Articles

Preparation and Properties of Metal Ion Derivatives of the Lentil and Pea Lectins†

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ABSTRACT: Lentil lectin (LcH) and pea lectin (PSA) belong to the class of D-glucose/D-mannose binding lectins and resemble concanavalin A (Con A) closely in physicochemical, structural, and biological properties. LcH and PSA, like Con A, are Ca^{2+}-Mn^{2+} metalloproteins that require the metal ions for their saccharide binding and biological activities. Studies of the relationship between the metal ion binding and saccharide binding activity in LcH and PSA have been difficult due to the problem of metal ion replacement in these proteins. We now report a method of metal ion replacement in both lectins that allows substitution of the Mn^{2+} in the native proteins with a variety of transition metal ions, as well as substitution of the Ca^{2+} with Cd^{2+} in a particular complex. The following metal ion derivatives of both LcH and PSA have been prepared: Ca^{2+}-Zn^{2+}, Ca^{2+}-Co^{2+}, Ca^{2+}-Ni^{2+}, and Cd^{2+}-Cd^{2+}. All of these derivatives are as active as the native lectins, as demonstrated by precipitation with specific polysaccharides, saccharide inhibition of precipitation, and hemagglutination assays. The yields of these derivatives are good (generally >70%), and the degree of metal ion incorporation is high (generally >90%). The method of preparation is quite different from that for metal ion substitution in Con A, which proceeds via the apoprotein. In contrast, the apoproteins of LcH and PSA are unstable, aggregate above pH 4.0, and cannot be remetallized once formed. In general, we have found that equilibration of either lectin in acetate buffer at pH 4.0, 37 °C, with 0.1 M of the appropriate metal salt plus 0.1 M $CaCl_2$, leads to the corresponding Ca^{2+}-M^{2+}-substituted derivative of both proteins, after approximately 2 weeks for LcH and 4 weeks for PSA. Only Cd^{2+} leads to replacement at both Mn^{2+} and Ca^{2+} sites. Evidence indicates that metal ion release and capture by LcH and PSA are very different from those of Con A, which are sequential. By a number of criteria, the intrinsic molecular properties of these metal ion derivatives of both lectins are the same as those of the native proteins.

Lentil lectin (LcH)[1] and pea lectin (PSA) have been used extensively in recent years in various biological areas [cf. Lis & Sharon (1981)]. Both lectins consist of mixtures of two isolectins, which can be separated by ion-exchange chromatography (Howard et al., 1971; Entlicher et al., 1970; Trowbridge, 1974). Like Con A, they are D-mannose/D-glucose-specific lectins and resemble Con A in physicochemical, structural, and biological properties (Goldstein & Hayes, 1978; Brown & Hunt, 1978; Lis & Sharon, 1981). These lectins are mitogenic and agglutinate transformed and protease-treated cell lines selectively (Brown & Hunt, 1978), and they have been used for the isolation and structural elucidation of glycopeptides and complex carbohydrates (Lotan & Nicolson, 1979), for the separation of intact cells (Kinzel et al., 1976), and in the study of cell surface morphology (Nicolson, 1978). All three lectins are metalloproteins containing Mn^{2+} and Ca^{2+} in the native state and exhibit a high degree of sequence homology (Leburn et al., 1983; Foriers et al., 1981). However, Con A can exist as a dimer or tetramer (Hardman & Ainsworth, 1972), depending upon pH, but LcH and PSA are found only as dimers with molecular weights of approximately 47 kDa (Leburn et al., 1983; Meehan et al., 1982). Although these lectins show general similarity in specificities toward simple sugars (Allen et al., 1976), they are found to behave differently toward complex oligosaccharides and glycopeptides (Debray et al., 1981; Kornfeld et al., 1981). In view of their structural similarity, their activity differences become more interesting and emphasize the need for a closer examination of the molecular properties of these lectins.

Metal ions play a key role in regulating the saccharide binding activities of a large number of lectins [cf. Goldstein

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[1] Abbreviations: LcH, *Lens culinaris* hemagglutinin, lentil lectin; CMLcH, native LcH mixture containing 1 equiv of Mn^{2+} and 1.7-2.0 equiv of Ca^{2+} per monomer; LcHA and LcHB, the two isolectins of native lentil lectin mixture in the order of elution from the CM-cellulose column (Howard et al., 1971); PSA, *Pisum sativum* agglutinin, pea lectin; CMPSA, native PSA mixture containing 1 equiv of Mn^{2+} and 1.7-2.0 equiv of Ca^{2+} per monomer; PSA A and PSA B, the acidic and basic isolectins of native pea lectin mixture, respectively (Trowbridge, 1974); Con A, concanavalin A with unspecified metal ion content; CMPL, Con A with Mn^{2+} and Ca^{2+} at S1 and S2, respectively, in the locked conformation; PGM, *O*-phosphonoglucomannan from *Pichia pinus*; GM, D-glucomannan from *P. pinus*; NMRD, nuclear magnetic relaxation dispersion, the magnetic field dependence of the solvent nuclear magnetic relaxation rates ($1/T_1$); PBS, 10 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.2; EDTA, ethylenediaminetetraacetic acid disodium salt; EPR, electron paramagnetic resonance.

