Biochimica et Biophysica Acta, 643 (1981) 220—232
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BBA 79195

INTERACTION OF MONOLAYERS OF CONCANAVALIN A WITH MONO- AND POLYSACCHARIDES

A STUDY BY MEANS OF INFRARED-ATTENUATED TOTAL REFLECTANCE SPECTROSCOPY

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(Received August 6th, 1980)
(Revised manuscript received December 10th, 1980)

Key words: Infrared spectroscopy; Concanavalin A monolayer; Polysaccharide; Monosaccharide

Summary

The effects of pH, Mn^{2+} and Ca^{2+} and urea denaturation on the interaction of monolayers of concanavalin A on saline with the polysaccharide dextran B-1355 and the monosaccharides methyl α-D-mannopyranoside and D-galactose have been investigated. Infrared absorption spectra of compressed monolayers of the protein and the protein-dextran complex coated on a germanium plate have been obtained by means of attenuated total reflectance spectroscopy. Except in one case of denaturation, the amide I absorption of concanavalin A peaked around 1631 cm^{-1}, indicating a predominance of the β-pleated sheet conformation, in agreement with its secondary structure in the solution and crystalline phases. The contribution to the absorbance of the concanavalin A-dextran films at 3300 cm^{-1} due to absorption by the O-H stretching modes of the polysaccharide is a measure of its binding. Increasing the pH from 6.1 to 7.5 appreciably reduced the dextran binding, at pH 9.3 the binding was zero. Adding 1 mM Mn^{2+} and Ca^{2+} to the subphase at pH 7.5 restored both the dextran binding and the affinity of concanavalin A for methyl α-D-mannopyranoside to that of the native protein at pH 6.1. At this latter pH, the weak binding of dextran to monolayers of demetallized concanavalin A (apo-concanavalin A) was also restored to that for the native molecule by the addition of these divalents. This indicates the requirement of concanavalin A for these ions to maintain the integrity of the saccharide-binding site. The loss of dextran binding with urea denaturation was also observed. These results parallel those for solu-

Abbreviation: ATR, attenuated total reflectance spectroscopy.

tions of the protein, indicating the validity of the monolayer system for the study of these interactions.

Introduction

In recent times there has been much interest in the plant protein, concanavalin A, due to its unusual effects on animal cells. This protein agglutinates erythrocytes [1] and transformed fibroblasts [2] and stimulates patching, capping [3] and mitosis [4] in rat thymocytes. The biological effects of concanavalin A appear to be related to its sugar-binding properties. The saccharide-binding specificity in solution has been shown to be directed toward the monosaccharides glucose and mannose, which contain similar hydroxyl configurations at the 3-, 4-, and 6-positions [5]. Since an equatorial hydroxyl at the 4-position is essential for this binding [6], D-galactose with an axial hydroxyl at this position does not bind to concanavalin A.

The present research was undertaken to extend the study of concanavalin A-sugar interactions from solution [5—9] and crystalline phases [10] to that of surfaces, namely monolayers of concanavalin A on saline. Since most of the biological effects of concanavalin A occur at cell membranes, this objective is of some interest even though the system chosen is quite removed from a biological membrane. The ideal model system would consist of unilamellar phospholipid vesicles containing either glycolipids or glycoproteins with the appropriate sugar groups oriented on the outer surface. For concanavalin A monolayers on saline, the concanavalin A-sugar interactions occur near the air/saline interface (non-ideal) but the protein molecules are in ordered arrays in which their orientation relative to the surface might be an approximation to that on cell membranes. The monolayer system should complement that of concanavalin A in solution where the protein is entirely in aqueous but randomly oriented. A similar objective has been expressed in a recent investigation of the interaction between concanavalin A and monolayers of glycolipids [11]. For monolayers of concanavalin A on saline to be an acceptable model system for the study of the protein-sugar interactions, the following conditions should be satisfied: (a) the saccharide-binding specificity observed in solution must be operative, namely, methyl α-D-mannopyranoside binds strongly but D-galactose not at all; (b) the effects of pH and protein denaturation on sugar binding should agree with those observed in solution; (c) the absence of Mn^{2+} and Ca^{2+} from their appropriate sites on concanavalin A should drastically reduce the binding of saccharides to the protein [12]. It will be demonstrated that these conditions are essentially met by monolayers of concanavalin A on saline.

