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Birch PR-10c interacts with several biologically important ligands

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Abstract

PR-10c is a unique member of PR-10 proteins in birch, since it is the only one known to be post-translationally modified by glutathione and is not constitutively expressed in pollen. Both reduced and S-glutathiolated forms of PR-10c show low ribonuclease activity. However, the major function of the protein is apparently not yet resolved. Our protein–ligand interaction studies with saturation transfer difference (STD) NMR revealed that PR-10c interacts with several biologically important molecules, including cytokinin, flavonoid glycosides, sterols and emodin. Competition study with deoxycholate and kinetin revealed no statistically significant binding interference, indicating that these ligands have different binding sites in PR-10c. Ligand docking studies with a molecular model of PR-10c support the STD NMR results of ligand binding and binding epitopes, suggesting that there are three potential binding sites in PR-10c: two in the hydrophobic cavity and one in the glycine-rich loop. Our docking calculations suggested that only kinetin interacts with the glycine-rich loop, the binding occurring through its adenine moiety. Clear ligand specificity could be observed in the binding of nucleotide derivatives. S-glutathiolation of PR-10c did not affect kinetin binding. The present results suggest that birch PR-10c is a multifunctional protein, which has diverse roles in plant stress responses.

Keywords: Saturation transfer difference NMR; Molecular modelling; Pathogenesis-related proteins; Glutathiolation; Cytokinins; Phytosterols; Flavonoids; Emodin

1. Introduction

The PR-10 (pathogenesis-related class 10) protein family consists of a large group of homologous proteins found from several plant species. The expression of PR-10 proteins is up-regulated as a response to various biotic and abiotic factors. Several food and pollen allergens, e.g., Bet v 1 (Breiteneder et al., 1989), Api g 1 (Breiteneder et al., 1995), Mal d 1 (Vanek-Krebitz et al., 1995; Pühringer et al., 2000), Pru av 1 (Scheurer et al., 1997) and hazelnut major allergen Cor a 1.04 (Lüttkopf et al., 2002) are included in the PR-10 family. Furthermore, the sequence and structural homology of PR-10 proteins with major latex proteins (MLP) suggest that these proteins are related and might share a similar function (Osmark et al., 1998).

Although several PR-10 proteins have been reported to possess RNase activity, including birch Bet v 1 (Swoboda et al., 1996; Bufe et al., 1996), birch PR-10c (Koistinen et al., 2002a), lupin root LaPR-10 (Bantignies et al., 2000), cotton GaPR-10 (Zhou et al., 2002), PR-10 protein from *Pachyrrhizus erosus* seeds (Wu et al., 2002) and pepper CaPR-10 (Park et al., 2004), also other functions have been ascribed to PR-10 proteins. Structural homology of Bet v 1 with the START domain of MLN64 (Tsujishita and Hurley, 2000) suggested a similar function for Bet v 1 as a steroid binding protein; until now, Pru av 1 (Neudecker et al., 2001), Bet v 1

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(Mogensen et al., 2002) and Bet v 1l (Marković-Housley et al., 2003) have been shown to interact with phytosteroids. Furthermore, Bet v 1 interacts with fatty acids, cytokinins and flavonoids (Mogensen et al., 2002). It has been reported recently that Hyp-1 protein from *Hypericum perforatum*, which shows high sequence similarity with PR-10 proteins, is able to convert emodin to hypericin in vitro (Bais et al., 2003).

Although PR-10c shares high sequence similarity with Bet v 1 and other PR-10 proteins in birch, it is a unique member of PR-10 proteins, and appears to belong to a different subfamily. It has much higher sequence identity with Cor a 1.04 proteins from hazelnut than with any other birch PR-10 protein. Furthermore, unlike the *Bet v* 1 genes, *PR-10c* is not constitutively expressed in pollen (Swoboda et al., 1995). The most interesting similarity between PR-10c and four Cor a 1.04 proteins is the presence of a cysteine residue in position 82-83, which is absent from the other PR-10 proteins in birch. This cysteine in PR-10c can be post-translationally modified by glutathione both in vitro and in vivo (Koistinen et al., 2002a). Even though the PR-10 proteins share a high sequence similarity, the different subfamilies may differ in their structure. Pasternak et al. (2005) reported that LlPR-10.2A, a member of a novel subclass of lupin PR-10, deviates structurally from LlPR-10.1A and LlPR-10.1B of another subclass of lupin PR-10 family by having a different shape and volume in the hydrophobic ligand-binding cavity.

