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Circular Dichroism Studies on the α-D-Galactopyranosyl Binding Lectin Isolated from the Seeds of *Bandeiraea simplicifolia*†

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ABSTRACT: The conformation of the α-D-galactopyranosyl binding lectin isolated from *Bandeiraea simplicifolia* seeds has been investigated over a broad range of pH in the presence of various solvents by circular dichroism (CD) spectroscopy in the region 200–300 nm. Analyses of the spectra obtained on the native protein show the lectin to contain a considerable proportion of β structure (30–40%). The native conformation was found to be largely insensitive to changes in pH, but was influenced by sodium dodecyl sulfate or trifluoroethanol. Alterations in conformation in the presence of these agents were reflected in the CD spectra and show the presence of α helix under these conditions. These changes in conformation are accompanied by a loss in polysaccharide-precipitating activity. The protein is irreversibly denatured in 8 M urea. Neither removal of the intrinsic calcium ions from the protein nor addition of methyl α-D-galactopyranoside induces any appreciable change in the CD spectra of the protein although the former treatment abolishes the polysaccharide-precipitating capacity of the lectin. The conformational data obtained in the present study are compared with data available from conformational studies of other lectins and leads to the hypothesis that most lectins probably contain β structure as the predominant conformational feature.

The isolation and characterization of an α-D-galactopyranosyl binding lectin from the seeds of *Bandeiraea simplicifolia* were recently reported from this laboratory (Hayes and Goldstein, 1974). The lectin consists of four similar subunits (molecular weight 28 500) held together by noncovalent forces. Each subunit contains one sulfhydryl group and two subunits contain one Ca(II). Both of these structural features are necessary for the lectin to exhibit polysaccharide precipitating activity. The protein molecule has been shown to contain four binding sites (Hayes and Goldstein, 1975).

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The lectin reacts specifically with polysaccharides and glycoproteins containing α-D-galactopyranosyl end groups and will also agglutinate types B and AB but neither types A nor O erythrocytes (Hayes and Goldstein, 1974; Judd et al., 1975).

The present study of the *B. simplicifolia* lectin was undertaken to assess (a) the nature and extent of secondary structure in the protein and (b) the existence of any correlation between the carbohydrate binding capacity of the lectin and the conformation of the protein.

Materials and Methods

The *B. simplicifolia* lectin was isolated according to the procedure previously reported (Hayes and Goldstein, 1974). Protein solutions were adjusted to the desired pH by exhaustive dialysis at 4° against the appropriate buffer (0.01 M sodium phosphate, pH 7.1; 0.06 M sodium acetate, pH 3.7; and 0.06 M glycine–NaOH, pH 9.8).

For the denaturation experiments the protein solution was exhaustively dialyzed against 8 M urea at 4°. The renaturation experiments were performed by similar dialysis of the urea solutions against 0.01 M sodium phosphate (pH 7.2).

Solutions of protein containing trifluoroethanol (Aldrich Chemical Co.) were prepared by the careful addition of the alcohol to a protein solution in sodium phosphate buffer at pH 7.1.

For experiments with Na_2EDTA, sodium dodecyl sulfate, or methyl α-D-galactopyranoside appropriate amounts of these reagents in phosphate buffer were added to protein solutions in the same phosphate buffer. Metal-free protein was prepared as described earlier (Hayes and Goldstein, 1974), and the CD spectrum was obtained on protein solutions in glass-distilled water.

All protein solutions were routinely centrifuged at 12000 *g* for 15 min at 4° to remove any particulate matter present.

Protein concentrations were determined in duplicate using the microbiuret assay (Janatova et al., 1968) with bovine serum albumin as the standard and/or the value of $E_{1\,cm}(1\%)$ (280 nm) = 14.1 to calculate the concentration.

Circular dichroism (CD) spectra were obtained using a JASCO spectropolarimeter modified to the SS-20 configuration. Each spectrum was scanned in triplicate and each measurement was generally performed on two separate occasions.

Attempts to extend the CD spectra below 200 nm were unsuccessful because of poor signal to noise ratio which prevented an accurate assessment of the peak position and intensity in this region. Cells were Opticell cylindrical cells of 1- and 20-mm path length for the ranges 200–300 and 250–300 nm, respectively. The measurements were done using a scan speed of 10 nm/min at 27 ± 1°. The protein concentrations were in the range of 0.3–0.5 mg/ml.