The method which will be used to study these films is that of attenuated total reflectance spectroscopy (ATR) in the infrared [13]. It can give the vibrational absorption spectrum of a monolayer attached to a substrate which is transparent and has a large index of refraction in the infrared. Monolayers of several proteins have been previously studied in this manner [14,15]. The infrared spectrum in the amide absorption region, 1700—1500 cm^{-1}, characterizes the secondary structure of the protein film [16]. The absorption of the O-H stretching mode at approx. 3300 cm^{-1} determines the amount of the polysac-

charide, dextran B-1355, bound to the concanavalin A film. The competitive binding between dextran and the monosaccharides, methyl α-D-mannopyranoside and D-galactose, for concanavalin A as determined by this absorption, will be a measure of the monosaccharide binding. The lack of sufficient O-H absorption by the monosaccharides precludes the direct determination of their binding to concanavalin A.

The principal purpose of this paper is to show the effects of the film pressure, pH, urea and Mn^{2+}, Ca^{2+} on the binding of dextran, methyl α-D-mannopyranoside and D-galactose to monolayers of concanavalin A. In addition, their effect on the conformation and orientations of the polypeptide chains will be investigated.

Materials and Methods

Chemicals. Native concanavalin A is from Miles-Yeda, Miles Laboratories (Elkhart, IN) as a lyophilized powder. Apo-concanavalin A was prepared as previously described [17]. Both proteins were gifts from Dr. C.F. Brewer, A. Einstein College of Medicine. Ultrapure urea was obtained from the Schwarz-Mann Division of Becton, Dickinson (Orangeburg, NY). Dextran B-1355 as lyophilized strips was a gift from Dr. Hehre, A. Einstein College of Medicine. Methyl α-D-mannopyranoside is from Pfanstiehl Laboratories (Waukegen, IL) and D-galactose is from Sigma Chemical (St. Louis, MO).

Solutions. All the solutions were prepared with doubly distilled water which was normally at pH 6.1. Stock solutions of the protein were at concentrations of 0.07—0.10 mg/ml in 0.15 M NaCl at pH 6.1. For some of the experiments, $CaCl_2$ and $MnCl_2$ were added to give 1 mM concentrations. Concanavalin A solutions, without divalents, were brought to pH 6.8 and 7.5 by adding HCl to 0.02 M Na_2HPO_4, for pH 9.3, NaOH was added to 0.03 M phosphate. For the subphase, 0.15 M NaCl solutions were prepared and also buffered with Na_2HPO_4 at pH 6.8, 7.5 and 9.3. For the experiments at pH 7.5 which required 1 mM Ca^{2+} and Mn^{2+}, the phosphate buffer was replaced by 0.01 M Tris (Sigma Chemical). Dispersions of dextran in water were prepared at concentrations of approx. 2 mg/ml and aqueous solutions of both monosaccharides at approx. 35 mg/ml.

For the denaturation of concanavalin A by urea, a 2.7 mg/ml protein solution in 1 M NaCl at pH 7.4 was treated with 5.4 M urea for 40 min. The protein solution was then diluted 1 : 20 for spreading as a monolayer.

Preparation of monolayers. Monolayers of concanavalin A were prepared by either spreading an appropriate amount of stock solution directly on the saline surface or by slowly depositing drops of stock solution on the surface of a 5 mm diameter glass rod immersed in the subphase (method of Trurnit [18]). The subphase filled a Teflon tray of 324 cm^2 (26 cm × 12.5 cm) area and 680 ml volume. Prior to spreading, the surface was cleaned by skimming with a Teflon barrier until compression of the surface with the barrier gave zero surface pressure (surface tension equals that of saline). The surface tensions were measured by the Wilhelmy balance method [19] using a sandblasted platinum blade suspended from a torsion balance.