Furthermore, different expression profiles have been described for *PR-10c* and *PR-10a* genes in birch in response to wounding (Poupard et al., 1998) and auxin treatment (Poupard et al., 2001). The different PR-10 proteins also respond differently to copper exposure (Koistinen et al., 2002b). Even though at least three possible functions have been proposed for PR-10 proteins, the exact role of these proteins in plant stress responses, defence, stress tolerance and pollen development is still unclear.

The aim of the present study was to examine the possible binding of various biologically important ligands to PR-10c, and to determine the binding epitope of the ligands using saturation transfer difference (STD) NMR spectroscopy (Mayer and Meyer, 1999) and molecular modelling in order to shed more light to the role of PR-10 proteins in stress response and plant defence.

2. Results

2.1. Binding of PR-10c to nucleotide derivatives

The structure, biological function and binding epitope of ligands interacting with PR-10c-His are presented in Table 1. Binding of cytokinin (kinetin) was studied both on the reduced and glutathiolated form of PR-10-His protein. The STD NMR results indicated that both forms interact with kinetin (Fig. 1). The calculated STD factors show that adenine rather than furan moiety of the molecule interacts with the protein (Fig. 2(a)). The interaction of kinetin was also tested with a higher concentration of another PR-10c-His protein construct (containing thrombin protease cleavage site) with similar results. Binding of another adenine derivative, i.e., ATP, to PR-10c-His protein could not be detected with STD NMR. The method has been used recently to characterize the binding of ATP to protein kinases (McCoy et al., 2005).

2.2. Birch PR-10c binds flavonoid glycosides

The interaction of PR-10c-His with flavonoids was studied with two quercetin glycosides, i.e., quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-rutinoside (rutin). Both hyperoside and rutin interact with PR-10c-His. The results clearly indicated that the interaction occurs through the B ring of quercetin moiety since no saturation transfer was observed for the glycosidic and A ring protons (Fig. 3).

2.3. Interaction of PR-10c-His with other biologically important molecules

PR-10 homologue Hyp-1 protein has been reported to form hypericin from emodin in vitro (Bais et al., 2003). The present results indicate that emodin clearly interacts with PR-10c-His protein (Fig. 4). However, formation of hypericin from emodin could not be demonstrated within 72 h with NMR experiments. Neither was any significant difference observed in STD factors for aromatic protons.

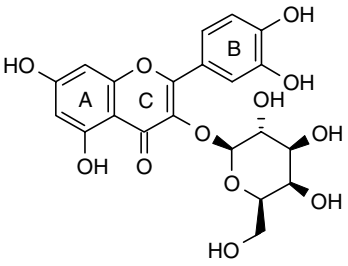
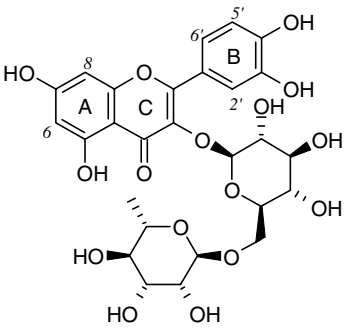
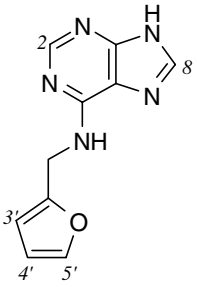
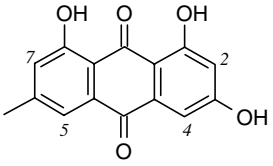
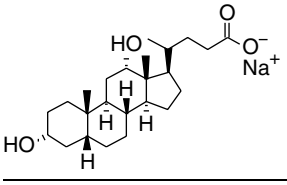
The PR-10c-His protein interacted also with deoxycholate. The CH_3-19 appears to have the largest relative STD-effect. STD-effects were also calculated for some other CH_3-groups; those were all smaller than the STD-% of CH_3-19. No significant effects were seen for aliphatic chain hydrogens. This could indicate, together with the fact that the molecule is roughly planar, that the steroid nucleus of the molecule is closer to the protein. Competition experiment with kinetin showed that these two molecules do not interfere with each other statistically significantly (Fig. 2), suggesting that deoxycholate and kinetin have different binding sites in the protein.

Mogensen et al. (2002) have reported that birch Bet v 1 does not interact with indole-3-acetic acid or gibberellic acid, and these molecules were thus used as negative controls for the STD NMR measurements. The present results indicated that, even though PR-10c bound several biologically important molecules, no interaction occurs between PR-10c-His and indole-3-acetic acid or gibberellic acid.