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& Hayes (1978)]. However, only in the case of Con A has the regulatory role of metal ions been studied in detail [cf. Brewer et al. (1983b)]. Each monomer of Con A possesses two metal ion binding sites: S1, the so-called "transition metal ion" site, and S2, the "calcium" site. Both sites must be occupied for full saccharide binding activity; the demetallized protein is essentially without activity. Mn^{2+} and Ca^{2+} (as well as other metal ions) bind to apo-Con A in a sequential manner and induce a first-order conformational transition in the protein that alters both the metal ion and saccharide binding properties of the lectin (Brown et al., 1977). Both thermodynamic and kinetic measurements have been used to characterize a variety of metal ion and saccharide complexes with two major conformational states of the protein, which has led to a detailed understanding of the molecular basis for the regulation of the activity of Con A by metal ions (Brewer et al., 1983b).

Little is known about the role of Mn^{2+} and Ca^{2+} in the lentil (CMLcH) and pea (CMPSA) lectins, which are their native forms, and the results available are conflicting in terms of their Mn^{2+} and Ca^{2+} content (Paulova et al., 1971a,b; Lonnderdal et al., 1983). Preliminary X-ray crystallography of CMPSA shows that the metal ions are located at positions similar to that in Con A (Meehan et al., 1982; Riskulov et al., 1984), while the primary sequence data show conservation of the amino acids in CMLcH and CMPSA that form the S1 and S2 sites in Con A, except that Tyr-12 at the S2 site of Con A is replaced by a phenylalanine residue in CMLcH (Foriers et al., 1981; Leburn et al., 1983). Yet, unlike Con A (Agrawal & Goldstein, 1968; Kalb & Levitzki, 1968; Koenig et al., 1973) attempts at demetallizing and remetallizing CMLcH and CMPSA with the full restoration of activity have not been successful (Paulova et al., 1971a,b).

In this paper, we report the preparation and characterization of a variety of metal ion derivatives of LcH and PSA, as part of a continuing investigation of the role of metal ions in the structure–function correlation of lectins. The procedure for metal ion substitution in both proteins is unique and quite different from that used for Con A (Agrawal & Goldstein, 1968; Kalb & Levitzki, 1968; Koenig et al., 1973), in that it does not involve formation of the apoprotein. The following derivatives of both LcH and PSA were prepared: Ca^{2+}–Zn^{2+}, Ca^{2+}–Ni^{2+}, Ca^{2+}–Co^{2+}, and Cd^{2+}–Cd^{2+}. The results show that all of these metal ion derivatives are as active as the native Ca^{2+}–Mn^{2+} lectins and that all possess similar physical and chemical properties. Several of these derivatives provide opportunities for spectroscopic studies of these two important but less well-characterized lectins.

Materials and Methods

Seeds of lentil (*Lens culinaris* Med. sub. *Macrosperma*) and pea (*Pisum sativum* L. var. Columbian) were purchased from a local food store. Salts of different metals were the highest purity products available from either Mallinckrodt or Fisher Scientific Co. Monosaccharides were obtained from Sigma Chemical Co. and Pfanstiehl Laboratories. Polysaccharides PGM and GM of *P. pinus* were gifts from Dr. M. Slodki, Northern Regional Research Center, U.S. Department of Agriculture.

Preparation of Lectins. CMLcH was purified by affinity chromatography on Sephadex G-100 (Ticha et al., 1970). The separation of the two isolectins LcHA and LcHB was done on a CM-cellulose column (Howard et al., 1971). CMPSA and the two isolectins A and B were obtained according to the procedure described by Trowbridge (1974).

Preparation of Metal Derivatives. CMLcH and CMPSA were dissolved at about 5 mg/mL in 10 mM sodium acetate buffer, pH 4.0, containing appropriate amounts of the salts of different metals. The following combinations of salts were used: 100 mM each of $CoCl_2$ + $CaCl_2$, nickel acetate + $CaCl_2$, and $ZnCl_2$ + $CaCl_2$, and 200 mM $CdSO_4$. The solutions were dialyzed at 37 °C against a 10-fold excess of the solvent buffer for 16 days for lentil lectin and 30 days for pea lectin. Any precipitate formed was removed by centrifugation, and the metal ion derivatives were then dialyzed against water at 4 °C and stored as salt-free lyophilizate.

Protein Concentrations. The concentrations of CMLcH, LcHA, and LcHB were determined spectrophotometrically by using the extinction coefficient $A^{1\%,1cm} = 12.6$ at 280 nm (Howard et al., 1971). The extinction coefficient of CMPSA was determined by weighing the salt-free lyophilized protein accurately and then dissolving it in 0.1 M potassium acetate/0.1 M KCl buffer, pH 6.4. The value found for $A^{1\%,1cm}$ at 280 nm was 15.0. The extinction coefficients of PSA A, of PSA B, and of different metal ion derivatives of LcH and PSA were found to be the same as those of the respective native mixtures. All protein concentrations are reported in terms of monomer concentration.