Calculations of the mean residue molecular ellipticity, ($[\theta]_{MRW}$), were done taking 122 as the mean residue weight of *B. simplicifolia* lectin. CD data were not corrected for the refractive index dispersion of the solvents.

The activity of the lectin was assayed qualitatively using micro hematocrit capillary tube tests against a bovine serum albumin–melibionate conjugate[1] or against guar gum. Quantitative assays of activity were performed using the precipitin technique (So and Goldstein, 1967; Hayes and Goldstein, 1974) with the aforementioned albumin–melibionate conjugate.

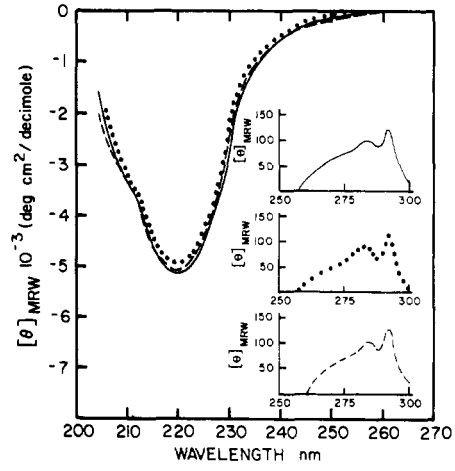

FIGURE 1: The far- and near-uv (inserted) CD bands of the *B. simplicifolia* lectin: (—) at pH 7.1 (sodium phosphate buffer); (···) at pH 3.7 (sodium acetate buffer); (- - -) at pH 9.8 (glycine–NaOH buffer).

Results and Discussion

The Structure of the Native Lectin and the Effect of pH and Urea. The CD spectra obtained from protein solutions at pH 3.7, 7.1, and 9.8 are illustrated in Figure 1. The position of the trough at 220 nm corresponds with the trough position (217–219 nm) obtained from proteins known to be in the β conformation (Timasheff et al., 1967). Although the 217–219-nm Cotton effect assignment was originally based on spectra obtained from poly(α amino acids) such as poly(L-lysine) (Greenfield and Fasman, 1969) and the contributions of aromatic side chains to the spectrum of proteins were not known, a sufficient number of proteins with β structure conformations have been examined so that support for the overall assignment appears to be justified. The spectra we have obtained appear unambiguous in assigning the major secondary structure of the *B. simplicifolia* lectin as that of the β conformation. The β structure might be present in one uninterrupted β pleated sheet or possibly in two or more minor ordered segments. No second Cotton effect or trough can be discerned in the region of 208 nm indicating the absence of any appreciable α-helical conformation. A quantitative assessment of the β-structural content of this protein must be taken with some reservation. Nevertheless, by use of the relationship of Chen et al. (1972), we estimate the content of β structure to be 30–40%, which is similar to the β-conformation content of concanavalin A (Con A) found by x-ray studies (Hardman and Ainsworth, 1972; Becker et al., 1975; Reeke et al., 1975).

It is noteworthy that the secondary structure of the lectin is quite stable to changes in pH. Previous results (Hayes and Goldstein, 1974) have revealed that the polysaccharide-precipitating capacity of the *B. simplicifolia* lectin decreases abruptly below pH 5 and more gradually above pH 10. The present study shows that the polysaccharide-precipitating capacity decrease in this pH range is not the consequence of changes in the secondary structure of the protein, but rather would appear to be a consequence of the titration of certain functional groups near the carbohydrate binding site. Moreover, the conformational stability of the *B. simplicifolia* lectin over a wide range of pH suggests that

[1] Lönngren, J., and Goldstein, I. J., to be published.

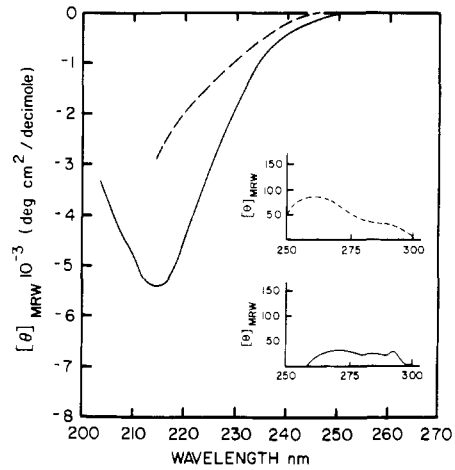

FIGURE 2: The far- and near-uv (inserted) CD bands of the *B. simplicifolia* lectin: (- - -) in 8 M urea; (—) after exhaustive dialysis of the urea solution against sodium phosphate buffer (pH 7.2).