2.4. Molecular modelling of PR-10c and ligand docking

Docking of kinetin, emodin, gibberellic acid and deoxycholate revealed two separate theoretical binding sites in the hydrophobic binding cavity of PR-10c (Fig. 5). The binding sites locate at the opposite corners of the cavity so that two molecules may bind to the cavity at the same

Table 1
Structure, biological function and binding epitope of ligands interacting with PR-10c-His protein

Structure	Ligand	Biological function	Binding epitope
	Hyperoside (quercetin-3-*O*-galactoside)	Flavonoid glycoside Plant defence Antioxidant Pigment	B-ring of quercetin moiety
	Rutin (quercetin-3-*O*-rutinoside)	Flavonoid glycoside Plant defence Antioxidant Pigment	B-ring of quercetin moiety
	Kinetin (N^6-furfuryladenine)	Cytokinin Cell division Bud growth Fruit and embryo development	Adenine moiety
	Emodin (1,3,8-trihydroxy-6-methylanthraquinone)	Anthraquinone (see Izhaki, 2002) Antioxidant Antimicrobial Feeding deterrent	No clear difference observed
	Deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid sodium salt)	Bile salt Not found in plants Structural homology with brassinosteroids (Marković-Housley et al., 2003)	Steroid nucleus

time. On the basis of ligand scoring values, which are rough measures of binding affinities, the two binding sites seem to possess similar affinities to the ligands.

Two bound emodin molecules are separated by ca. 6 Å. The binding orientation and separation of emodin molecules do not particularly favour their interaction. However, as the molecules are not tightly bound to the protein but may freely move around in the cavity, the conversion of emodin to hypericin appears plausible.

In addition to the hydrophobic cavity, a third binding site was found for kinetin in the glycine-rich loop. The docking results of kinetin are in agreement with NMR measurements: adenine moiety of kinetin interacts more closely with the protein than does the furan moiety. On the basis of the scoring values the affinities of these three sites are similar.

The two binding sites found in the docking analysis for deoxycholate are close to those observed in the crys-

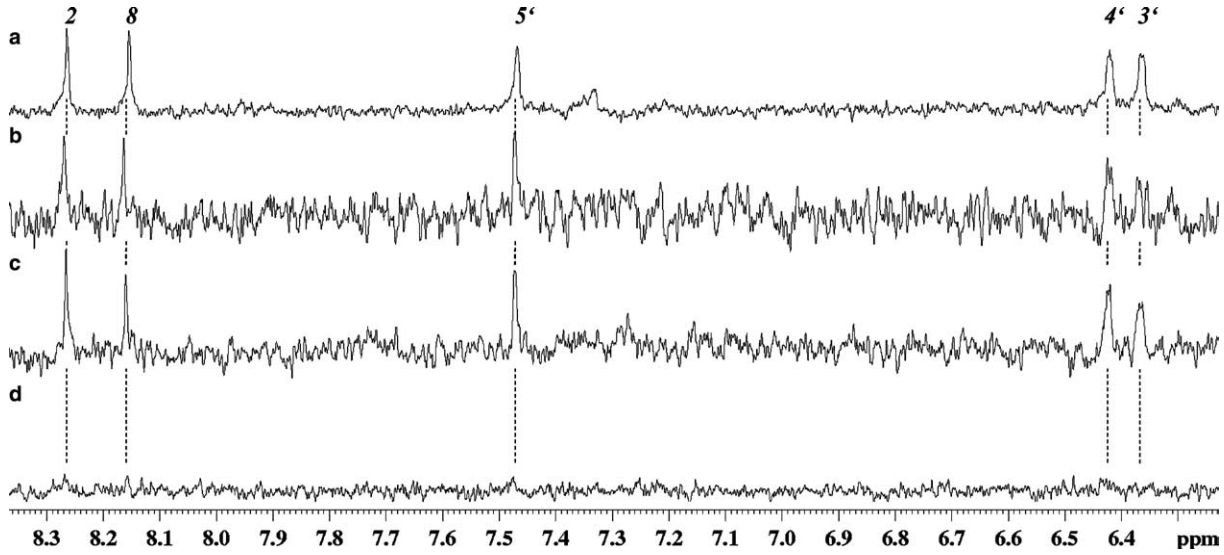


Fig. 1. Binding of kinetin to reduced and glutathiolated form of PR-10-His fusion protein observed with STD NMR. (a) Part of a standard proton NMR spectrum of kinetin for reference. STD NMR spectrum of kinetin with (b) glutathiolated and (c) reduced PR-10-His fusion protein showing signals from bound kinetin. (d) STD NMR spectrum of kinetin without protein; all signals are cancelled. For assignments, see the structure in Table 1.

