Beyond carbohydrate binding: new directions in plant lectin research

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Although for a long time carbohydrate binding property has been used as the defining feature of lectins, studies carried out mostly during the last two decades or so demonstrate that many plant lectins exhibit specific interactions with small molecules that are predominantly hydrophobic in nature. Such interactions, in most cases, appear to be at specific sites that do not interfere with the ability of the lectins to recognise and bind carbohydrates. Further, several of these ligands have binding affinities comparable to those for the binding of specific carbohydrates to the lectins. Given the ability of lectins to specifically recognise the glycocode (carbohydrate code) on different cell surfaces and distinguish between diseased and normal tissues, these additional sites may be viewed as potential drug carrying sites that could be exploited for targeted delivery to sites of choice. Porphyrin–lectin complexes are especially suited for such targeting since porphyrins are already under investigation in photodynamic therapy for cancer. This review will provide an update on the interactions of plant lectins with non-carbohydrate ligands, with particular emphasis on porphyrin ligands. The implications and potential applications of such studies will also be discussed.

1. Introduction

The specific recognition of carbohydrates by lectins is so striking that they are commonly defined as carbohydrate binding proteins (other than enzymes or antibodies) capable of specific and reversible interaction with these ligands.[1,2] There have been excellent reviews from time to time that captured the various aspects of developments in the field,[3] summarized the importance of carbohydrates and lectins in immunology, oncology or medicine,[2,4–7] discussed various methods of lectin classification[3,9,10] or gave detailed accounts of specific lectins and their properties.[11–13] There have also been two excellent books on lectins with a wealth of information on various aspects of lectins, especially regarding their occurrence and carbohydrate binding specificity, structural features and applications.[14,15] This review does not therefore attempt to repeat what has already been so ably achieved by others in the field. We instead intend to focus on non-carbohydrate ligands that have been reported to interact with lectins and the possible implications that this could have on the directions of research in this field, with specific focus on porphyrin binding by plant lectins, a subject of detailed investigations in our laboratory during the last decade. These aspects have not been the main subject of any review so far, although a brief mention has been made in the recent book by Sharon and Lis.[15]

2. Lectin research: origins and developments

Although it seems apparent now that Weir Mitchell had already observed lectin activity in rattle snake venom before 1860,[11] it wasn't until at least six years later, when Stillmark reported the dramatic action of ricin on red blood cells and then Helin followed it up by a similar report on abrin, that agglutinins caught the attention of the medical community. Reports of hemagglutinins from a wide variety of sources were quick to follow. Besides plants, agglutinins were discovered in fungi, bacteria, viruses, invertebrates and vertebrates. Although this early period established, beyond any doubt, the proteinaceous nature of lectins and their cell-agglutination and precipitation capabilities, lectin research thereafter was beset with problems and difficulties for the next quarter of a century or so.[16]

Studies, by Sugishita, Jonsson, Boyd and Renkonen, provided the proverbial 'shot in the arm' for research on lectins by identifying lectins as cell-recognition molecules that could have practical applications.[16] Reports of blood-group specificity, mitogenicity and tumour cell-binding of lectins followed almost immediately. The number of known properties and possible applications of lectins grew rapidly. Concanavalin A (Con A), a lectin from jack beans, became the first lectin to be crystallized and then extensively characterized by Sumner and Howell who also showed for the first time that sucrose could inhibit its agglutination activity.[17] Two other major discoveries set the tone of the research that was to follow. Funatsu and his collaborators isolated the first non-toxic lectin from *Ricinus communis*, shattering the prevalent notion at that time that lectins were necessarily toxic proteins.[18–20] Secondly, it was shown that several of these lectins, such as that from soybean, were glycoproteins.[21] Lectin research had progressed beyond the serological level and it was but a matter of time before molecular level analysis of lectin activity would begin in earnest.

However, it may be useful to emphasise here that the trajectories of research in the field have been neither smooth nor unidirectional. The field of plant and animal lectins grew from the very different interests and concerns of researchers working on them. While it is certainly undeniable that reports on plant lectins far outnumbered those from microbial or animal sources, owing as much perhaps to their relative ease of availability as to their

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ease of purification, yet to a new entrant in the field it would be more than a little puzzling that much less is known about the *in vivo* function of the former than the latter. Interest in studying microbial and animal lectins grew directly from interest in the biological functions of these proteins, with lectin activity being discovered subsequently, or almost coincidentally, in many cases. On the other hand, the effect of plant lectins on different cell types had already set the agenda for early research on them, leading to an extensive search for lectins in plant extracts and identification of a large number of lectins with practical applications. Such an objective did not require identification of the biological function of the protein *per se.* Indeed, in several cases where biological functions have been hypothesised or proven, the effect of the plant lectins on microbial or animal cells has provided clues to their putative function *in vivo.* Research on the endogenous roles of plant lectins has therefore been a late starter although some progress has been made in this direction in recent years.[22,23] Despite this, interest in studying plant lectins has been sustained owing to the fact that their natural abundance makes their applications in a large number of areas much more feasible.[12,24]

3. Lectin–carbohydrate interaction

Glycosylation is the key step in a number of processes at the cellular level. Cell-surface oligosaccharides get altered in various kinds of pathological conditions including malignant transformations.[25] With developments in the closely-related field of glycobiology, it has now become evident that oligosaccharide-mediated recognition plays a very important role in a variety of biological processes such as fertilization, immune defence, viral replication, parasitic infection, cell–matrix interaction, cell–cell adhesion and enzymatic activity. Lectins have been implicated in most, if not all of these recognition events.[12,25–28] The strict selectivity that this kind of recognition requires, imposes a stringent geometry upon both the ligand and the corresponding receptor, thus conferring unique sugar-specificities upon lectins.[13,28–33] Carbohydrates can interact with lectins *via* hydrogen bonds, metal coordination bonds and van der Waal's and hydrophobic interactions. Selectivity results from specific hydrogen bonding and/or metal coordination bonds with key hydroxyls of the carbohydrates which can act as both acceptors and donors of hydrogen bonds. Water molecules often act as bridges in these interactions. The hydroxyl at the C4 position, in particular seems to be a decisive player in these events. Steric exclusions minimise unwanted recognition, further fine-tuning the saccharide specificity of the lectin. Subsite binding and subunit multivalency, where possible, increase the binding selectivity manifold.[31,34] In subsite binding, the primary binding site appears critical for carbohydrate recognition, but secondary binding sites contribute to enhanced affinity of the lectin towards specific oligosaccharides. For example, the legume lectins *Lathyrus ochrus* isolectin II (LOL II) and Con A are both Man/Glc specific lectins, but their oligosaccharide preferences are very different. LOL II has several-fold higher affinity for oligosaccharides that have additional α(1–6)-linked fucose residues while Con A does not (for reviews see[28,31]). In subunit multivalency, several subunits of the same lectin contribute to the binding by recognising different extensions of the carbohydrate or different chains of a branched oligosaccharide. This kind of binding is exhibited, among others, by the asialoglycoprotein receptor, the mannose binding protein (MBP) from the serum, the chicken hepatic lectin and the cholera toxin.[29,30]

It appears that the monosaccharide specificity of a lectin, although useful, need not necessarily tell the complete story. It has become evident in a large number of cases, particularly in the case of those lectins with proven or putative biological functions, that multivalency of the receptor is a prerequisite for recognition. Thus the MBP, for example, binds to monomeric mannose units and simply releases them but, when it binds to the oligomannosides on a pathogen that has the same spacing as the trimers of MBP, it triggers off a biological response that results in complement fixation.[35,36]

4. Definition of lectins and their classification: the ongoing debate

As a group of proteins, lectins exhibit considerable specificity in binding oligosaccharides and yet possess enormous structural diversity.[13,25] For a group so varied both in structure and function, arriving at a common definition that suitably describes all their characteristics is obviously not easy. The initial definition of lectins as synonymous with agglutinins[37] made way for a newer and more general definition of lectins: proteins that possess at least one non-catalytic domain, which binds reversibly to specific carbohydrates.[38,39] By this definition, agglutination no longer remained the pivotal property by which a lectin is defined, although in practice it continued to be a useful marker. Carbohydrate binding and specificity became the new criteria for the definition of a lectin. A useful and popular method of classifying lectins, particularly from plant sources, is based on the mono- or disaccharide specificities of the lectins, although as mentioned earlier, amongst lectins where subsite binding is important, the oligosaccharide specificities may be very different despite similar monosaccharide specificities.[28,31] Yet perhaps due to both historical and practical reasons, this method of classification has continued to be popular.

However, it is difficult to apply such a classification universally. For example, using such a framework it would simply not be possible to classify a lectin like galectin-10, whose soluble form apparently binds galactose but the crystalline state recognises mannose instead.[40] Similarly, there has been mounting evidence to suggest that jacalin, the T-antigen binding lectin from jackfruit seeds, is capable of binding both galactose and mannose sugars.[41] Research work from a number of groups, carried out mostly during the eighties and early nineties,[42–49] also shows that plant and animal lectins could have one or more hydrophobic binding sites different from their carbohydrate binding site(s) and these could play a role in protein–protein interactions. A number of new questions arise from these studies. Are lectins multi-functional proteins invested with the power to trigger biological activity by using additional binding sites to their carbohydrate binding ones? What functions do these recognitions confer upon lectins and how important were they to the role of these proteins in biological systems?