Metal Ion Analysis. The protein solutions were acidified to pH 1.2 with concentrated HCl and allowed to stand overnight; the precipitates were removed by centrifugation. These solutions were used for metal ion analysis by atomic absorption measurements using a Perkin-Elmer Model 603 spectrophotometer as in Brewer et al. (1983a). Mn^{2+} in these solutions was also determined by proton NMRD techniques according to Brewer et al. (1983a).

Ultraviolet Absorption Spectra. Spectra were taken at room temperature in a Cary 14 recording spectrophotometer in the range 240–320 nm in PBS at concentrations of 0.56 mg/mL for CMLcH, its metal derivatives, and the isolectins LcHA and LcHB and 0.47 mg/mL for CMPSA, its metal ion derivatives, and the isolectins PSA A and PSA B.

NMRD Measurements. Data were obtained by using the field-cycling instrumentation and procedures previously referenced (Koenig & Schillinger, 1969; Hallenga & Koenig, 1976; Brown et al., 1977).

Hemagglutination Assays. Assays were done at room temperature in PBS at 1 mg/mL protein concentrations using 3% suspensions of erythrocytes from the same rabbit (Osawa & Matsumoto, 1972).

Turbidity Reaction and Inhibition by Monosaccharides. A modification of the method of Poretz & Goldstein (1968) was used. To 0.1 mL of PBS containing 0–30 mM monosaccharide was added 0.8 mL of protein solution at room temperature. The reaction was initiated by adding of 0.1 mL of polysaccharide solution and mixing immediately in a vortex mixer. Development of turbidity was monitored spectrophotometrically at 420 nm at various time intervals by use of a Gilford 250 spectrophotometer. The appropriate blank readings were obtained by adding methyl α-D-glucopyranoside, an excellent inhibitor of both lectins, to parallel sets of tubes containing the corresponding amounts of lectins and polysaccharides.

Results

Metal Ion Content of Native LcH and PSA. The stoichiometries of Mn^{2+} and Ca^{2+} in CMLcH and CMPSA and in their isolectins, obtained by the use of atomic absorption and proton NMRD measurements, are given in Tables I and II, respectively. The results are the average of more than five determinations. The metal contents are expressed in Tables I and II as mole percents of the monomeric units [23.5 kilodaltons (kDa) for either protein] and show the presence of one Mn^{2+} and 1.8 Ca^{2+} per monomer. The stoichiometries of

Table I: Metal Ion Content of Lentil Lectin (LcH) Derivatives

LcH derivatives	yield (%)	mol % Mn	Ca	Zn	Co	Ni	Cd
native		95 98[a]	185 ± 15				
LcHA		94 97[a]	180				
LcHB		96 98[a]	190				
Ca^{2+}–Zn^{2+}	78	<0.1	170	77			
Ca^{2+}–Co^{2+}	87	<0.1	200		90		
Ca^{2+}–Ni^{2+}	72	<0.1	200			90	
Cd^{2+}–Cd^{2+}	67	<0.1	1.2				167

[a] By NMRD measurement. All other results are by atomic absorption analysis.

Table II: Metal Ion Content of Pea Lectin (PSA) Derivatives

PSA derivatives	yield (%)	mol % Mn	Ca	Zn	Co	Ni	Cd
native		92 96[a]	180 ± 15				
PSA A		94 98[a]	185				
PSA B		92 95[a]	180				
Ca^{2+}–Zn^{2+}	82	<0.1	160	90			
Ca^{2+}–Co^{2+}	90	<0.1	160		95		
Ca^{2+}–Ni^{2+}	78	<0.1	200			92	
Cd^{2+}–Cd^{2+}	76	<0.1	2.4				182

[a] By NMRD measurement. All other results are by atomic absorption analysis.

Mn^{2+} and Ca^{2+} are the same for LcHA and LcHB. The same results are also found for PSA A and PSA B.

Preparation of Metal Derivatives. Both CMLcH and CMPSA are found to lose metal ions irreversibly when dialyzed against 10 mM sodium acetate buffer, pH 4.0, at 37 °C. The half-lives were approximately 2 and 4 days, respectively. On dialysis against the above buffer containing 100 mM $MnCl_2$ and 100 mM $CaCl_2$, both lectins are found to remain stable. It appears that Mn^{2+} and Ca^{2+} in these lectins exchange with Mn^{2+} and Ca^{2+} in solution. However, the presence of 10 mM $MnCl_2$ and 10 mM $CaCl_2$ fails to protect the two proteins from loss of their metal ions. Use of 100 mM $ZnCl_2$, $CoCl_2$, or nickel acetate, instead of $MnCl_2$, together with 100 mM $CaCl_2$, in pH 4.0 buffer, leads to the selective replacement of Mn^{2+} in either lectin by these metal ions. $CdSO_4$ (200 mM) added to the above buffer results in the displacement of both Ca^{2+} and Mn^{2+} in either lectin to form Cd^{2+}–Cd^{2+} derivatives in significant yields. The lectins are found to have poor stability (20–30% yields) when dialyzed against 100 mM $CdSO_4$. The amount of metal ion incorporation and the respective yields of different metal ion derivatives of LcH and PSA are shown in Tables I and II, respectively, and are the average of two independent determinations. Slightly higher yields are obtained with PSA.