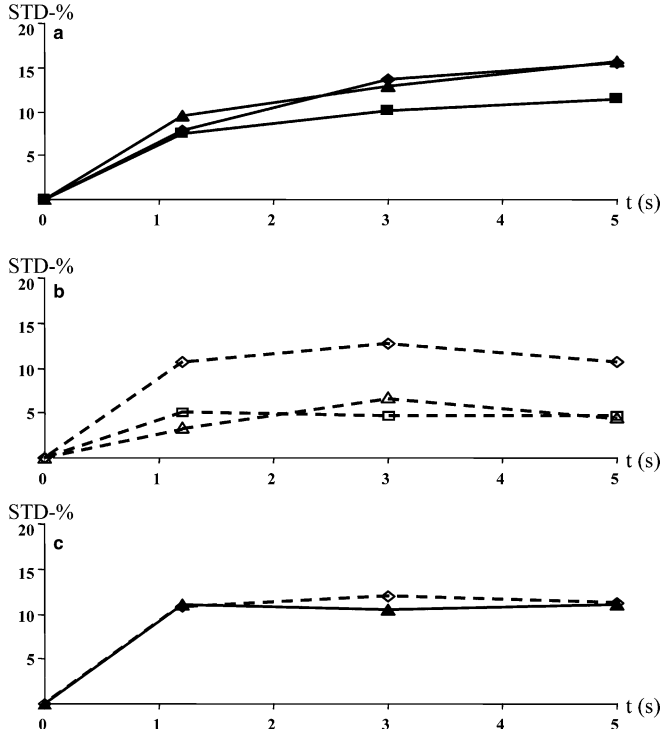


Fig. 2. (a) Relative STD effects (STD-%) for different hydrogens of kinetin as a function of saturation time (s). Solid square corresponds to H-5 in furan ring, triangle to H-8 and diamond to H-2 in purine moiety. As the saturation time increases, also relative STD-effects grow larger. Since the STD-effect is larger for those parts of ligand which are closest to the protein, it is possible to conclude that the purine ring of kinetin is closer to the protein than the furan ring. (b) Relative STD-effects (STD-%) for selected signals of the deoxycholate hydrogens as a function of saturation time (s). Open square corresponds to H-12, triangle to H-3 and diamond to CH_3-19. (c) Relative STD-effects for kinetin (solid triangle) and deoxycholate (open diamond) measured from the same sample. Since both molecules have equal STD-% and there is no major change in STD-% when compared to those of each molecule alone with the protein, it can be proposed that these two molecules do not compete for the same binding site.

tal structure of Bet v 1l-deoxycholate complex. The docking of deoxycholate was done to the protein model refined without a bound ligand, i.e., the protein structure was not adapted for deoxycholate binding and indicates that docking provides meaningful binding modes. An additional molecular dynamics (MD) simulation was done for PR-10c complexed with two deoxycholates. This simulation was structurally and energetically stable and the bound molecules stayed close to the binding sites observed in the crystal structure of Bet v 1l. Thus, in terms of deoxycholate binding, PR-10c resembles Bet v 1l.

Two hyperoside molecules and one rutin molecule were also found to fit into the binding pocket. However, no clear binding conformation or site was found for these molecules. Docking suggested that PR-10c is also able to bind betulin and sitosterol.

3. Discussion

NMR spectroscopy offers several different techniques to monitor protein–ligand interactions (Stockman and Dalvit, 2002; Meyer and Peters, 2003). One of the advantages of NMR is that it conserves the sample, enabling its use for several consecutive measurements. Depending on protein, concentrations and information seeked for, the most efficient technique can be chosen. The STD NMR technique, which is based on saturation transfer from protein to bound ligand (Mayer and Meyer, 1999), is a fast and informative way to explore ligand binding to protein. It has been used for several applications, e.g., to characterize carbohydrate binding to proteins (Klein et al., 1999), peptide liposome-integrated integrin interactions (Meinecke and Meyer, 2001), ligand binding to viruses (Benie et al., 2003) and flavonoid binding to transporter