Moreover, there are also proteins that have single or weak carbohydrate recognition domains (CRDs). The aggrecans and versicans are, for example, two groups of proteoglycans whose primary sequence was used to predict the presence of carbohydrate recognition domains in them.[29] It was shown that these macromolecules have weak saccharide binding, although the endogenous

functions of such a binding, if any, have not been identified yet. Similarly, interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-6, IL-7, IL-12 and the tumour necrosis factor α are all reported to exert their activity by lectin-like interactions with the substrate.[50–53] One study has shown that the receptor for insulin-like growth factor-II is a multifunctional binding protein,[54] and another has shown that the receptor binds to the growth factor at the same site at which it binds mannose-6-phosphate implying that the receptor exhibits lectin-like behaviour.[55] The envelope glycoprotein of human immunodeficiency virus type-1 also has an *N*-acetyl-β-D-galactosamine binding site that is able to clearly distinguish between glycoproteins carrying this oligosaccharide and others that don't.[56]

Yet another group of proteins which could lay claims to being classified along with lectins are those that appear to share extensive primary sequence homology with certain lectins despite not exhibiting any marked saccharide binding ability. For example, curculin, a sweet-tasting and taste modifying protein has a primary sequence very similar to that of the mannose binding lectin (GNA) from the snow drop bulb (*Galanthus nivalis*). Three-dimensional modelling of the structure of curculin using data available on GNA suggested that none of the three putative mannose binding pockets could bind a mannose unit.[57]

Of course one way to work around this problem would be to use topological similarities rather than carbohydrate-recognition or primary sequence homology in order to classify lectins. Although carbohydrate binding specificity is no longer pivotal for the classification, the property of carbohydrate binding would still be a necessary feature to include any protein in the category of lectins. Thus based primarily on topological features, some scientists have preferred to classify carbohydrate-binding proteins into two groups. Group-I, comprising enzymes and periplasmic binding proteins, have proteins that possess deep binding pockets in which the carbohydrates are completely engulfed while group-II contains proteins that are neither enzymes nor antibodies and which have shallow/superficial binding sites. The term lectin is associated by these researchers only with the second group of carbohydrate-binding proteins.[30,31] Some completely unrelated lectins, such as the pea lectin and galectin-2, show striking topological similarities despite sharing no significant sequence homology or carbohydrate specificity.[31] Similarly, some Type-II antifreeze proteins have a global fold homologous to the carbohydrate binding domain of C-type lectins.[58,59] Should these proteins be grouped together and if so, what advantage does such a classification afford us in terms of our understanding of the structure and function of these proteins?

Lis and Sharon[12] proposed a method of classification based on structural features of the proteins of interest. Thus they classified lectins into three groups: simple, mosaic and macromolecular assemblies. Within each group there could be further classifications based on sequence similarities and structural properties. Simple lectins in this method of classification have a small number of subunits, each with a carbohydrate recognition domain (CRD) with possible additional domains for other types of ligands. Most plant lectins would be treated as simple lectins. Mosaic proteins have more diverse sources of origin, have multi-functional domains and only one CRD. Viral hemagglutinins and animal C-, P- and I-type would all belong to this category. Macromolecular assemblies consist of filamentous proteins, mostly of bacterial origin, that have several different subunits assembled together in a defined order. Only one of the subunits, usually a minor component is associated with carbohydrate recognition. The emphasis appears to be on a combination of structure and carbohydrate recognition.

However, not all carbohydrate binding proteins are accommodated in this categorization. For example, toxins have been excluded although historically some of the earliest 'lectins' to be studied were toxins. Further, carbohydrate binding receptors with CRDs that are buried in the interiors of the proteins or proteins with lectin-like structural organization but with no proven CRD are not included in this classification.

As new terrains are explored, new questions come up too. Should lectins be classified based on their carbohydrate specificities, or by comparing with other lectin-like proteins which share sequence homology with them, or with those with whom they share a common fold? Or should it be a judicious combination of the three? Or should the most significant biological function of the protein be the ultimate consideration? These questions need to be addressed in order to arrive at a classification that can be followed by all workers in this field.

5. Multifunctionality in plant lectins

While a number of animal lectins have clearly been shown to be bifunctional,[42] similar correlations between biological function and non-carbohydrate ligand binding have been harder to come by for plant lectins. To begin with, despite much work done aimed at elucidating their functions in native tissues, the actual roles of lectins in plants are still a matter of much speculation, and wherever such roles have been proposed the evidence available is much less certain than in the case of animal lectins. Secondly, in most cases where biological roles have been inferred, the targets of study have been the carbohydrate binding domains of lectins. Several excellent reviews have highlighted the probable endogenous roles of plant lectins.[12,22–24]

Since a number of lectins have been isolated from storage tissues in plants (seeds or vegetative storage tissues) where they make for a very large proportion of the total protein content in the tissue, it has been speculated that lectins might serve as plant storage proteins. Many of these lectins have been shown to also exhibit behaviour similar to other storage proteins. For example, they are developmentally regulated in a manner very similar to other storage proteins and, during germination some of these are degraded and appear to be important sources of nitrogen for the development process.[60,61] What is not known is whether these lectins are merely storage proteins with lectin-like behaviour or whether they serve more than one purpose in the storage tissue. Direct evidence for the binding of auxins, gibberlins or other plant growth factors to lectins *in vivo*, which can be correlated to their role in plant development, is still lacking.

However, one question that is often raised is why such storage proteins exhibit exquisite specificity for carbohydrates that do not appear to be present within the plant itself? This has led to the speculation that these lectins may double up as defence proteins against pathogenic invasion, if such a situation arose.[12,24,38,62] Defence against pathogens and predators in plants could occur either *via* a passive defence mechanism or an active one.

Passive defence in plants include physical barriers, biochemical adaptations and morphological adaptations. Biochemical adaptations in turn include accumulation of toxic low molecular

weight compounds in the whole plant or susceptible tissues.[62] Toxins could also be lectins, lectin-like proteins or ribosome inactivating proteins (RIPs) whose target may even be a specific group of organisms.[24,38,63–66] It is not known, however, whether lectins interact with phytoalexins or other toxic low molecular weight compounds and therefore enhance the plant's defence capabilities. An active defence system is one in which cells in the vicinity of the affected area are triggered to synthesise specific pathogenesis-related proteins or low molecular weight compounds like phytoalexins which have antibiotic activity. Lectins too could perhaps get triggered in such an active defence system. For instance, it was shown that barley lectin and wheat germ agglutinin preferentially accumulate in nematode-infested roots of these plants.[67] In healthy roots their levels drop three days after germination while in infested roots their levels fall-off much less rapidly. Lectins were found at the nematode feeding site and, interestingly, did not accumulate if the plant was inoculated with a nematode not specific for it. There are also reports of induction of lectins in response to abiotic stress in plants. For example, it has been shown that the SalT gene product in rice is a mannose binding lectin which may be induced by different kinds of stress inducing agents including high salt.[68–70]

Lectins have also been implicated in rhizobium-plant root nodule interaction. Rhizobia can bind to both host and non-host tissues but, apparently, the specificity of the binding determines the symbiotic nature of the interaction between the bacteria and the host plant. When specific binding occurs, lectins from the bacteria as well as from the plant roots are involved, resulting in close proximity that can promote physiological and biochemical responses.[12,24,71,72] Lectins also appear to be involved in the production of lipochitooligosaccharides or nodulation (Nod) factors by the bacteria in response to flavanoids produced by the plant.[73,74]

Some galactose specific lectins in plants have been correlated with cryoprotective properties in the tissues that they accumulate in.[75–77] For example, the leaves of mistletoe (*Viscum album* L.) contain three Gal/GalNAc specific lectins, two of which show strong cryoprotectivity during freezing and thawing of isolated spinach thylakoid membranes.[76] It has also been shown that the accumulation of these lectins in mistletoe leaves is seasonally regulated. Further, these cryoprotective lectins bind to the head groups of digalactolipids in thylakoid membranes and the efficiency of this binding depends primarily upon hydrophobicity.[76]

6. Non-carbohydrate and hydrophobic ligands for plant lectins

6.1 Hydrophobic sites in carbohydrate binding domains

It has not escaped the notice of most researchers in the field that the highly specific saccharide recognition sites of many lectins bind to sugar derivatives containing large hydrophobic or aromatic rings much better than they do to simple mono- or disaccharides. Fluorescently labelled sugars with either a methylumbelliferyl moiety or a dansyl group as the reporter have proved to be extremely useful for studying lectin–carbohydrate interactions. Thermodynamic studies on carbohydrate binding have suggested that in general, many lectins have enthalpically driven associations with carbohydrates. In many cases, the presence of hydrophobic substituents enhance the binding of the carbohydrate to the lectin, indicating either that the binding pocket itself is hydrophobic or additional hydrophobic sites exist close to the primary carbohydrate binding site. For example, for Con A as well as pea lectin (PSL), it has been shown from single crystal X-ray diffraction data of the carbohydrate–lectin complexes in the presence of Ca^{2+} and Mn^{2+} that not only conserved Asp, Asn and Gly are involved in the carbohydrate binding sites of the lectins but stacking interactions of the sugar with a conserved Tyr is important for the stability of this interaction.[32,78]