The monolayers formed by direct spreading and used for most of the studies required approx. 4 h to reach a stable surface tension. The monolayers

prepared by the method of Trurnit [18] attained a steady-state in less than 1 h but were not as stable as the spread films. To attain comparable initial surface pressures of approx. 10 dynes/cm at pH 6.1, the films prepared by the method of Trurnit [18] required approx. 0.16 mg of concanavalin A compared to 0.35 mg for the spread films. In the former, part of the monolayer is already formed on the glass rod prior to sliding onto the saline surface, for the latter there is a slow partitioning of the protein between the solution phase and the surface. The monolayers were compressed by the slow manual movement of the Teflon barrier. Both types of films gave similar isotherms and spectra and showed comparable interactions with saccharides.

The sugars were added by injecting a total of 6 ml of solution under approx. 30 regions of the compressed monolayers. After 40 min the subphase was gently stirred several times with a U-shaped glass frame. The film was lifted for spectroscopic examination 1 h after the stirring.

Spectroscopic measurements. The monolayers were coated on a freshly cleaned [20] Ge plate (50 mm × 20 mm × 2 mm) which served as the optical medium for the totally reflected beam. The film deposition was done by the Langmuir-Blodgett method [21]; the immersed hydrophilic plate is slowly lifted (approx. 1 mm/min) through the surface at the desired surface pressure, thereby depositing a monolayer of concanavalin A on all Ge surfaces with the hydrophobic surfaces of the protein oriented away from the plate. The thin opposite edges of the trapezoidal Ge plate are cut to give an incidence angle of 30° for the light beam, yielding 45 internal reflections. During the deposition, the film pressure is kept approximately constant by manually adjusting the barrier. This adjustment always corresponded to a reduction in film area approximately equal to the area of the Ge plate. The spectroscopic arrangement for obtaining both unpolarized and polarized spectra has been previously described [20].

Results

Isotherms

Typical isotherms obtained for the concanavalin A monolayers are presented in Fig. 1. They are characterized by a linear region at medium surface pressures, with the slope decreasing with increasing compression. This latter behavior, in the case of monolayers of synthetic polypeptides, has been shown to be due to the initiation of a second layer of molecules [22]. A corresponding non-linear increase in the absorbance of the 1631 cm^{-1} band at large surface pressures, observed in this study at pH 6.1 and 7.5, appears to support this interpretation for these cases. It should be mentioned, however, that the flattening of the isotherm at high film pressures, which has also been observed for other proteins [14,23], has been explained differently. For β-lactoglobulin, it was attributed to the observed change in conformation from an α-helix to β-pleated sheets [15]. For bovine serum albumin it has been attributed to a desorption of the protein from the film phase [23]. These alternate explanations do not appear to be applicable to concanavalin A. A decrease in the surface pressure at which the isotherms flatten appears to be characteristic of high pH and urea treatment, conditions associated with protein denaturation. As will be shown later, these