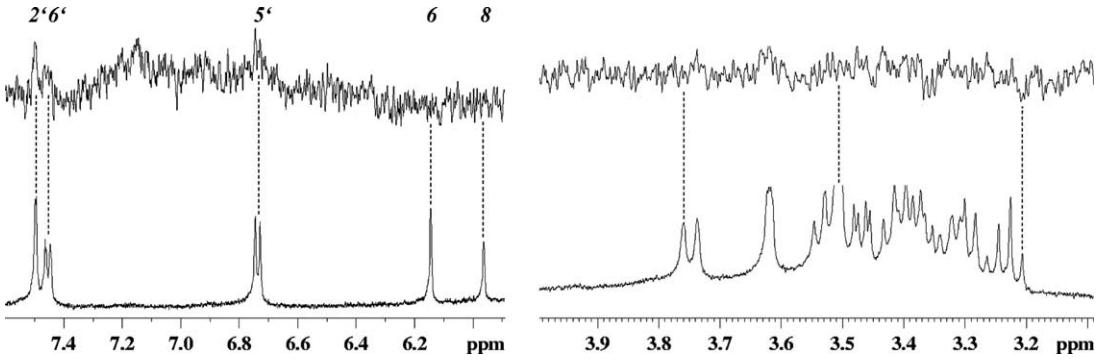


Fig. 3. Binding of rutin to PR-10-His protein. Two parts of the reference ^{1}H NMR spectrum (below) and parts of the STD NMR spectrum (above) are shown. In the STD spectrum, only the signals arising from the B-ring of quercetin moiety are visible, indicating that this part of the molecule is closest to the protein. For assignments, see the structure in Table 1.

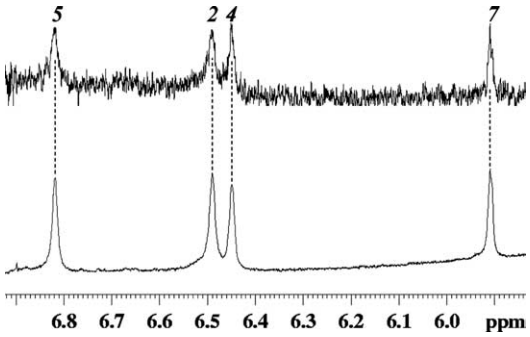


Fig. 4. Binding of emodin to PR-10-His protein. Part of the reference ^{1}H NMR spectrum (below) and part of the STD NMR spectrum are shown. No clear difference in STD signals could be observed. For assignments, see the structure in Table 1.

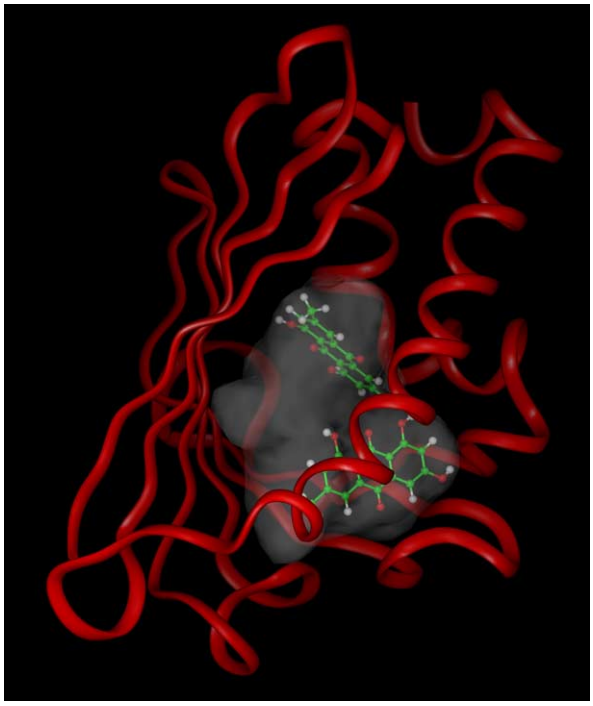


Fig. 5. Ribbon representation of the PR-10c model showing the hydrophobic ligand-binding cavity and two bound emodin molecules. Ligands were docked to the cavity using the GOLD program.

protein (Nissler et al., 2004). One of the advantages of this technique is the relatively small amount of protein needed compared to other NMR techniques. It also reveals the binding epitope of the bound ligand (Maaheimo et al., 2000; Mayer and Meyer, 2001; Johnson and Pinto, 2004; Wen et al., 2005), since the saturation transfer will be more efficient for those parts of the ligand which come into close contact with the protein, and thus the relative intensities of ligand signals indicate its binding epitope. In addition, the method is relatively fast since only two 1D spectra of one sample, i.e., off- and on-resonance, are needed. The STD difference spectrum, resulting from subtracting of on-resonance from off-resonance spectrum, contains signals resulting only from saturation transfer from the protein to the bound ligand. Depending on the concentrations used, the binding information can be obtained during one day or even in one hour. This method can also be combined to any NMR pulse sequences, resulting in a set of experiments for selection (Mayer and Meyer, 1999, 2001; Maaheimo et al., 2000). However, no information about the binding site of the protein can be gained with STD NMR. For these purposes one should use, e.g., ^{15}N-labeled protein with SAR by NMR (Shuker et al., 1996).