6.2 Peptides as carbohydrate mimetics

An exciting new dimension was added to the lectin–ligand interactions by reports from two groups that explored the possibilities of highly selective peptide binding to lectins that act as sugar-mimics for them.[79,80] These studies suggest the possibility of using peptides to study the topological relationships that are shared by the peptides and the carbohydrates specific for a given lectin. Using phage-display libraries of hexapeptides, Goldstein's group identified the consensus hexameric peptide containing the sequence YPY capable of highly specific interaction with Con A as compared to other mannose binding lectins, with affinity constants comparable to that of the lectin for methyl α-D-glucopyranoside.[79] It had already been shown that Con A binds to phenyl α-D-mannopyranoside with much greater affinity than methyl α-D-mannopyranoside (MeαMan), suggesting the presence of amino acids capable of hydrophobic interactions within or very close to the sugar-binding site, a hypothesis that was well supported by subsequent studies on the crystal structure of the lectin–carbohydrate complex as well. The possibility therefore that the peptide might just bind, *via* hydrophobic interactions, close to the sugar binding site and sterically inhibit saccharide binding to the lectin could not have been ruled out conclusively in this study. But Salunke's group showed that polyclonal anti-α-D-mannopyranoside recognised various peptide ligands of Con A and polyclonal antibodies generated against the specific 12-mer peptide recognised Con A-specific carbohydrates, thus settling the debate on whether indeed the peptides bind to the same site as the carbohydrates.[80] While these studies do not suggest that such recognitions are significant biologically, it would be an interesting hypothesis. Could it be, for example, that conserved lectin-like domains perform a function analogous to carbohydrate-binding? Are there small peptides *in vivo* that regulate the function of lectins, as are known to exist for many enzymes? When Con A was co-crystallised with the peptide DVFYPYPYASGS in the presence of Mn^{2+} and Ca^{2+} it was observed that two kinds of binding site were generated for the peptide as a result of crystal packing.[81] The primary binding site was in fact one that was independent of the sugar binding site in which the peptide bound to the lectin in a shallow crevice on the monomeric subunit with one side largely exposed to solvent. In the secondary binding mode, the peptide was located in a site between the mannose binding site and the primary peptide binding site and interacted with the lectin through predominantly hydrophobic interactions (Fig. 1). Further, these hydrophobic sites did not correspond to the ones identified by Hardman and Ainsworth[45] for hydrophobic binding to Con A. The tyrosine residues in the conserved YPY motif appear to be important for maintaining the hairpin bend in the peptide structure while only the second tyrosine seems

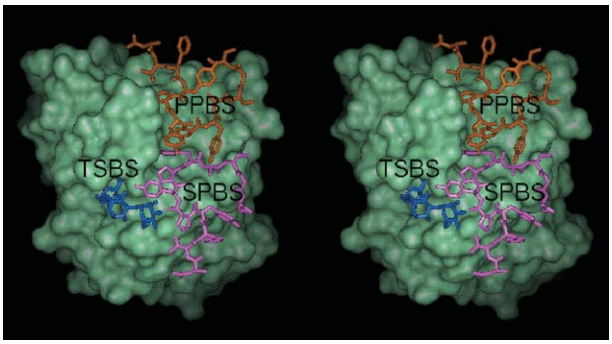

Fig. 1 Crystal structure of a peptide–Con A complex. A stereoview of the surface representation of a Con A subunit is shown with the bound peptide in the primary peptide binding site (*PPBS*) and the symmetry-related peptide in the secondary peptide binding site (*SPBS*). Comparison of the two sites has been made with respect to the trisaccharide binding site (*TSBS*) on Con A. The peptide molecules at the primary site (*orange*) and at the secondary site (*pink*) and trimannose (*blue*) are shown as *stick drawings.* Reproduced from.[81] (Copyright (2000) American Society for Biochemistry and Molecular Biology, Inc.).

to be involved in direct interaction with the lectin. While the peptide binding site does not seem to in any way overlap with the carbohydrate binding site, it appears that the former is in close proximity to the mannose and trimannose binding site. Interestingly, the mitogenicity of the lectin, which has been attributed to its carbohydrate recognition of the glycoproteins on the cell surface, was found to be inhibited by this 12-mer peptide. The YPY motif apparently plays an important role in this inhibition. While a conservative replacement of either of the two tyrosine residues with a phenylalanine showed only a marginal alteration in the inhibition of mitogenicity, a mutation to Ser resulted in drastic reduction in such inhibition. The recognition of the peptides by polyclonal anti-α-D-mannopyranoside antibodies was likewise affected.

One possible argument for rationalizing these rather contradictory results is to begin with the assumption that the peptide mimics the carbohydrate only under certain specific conditions. The interaction observed in the solid state and that in solution might be two completely unrelated phenomena. Peptides have often been shown to bind to receptor sites *via* an induced-fit mechanism. Thus a peptide with the consensus YPY motif, first identified by its specificity for Con A, would be expected to bind to the lectin in solution as was indeed reported by Goldstein's group.[79] Their observation that the carbohydrate binding ability of the lectin could be inhibited by the peptide was corroborated by the initial report from Salunke's group.[80] Indeed, the studies on effect of the peptide on the mitogenicity of the lectin too were carried out in solution state and must therefore be indicative of the same kind of interaction.[81] Taken together, their results seem to indicate that in solution the peptide binds to the lectin at or close to the carbohydrate binding site.

The crystal structure presents a very different picture. It must be kept in mind that the concentrations of lectin and ligand used for obtaining single crystals are extremely high when compared to studies carried out in the solution state. Not only are peptides less likely to remain soluble at such concentrations but the likelihood of their aggregation and therefore adopting 'non-native' conformations significantly increases. The very fact that the peptide 'fits' into both the primary and secondary binding sites of the lectin (see Fig. 1) could be a manifestation of such an effect. Thus the remarkable specificity of the peptide for the lectin that was observed in solution state appears compromised as a result of the crystallisation conditions. The fact that the peptide binds at the secondary binding site to a crystallographic equivalent of the primary binding site using a completely different set of contacts and mode of interactions, could be a reflection of this fact. Extending this argument further it would be interesting to speculate that the tyrosines in the conserved YPY motif might not play as significant a role in the solid-state binding of peptide to the lectin as was observed in the solution state, as long as the hairpin bend is preserved. However relevant such a picture might appear from the point of view of physiological function, the very fact that such interactions can occur under appropriate conditions is nonetheless significant.

6.3 Binding of ANS and TNS at hydrophobic sites in lectins

In a series of very interesting papers, some groups have explored the possibility that carbohydrate-independent hydrophobic sites for specific ligands could also exist in plant lectins. Using known hydrophobic fluorescent probes such as 1,8-anilinonaphthalenesulfonic acid (ANS) and 2,6-toludinylnaphthalenesulfonic acid (TNS), whose fluorescence quantum yields show marked enhancement upon binding to a hydrophobic site as compared to their fluorescence in aqueous solution, they showed that ANS and TNS bind to a large number of legume lectins as well as some non-legume lectins[48,82,83] Roberts and Goldstein[48] showed that most legume lectins also have a conserved specific hydrophobic binding site for ANS and this site was completely independent of the carbohydrate binding site. Further, both ANS and TNS bind to proteins at sites that recognise cofactors or hormones. Association constants reported in literature for the interaction of various lectins with ANS and TNS are listed in Table 1.

6.4 Binding of adenine and phytohormones to lectins

In 1971 Jaffe and Palozzo reported the isolation of nonpolar substances (most likely steroids) from Con A preparations using non-polar solvents (as mentioned in[45]). Hardman and Ainsworth[45] reported the binding of non-polar molecules to crystalline Con A and postulated that the lectin might function in regulation of cell-division or germination by binding some non-polar molecules such as growth factors or cytokinins. Edelman and Wang showed that the phytohormone indoleacetic acid bound to Con A very weakly.[86] With the assumption that other plant hormones and growth factors might also bind to legume lectins and this binding might occur at the ANS or TNS binding sites, Roberts and Goldstein further examined the binding of several lectins by adenine derivatives, cytokinins, and other naturally occurring hydrophobic plant molecules.[49] They concluded that the lectins from lima bean (LBL), *Dolichos biflorus*, kidney bean and soybean had adenine binding sites of varying affinities. LBL bound to cytokinins besides adenine. Adenine derivatives possessing hydrophobic substituents bound to LBL with better affinities and this binding was in all probability mediated by the high-affinity TNS binding site of the lectin. When the same group probed the adenine binding site of the lectins from lima bean

Table 1 Association constants for the interaction of ANS, TNS and other hydrophobic probes with different lectins

Lectin	Ligand	$K_a \times 10^{-4}$ (M^{-1})	Reference
Concanavalin A	TNS	1.9[a]	82
	ANS	0.28 ± 0.04	48
Dolichos biflorus CRM	ANS	0.11 ± 0.02	48
	TNS	2.8 ± 0.08	
Dolichos biflorus	ANS	0.45 ± 0.02	48
	TNS	1.9 ± 0.02	
Griffonia simplicifolia I–A_4	ANS	0.74 ± 0.06	48
	TNS	6.7 ± 1.5	
G. simplicifolia II	ANS	0.51	48
G. simplicifolia III	ANS	0.14 ± 0.01	48
G. simplicifolia IV	ANS	0.61 ± 0.12	48
Lentil lectin	ANS	0.39 ± 0.02	48
	TNS	12.0 ± 3.0	
Lima bean lectin	ANS	0.45 ± 0.01	47
		0.39[b]	
		0.365[c]	
	TNS	7.9 ± 1.2 and 2.2 ± 0.8	
	N-phenyl-1-naphthylamine	6.0	
	Rose bengal	60.0[d]	
		80.0[e]	
Lotus tetragonolobus lectin	ANS	0.55 ± 0.05	48
Pea lectin	ANS	0.31 ± 0.02	48
	TNS	2.3 ± 0.7	
Peanut agglutinin	ANS	0.77 ± 0.02	48
Phaseolus vulgaris E_4	ANS	+cooperative	48
	TNS	2.2 ± 0.6	
P. vulgaris E_3L_1	ANS	+cooperative	48
P. vulgaris E_2L_2	ANS	0.46 ± 0.01	48
P. vulgaris E_1L3	ANS	0.59 ± 0.01	48
P. vulgaris L_4	ANS	0.52 ± 0.01	48
	TNS	8.5 ± 2.3	
Soybean agglutinin	ANS	0.45 ± 0.01	48
	TNS	4.7 ± 0.4	
R. communis agglutinin I	ANS	0.16 ± 0.01	48
	TNS	7.0 ± 0.3	
Potato lectin	ANS	0.43 ± 0.06	48
Wheat germ agglutinin	ANS	0.52 ± 0.2	84
	TNS	7.0 ± 0.3	
		8.33 ± 1.38	
Pseudomonas aeruginnosa PA-1 lectin	TNS	11.4 ± 1.6	85