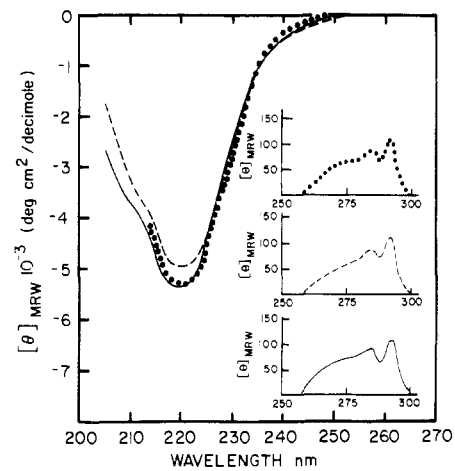

FIGURE 3: The far- and near-uv (inserted) CD bands of the *B. simplicifolia* lectin: (—) at pH 7.1 (sodium phosphate buffer) in the presence of methyl α-D-galactopyranoside (30 mM solution); (- - -) demetallized lectin; (· · ·) in the presence of Na_2EDTA (0.1 M).

charge-charge interactions almost certainly do not play a significant role in the structural stabilization of this protein. This pH stability of the secondary structure of the *B. simplicifolia* lectin stands in sharp contrast to our own and other findings (Zand et al., 1971; Pflumm et al., 1971) on the pH dependence of the jack bean lectin, Con A. This lectin exhibits pH-dependent conformational behavior that is reflected in the CD spectra, particularly above pH 9.

Available data do not allow a detailed analysis of the Cotton effects in the near-uv region. However, this region provides a probe for changes in the tertiary structure in the environment of tryptophan and tyrosine residues in the present protein since no disulfide linkages are present (Hayes and Goldstein, 1974). As shown in Figure 1, the near-uv region does not display any major pH-dependent changes.

As expected, the protein is randomized in the presence of 8 M urea; the trough at 220 nm is abolished as shown in Figure 2. Attempts to renature the protein to its original conformation by removal of urea by dialysis against phosphate buffer (pH 7.2) failed to restore the native conformation. These renaturation experiments were accompanied by some loss of protein by precipitation. Furthermore, the polysaccharide-precipitating activity of the lectin was not restored. Instead the spectra indicate that structural alterations induced in the presence of urea followed by exhaustive dialysis against phosphate buffer represent a form of the protein that is different from the native form. These results suggest that at least one other conformational state of the protein is of equivalent or of lower free energy. By way of comparison, studies of urea-denatured Con A have shown that the native β conformation of this protein is also not amenable to restoration by removal of the denaturant (Pflumm and Beychok, 1974).

The Effect of Added Methyl α-D-Galactopyranoside and Calcium Ions. The CD spectra of the lectin in pH 7.1 in the presence of methyl α-D-galactopyranoside (30 mM) (Figure 3) indicate that the binding of this glycoside by the lectin is not accompanied by any significant conformational change in the protein. The position of the Cotton effect at 220 nm is unchanged while the intensity increases by only 5%, indicating the conformational stability of the lectin under these conditions. This may be a reflection of the noninvolvement of the β structure at the active site or the rigidity of the active site geometry upon sugar binding. The near-uv spectrum (250–300 nm) is largely unaltered upon sugar binding demonstrating that virtually no perturbation of aromatic amino acid side chains has occurred. The perturbation of these amino acids in the active site region would be expected to give rise to significant changes in the CD spectrum upon sugar binding.