The STD NMR method was chosen here to study the possible interaction of birch PR-10c protein with biologically important ligands. Of the ligands studied, cytokinins are plant hormones that regulate several processes in plant growth and development, e.g., cell division, bud growth, fruit and embryo development. The present results indicate that kinetin interacts with both glutathiolated and reduced forms of PR-10c-His protein through adenine moiety. The observed binding is in line with previous findings on the interaction of the birch major allergen Bet v 1 with the cytokinins kinetin and IPA (Mogensen et al., 2002), and on the homology of PR-10 proteins with cytokinin binding protein of mung bean (*Vigna radiata*) (Fujimoto et al., 1998). Furthermore, several PR-10 proteins have been reported to possess RNase activity. The glycine rich loop, which resembles the P-loop motif found from many nucleotide-binding proteins (Saraste et al., 1990) is highly conserved

in the PR-10 family. It is probable that kinetin and RNA interact with PR-10 proteins through the P-loop-like motif, which has been shown to be critical for the RNase activity of *Pachyrrhizus erosus* SPE-16, a protein belonging to the PR-10 family (Wu et al., 2003). It has been shown recently that glutathiolation of PR-10c-His does not affect the RNase activity of the protein (Koistinen et al., 2002a). This supports the present results that glutathiolation of PR-10c-His did not affect kinetin binding. Interestingly, the present results indicated that ATP does not interact with PR-10c-His, even though kinetin is bound through its adenine moiety, suggesting specificity in the interaction of the nucleotide derivatives with PR-10c.

Competition experiment with kinetin and deoxycholate demonstrated that these ligands do not interfere with each other, indicating that they have a different binding site. The docking results partly supported this observation; kinetin and deoxycholate both have two binding sites where they can bind without interfering with each other. Deoxycholate shares structural similarity with brassinosteroids, which have been reported to bind to Bet v 1l (Marković-Housley et al., 2003). Also the major cherry allergen Pru av 1 has been reported to bind the brassinosteroid homocastasterone (Neudecker et al., 2001). Brassinosteroids are plant hormones that participate in several developmental responses, e.g., cell expansion and division, vascular differentiation, senescence and modulation of stress responses (reviewed by Clouse and Sasse, 1998). Our docking experiments indicate that PR-10c is also able to bind sitosterol as well as the triterpene betulin that gives the white colour to birch bark.

The flavonol glycoside quercetin-3-*O*-galactoside (hyperoside), one of the main phenolic compounds in birch (Laitinen et al., 2000), also interacts with PR-10c-His. The biological importance of flavonoid binding is strongly supported by the observation that PR-10c-His is able to bind both tested glycosides, i.e., hyperoside and rutin. Indeed, even quercetin-3-*O*-rutinoside (rutin), which contains a disaccharide moiety and is, therefore, a relatively large molecule, fits into the binding cavity of PR-10c. Binding of flavone and flavanone (naringenin) to birch Bet v 1 has been reported previously (Mogensen et al., 2002). On the other hand, flavonoids are often present as glycosides in plants. Flavonols (e.g., quercetin), a subclass of flavonoids, are generally present as diglycosides in pollen (reviewed by Taylor and Hepler, 1997). Flavonoids are usually located in the vacuoles, but flavonoid glycosides are also found from the cell wall (Markham et al., 2000).

Vacuolar transport of flavonoids has been suggested to be similar to the detoxification mechanism of herbicides. However, studies on petunia glutathione-S-transferase (GST) AN9 indicated that, instead of forming glutathione conjugates with flavonoids, AN9 binds flavonoids and was suggested to serve as a cytoplasmic flavonoid carrier protein (Mueller et al., 2000). Somewhat similar role could be proposed for PR-10c in the storage of flavonoids and their transport to the site where they are needed most. Phenylpropanoids are a group of structurally divergent compounds derived from cinnamic acid, and several phenylpropanoid compounds are induced in plants by various abiotic and biotic stresses (reviewed by Dixon and Paiva, 1995). Interestingly, the expression of *PR-10* genes has been previously related to phenylpropanoid accumulation. Asparagus *AoPR1* promoter has been reported to be active in majority of cell types known to accumulate different classes of phenylpropanoid derivatives (Warner et al., 1994). Furthermore, sorghum PR-10 transcripts accumulate in *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*-inoculated parts of the plant along with the accumulation of phytoalexins (Lo et al., 1999).