[a] Calculated from the K_d value of 5.2×10^{-5} M. [b] Determined by monitoring fluorescence enhancement of the probe. [c] Determined by equilibrium dialysis. [d] Calculated from enhancement of ligand fluorescence intensity. [e] Calculated from protein fluorescence quenching.

and kidney bean by using photoaffinity labelling,[46] they came up with very interesting results suggesting that adenine perhaps binds at a site that is sandwiched between two Con A dimers and not in a hydrophobic cavity of a Con A monomer. This is similar to that observed in the pea lectin as well, although in the pea lectin it does not make inter-molecular contacts. Since the binding of 2,3-diphosphoglycerate to haemoglobin is in a similar site between the β_1 and β_2 subunits and is responsible for the allosteric regulation of oxygen binding to haemoglobin, they raised the speculation that adenine or cytokinins could serve a similar purpose with respect to hydrophobic binding in lectins. Using detailed characterisation of the adenine binding sites of the *Dolichos biflorus* lectins Marilyn Etzler's group[43,44] too suggested that perhaps these could act as physiologically relevant ligands for lectins in plants. The crystal structure of DBL–adenine complex was subsequently determined,[87] which clearly showed that a total of four adenine molecules bind to the DBL tetramer (Fig. 2); however, the adenine binding sites are distinctly different from the carbohydrate binding sites.[87] In a recent study the interaction of TNS, adenine and phytohormones with WGA was investigated and it has been shown that the lectin has two types of binding sites for TNS, a high-affinity site and a low-affinity site. Adenine and adenine-related phytohormones such as zeatin, kinetin, as well as abscissic and gibberillic acid bind to WGA with affinities in the range of $K_a = 1.6–2.3 \times 10^6$ M^{-1}, which are higher than the affinity exhibited by this lectin towards different saccharides.[84] WGA is the first cereal lectin that has been shown to bind adenine and other plant growth hormones. The association constants for the interaction of adenine, its derivatives and various plant growth hormones are listed in Table 2.

6.5 Porphyrin binding by lectins

Porphyrins are primarily hydrophobic molecules normally present in biological systems bound to polypeptide chains, as in the case of hemoglobin, myoglobin or chlorophyll. Of the several uses that porphyrins have been put to clinically, photodynamic therapy has been particularly attractive (for reviews, see ref. 95). There have been numerous reports and studies on the binding of porphyrins to proteins, lipids and other components of tissue.[96,97] Porphyrins

Table 2 Association constants, K_a for the interaction of adenine, phytohormones and related compounds with different lectins

Lectin	Ligand	$K_a \times 10^{-4}(M^{-1})$	Reference
Dolichos biflorus	Adenine	50.0[a]	49
	Adenine	70.3 ± 6.4	43
	Kinetin	18.1 ± 1.0	
	Isopentyladenine	11.5 ± 1.2	
	Benzyltetrahydropyranyl-adenine	8.38 ± 0.52	
	Zeatin	7.36 ± 0.46	
	Benzyladenine	7.09 ± 0.49	
	Dihydrozeatin	4.90 ± 1.22	
	Benzoyladenine	2.94 ± 0.64	
	Adenosine	0.865 ± 0.189	
	Xanthine	0.451 ± 0.327	
	Hypoxanthine	0.251 ± 0.179	
	Ferulic acid	0.180 ± 0.103	
	Diphenyl urea	0.153 ± 0.011	
	Guanine	0.129 ± 0.022	
	N^6-2,2,6,6 tetra-methyl 1-oxypiperi-dine 4-yl adenine	50	88
DB58 (*Dolichos biflorus* stem and leaf lectin)	Adenine	156 ± 20	43
	Kinetin	19.6 ± 4.5	
	Isopentyladenine	20.3 ± 4.6	
	Zeatin	11.1 ± 1.0	
	Adenosine	2.25 ± 1.63	
	Xanthine	0.962 ± 0.064	
Lima bean lectin	Adenine	8.33 ± 0.69[a]	49
	N^6-Benzyladenine	4.17 ± 0.17[a]	
	Zeatin	1.10 ± 9.0[a]	
	Hypoxanthine	0.83 ± 0.2[a]	
	8-Azidoadenine	4.7	46
	N^6-2,2,6,6 tetra-methyl 1-oxypiperi-dine 4-yl adenine	3.1	88
Phytoheamagglutinin-E_4	Adenine	11.63[a]	49
	8-Azidoadenine	1.7	45
	N^6-2,2,6,6 tetra-methyl 1-oxypiperi-dine 4-yl adenine	2	88
PHA–L_4		0.52	88
Soybean agglutinin	Adenine	7.69[a]	49
Winged bean basic lectin	Adenine	1.50	89
Winged bean acidic lectin	Adenine	42.0	90
	Adenosine	0.15	
concanavalin A	Indole acetic acid	0.07	86
Peanut agglutinin	N6-benzylaminopurine	n.d.[b]	91
Hog peanut lectin (*Amphicarpaea bracteata*)	Adenine	130	92
Wheat germ agglutinin	Adenine	~166.7[a]	84
	Kinetin	~166.7[a]	
	Isopentyladenine	232.5 ± 11.6	
	Zeatin	232.5 ± 11.6	
	Abscisic acid	227.3 ± 20.7	
	Gibberellic acid	153.8 ± 47.3	
Pseudomonas aeruginosa PA-1 lectin	Adenine	27 ± 6.6 2.3 ± 0.83	85
Ricin A chain	Adenine (at pH 4.5) (at pH 7.3)	1.79 0.29	93
	Adenine	0.1	94

[a] Calculated from the reported K_d values, [b] Crystallization of PNA-N6-benzylaminopurine is reported. The K_a value was not determined.

have also been in the news in recent times for their use as therapeutic agents to slow down the progression of transmitted bovine spongiform encephalopathy due to their ability to bind to normal protease sensitive prion proteins as opposed to the protease resistant rogue ones in tissue culture studies.[98] Porphyrins are also being investigated as possible anti-microbial agents due to their potential to inhibit microbial growth in cell cultures.[99,100] In patients suffering from porphyrias, a genetic disorder involving biosynthesis of porphyrins, accumulation of porphyrin and/or its precursors occurs in the liver and other organs. Some studies have been aimed at understanding these phenomena and finding suitable ways of effectively transporting drugs to such tissues. In view of the fact that some lectins are already under investigation for the targeted delivery of drugs, finding lectins with high affinity for porphyrins can expand the scope of lectin-mediated drug delivery.[8,95] Whether porphyrins are physiologically relevant ligands for lectins is as yet unknown.

6.5.1 Binding of porphyrins to concanavalin A and pea lectin. In the first report of its kind, we showed that Con A and pea (*Pisum*

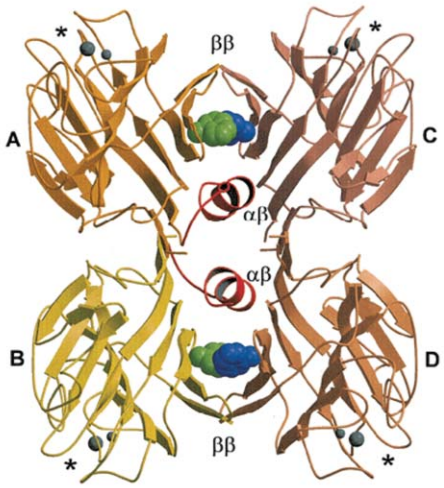

Fig. 2 Structure of the *Dolichos biflorus* lectin tetramer complexed with adenine, determined by single-crystal X-ray diffraction studies.[87] Each subunit is shown in a different color. The four observed adenine molecules are shown as space-filling models in green and blue. The two types of dimer–dimer interfaces are indicated as αβ and ββ. Ca^{2+} and Mn^{2+} ions are shown as large and small gray spheres, respectively. The locations of the four sugar-binding sites are indicated with an asterisk (*). Reproduced from.[87] (Copyright (1999) Academic Press).

sativum) lectins bind to porphyrins with considerable affinity.[101] As stated earlier, Con A and pea lectin are two of the most well characterised plant lectins that specifically recognise mannose and glucose, as well as some of their derivatives. They share extensive sequence homology and single crystal X-ray diffraction studies have shown their 3-dimensional structures to be very similar.[78] Biochemical evidence as well as data obtained from solving the crystal structures of these lectins bound to their specific sugars suggested that a conserved Asp, Asn, and Gly are directly involved in carbohydrate binding by the lectins. Hydrophobic stacking interactions of the carbohydrate with a conserved Tyr further enhance the stability of this interaction.[32,78] X-ray diffraction studies have revealed that *o*-iodophenyl-β-D-glucopyranoside and *o*-iodophenyl-β-D-galactopyranoside bind to Con A in a non-polar binding site that is adjacent to the saccharide binding site.[45] Binding of hydrophobic ligands to the two lectins have already been described in the previous section although, to the best of our knowledge, no crystallisation studies of these lectins with such hydrophobic ligands have been reported.

We investigated the interaction of a free base porphyrin, *meso*-tetrasulfonatophenyl-porphyrin (H_2TPPS) and the corresponding metal derivative, *meso*-tetra(4-sulfonatophenyl)porphyrinato-zinc(II) (ZnTPPS) to these lectins.[101] Structures of these and other porphyrins investigated for lectin binding are given in Fig. 3. Each lectin subunit was found to bind one porphyrin molecule and the association constant, K_a (in the range of 1.2×10^4 M^{-1} to 6.3×10^4 M^{-1}), estimated from absorption and fluorescence titrations were in the same range as that for ANS or TNS binding to these lectins or that for porphyrin interactions with serum albumins.[47,48,97] Both free lectin and lectin saturated with the specific saccharide were found to bind the porphyrin with comparable binding strength indicating that porphyrin binding takes place at a site different from the sugar binding site. A representative double logarithmic plot depicting the analysis of porphyrin binding to Con A is shown in Fig. 4. The K_a values obtained for the interaction of cationic and anionic porphyrins with different lectins are given in Table 3.