Treatment of the lectin with Na_2EDTA (0.1 M) or removal of the intrinsic calcium ions by dialysis against aqueous acetic acid does not induce any considerable conformational changes in the conformation of the lectin as seen in Figure 3. Earlier studies (Hayes and Goldstein, 1974) have shown that EDTA does not remove the calcium ions but binds to the protein. This CD study suggests that although the metal ions are necessary for the lectin to bind carbohydrate it does not function as a stabilizing factor for its β structure. It is noteworthy that in the cases of Con A (Barber and Carver, 1975) and the *Limulus polyphemus* agglutinin (Finstad et al., 1974), removal of the calcium ions appears to induce considerable conformational changes, but in the case of the former removal of the manganese ions necessary for sugar binding ability do not change the CD appreciably (Barber and Carver, 1975).

Effects of Sodium Dodecyl Sulfate and Trifluoroethanol on the Structure and Function of the Protein. The conformational stability of the protein was investigated in the presence of agents which are known to promote the formation of α helix in many proteins.

The CD spectra obtained in the presence of 5 and 25 mM dodecyl sulfate at pH 7.1 are presented in Figure 4. At 25 mM dodecyl sulfate, the CD spectrum is unambiguous in showing the presence of a moderate amount of α-helical conformation. Using the Greenfield-Fasman equation (Greenfield and Fasman, 1969), the α-helix content under these conditions is estimated to be 19%. At 5 mM dodecyl sulfate, the spectrum shows an overall shape and trough intensity intermediate to that of 25 mM dodecyl sulfate and no dodecyl sulfate. At 5 mM the 220-nm trough is more intense than at zero dodecyl sulfate concentrations with only the hint of a broadening at 208 nm. Virtually no change in the near-uv region is discernible at 5 mM dodecyl sulfate but an extensive change in the intensity of the spectra in this region is apparent at 25 mM dodecyl sulfate. The polysaccharide-precipitating ability of the lectin is lost at 5 mM dodecyl sulfate. The loss in precipitating ability may be a

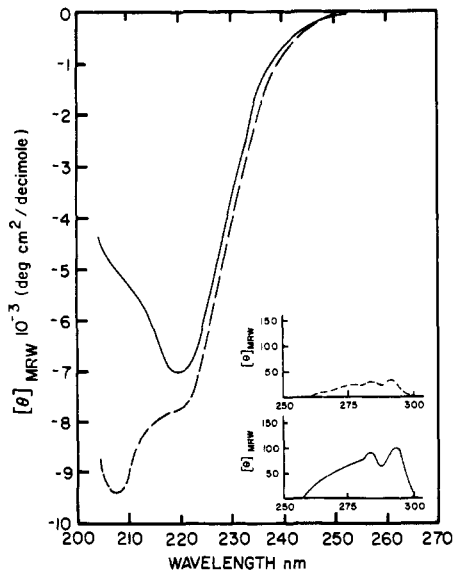

FIGURE 4: The far- and near-uv (inserted) CD bands of the *B. simplicifolia* lectin: (—) in 5 mM dodecyl sulfate solution (pH 7.2) (sodium phosphate buffer); (- - -) in 25 mM dodecyl sulfate solution (pH 7.2) (sodium phosphate buffer).

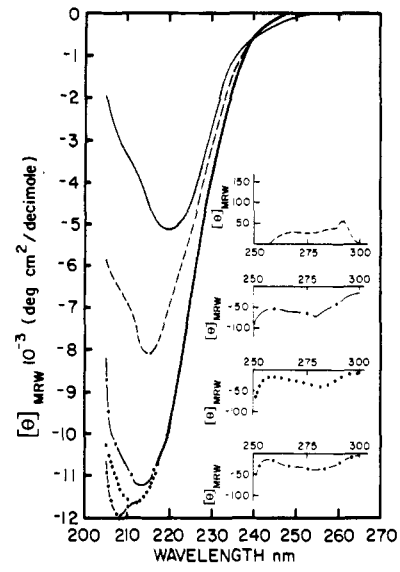

FIGURE 5: The far- and near-uv (inserted) CD bands of the *B. simplicifolia* lectin: (—) at 0 and 10% (v/v) trifluoroethanol concentration; (- - -) at 20% (v/v) trifluoroethanol concentration; (· — ·) at 25% (v/v) trifluoroethanol concentration; (· · ·) at 35% (v/v) trifluoroethanol concentration; (· – · – ·) at 45% (v/v) trifluoroethanol concentration. All trifluoroethanol titration experiments were performed with stock solutions of the lectin in phosphate buffer (pH 7.2).

reflection of the dissociation of the lectin into four subunits (Hayes and Goldstein, 1974), or it may reflect the consequence of a small conformational change in the region of the binding site or a combination of these factors. Similar CD behavior for Con A in the presence of dodecyl sulfate has been reported (Kay, 1970; Jirgensons, 1973).