Emodin is an anthraquinone, which has been suggested to act as feeding deterrent, to be an allelopathic compound, to have antimicrobial activity and to participate in seed germination and dispersal (reviewed by Izhaki, 2002). It has been reported that Hyp-1, a member of PR-10 family, is able to convert emodin to hypericin in vitro (Bais et al., 2003). Our results show that PR-10c-His interacts with emodin and, on the basis of ligand docking, is able to bind two emodin molecules at the same time. Although formation of hypericin could not be demonstrated by NMR experiments, the possibility that PR-10c may participate in it cannot be excluded. It should be noted that the experimental conditions in the present study were slightly alkaline due to the poor solubility of emodin, and the catalytic activity of Hyp-1 has been shown to have optimum between pH 6.5 and 7.0 (Bais et al., 2003). Further evidence for the role of PR-10 proteins in the condensation reaction has been obtained from the sequence similarity between Hyp-1 and norcoclaurine synthase, which catalyses the first committed step in the biosynthesis of benzylisoquinoline alkaloids (Samanani et al., 2004). However, Liscombe et al. (2005) reported recently that PR-10 homologous proteins PsPR10-1, PsPR10-2, OsPR10, VrCSBP, HpHYP1, BbMAP (Bet v 1a) and PmPR10 do not show detectable NCS activity.

Based on the present results, PR-10c is capable of binding several different types of ligands using different binding sites for different ligands. This suggests that PR-10c has a role in the storage and transport of biologically important molecules. Although glutathiolation of PR-10c does not affect the interaction with kinetin, it may have some other role in the transport or storage of ligands. In our previous studies, PR-10c was shown to possess RNase activity (Koistinen et al., 2002a). It has been proposed that RNase activity of PR-10 proteins may be incidental and of no biological importance (Biesiadka et al., 2002). On the other hand, recent reports on the antifungal and antibacterial activity of ocatin (PR-10) from Andean tuber oca (Flores et al., 2002) and antifungal and antiviral activity of CaPR-10 from pepper (Park et al., 2004) suggest that RNase activity plays an important role in plant defence responses. Therefore, it appears that members of PR-10 protein family have several different activities and functions in various tissues and conditions.

4. Experimental

4.1. Expression and purification of PR-10c protein

Primers for amplification and cloning of PR-10c into pQE31 expression vector (Qiagen, Valencia, CA, USA) contained restriction enzyme cleavage sites for SalI (5′ end) and PstI (3′ end), and thrombin protease cleavage site for removal of the N-terminal 6 × HisTaq from the fusion protein. The PR-10c construct was transferred into *E. coli* M15 [pREP4] protein expression strain. Colonies expressing PR-10c-His fusion protein were screened with colony-lift using a monoclonal antibody against 6 × His taq (Pharmacia, Uppsala, Sweden).

Large-scale culture was performed as described previously by Koistinen et al. (2002a). PR-10c-His protein was purified with Ni-NTA agarose (Qiagen) following the manufacturer's instructions, except that *E. coli* lysate was mixed with Ni-NTA agarose in PD-10 column. Purified PR-10c-His was incubated overnight with 25 mM DTT and dialysed against H_2O.

Interaction of glutathiolated and reduced forms of PR-10c-His with kinetin was tested with HPLC-purified protein without thrombin protease cleavage site (Koistinen et al., 2002a).

4.2. Chemicals

Quercetin-3-*O*-rutinoside (rutin), kinetin, gibberellic acid, emodin and deoxycholic acid sodium salt were purchase from Sigma Chemicals (St. Louis, MO, USA). Quercetin-3-*O*-galactoside (hyperoside) was from Fluka (Buchs, Switzerland), adenoside-5′-triphosphate (ATP) from Boehringer Mannheim (Germany) and indole-3-acetic acid from Duchefa (Haarlem, the Netherlands).

4.3. Sample preparation for NMR studies

All samples were prepared using deuterated phosphate buffer (100 mM K_2DPO_4/KD_2PO_4, 100 mM NaCl in 99.8%-*d* D_2O, pH* = 7.0) as the solvent. For preliminary studies, HPLC-purified glutathiolated and reduced forms of PR-10c-His were tested using 2 μM protein and 200 μM kinetin as a ligand. To achieve a higher protein concentration, the purified PR-10c-His was lyophilised and the dried protein was dissolved in the buffer to a final concentration of 20 μM. Kinetin, gibberellic acid, rutin, hyperoside, emodin, indole-3-acetic acid and deoxycholic acid used as ligands in protein–ligand binding studies were dissolved in D_2O, using NaOD to adjust the pH when necessary for dissolution. Ligand concentration in each sample was 2 mM, resulting in 1:100 protein to ligand ratio. When deoxycholate and kinetin were studied together, 2 mM concentration was used for both ligands. The final volume of samples in all studies was 400 μl.

4.4. Saturation transfer difference NMR (STD NMR) spectroscopy

All measurements were performed on Bruker AVANCE 500 DRX spectrometer operating at 500.13 MHz and equipped with 5 mm inverse triple resonance probe. The spectra were measured at 300 K without spinning of the sample.