Cu^{2+} and Gd^{3+} did not replace Mn^{2+} in the native lectins at 200 mM metal ion concentrations in the above buffer. The failure of Cu^{2+} to replace Mn^{2+} may be due to its stereochemical preference for planar geometry, unique among the bivalent transition-metal ions.

Gel Filtration. Figure 1 shows the results of gel filtration of a mixture consisting of CMLcH and all the metal ion derivatives of LcH on a Bio-Gel P-100 column (for details see the figure legend). The mixture coelutes in a single symmetrical peak, which indicates that the various derivatives of LcH have the same shape and size (molecular weight) as the native lectin. The same results were obtained with a mixture of

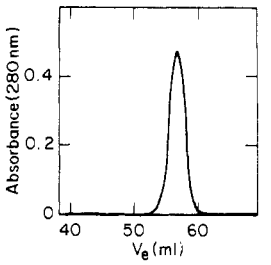

FIGURE 1: Bio-Gel P-100 chromatography (50 × 1.6 cm) of a mixture of 1 mg each of native Ca^{2+}–Mn^{2+}–LcH and all other metal ion derivatives of LcH (Table I) in pH 6.4 buffer, 0.1 M potassium acetate and 0.1 M KCl, at room temperature. The flow rate was 10 mL/h.

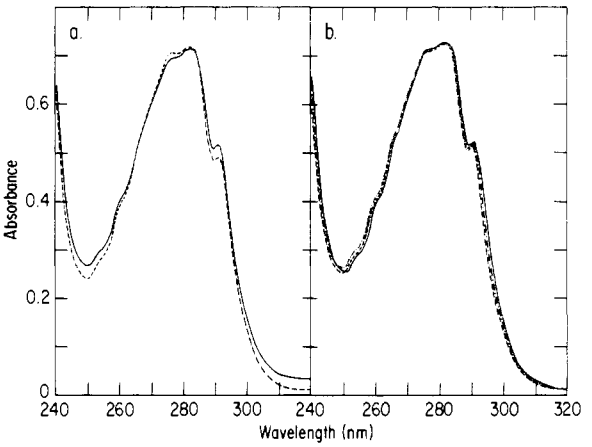

FIGURE 2: Ultraviolet absorption spectra (a) of CMLcH (—) and CMPSA (---) and (b) of CMLcH (—), Ca^{2+}–Zn^{2+}–LcH (---), and Cd^{2+}–Cd^{2+}–LcH (···) in PBS at room temperature. The spectra of CMLcH and CMPSA did not change upon addition of 0.1 M methyl α-D-mannopyranoside. The protein concentrations were 0.56 mg/mL each for CMLcH and its derivatives and 0.47 mg/mL for CMPSA.

CMPSA and its metal derivatives (not shown).

CMLcH, demetallized in either 1 M acetic acid buffer, pH 2.2, or 10 mM sodium acetate buffer, pH 4.0, is found to give the identical elution profile (not shown) on gel filtration on a Bio-Gel P-150 column using 10 mM sodium acetate buffer, pH 4.0. The demetallized protein eluted within the void volume, showing that it contained only high molecular weight species. Similar results were obtained with CMPSA demetallized by both of the above methods. The results indicate that both lectins polymerize on demetallization (when the pH was raised to 4.0 or above) and that the final states are essentially independent of these two methods of demetallization.

Ultraviolet Absorption Spectra. Figure 2a shows that the ultraviolet spectra of CMLcH and CMPSA in the range 240–320 nm are very similar. Spectra of the two isolectins (not shown) of either lectin are superimposable at the same concentrations. Matsumoto et al. (1980) obtained a UV difference spectrum of LcH in the presence of methyl α-D-mannopyranoside in the range 270–300 nm. In our studies, however, we observed that the spectra of CMLcH and CMPSA (Figure 2) did not change in the presence of 0.1 M of the sugar in the range 240–320 nm.

Figure 2b shows that the ultraviolet spectra of CMLcH and its Ca^{2+}–Zn^{2+} and Cd^{2+}–Cd^{2+} derivatives are essentially identical. The spectra of the other metal derivatives of LcH (not shown) are also identical with that of CMLcH. The same results have also been found for CMPSA and all the metal derivatives of PSA (not shown). These results indicated that the prolonged exposure to pH 4.0 at 37 °C, needed for exchange of metal ions, did not alter the environment of the aromatic amino acid residues of these lectins and that the

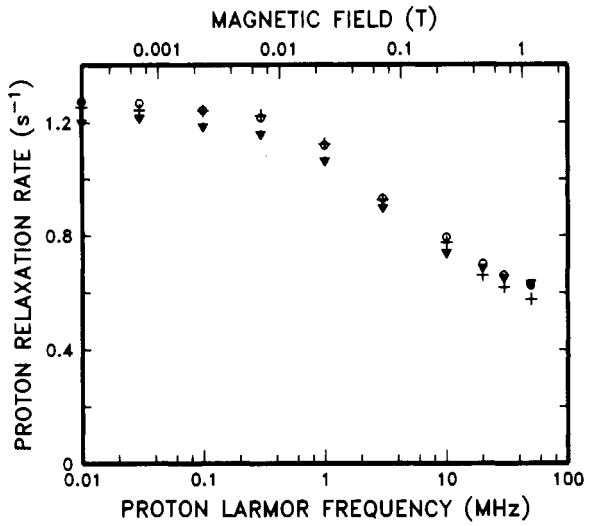

FIGURE 3: NMRD profiles of Ca^{2+}–Zn^{2+}–LcH (+), Ca^{2+}–Co^{2+}–LcH (○), and Ca^{2+}–Ni^{2+}–LcH (▼) in pH 6.4 buffer, 0.1 M potassium acetate, 0.1 M KCl, and 10 μM EDTA, at 5 °C. Protein concentrations were 25.1 mg/mL (1.07 mM with respect to monomer).