The CD spectra obtained in the presence of five different concentrations of trifluoroethanol are given in Figure 5. At 10% (v/v) trifluoroethanol no change in conformation of the protein is observed. Also, at this concentration of trifluoroethanol 95% of the precipitating activity of the protein is still present. However, at a concentration of 20% trifluoroethanol, the CD spectrum shows changes in shape and intensity in the 200–300 and 250–300 nm regions. At this concentration of trifluoroethanol there is a complete loss of polysaccharide-precipitating capacity. At 25, 35, and 45% trifluoroethanol concentration there is a more extensive change in the shape and intensity of the far-uv CD spectra. At the highest concentration of trifluoroethanol there is a trough at 208 nm. Since there is no pronounced shoulder or trough around 222 nm other conformational changes might also have been induced although the formation of α helix is the predominating structural change. Estimates of α-helical content using the intensity at 208 nm give 28%. The α-helical content of the native lectin, unperturbed by dodecyl sulfate or trifluoroethanol, was calculated by the procedure of Chen et al. (1972) to be in the order of 0–5%. Conformational changes are also reflected in the appearance of the near-uv CD spectra. At 25, 35, and 45% trifluoroethanol concentrations the Cotton effects are inverted relative to the native state and the 10% concentration of trifluoroethanol. Thus the more extensive conformational changes at higher trifluoroethanol concentrations place the aromatic chromophores in a different asymmetric environment.

The dodecyl sulfate and trifluoroethanol titration experiments suggest that β-pleated sheet regions are gradually transformed to α helices or that folding of random regions in the protein occurs with a concomitant formation of α-helical segment(s). It is not possible to distinguish between these possibilities using present techniques.

The present study has demonstrated the presence of β structure as the major ordered structure of the *B. simplicifolia* lectin. This structure can be disturbed by several agents such as urea, dodecyl sulfate, and trifluoroethanol. However, the β structure is stable to changes that have been shown to affect the sugar binding site, e.g., changes in pH, removal of the calcium atom, and binding of methyl α-D-galactopyranoside. Although not actually proven this serves to suggest that a β-pleated sheet may not be part of the sugar combining site of the lectin, per se, but may be situated in another region of the molecule.

CD data have been reported for several carbohydrate binding proteins such as Con A (Pflumm et al., 1971; Zand et al., 1971), the *Limulus polyphemus* agglutinin (Finstad et al., 1974), the *Helix pomatia* agglutinin (Hammarström, 1974), the *Pisum sativum* lectin (Bureš et al., 1972), the *Ricinus communis* lectins (Shimazaki et al., 1975), and the *Dolichos biflorus* and *Robinia pseudoacacia* lectins (Père et al., 1975). It is remarkable that all these studies, including the present one, have indicated that the major ordered structure of these proteins is β structure. This also appears to be valid for the *Phaseolus lunatus* lectin[2] and for a second lectin isolated from *B. simplicifolia* that binds 2-acetamido-2-deoxy-D-glucopyranosyl end groups.[2]

For the most extensively studied lectin, Con A, results of CD investigations have been confirmed by x-ray crystallographic studies (Hardman and Ainsworth, 1972; Becker et al., 1975; Reeke et al., 1975). These studies have, inter alia, demonstrated the presence of two β-pleated sheet structures in each subunit. One of these regions, situated at the interface between subunits, is involved in the formation of di- and tetramers.

Oligomer formation is a prerequisite for the function of these proteins as polysaccharide and glycoprotein precipitating agents. In most cases, the aggregation of monomeric

[2] Unpublished results.

protein units has been shown to be mediated by noncovalent forces and it seems entirely possible that β-pleated sheets may be responsible for these interactions. In fact β structure might be a general conformational feature of all lectins.

It is noteworthy that immunoglobulins, e.g., IgG (Cathou et al., 1968) to which the lectins bear a relationship so far as their immunochemical behavior is concerned, also have been considered to have β structure as the major ordered secondary structure.

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