One-dimensional STD NMR experiments (Mayer and Meyer, 1999) were recorded typically with 4096 scans for both off- and on-resonance spectra. Difference spectrum was obtained internally via phase cycling. For samples with higher concentration of H_2O, water suppression was achieved using WATERGATE pulse sequence (Piotto et al., 1992). Irradiation frequency was set to 15 kHz for off-resonance and 0 Hz for on-resonance. Selective saturation of the protein was achieved using a pulse train of selective Gaussian pulses. Duration of each pulse was 50 ms and the pulses were separated by 1 ms delay. Saturation time was 2 s. In addition, when build-up of saturation transfer was explored, saturation times of 1.2, 3 and 5 s were used. For these studies, off- and on-resonance spectra were recorded and quantified separately to obtain build-up curves. The relative STD effects for individual protons were calculated by determining the individual signal areas in the on- (A_{on}) and off-resonance (A_{off}) spectrum and subtracting them ($A_{off} - A_{on}$). This corresponds to the signal area in STD spectrum (A_{STD}). To calculate STD-% values, the resulting area A_{STD} was divided by the signal area in the off-resonance spectrum and finally multiplied by 100% (STD-% = $100\% \times (A_{off} - A_{on})/A_{off}$).

All spectra were processed using PERCH NMR Software (PERCH Solutions Ltd., Kuopio, Finland). Prior to Fourier transformation, the FIDs were multiplied by an exponential window function with line-broadening of 1 Hz. Quantifications were performed using the TLS program (Laatikainen et al., 1996) of PERCH NMR Software on a standard desktop PC.

4.5. Protein modelling and ligand docking

Comparative protein modelling approach was applied to construct a structural model of birch PR-10c by using the COMPOSER program (Sutcliffe et al., 1987a,b) as implemented in the SYBYL (version 6.91, Tripos Associates, St. Louis, USA) molecular modelling software. The crystal structure of Bet v 1l in complex with deoxycholate determined to a resolution of 1.9 Å (Brookhaven Protein Data Bank code 1FM4) (Marković-Housley et al., 2003) was used as a template in the model building. The sequences of PR-10c and Bet v 1l were aligned with the sequence alignment program ClustalW (Thompson et al., 1994). Due to the high (72%) sequence identity shared by PR-10c and Bet v 1l, the protein model is expected to be reliable (Martí-Renom et al., 2000).

Initial PR-10c model obtained from the modelling was refined by molecular dynamics (MD) simulations. The refinement was done for the ligand-free PR-10c and for

PR-10c complexed with two deoxycholate molecules docked using the Bet v 1l crystal structure as a model. For the MD simulations, PR-10c was solvated by 8174 TIP3P water molecules in a periodic box of $58 \times 61 \times 76$ Å. The water molecules of the systems were first energy-minimized for 1000 steps, heated to 300 K in 5 ps and equilibrated by 10 ps at a constant temperature of 300 K and pressure of 1 atm. After that the simulation systems were minimized for 1000 steps, the temperature of the systems was increased to 300 K in 5 ps and equilibrated for 100 ps while keeping the protein backbone atoms (N, Cα, C) restrained by an atom-based harmonic potential of 1 kcal mol^{-1} $Å^{-2}$. The purpose of these simulation steps was to remove atom–atom clashes and let the protein side chains pack efficiently. After that the restraints were removed and 300 ps MD simulations were carried out. In the simulations, the electrostatics were treated using the particle-mesh Ewald method. A timestep of 1.5 fs was used and bonds involving hydrogen atoms were constrained to their equilibrium lengths using the SHAKE algorithm. The simulations were done using the AMBER8.0 simulation package (University of California, San Francisco, USA) and the parm99 parameter set of AMBER. The parameters of deoxycholate were generated with the Antechamber suite of AMBER8.0 in conjunction with the general amber force field. The atomic point charges of the ligand were calculated with the two-stage RESP (Bayly et al., 1993) fit at the HF/6-31G* level using ligand geometries optimized with the semi-empirical PM3 method using the Gaussian03 program (Gaussian Inc., Pittsburgh, USA). The quality of the refined PR-10c model was checked using the PROCHECK program (Laskowski et al., 1993). In the model, 87.5% of the residues were in most favoured regions of the Ramachandran plot and none in disallowed regions.

Ligands were docked to the ligand-binding cavity of ligand-free PR-10c structure obtained from the 300 ps MD simulation using the GOLD (version 2.1) protein–ligand docking program (Verdonk et al., 2003). For each ligand, 10 dockings with the gold score-scoring function were performed.

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