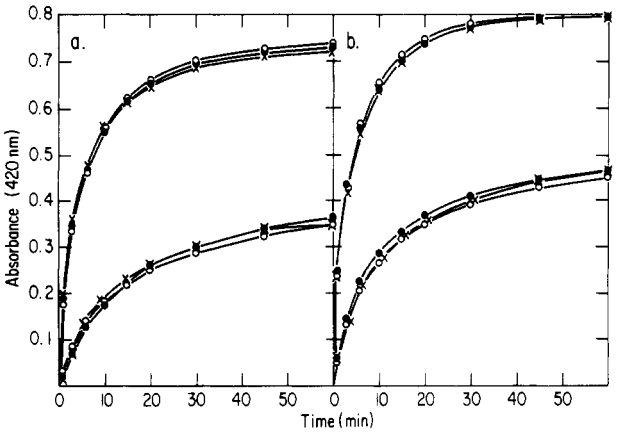

FIGURE 4: Development of turbidity as a function of time for (a) native Ca^{2+}–Mn^{2+}–LcH (●), Ca^{2+}–Zn^{2+}–LcH (×), and Cd^{2+}–Cd^{2+}–LcH (○) and (b) native Ca^{2+}–Mn^{2+}–PSA (●), Ca^{2+}–Zn^{2+}–PSA (×), and Cd^{2+}–Cd^{2+}–PSA (○), with polysaccharides from *P. pinus* in PBS at room temperature. The higher data set in each panel represents the reactions with PGM and the lower set the reactions with GM. In each case, the protein concentration was 400 μg/mL and the polysaccharide concentration was 40 μg/mL in the final mixture.

structures and conformations of the metal ion derivatives are essentially identical with those of the native lectins.

Proton NMRD Profiles of Metal Ion Derivatives. The NMRD profiles were measured over a wide range of magnetic fields, corresponding to proton Larmor frequencies of 0.01–50 MHz. Measurements were made on solutions of Ca^{2+}–Zn^{2+}, Ca^{2+}–Co^{2+}, and Ca^{2+}–Ni^{2+} derivatives of LcH and PSA at pH 6.4, at 5 and 25 °C; the protein concentrations were nominally 1.07 mM for the derivatives. Figure 3 shows the profiles of the magnetic field dependence of the relaxation rates for the derivatives of LcH at 5 °C. These rates include, in addition to the protein contribution, a constant buffer contribution of 0.52 s^{-1} at this temperature. In all cases, the values of $1/T_1$ decrease monotonically as the magnetic field is increased from a very low value. Each curve has a point of inflection; the value of the field at the inflection point (ν_c) relates to the orientational relaxation time of the proteins (τ_R), which, in turn, depends upon the size, shape, concentration, temperature, and solvent composition (Hallenga & Koenig, 1976). Since measurements were made under the same conditions, the superimposable profiles indicate that these metal derivatives of either lectin have the same hydrodynamic properties and are characteristic of diamagnetic globular proteins of 47 kDa as observed by Hallenga & Koenig (1976), when account is taken of the temperature difference of the two studies. Similar results were also obtained with the same metal ion derivatives of PSA (not shown).

NMRD measurements were also made on samples of LcH in 10 mM sodium acetate/10 μM EDTA buffer, pH 5.0, that had been demetallized by 1 M acetic acid buffer, pH 2.2, or 10 mM sodium acetate buffer, pH 4.0. The profiles (not shown) obtained with the lectin demetallized by either method were very similar and totally unlike those of globular proteins. The relaxation rates decreased rapidly as the magnetic field was increased from 0.01 to 1 MHz proton Larmor frequency, passed through a plateau region between 1 and 10 MHz, and decreased further at higher fields. Such profiles are diagnostic of protein association in solution (Lindstrom & Koenig, 1974). The results suggest that LcH aggregates on demetallization and that the final state does not depend upon the demetallization procedure. Similar results were also obtained from the gel filtration experiments. The profiles remained unchanged after 24-h incubation of the demetallized lectin with a mixture of 10 mM $MnCl_2$ and 10 mM $CaCl_2$ followed by removal of the excess metal ions by dialysis against the pH 5.0 buffer, which indicated that there was no uptake of metal ions by LcH once the apoprotein was formed.

Hemagglutination, Precipitation, and Precipitation-Inhibition Assays. CMLcH, LcHA, LcHB, and the metal ion derivatives of LcH in Table I are found to be equally active, each having a titer of 1024 at 1 mg/mL protein. The same results were also obtained with CMPSA, PSA A, PSA B, and the metal ion derivatives of PSA (Table II), each having titers of 2048 at 1 mg/mL protein.