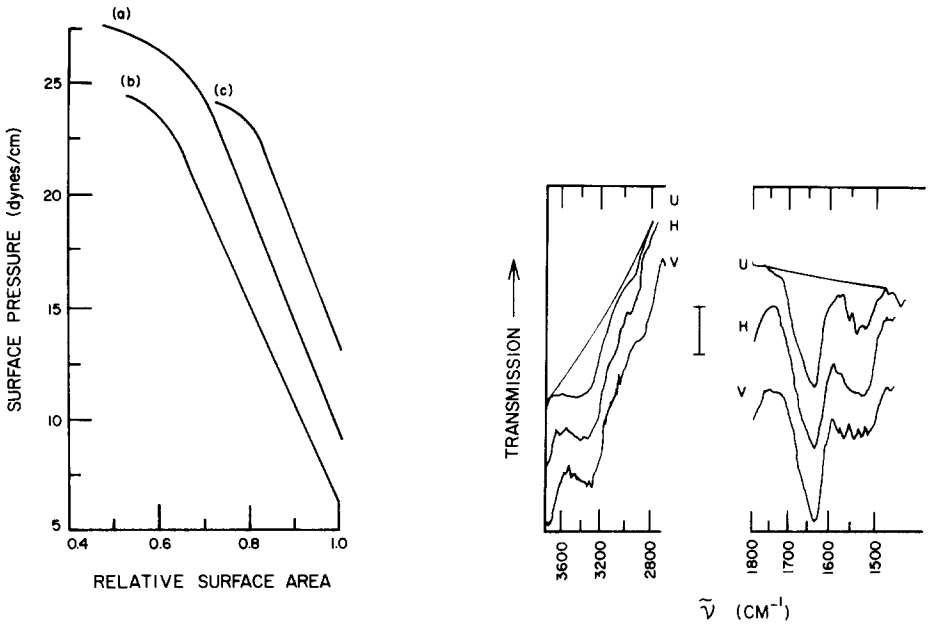

Fig. 1. Isotherms of concanavalin A monolayers on 0.15 M NaCl: (a) 0.35 mg concanavalin A at pH 6.1 spread directly on saline; (b) 0.27 mg concanavalin A treated with urea at pH 7.5 and spread by the method of Trurnit [18] (concanavalin A treated by 5.4 M urea for 40 min, then diluted to 0.27 M for spreading); (c) 0.16 mg concanavalin A at pH 9.3 spread by the method of Trurnit.

Fig. 2. Infrared spectra of compressed monolayers of concanavalin A at pH 6.1 on 0.15 M NaCl. U, unpolarized; V, incident radiation polarized perpendicular to incident plane; H, incident radiation polarized parallel to incident plane. 100% transmission lines used for determining the absorbances for the 3250 cm^{-1} and 1631 cm^{-1} bands are shown for the U spectra. The vertical bar indicates an absorbance of 0.01. The surface pressure for these films was 26 dynes/cm. The subphase contained 1 mM $MnCl_2$ and $CaCl_2$. (These spectra and those shown in the following figures were recorded with an expanded scale giving a magnification of five in the percent transmission mode.)

denatured films are unable to appreciably bind dextran. In addition, monolayers of apo-concanavalin A, the isotherm of which resembles Fig. 1c, have lost most of the dextran-binding capability of the native protein. Consequently, we have the interesting case of being able to relate the behavior of the isotherm of a protein monolayer to the integrity of the small saccharide-binding site. The shift in the isotherm for the urea-treated protein relative to the native molecule has also been observed in the case of bovine serum albumin [23]. The binding of methyl α-D-mannopyranoside and dextran did not affect curve (a).

Spectra

Typical spectra of compressed monolayers of concanavalin A in two regions of amide absorption are shown in Fig. 2. The spectra are expanded by a factor of 5 in the percent transmission mode. The peak of the 1631 cm^{-1} band in the unpolarized spectrum has an absorption of 5.3%, corresponding to an absorbance of 0.023. The three major bands are located at approx. 3260 cm^{-1} (amide A), 1631 cm^{-1} (amide I) and 1528 cm^{-1} (amide II). The motions mainly responsible for these absorptions are the N-H stretch, the C=O stretch and a