As in the case of peptide ligands for Con A, the crystal structure of Con A bound to H_2TPPS at 1.9 Å resolution presented a very different story from that in solution state.[106] Unexpectedly, when the lectin was co-crystallised along with the porphyrin, the sulfanatophenyl group of H_2TPPS occupied the same site in the lectin as methyl α-D-mannopyranoside in the lectin–sugar complex (Fig. 5). Besides, a pair of stacked porphyrins cross-linked together molecules of Con A using two of their side groups each, resulting in a pattern not very different from that observed during agglutination of cells by lectins. The sulfanatophenyl group mimics seven of the eight hydrogen bonds that are involved in the interaction between Con A and MeαMan. The hydrogen bonds involving the C4 hydroxyl of the sugar are replaced by those involving a molecule of water. Although π–π stacking is common for porphyrins, it has not been observed before in the

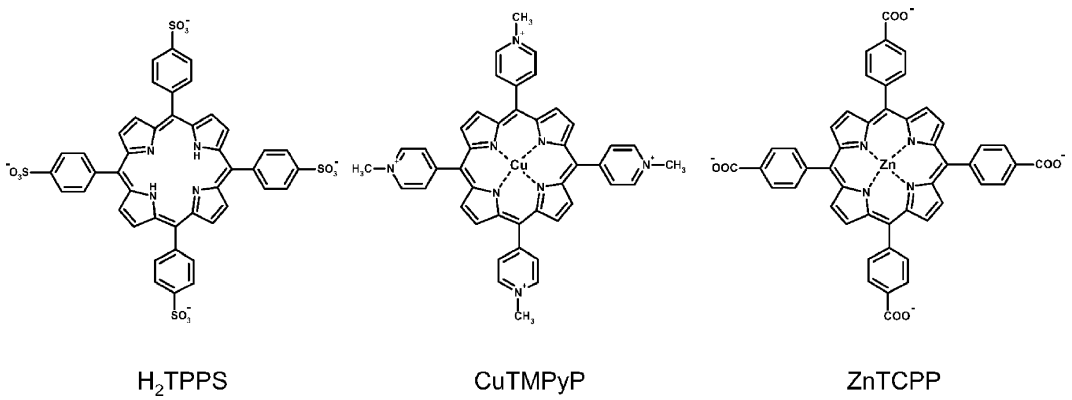

Fig. 3 Structures of porphyrins used in lectin binding studies. The structures shown are: H_2TPPS (*meso*-tetra-(4-sulfonatophenyl)porphyrin), CuTMPyP (*meso*-tetra-(4-methylpyridinium)porphyrinato copper (II)) and ZnTCPP (*meso*-tetra-(4-carboxyphenyl)porphyrinato zinc(II). Replacing the two hydrogen atoms in H_2TPPS with divalent cations Cu^{2+} or Zn^{2+} would give the corresponding metal derivatives, CuTPPS and ZnTPPS. Similarly replacement of the metal ions in the porphin core of CuTMPyP and ZnTCPP with Zn^{2+} and Cu^{2+}, respectively will give the corresponding metal derivatives, whereas replacing them with two hydrogen atoms will give the corresponding free base porphyrins, H_2TMPyP and H_2TCPP, respectively.

Table 3 Association constants, K_a for lectin–porphyrin interaction

Lectin	Porphyrin	$K_a \times 10^{-4}$ (M^{-1})	Reference
Concanavalin A	H_2TPPS	1.22	101
	ZnTPPS	5.64	
Pea lectin	H_2TPPS	2.96	101
	ZnTPPS	2.58	
Jacalin	CuTPPS	3.98 (±1.46)	102
	CuTCPP	1.34 (±0.41)	
	CuTMPyP	6.80 (±0.27)	
	H_2TPPS	0.65 (±0.05)	
	ZnTPPS	0.65 (±0.14)	
	H_2TCPP	0.41 (±0.05)	
	ZnTCPP	2.10 (±0.20)	
	H_2TMPyP	7.30 (±0.33)	
Snake gourd seed lectin	CuTPPS	50.0 (±13.0)	103
	CuTCPP	2.19 (±0.04)	
	CuTMPyP	5.02 (±0.51)	
	ZnTPPS	4.28 (±0.78)	
	ZnTCPP	3.85 (±0.13)	
Trichosanthes cucumerina seed lectin	CuTPPS	12.11 (±6.23)	104
	ZnTPPS	1.90 (±1.086)	
	CuTCPP	0.22 (±1.5)	
	ZnTCPP	0.58 (±0.3)	
	CuTMPyP	6.11 (±0.5)	
	ZnTMPyP	46.8	
Momordica charantia seed lectin	CuTMPyP	6.36	105
	H_2TMPyP	4.49	
	CuTCPP	2.97	
	H_2TCPP	2.84	
	ZnTPPS	1.10	
	H_2TPPS	0.58	

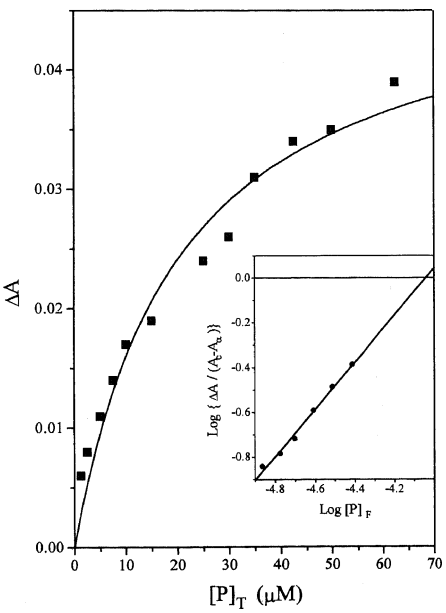

Fig. 4 Binding curve for Con A–H_2TPPS interaction. The change in the fluorescence intensity of H_2TPPS was plotted as a function of Con A concentration. The inset shows determination of the association constant, K_a from the binding data. The X-intercept yields the pK_a of the binding equilibrium. Reproduced from.[101] (Copyright (1997) Indian Academy of Sciences).

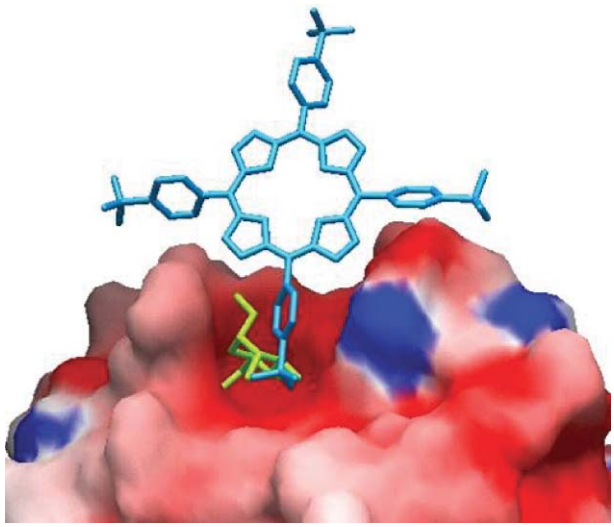

Fig. 5 Crystal structure of Con A–H_2TPPS complex. A superimposition of the ligand binding region of the complexes of the lectin with bound H_2TPPS and methyl-α-D-mannopyranoside is shown. Molecular surface of Con A is decorated with color to indicate charge distribution (*red*, negative; *blue*, positive). The structure shown clearly indicates H_2TPPS binds to Con A through the sulfonatophenyl group in a groove in which methyl-α-D-mannopyranoside is known to bind. Reproduced from.[106] (Copyright (2001) American Society for Biochemistry and Molecular Biology, Inc.).

crystal structure of proteins containing porphyrins. The fact that the porphyrin is not covalently bound to a polypeptide chain could encourage such stacking to occur much more readily in this case. What is really noteworthy is the fact that despite sharing no obvious similarities with MeαMan, H_2TPPS is able to mimic

the role of the sugar molecule with the aid of water molecules to cement such an interaction.

There are, however, many larger issues that could be raised in this context too as in the case of lectin–peptide complexes. The results from solid state structural analysis clearly do not add up to those obtained from solution state studies. The structure of Con A in the crystal structure was unaltered when compared to the lectin–MeαMan complex. It is well known that interactions of Con A with its ligands can influence the crystal packing.[81,107,108] But this does not seem to have occurred in either its complex with the peptide or with the porphyrin as compared to its crystal packing in the presence of the sugar.

One possible explanation of the crystallographic data is that both these ligands do indeed closely mimic the interaction of MeαMan with Con A.[106] The other reasoning would be to suggest that these ligands under the experimental conditions are unable to strongly influence the packing of the lectin in the crystal. Porphyrins, unlike many carbohydrates, are not highly soluble in aqueous solutions and are known to readily aggregate in aqueous solutions. At the much higher concentrations required for crystallisation as compared to that used in the solution state studies, it is not inconceivable that the porphyrins are already stacked together and therefore interact with the lectin as dimers rather than as monomers. The hydrogen bonds involving the hydroxyl at the C4 position of the sugar which is so critical to the specificity of the lectin's carbohydrate interactions get replaced by those from a water molecule, implying thereby that the discrimination that the lectin is capable of in solution might not exist in the crystallised state with respect to porphyrin binding. Nevertheless, that porphyrins in the aggregated state could mimic carbohydrate structure is a remarkable observation. It would be extremely interesting to see what kind of a binding would occur if the lectin was co-crystallised in the presence of the specific sugar along with the peptide or porphyrin.