Figure 4 shows the development of turbidity as a function of time for the Ca^{2+}–Zn^{2+} and Cd^{2+}–Cd^{2+} derivatives of LcH and PSA, and for the corresponding native lectins, with two polysaccharides from *P. pinus* PGM and GM (curves with other derivatives are not shown). CMPSA is found to produce stronger turbidity than CMLcH at the same protein concentration. The results are in accordance with the higher carbohydrate binding affinity of CMPSA (Allen et al., 1976; Debray et al., 1981). The native and the metal ion derivatives of each lectin are found to give overlapping curves. The curves for the two isolectins (not shown) are also identical with the native mixture for either lectin.

It has been reported by Paulova et al. (1971a,b) that the hemagglutinating and mannan-precipitating activities of CMLcH and CMPSA increase in the presence of $MnCl_2$ and $CaCl_2$. In contrast, we have found that overnight incubation of the native lectins with 10 mM $MnCl_2$ and 10 mM $CaCl_2$ followed by removal of excess metal ions by dialysis did not alter hemagglutinating and precipitating activities of the lectins. The enhancement of activities observed by these authors is most likely due to the interaction of the metal ions with the erythrocyte membranes (Long & Mouat, 1971) and polysaccharides.

For the determination of the concentrations of monosaccharides required for 50% inhibition of precipitation, readings were taken 45 min after mixing, a time in which the turbidity of the solution became virtually constant (Figure 4). For 50% inhibition of the precipitation of CMLcH, LcH A and LcH B, and the metal ion derivatives of LcH by PGM, the concentrations of monosaccharides required are as follows: methyl α-D-glucopyranoside, 2.2 mM; methyl β-D-glucopyranoside, 20.5 mM; and 3-*O*-methyl-α-D-glucopyranose, 0.40 mM. For 50% inhibition of the precipitation of CMPSA, PSA

A and PSA B, and the metal ion derivatives of PSA by the same polysaccharide the concentrations of monosaccharides required are as follows: methyl α-D-glucopyranoside, 1.1 mM; methyl β-D-glucopyranoside, 13.1 mM; and 3-*O*-methyl-α-D-glucopyranose, 0.20 mM. Essentially the same values were obtained for the corresponding concentrations of these sugars for inhibition of precipitation of the native forms and different metal derivatives of LcH and PSA by GM. The results show that the metal ion derivatives have the same sugar affinities as the respective native lectins and that the isolectins are also identical in this respect. In all cases, methyl α-D-glucopyranoside is about 10 times stronger an inhibitor than its β-anomer and about 5 times weaker than 3-*O*-methyl-α-D-glucopyranose.

Discussion

Properties of the Isolectins of Native LcH and PSA. Native CMLcH and CMPSA consist of mixtures of two isolectins, A and B, which have the same molecular weights but differ slightly in charge and amino acid compositions (Ticha et al., 1970; Howard et al., 1971; Entlicher et al., 1970; Trowbridge, 1974). Tables I and II show that the metal ion contents of the isolectins are the same. We have also observed that both isolectins of LcH have identical ultraviolet spectra, in agreement with Howard et al. (1971). The same results were found for the isolectins of PSA. The NMRD and EPR data (Bhattacharyya et al., 1985b) for the isolectins of both proteins are also essentially identical. Our results from hemagglutination, precipitation, and precipitation-inhibition studies indicate that the isolectins of LcH and PSA have identical sugar binding affinities (Bhattacharyya et al., 1985b) and cytotoxic activities (J. Ripka, personal communication). Two reports in the literature (Ticha et al., 1970; Young et al., 1971) indicate that LcHA and LcHB differ slightly in their sugar binding affinities, which contrasts with our findings. Such identity in structural and spectral properties of the isolectins, together with their identical biological and sugar binding properties, justifies the use of the native mixture in all our studies of both lectins.

Mn^{2+} and Ca^{2+} Contents of Native LcH and PSA. Our results, Tables I and II, show that there are approximately 2:2 and 4:2 stoichiometries between the bound Mn^{2+} and Ca^{2+}, respectively, and protein dimers, for either lectin. The Mn^{2+} contents of these lectins are similar to those of Con A, but they bind almost twice as much Ca^{2+}. Reports appear in the literature that conflict with our results and also differ among thermselves. CMLcH was found to contain 1 (Paulova et al., 1971a) to 2.6 mol (Lonnderdal et al., 1983) of Mn^{2+} and 1.8 (Paulova et al., 1971a) to 4.3 mol (Lonnerdal et al., 1983) of Ca^{2+} per mole (dimer). CMPSA was reported to have 1 (Paulova et al., 1971b) to 2 mol (Lonnerdal et al., 1983) of Mn^{2+} and 2 (Paulova et al., 1971b) to 2.5 mol (Lonnerdal et al., 1983) of Ca^{2+} per dimer. The low content of Mn^{2+} in the native lectins as found by Paulova et al. (1971a,b) is probably due to exposure of the solutions to pH 4.5 overnight during purification, a condition under which the lectins lose metal ions slowly, as shown in this study.