combination of N-H deformation and C-N stretch, respectively. Of most interest is the strongest of these bands, that of the amide I absorption, since its frequency is a measure of β-pleated sheet conformations in the secondary structure of the protein. Except in the case of one denaturation treatment to be discussed in a later section, the amide I peak was at approx. 1631 cm^{-1}, indicative of β-pleated sheet configurations [16]. This frequency was independent of pH for values ranging from 5.5 to 9.3, and of surface pressures varying from 8 to 30 dynes/cm. The dichroism of this band determines the average orientation of the C=O transition moment which approximates this bond's orientation. For the spectra in Fig. 2, the absorbance for the vertical polarization relative to that for the horizontal polarization was 1.10 for this band and 0.94 for the amide A band. These values indicate that the average orientations of both the C=O and N-H bonds are parallel to the air/aqueous interface [20]. These orientations were found to be independent of pH, pressure and sugar binding. The poorer quality of the vertical spectrum in Fig. 2 is due to the smaller aperture of the Brewster's angle Ge polarizers for this orientation. The narrow bands between 1500 and 1600 cm^{-1} are due to uncompensated H_2O vapor absorption because of incomplete N_2 purging of the optical paths.

Binding of sugars

Since the sensitivity of the spectrometer was insufficient to observe the binding of monosaccharides to concanavalin A, two indirect methods were used for this determination. They both depend on the observable absorption of the 3300 cm^{-1} O-H stretching mode of the polysaccharide, dextran, bound to the concanavalin A monolayer. The first method depends on the displacement of bound dextran by monosaccharides according to their binding affinity for concanavalin A [24]. The second depends on the inhibition of dextran binding by monosaccharides bound to the protein [6]. An example of such a study is illustrated in Fig. 3. In Fig. 3a is shown the enhanced absorption at 3300 cm^{-1} due to the binding of dextran and the effect on this absorption of subsequent additions to the subphase of either methyl α-D-mannopyranoside or D-galactose *. It is clearly seen that the addition of the former displaces some of the bound dextran while that of D-galactose has no appreciable effect. The absorbance of the O-H absorption of the bound dextran was obtained in the following manner: first, base lines for 0% absorption were estimated in the 3300 cm^{-1} region of both the unexpanded and expanded spectra; examples of the latter are shown for concanavalin A and concanavalin A + methyl α-D-mannopyranoside in Fig. 3a and b, respectively. Using these baselines, the percent transmission and absorbance at 3300 cm^{-1} was determined for the concanavalin A + dextran films. Next, the absorbance at this frequency for the N-H stretching mode of concanavalin A was obtained from the spectrum of the concanavalin A monolayer. Subtracting this value from that obtained from the concanavalin A + dextran film yields the absorbance of the dextran absorption. This analysis applied

* To obtain these spectra the sequence of operations were as follows: (1) Initial concanavalin A monolayer deposited on Ge plate and spectrum recorded; (2) Ge plate cleaned and film of concanavalin A + dextran deposited on plate and its spectrum recorded, and (3) Ge plate cleaned and film of concanavalin A + dextran + methyl α-D-mannopyranoside or D-galactose deposited on plate and spectrum recorded.