6.5.2 Porphyrin binding to peanut agglutinin. Peanut agglutinin (PNA) is a galactose-specific, nonglycosylated homotetrameric protein of M_r110 kDa. Each subunit of this lectin has one carbohydrate binding site that specifically recognizes the tumor-associated T-antigen.[109] The three dimensional structure of PNA has been solved without any ligand[110] as well as upon complexation with a variety of bound sugars such as methyl-β-D-galactopyranoside, lactose, *N*-acetyllactosamine and the T-antigenic disaccharide, Galβ1-3GalNAc.[111–114] The ability of PNA to specifically recognise the T-antigen and to distinguish it from the more abundant cryptic T-and Tn antigens makes it a useful diagnostic tool.[115,116] The quaternary structure of PNA is rather unusual in that the four subunits of this homotetramer are not related to each other by a conventional 222 or 4-fold symmetry, although they have similar tertiary structures and contain equivalent sugar-binding sites.[110] This unusual 'open' quaternary structure results in the exposure of a hydrophobic region on the surface of the protein, which is probably why PNA aggregates in solution, especially at low temperatures.[117]

Solution studies on the interaction of porphyrins with PNA revealed that the lectin binds both cationic (CuTMPyP) and anionic (CuTPPS and CuTCPP) porphyrins with affinities in the range of $\sim 10^3$–10^5 M^{-1} (R. S. Damai and M. J. Swamy, unpublished observations). The binding affinities were not significantly affected by the presence of 0.1 M lactose, the disaccharide that is specifically recognized by PNA. Very recently the structures of the complex of H_2TPPS with PNA as well as the ternary complex of PNA with lactose and H_2TPPS were published.[118] These structures reveal that unlike Con A even in the crystalline state the porphyrin does not associate with the protein at the carbohydrate recognition site. In both H_2TPPS–PNA binary complex and H_2TPPS–PNA–lactose ternary complex four porphyrin dimers and one porphyrin trimer bind to the PNA tetramer at sites that are different from the carbohydrate binding sites (Fig. 6). Two of the porphyrin dimers bind at equivalent sites on subunits A and B, and the other two porphyrin pairs also bind at equivalent sites on subunits C and D. However, the binding sites on subunits A and D (or B and D) are not equivalent. This is in contrast to the crystal structures of Con A–H_2TPPS and jacalin–H_2TPPS complexes, where the porphyrin dimers bind at equivalent sites on all four subunits of the lectin tetramer.[119] The unusual 'open' quaternary structure of PNA also plays a role in the interaction of porphyrins with PNA. The porphyrin trimer binds to the A and B subunits of PNA *via* the exposed hydrophobic site, the equivalent site of which is involved in intersubunit interaction in other legume lectins. In addition, two amino acid residues belonging to an exposed hydrophobic site in subunits C and D are involved in the interaction of the porphyrin dimers with the protein surface.

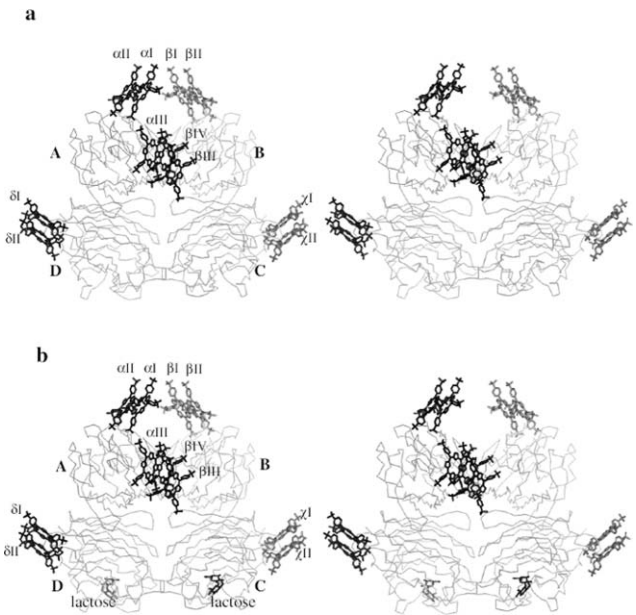

Fig. 6 Crystal structures of H_2TPPS–PNA binary complex (a) and H_2TPPS–PNA–lactose ternary complex (b). The stereo drawings depict PNA molecules in gray and H_2TPPS molecules in black. Lactose (black) occupies the carbohydrate-binding site in subunits C and D of the PNA ternary complex but not in subunits A and B. Reproduced from.[118] (Copyright (2005) American Chemical Society).

The binding of porphyrin to PNA at sites that are distinctly outside the carbohydrate binding site in at least two of the subunits, thereby not affecting the carbohydrate binding ability of the lectin is of considerable interest in photodynamic therapy (PDT), which is a relatively new approach for the treatment of cancer. Due to its specific recognition of the tumor-associated Thomsen–Friedenreich antigen,[115] PNA appears to be an appropriate choice

for targeting porphyrins to tumor cells. Indeed, the structure of the H_2TPPS–PNA–lactose ternary complex clearly shows that the carbohydrate-binding ability of the lectin is not affected by the porphyrin binding and suggests that this lectin can potentially be employed for the targeting of the porphyrin photosensitizer to tumor cells.[118] Further studies with cultured tumor cells and animal models are required to explore this exciting possibility further.

6.5.3 Porphyrin binding to jacalin. Jacalin is a galactose-specific, homotetrameric lectin purified from *Artocarpus integrifolia* (Moraceae family) with an M_r 66 kDa and subunit mass of 16.5 kDa.[120,121] The lectin has one carbohydrate-binding site per subunit that recognises the α-anomer of galactose.[122] Jacalin has attracted a great deal of attention due to its ability to selectively stimulate T and B lymphocytes of human origin,[123] as well as its specific recognition of the T-antigenic disaccharide, Galβ13GalNAcα.[124,125] Solution state studies on the carbohydrate binding properties of this lectin have shown that it binds better to sugars that are derivatized by attaching a hydrophobic moiety such as 4-methylumbelliferyl glycosides of galactose as compared to simple methyl galactosides.[122] These observations suggest that there might be a hydrophobic region on the lectin surface that is presumably in the vicinity of the saccharide binding site. The three dimensional structure of this lectin complexed with the ligand, MeαGal, has also been solved by single-crystal X-ray diffraction,[126] thus giving us a clue regarding the amino acids that play a crucial role in the lectin–sugar interaction. Aromatic amino acids Phe47, Tyr78, Tyr122 and Trp123 form part of the binding pocket, in agreement with the solution state studies that indicated that the carbohydrate binding site might involve side chains of hydrophobic residues.[126,127] The carbohydrate forms two hydrogen bonds with Gly1 of the α chain of the lectin. The side chain of Asp125 also interacts with the C4 hydroxyl of the carbohydrate. Model building studies have indicated that even if the hydroxyl at the C4 position were equatorial, as it is in glucose or mannose, it would continue to interact with Asp125, but not with Gly1. Thus unusually, jacalin is a lectin whose terminal amino acid group is critical for its specificity determination.[12,126] Crystal structure of jacalin–T-antigen complex has also been solved at 1.62 Å resolution. The predominant interactions in this case are through the GalNAc moiety with Gal only interacting *via* water molecules. It includes hydrogen bonding between anomeric hydroxyl of GalNAc with the pi electrons of an aromatic residue.[128]

Of late there has been mounting evidence pointing to the plasticity of the carbohydrate binding site of jacalin, and its saccharide specificity does not appear to require stringent recognition of the axial C4 hydroxyl. Derivatives of galactose that have the crucial C4 hydroxyl altered as in 4-methoxygalactose are also well accommodated by the binding site of the lectin.[40] Further, biochemical analyses based on surface-plasmon-resonance measurements, combined with the X-ray-crystallographic determination of the structure of a jacalin–methyl-α-D-mannopyranoside complex at 2 Å resolution, demonstrated that jacalin is fully capable of binding mannose and oligomannosides albeit with weaker affinities. Apparently the relatively large size of the carbohydrate-binding site enables jacalin to accommodate monosaccharides with different hydroxyl configurations.[41]

Solution state binding studies using various free base and metalloporphyrins demonstrated that the lectin bound to porphyrins in a carbohydrate-independent manner. The interaction of the porphyrin with the lectin appeared to be hydrophobic since charged as well as uncharged porphyrins bound to the lectin with comparable affinities. The K_a values estimated from both absorption as well as fluorescence spectroscopic titrations at room temperature were found to be in the range of 2.4×10^3 M^{-1} to 1.3×10^5 M^{-1}, comparable to those obtained for other protein–porphyrin as well as lectin–porphyrin interactions.[102,103]

Once again, crystal structure tells a different story. The crystal structure of (*meso*-tetrasulfanatophenylporphyrin)–jacalin complex has been recently reported at 1.8 Å resolution.[119] Unlike in the case of Con A–H_2TPPS complex where the cross-linking between four monomers of the lectin was a consequence of interactions between two stacked porphyrin molecules, in the jacalin–H_2TPPS complex a porphyrin pair is sandwiched between two symmetry related jacalin monomers leading to the cross-linking of the protein molecules in the crystal (Fig. 7). Besides stacking interactions which predominate in the porphyrin–lectin complex, H_2TPPS also forms hydrogen bonds with the protein. Jacalin exhibits no major backbone conformational change upon binding the porphyrin *vis-à-vis* carbohydrate binding. But the side chains of residues involved in imparting galactose specificity to jacalin are conformationally altered in order to accommodate the H_2TPPS. The reorientation of a Phe47 appears critical in the ability of the carbohydrate binding site of jacalin to accommodate the porphyrin. The porphyrin does not enter the carbohydrate binding site *per se.* Two of the three water molecules that are present in the carbohydrate binding site of jacalin in its ligand free state are retained while the third is absent in the porphyrin–lectin complex. These water molecules continue to interact with the lectin while also interacting with the porphyrin. Perhaps due to these water-mediated contacts, the interactions of porphyrin to jacalin appear to be relatively weaker than those observed between galactopyranoside and jacalin.[119]

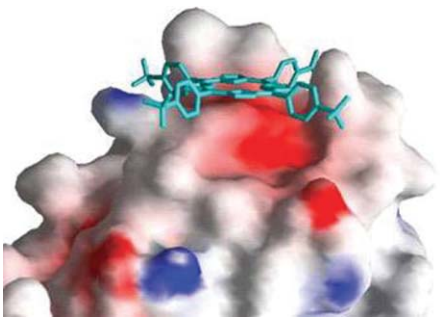

Fig. 7 Crystal structure of jacalin–H_2TPPS complex. The porphyrin molecule is shown in stick form and the ligand binding region of jacalin is shown in a molecular surface representation colored according to charge (red, negative; blue, positive). Reproduced from.[119] (Copyright (2004) International Union of Crystallography).