Effects of Demetallization on LcH and PSA. In the course of our initial attempts to develop a procedure for metal ion exchange in CMLcH and CMPSA, we began by considering the use of chelators and/or acidification of the protein solutions to remove the metal ions, as was done in the case of Con A [cf. Koenig et al. (1973)]. Conflicting reports of such attempts for both CMLcH and CMPSA have been described in the literature. Paulova et al. (1971a) reported that dialysis of CMLcH against 0.1 M EDTA and 1 M acetic acid led to complete removal of Mn^{2+} while the Ca^{2+} content remained unaffected. The molecule dissociates under this condition to α and β chains, which can be separated by Bio-Gel P-100 chromatography (Fliegerova et al., 1974). Paulova et al. (1971a) also reported that attempts to remetallize the lectin so treated by exposing it to a mixture of 5 mM $MnCl_2$ and 5 mM $CaCl_2$ led to uptake of Mn^{2+} to 74% of that of the native preparation although the lectin did not regain its biological activities, which was attributed to the aggregation of the heavier subunit (Fliegerova et al., 1974).

In a similar study on CMPSA, Paulova et al. (1971b) reported that dialysis of the lectin against 0.1 M EDTA and 1 M acetic acid left the Mn^{2+} content unaffected, but that the Ca^{2+} content decreased to about 11% of that of the native preparation, and that the biological activities decreased significantly. However, on subsequent exposure to 5 mM $MnCl_2$ and $CaCl_2$ the hemagglutination activity of the lectin was almost completely recovered, and the polysaccharide-precipitating activity increased.

By contrast, we observed that CMLcH and CMPSA behaved very similarly with respect to the loss of metal ions at low pH although CMLcH is more sensitive. Dialysis of CMLcH against 1 M acetic acid (pH 2.2) for 3 days at 4 °C resulted in complete removal of Mn^{2+} and 90% loss of Ca^{2+}. CMPSA requires dialysis against 1 M acetic acid at 37 °C for 5 days for 100% removal of Mn^{2+}, and the Ca^{2+} content decreased to about 50%. The two lectins also lose metal ions on dialysis against 10 mM sodium acetate buffer, pH 4.0, but the rates of loss are appreciably slower and sensitive to temperature, as expected. The half-lives for the loss of Mn^{2+} from CMLcH and CMPSA were estimated by NMRD measurements in the pH 4.0 buffer at 37 °C to be approximately 2 and 4 days, respectively.

LcH and PSA demetallized either by 1 M acetic acid at pH 2.2 or at pH 4.0 in a 0.1 M sodium acetate buffer are found to behave identically in terms of aggregation of the respective apoproteins between 4 and 37 °C. They precipitate in 0.1 M NaCl above pH 4.0, and gel filtration on Bio-Gel P-150 and NMRD measurements of the demetallized lectins clearly indicate similar aggregation of the apoproteins in solution irrespective of the method of demetallization. Succinylation prior to demetallization could not prevent association of the apolectins. Demetallized LcH gave indistinguishable NMRD profiles at pH 5.0 in 10 mM sodium acetate buffer before and after incubation with a mixture of $MnCl_2$ and $CaCl_2$, indicating no uptake of Mn^{2+}. That there was no uptake of metal ions was confirmed by the results of atomic absorption measurements. These results are in contradiction with the findings of Paulova et al. (1971a), who observed an increase in Mn^{2+} content of the demetallized LcH under the same condition.

In summary, we find that, under a variety of conditions, the demetallized forms of LcH and PSA aggregate at and above pH 4.0, and they cannot be remetallized. Therefore, a method of metal ion substitution had to be developed that did not involve the formation of significant amounts of the apoproteins of the two lectins.

Preparation and Properties of Metal Ion Derivatives of LcH and PSA. The loss of metal ions from CMLcH and CMPSA at pH 4.0 and 37 °C can be prevented by the presence of a large excess (at least 0.1 M) of Mn^{2+} and Ca^{2+} in the buffer. It appears, therefore, that Mn^{2+} and Ca^{2+} in the proteins exchange with the Mn^{2+} and Ca^{2+} in solution in a time too short for the intermediate apoproteins to aggregate; thus the process is kinetically controlled. Interestingly, the presence of the buffer is required to stabilize the exchange reaction of

the lentil protein but not that of the pea protein. Lower concentrations of both ions (10 mM each) failed to stabilize the proteins.

That metal ion exchange occurs under the above conditions is confirmed by the observation that substitution of 0.1 M $MnCl_2$ by either $ZnCl_2$, $CoCl_2$, or nickel acetate, in the presence of 0.1 M $CaCl_2$, leads to the corresponding S1 metal ion substituted derivatives of both lectins in good yields (Tables I and II). Only $CdCl_2$ alone can prevent the formation of the apolectins, leading to the corresponding ternary Cd^{2+} complexes. This property of LcH and PSA is similar to that of Con A, which also forms a ternary Cd^{2+} complex, CdCdPL, with Cd^{2+} at both the S1 and S2 sites (Shoham et al., 1973).