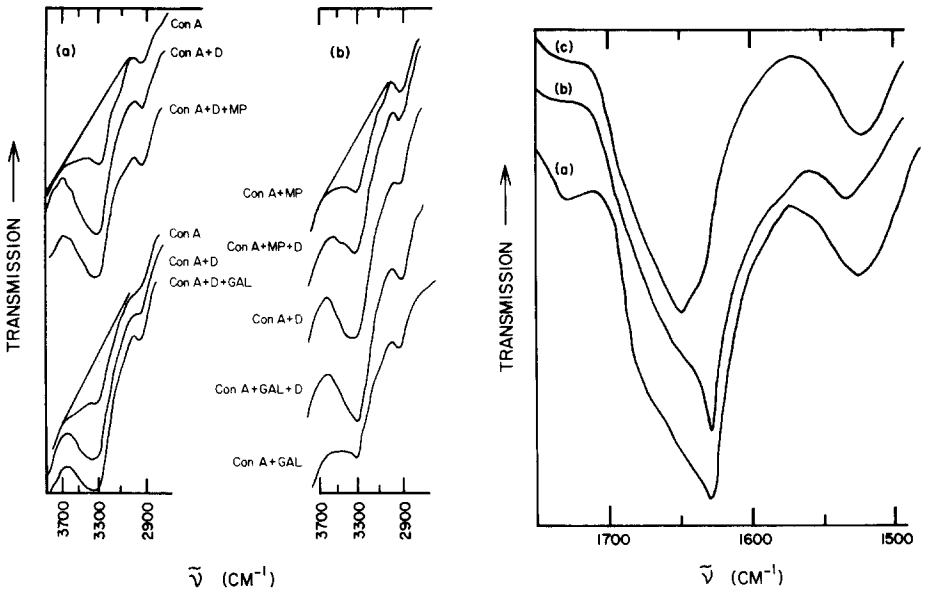

Fig. 3. Infrared spectra of compressed monolayers of concanavalin A at pH 6.1 and after interacting with dextran, methyl α-D-mannopyranoside and D-galactose (D-Gal). (a) exchange of bound dextran by methyl α-D-mannopyranoside and D-galactose; (b) inhibition of dextran binding by methyl α-D-mannopyranoside and D-galactose. 100% transmission lines used for determining the absorbances at 3300 cm^{-1} for the concanavalin A and concanavalin A + methyl α-D-mannopyranoside spectra are shown. Surface pressure of monolayers produced by spreading 0.35 mg of protein was 26 dynes/cm. The subphase contained 1 mM $MnCl_2$ and $CaCl_2$. Approx. 10 mg of dextran in suspension and 200 mg of monosaccharides in solution were injected into subphase. Interaction time for the saccharides was 1 h 40 min. D, dextran; MP, methyl α-D-mannopyranoside; GAL, D-galactose.

Fig. 4. Effects of denaturation on the amide I, II spectra of compressed monolayers of concanavalin A. (a) pH 6.1 (normal); (b) pH 9.3; (c) after Ge plate coated with normal film is immersed in water for several minutes and dried. For (a and c) surface pressure was 26 dynes/cm; for (b), it was 24 dynes/cm.

to the example shown in Fig. 3a showed that the addition of methyl α-D-mannopyranoside produced a 25% loss in the amount of bound dextran while that of D-galactose produced a surprising 37% increase. However, it turns out that during the interaction time (approx. 100 min) of the monosaccharides with the concanavalin A + dextran monolayer, additional dextran binds to the film. This was determined by comparing the binding of dextran for the original 100 min with that for an additional 100 min. When this is taken into account for the case illustrated in Fig. 3a, the addition of methyl α-D-mannopyranoside displaced 55% of the bound dextran while D-galactose had a negligible effect. If sufficient time were allowed for the dextran binding to saturate, the experiments would become impractically lengthy.

In Fig. 3b is displayed the effect of these two monosaccharides on the subsequent binding of dextran. An analysis of the top three spectra at 3300 cm^{-1} shows that the addition of methyl α-D-mannopyranoside to the subphase under a concanavalin A monolayer produced a 59% inhibition in the subsequent binding of dextran. The bottom three spectra show that D-galactose has no such effect. In fact, the intensity of the 3300 cm^{-1} band is increased by 18% in the latter experiment. Possible explanations for this unexpected result will be dis-

cussed later. These results on the specificity of sugar binding at film pressures of approx. 26 dynes/cm at pH 6.1 together with those for similar experiments at pH 6.8 and 7.5 are given in Table I. The consistency of these experiments is illustrated by the agreement between the results for the exchange of bound dextran and for the inhibition of dextran binding at pH 6.1 with divalents added to the subphase. Raising the pH to 6.8 and 7.5 appreciably decreased the binding of dextran unless additions of Ca^{2+} and Mn^{2+} were made to the subphase. At pH 7.5 the addition of these ions were also found necessary for maintaining the specificity of the binding.

