols with moderate affinity. This conforms to the recent observation that Pru av 1 binds the phytosteroid homocastasterone, although this study provided no quantitative data (21). Modeling studies of Pru av 1 suggested that up to two molecules of sterol could bind in the cavity. Also, binding studies with the fluorescent cholesterol analogue (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-cholesterol (NBD)-cholesterol on StAR, a protein containing a START domain, showed two sterol binding sites (54). The apparent dissociation constant of the Bet v 1-DHE complex (20 μM) may well represent an average of several dissociation constants. However, the Bet v 1-DHE data failed to yield consistent results with binding models employing several binding sites (data not shown). The STAR protein stimulates exchange of DHE between mitochondrial membranes in vitro (54). DHE exchange experiments (55) reveal no such activity for Bet v 1 in small unilamellar vesicles (data not shown) indicating that Bet v 1 is not a sterol carrier protein but rather a sterol binding protein.

**Implications of the Identified Ligands on the Biological Function of Bet v 1**

The sequence similarity and wide distribution of the PR-10 proteins throughout the plant kingdom is an indication of an indispensable function in plants. It is, however, difficult to assign a unique function to all PR-10 members, because no coordinated expression occurs. In birch, for example, Bet v 1 is expressed from several loci. Certain isoforms are constitutively expressed in roots (56) or in pollen. Others are only expressed upon infection with fungi or bacteria (57, 58) or upon copper stress (59). This study points to ligand binding activity as an important aspect of the biological function of Bet v 1. The multiplicity of ligands identified, however, hampers the assignment of a unique function. The Bet v 1 isoform used in this study is expressed in pollen, and the following discussion is therefore restricted to pollen physiology.

The processes leading to plant reproduction, i.e. germination of the pollen grain on the stigma, directional pollen tube growth, and fertilization, involve a myriad of cell-cell recognition and signaling events (60). The fact that Bet v 1 is present in large quantities in the pollen grain and readily extracted upon hydration (61) suggests that Bet v 1 is involved in these processes. The majority of ligands that have been identified in this study, fatty acids, flavonoids, and cytokinins, theoretically could be involved in such processes. Lipids are important for hydration of the pollen grain (62). They are thought to form a watertight seal between pollen and stigma, facilitating the rapid transport of water into pollen through channels in the stigma and pollen membranes. In petunia, the flavonoid kaempferol is required for pollen fertility (63). The enzyme chalcone synthase catalyzes the first step in the biosynthesis of flavonoids, and chalcone synthase-deficient petunia cannot form the pollen tube (63). In this study we have shown that Bet v 1 binds flavone and naringenin, compounds very similar to kaempferol. The ability of Bet v 1 to bind long-chain fatty acids and flavonoids may suggest a role for Bet v 1 in ensuring proper hydration and germination of pollen, for instance by transporting the lipids or flavonoids to the stigmatic surface and releasing them there.
Cytokinins are plant growth hormones that control differentiation and proliferation of plant cells. Certain proteins have evolved to bind these hormones, however, their specific function has not been established (64). It has been suggested that cytokinin binding proteins may act as storage compartments for cytokinins in seeds allowing rapid release of cytokinins upon germination (64). This could be compatible with a role for Bet v 1 in cytokinin binding.

In addition, it could be speculated that Bet v 1 plays a role in plants similar to that of serum albumin in animals. Serum albumin is a general transporter of endogenous ligands such as non-esterified fatty acids, bilirubin, and thyroxine (65). It binds fatty acids in an asymmetric fashion (66) with dissociation constants from low nanomolar to low micromolar concentrations (67). Another protein to bind a wide range of ligands with similar affinities without strict complementarity is adipocyte lipid binding protein. The binding affinity is in the nanomolar range according to ANS displacement assays (68, 69).

Several of the PR-10 proteins have been proposed to possess RNase activity (22–25), which may constitute a common trait of this protein family (50). The present study clearly demonstrates that Bet v 1 is capable of binding several types of ligands, most of which bind in the cavity suggesting a transport or storage function of Bet v 1, a role that is difficult to reconcile with RNase activity. It is generally accepted that plant RNases utilize two surface-exposed histidines for catalytic activity (70). Bet v 1 has no such residues in a similar spatial arrangement. Also, a naturally occurring RNase-like protein from resting rhizomes of Hedge bindweed (**Bet v 1**) has no such residues in a similar spatial arrangement. Additionally, it could be speculated that Bet v 1 plays a role in plants similar to that of serum albumin in animals.

**Ligand Binding Properties of the Major Birch Allergen**

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