By a variety of criteria, the metal ion derivatives of LcH and PSA are identical in their intrinsic molecular properties when compared to the corresponding native Ca^{2+}–Mn^{2+} lectin. UV spectra (Figure 2b) indicate that the aromatic groups in the proteins that give rise to the spectra have unchanged environments in the derivatives. Gel filtration chromatography (Figure 1) and NMRD results (Figure 3) show that the derivatives of LcH and PSA have the same hydrodynamic properties as the parent lectins (dimer), results that are of paramount importance for our NMRD studies of these lectins (Bhattacharyya et al., 1985a). The results of precipitation studies with two polysaccharides (Figure 4), sugar inhibition of the precipitation reactions, and hemagglutination data indicate that the saccharide binding activities of the metal ion derivatives of both lectins are equal to those of the native proteins. These results are similar to those observed for metal ion substituted derivatives of Con A that are equally active [cf. Brewer et al. (1973)], indicating that the saccharide binding properties of all three lectins are not sensitive to the nature of the metal ions that occupy the so-called transition-metal site (S1) and the calcium site (S2) in the proteins. Preliminary X-ray crystallographic analysis of PSA shows the presence of high electron density at regions in each monomer where the S1 and S2 sites of Con A are located (Riskulov et al., 1983). Thus there is good reason to expect that PSA (and LcH) will have corresponding S1 and S2 sites.

Summary

A method has been found that allows for the substitution of a variety of different transition-metal ions into the transition-metal binding sites (S1) of LcH and PSA as well as the preparation of ternary Cd^{2+} complexes of both lectins (Cd^{2+} at S1 and S2). All of the metal ion derivatives of LcH and PSA have the same intrinsic molecular properties as the respective native lectins, and they also have the same carbohydrate binding properties and biological activities. The preparation of these derivatives now permits a variety of spectroscopic techniques to be employed in order to investigate the relationship between the molecular properties of the lectins and their biological activities.

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Proton and Deuteron Nuclear Magnetic Relaxation Dispersion Studies of Ca^{2+}–Mn^{2+}–Concanavalin A: Evidence for Two Classes of Exchanging Water Molecules†

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ABSTRACT: We have measured the magnetic field dependence of the nuclear magnetic relaxation rates (NMRD profiles) of solvent protons and deuterons in solutions of Ca^{2+}–Mn^{2+}–concanavalin A (Con A) with and without saccharide present. Data were obtained over the range −8 to 35 °C; the extension to the lowest temperature was made possible by the presence of 5 M salt. Since previous theoretical analyses, using accepted relaxation theories of ^1H NMRD profiles alone, led to unsatisfactory conclusions, we have attempted to take advantage of the fact that the residence lifetime of a water ligand of the metal ions can influence the relaxation behavior of protons and deuterons differently. From a comparison of the present proton and deuteron results, we find that Ca^{2+}–Mn^{2+}–Con A has two classes of binding sites: one, associated with the inner coordiation sphere of the Mn^{2+} ions, having a resident lifetime for solvent water of $\sim 10^{-5}$ s that is reduced by the presence of saccharide and another having a lifetime of $\sim 5 \times 10^{-9}$ s, located with the protons of the bound waters ~4.4 Å from the Mn^{2+} ions (assuming two equivalent water molecules in this class), which is well beyond the coordination environment of the Mn^{2+} ions. The relaxation contribution of these more distant sites is unaffected by saccharide. The conclusions are corroborated by measurements of the temperature dependences of the proton NMRD profiles, which show quite clearly that the profiles are composite, containing two contributions with opposite dependences on temperature. The more slowly exchanging water molecules dominate proton relaxation above about 25 °C and dominate deuteron relaxation throughout. The more rapidly exchanging water molecules dominate proton relaxation at lower temperatures but make no detectable contribution to the deuteron data under these conditions. These results are the first in which it can be said with some confidence that exchanging water molecules liganded in two disparate classes of sites, with different exchange rates and different contributions to the NMRD profiles, have been identified.

Measurements of NMRD[1] profiles of solvent protons in solutions of Con A and its complexes with Mn^{2+}, Ca^{2+}, and saccharides have yielded a wealth of biochemical information about the complexes (Brown et al., 1977; Koenig et al., 1978; Brewer et al., 1983a,b). In these investigations, observed NMRD profiles were used as indicators of the chemical state of Mn^{2+} and of the conformation of the protein and its change with time. The conclusions reached depended only on complex-specific contributions to the observed NMRD profiles derived from the interactions of solvent protons with the (paramagnetic) Mn^{2+} ion in its many complexed forms. The details of relaxation theory played little role in the interpretation of the biochemistry of Con A, and indeed, an accurate theory of relaxation from which, for example, one could deduce the number q of exchanging solvent mlecules liganded to Mn^{2+} ions complexed with protein did not exist at the time [cf.

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[1] Abbreviations: NMRD, nuclear magnetic relaxation dispersion, the magnetic field dependence of the solvent nuclear magnetic relaxation rates (in protein solutions); Con A, concanavalin A with unspecified metal ion content; MP, the Mn^{2+}–Con A complex in the unlocked conformation; CMPL, Con A with 1 equiv per monomer of bound Mn^{2+} and Ca^{2+} in the locked conformation; SCMPL, CMPL complexed with saccharide; CZPL, the analogous Zn^{2+} complex; α-MDM, methyl α-D-mannopyranoside.

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