Apart from the question of whether or not one is seeing a physiologically likely event and whether indeed one is dealing with the same situation in solution state and solid-state investigations, there are several interesting questions that these data raise. For example, does the binding site of the lectin that apparently has some flexibility in carbohydrate specificity truly accommodate a

molecule so fundamentally different as a porphyrin? Is this the flexibility of the lectin's recognition or is the porphyrin molecule, that is normally regarded as a hydrophobic ligand, acting as a carbohydrate mimic? Or is this a hybrid situation where both ligand and receptor show a certain degree of flexibility? Does the availability of hydrophobic residues in the carbohydrate recognition pocket make such an interaction far more feasible? In the jacalin–carbohydrate complex, the three water molecules found in the binding site of the lectin in its ligand-free state are displaced by the carbohydrate. While hydrogen bonding involving water molecules does stabilize the interaction, the primary contacts of the ligand with the binding site are clearly the ones dictating the interaction. But in the porphyrin–lectin complex, the interaction of the ligand is weak and water molecules at the binding site appear to be playing a vital role in mediating the contact between receptor and ligand. Such binding imparts flexibility to the interaction and is unlikely to be highly specific. In the solution state too, despite porphyrin binding being carbohydrate-independent, there is nothing to suggest significant variations in binding of various porphyrins to the lectins, a point that could be interpreted as a sign of flexibility in the interaction. Could it be that in solution state the carbohydrate at the binding site mediates interaction between the lectin and the porphyrin very much in the manner that water does in the jacalin–H_2TPPS crystal? The other likely situation is that under conditions of crystallization, porphyrin molecules at high concentrations tend to aggregate and bury their hydrophobic surfaces in an aqueous environment, resulting in a very different presentation of the ligand to the protein. Thus whether the porphyrin binds the protein as a monomer or a higher order aggregate will depend on the thermodynamic and kinetic parameters of self aggregation competing with the binding event under crystallization conditions. Although H_2TPPS is, relatively speaking, a water-soluble porphyrin that exists as a monomer in the pH range of 5–12, its aqueous solutions deviate from Beer–Lambert's law at concentrations above 1 μM, suggesting self aggregation. In the event of significant aggregation occurring under crystallization conditions porphyrin binding would be different from that seen in solution and the two situations would not be comparable at all.

6.5.4 Porphyrin binding to Cucurbitaceae lectins. What makes seed lectins from cucurbits particularly interesting is the fact that many of them show extensive structural homology to Type-II ribosome inactivating proteins (RIPs) yet do not inactivate ribosomes or do so only weakly.[129,130] Type-II RIPs like ricin and abrin use the carbohydrate binding domain of their B-chain to recognise and bind to the target cell and thereby promote endocytosis of the A-chain whose *N*-glycosidase activity on ribosomal RNA is responsible for their ability to inhibit protein synthesis in these cells. One strategy for targeting drugs to specific cells therefore involves using drugs linked up to truncated versions of the toxin that lack *N*-glycosidase activity.[131–133] Cucurbit seed lectins could perhaps also perform well as drug carriers without complications resulting from toxicity of the lectins for the target cells. Porphyrin binding studies with three lectins from this family have yielded some interesting results.

The snake gourd seed lectin (SGSL) is a β-galactose-specific glycoprotein of Mr ~60 kDa. It has two non-identical subunits of M_r 32 kDa and 23 kDa respectively linked by disulfide bridges and has a histidine residue in its sugar-recognition site. Saccharide binding studies for this lectin have concluded that the lectin binds sugar derivatives containing a hydrophobic residue in the β-position of the anomeric carbon bind better than simple methyl derivatives of galactose suggesting that the binding pocket of the lectin might involve hydrophobic residues as well.[134] It was found that the absorption and fluorescence intensity of free base porphyrins was not altered significantly upon titration with SGSL.[103] The Cu- and Zn-porphyrins on the other hand experienced significant decrease in their absorbance and fluorescence intensities, respectively. The binding of porphyrins by SGSL also occurs *via* hydrophobic interactions. The presence of the specific sugar, lactose, does not significantly alter the extent of change in the fluorescence intensity or the association constant for the lectin–porphyrin complex in most cases. Agglutination activity of the lectin also remains unaffected in the presence of the porphyrins. Thus the saccharide-binding is distinct from porphyrin-binding and the one does not appear to interfere with the other. For CuTCPP a ten-fold increase in binding affinity in the presence of the carbohydrate is noticed, it is possible that conformational changes upon saccharide-binding favourably alters the hydrophobic binding site for this porphyrin. Interestingly, SGSL binds to both kinds of metallo-porphyrins investigated, but does not show any apparent interaction with free base porphyrins. When compared to Concanavalin A, pea lectin and jacalin, SGSL shows slightly higher binding affinities for porphyrins. The association constants for the SGSL–porphyrin complexes seem to be in the same range as that for the lectin–saccharide complexes. Clearly, the lectin has a very prominent and distinct site per subunit for these hydrophobic ligands.

The *T. cucumerina* seed lectin (TCSL) and *Momordica charantia* lectin (MCL) are the only lectins whose themodynamics of porphyrin binding in solution state have been investigated in some detail.[104,105] Like SGSL, TCSL and MCL are β-galactose-specific glycoproteins from the Cucurbitaceae family[135–137] TCSL exhibits immunological cross-reactivity with anti-SGSL antiserum while MCL does not show immunological cross-reactivity with either anti-TCSL or anti-SGSL antiserum.[136,138]

Like in the case of other lectins, porphyrin binding to MCL appeared to be at a site different from the carbohydrate binding site and appeared to be independent of the charge on the porphyrin, suggesting mainly a hydrophobic mode of interaction. Such binding also appeared to not significantly affect the secondary and tertiary conformation of the protein. Thermodynamic parameters, derived from van't Hoff analysis of the association constants (Fig. 8), suggest a role for polar forces in the interaction, perhaps *via* hydrogen-bonding of water molecules in the binding pocket since significant enthalpy–entropy compensation is observed in the overall binding process.[105]

Absorption spectroscopic studies on the binding of metallo-porphyrins with TCSL indicated that TCSL also bound negatively or positively charged porphyrins in a carbohydrate-independent manner, with affinity constant (K_a) values in the same range as for other lectins and proteins studied.[104] Using the spectra obtained for the porphyrins in the presence of surfactants, it was concluded that in all probability, besides hydrophobic interactions, polar interactions *via* hydrogen bonding may also be involved in the binding of porphyrins to TCSL (see Fig. 9). Thermodynamic parameters indicate that unlike saccharide binding to many lectins

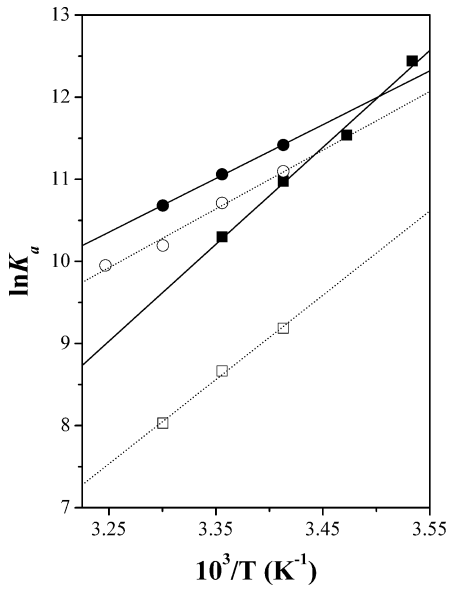

Fig. 8 van't Hoff plot of MCL-porphyrin binding. (□) H_2TPPS (*meso*-tetra-(4-sulfonatophenyl)porphyrin), (■) CuTCPP (*meso*-tetra-(4-carboxyphenyl)porphyrinato copper(II)), (●) CuTMPyP (*meso*-tetra-(4-methylpyridinium)porphyrinato copper (II)), (○) H_2TMPyP (*meso*-tetra-(4-methylpyridinium)porphyrin). Reproduced from.[105] (Copyright (2004) Federation of European Biochemical Societies).

that are enthalpically driven, porphyrin binding to TCSL is primarily entropically governed (Table 4). Stopped flow studies have been carried out to investigate the kinetics of the interaction of the tetracationic porphyrin CuTMPyP with TCSL. These studies have indicated that porphyrin binding to TCSL is four times slower (k_{+1} of 1.89×10^4 M^{-1} s^{-1}) than a diffusion-controlled process and probably involves formation of an intermediate. The dissociation rates obtained were comparable to the slow dissociation also observed for fluorescently labelled saccharides bound to various lectins.[139–141]

If porphyrin binding by TCSL is in anyway representative of porphyrin binding by other lectins as well, it would appear that in solution state the process is slow and is primarily driven by entropic considerations despite the likelihood of some polar hydrogen bonds being involved in the interaction. If this holds true in principle for solid state interaction as well, it seems more than likely that porphyrins interact with the lectin in the crystal as aggregates rather than as monomers and caution must be exercised in interpreting the two different sets of results obtained as a result of the very different experimental conditions. This appears to be particularly pertinent when dealing with hydrophobic ligands with a tendency for self aggregation in aqueous solutions and must be kept in mind while evaluating the efficacy of a molecule as a drug candidate.

6.5.5 Potential applications of porphyrin–lectin interaction. As mentioned before, porphyrins have excited a great deal of interest for their usefulness in photodynamic therapy.[95,142] Porphyrins preferentially accumulate in dividing cells and consequently their concentration in tumour tissue is much higher than in normal ones.[143] When excited by irradiation with light, porphyrins can react with molecular oxygen sending it into its excited singlet state which in turn causes irreparable tissue damage. However, the selectivity of these sensitizers towards tumour cells is not always sufficient for PDT to be efficient. Although *in vitro* studies and some animal studies have shown that these levels are eight to nine times higher in tumour tissues than in surrounding normal tissues, in most of the cases the concentration of the photoactive drug in tumour tissues is only about two times higher as compared to that in the surrounding tissues.[144] Invariably, therefore, killing tumour cells also implies damage to normal tissues. Hence, it is necessary to improve the ability of PDT agents to interact specifically with tumour tissues by coupling another agent that can preferentially interact with malignant cells. Among the many known cellular recognition agents, lectins seem to be attractive candidates for coupling with photosensitizers. The likelihood that lectin binding to porphyrins might further enhance their specific targeting and partitioning into malignant tissue is an interesting possibility. Further, several lectins have been previously tested for application as drug delivery agents, with some of them exhibiting considerable promise.[131–133,145,146] For example, conjugates of Con A with daunomycin and the α-chain of diphtheria toxin or ricin[131,132,146] have been prepared and tested for targeting drugs to tumor cells. Doxorubcin-wheat germ agglutinin and peanut agglutinin-ricin A-chain conjugates have also been explored in potential drug delivery applications.[133,147]

Owing to their remarkable specificities, plant lectins with affinities for the carbohydrates on microbial cell surface are already well characterised. Given the potential of porphyrins to act as antimicrobials,[99,100] it is pertinent to ask whether lectins could be used *in vivo* to specifically deliver porphyrins into pathogenic microbial cells, thereby improving the efficacy of the treatment, reducing the concentration of the drug required to be introduced into the system and thereby reducing the possible side-effects. In particular, lectins could be successful oral and mucosal drug-delivery agents. Not only are a large number of lectins part of our everyday diet, but also several of them are known to survive the harsh conditions of human gastro-intestinal tract.[148] Similarly, attempts have been made to use lectins in ocular drug delivery.[149,150] Specific hydrophobic binding sites on lectins provide the ideal opportunity to expand the use of these molecules in targeted therapy.

Table 4 Thermodynamic parameters for porphyrin–lectin interaction

Lectin	Porphyrin	$\Delta G°$ (KJ. mol^{-1})	$\Delta H°$ (KJ. mol^{-1})	$T\Delta S°$ (KJ. mol^{-1})	Reference
Trichosanthes cucumerina seed lectin	CuTPPS	−29.00	15.06 (±18.41)	−13.09	104
	CuTMPyP	−27.32	−7.53 (±11.72)	−20.198	
Momordica charantia seed lectin	CuTMPyP	−27.40	−54.4	−27.06	105
	H_2TMPyP	−26.55	−59.5	−33.02	
	CuTCPP	−25.53	−98.1	−72.69	
	H_2TPPS	−21.48	−85.3	−63.98	

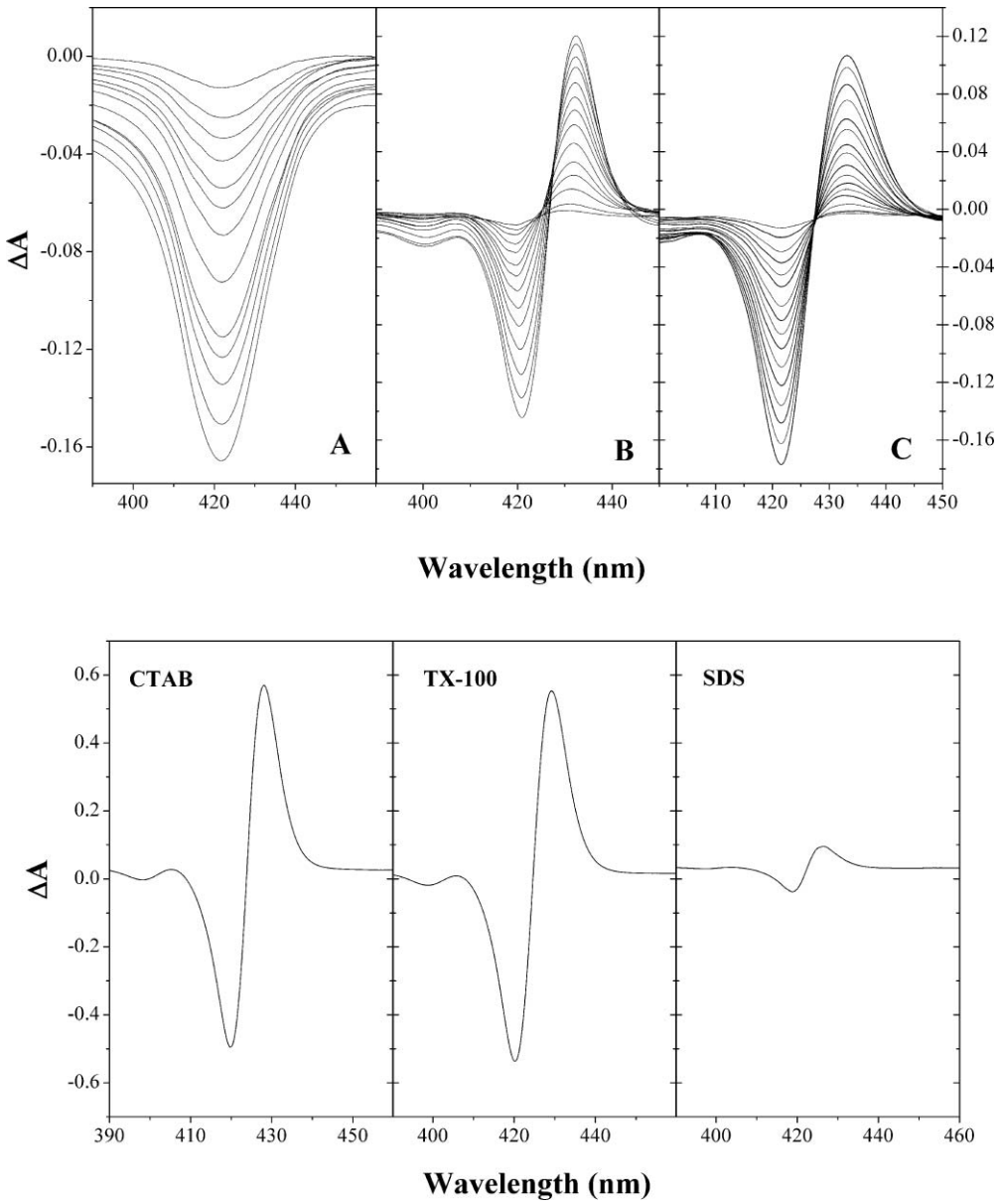

Fig. 9 Difference absorption spectra for TCSL–porphyrin interaction (upper panel) and surfactant–porphyrin interaction (lower panel). Upper panel: (A) CuTMPyP, (B) ZnTPPS, (C) ZnTCPP. The difference spectra were obtained by subtracting the spectrum of the porphyrin alone from the spectra obtained in the presence of different concentrations of the lectin. The Y-scale on the left relates to A and that on the right corresponds to B and C. Lower panel: Difference absorption spectra of ZnTPPS in the presence of different surfactants: CTAB, cetyltrimethylammonium bromide; TX-100, triton X-100; SDS, sodium dodecyl sulfate. Reproduced from ref. 104. (Copyright (2001) Federation of European Biochemical Societies).

7. Future outlook and concluding remarks

For well over a century, plant lectins have been studied with much interest due to their unique carbohydrate binding property. Apart from a detailed understanding of the structure–function relationships as well as evolutionary relationships between lectins, these studies have also led to the application of lectins in a variety of fields including cell biology and medicine. Lectins are now routinely used in the identification and purification of glycoproteins. Their use in blood typing as well as in clinical diagnostics is well established. Given their ability to specifically target different cell types, they have always been looked upon as useful candidates for targeted drug delivery. That several plant lectins appear to possess additional binding sites for hydrophobic ligands, including porphyrins, increases the versatility of these molecules for future applications. For example, it is possible to envisage porphyrins or other similar hydrophobic drugs specifically complexed to mammalian lectins such as selectins or galectins for targeted treatment at sites of inflammation or malignancy. The easy availability of molecular biological tools and the crystal structure information for several lectins, complexed with carbohydrates as well as non-carbohydrate ligands, makes it possible to envisage engineering the ligand binding sites for enhanced specificity and affinity with respect to specific drugs. Although *in vitro* studies of hydrophobic ligand interactions with lectins have been discussed for over two decades and porphyrin–lectin interactions have been

around for about eight years now, studies using animal models in this area are as yet lacking. Given the prospect of improved drug delivery candidates that such investigations could offer to clinical chemistry, it seems to be only a matter of time before such experiments will begin in earnest.

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