A remark should be made here concerning the possibility of absorption by the O-H stretching modes of any water bound to concanavalin A in the 3400—3200 cm^{-1} region. The ratio of the absorbance of the amide I band to that of the amide A was 2.1 ± 0.2 for monolayers of concanavalin A for several values of surface pressure and pH as well as different denaturation treatments. This ratio also had the same value for absorbed films of concanavalin A (not reported here), ovalbumin, bovine serum albumin and rabbit serum albumin [20]. Since the amide A band is in the region of maximum water absorption its intensity would be sensitive to changes in the water content of these proteins. The constancy of this ratio for these different cases, where the amount of bound water would not be expected to be the same, indicates an insufficient quantity of bound water to contribute to this spectral region.

TABLE I

EFFECTS OF pH, Ca^{2+} AND Mn^{2+} AND UREA ON THE BINDING OF DEXTRAN AND CONCANAVALIN A-MONOSACCHARIDE-BINDING SPECIFICITY FOR COMPRESSED MONOLAYERS OF CONCANAVALIN A ON SALINE

Monolayers of concanavalin A were prepared by spreading 0.16—0.35 mg either directly or by the method of Trurnit [18]. They were compressed to surface pressures of 24—26 dynes/cm. Solutions containing 10 mg of dextran B-1355 and 200 mg of either methyl α-D-mannopyranoside (α-D-MP) or D-galactose were then injected into the subphase in this order or the reverse one. Phosphate buffer varying from 0.02 M to 0.03 M maintained the pH above 6.1. The absorbance of the dextran absorption at 3300 cm^{-1} was obtained by subtracting the contribution of the concanavalin A absorption from that of the concanavalin A-dextran films. S.D. are given when more than one measurement was taken, the number is indicated in parenthesis. Percent specificity = (1 — bound dextran (after monosaccharide exchange or binding)/bound dextran (without monosaccharide interaction)) × 100%. 100% implies high monosaccharide affinity, 0% no binding; a large difference between values for methyl α-D-mannopyranoside and D-galactose implies high specific binding; negative values indicate increase in absorbance at 3300 cm^{-1} after sugar interaction (see text for explanation).

pH	Absorbance of bound dextran	Percent specificity			
		Exchange of bound dextran		Inhibition of dextran binding	
		α-D-MP	D-galactose	α-D-MP	D-galactose
6.1	0.010 ± 0.002 (10)	70 ± 8 (3)	5		
6.1 *	0.011 ± 0.002 (3)	49 ± 9 (2)	—15 ± 24 (2)	59	—18
6.8	0.004 ± 0.000 (2)			100	—25
7.5	0.004 ± 0.001 (3)			62 ± 12 (2)	52 ± 10 (2)
7.5 *	0.013 ± 0.002 (2)			64	—1
7.5 **	0.001				
9.3	0.000				

* 1 mM Ca^{2+} and Mn^{2+} in subphase.

** Concanavalin A treated with 5.4 M urea.

To further test the applicability of concanavalin A monolayers as a model system for studying concanavalin A-sugar interactions, the effect of various denaturation procedures on the binding of dextran was investigated. It had been previously shown that the CD spectra of solutions of concanavalin A at pH 9.3 [9,25,26] and of concanavalin A treated with 8 M urea at pH 7.1 [26] exhibited large blue shifts of the 223 nm minimum characteristic of the amide structure of the native molecule. Accompanying this perturbation of the secondary amide structure were losses in the hemagglutination activity. It had also been shown that the binding of dextran sharply decreases as the pH increases above 8 [27]. Similar treatments of the concanavalin A monolayers perturbed the isotherm (see Fig. 1) and greatly reduced dextran binding (see Table I). In addition, at pH 9.3, the spectrum of the amide I and amide II bands gives an indication of a change in amide structure (see Fig. 4).

If the Ge plate, coated with a monolayer of concanavalin A, is immersed in water or 0.15 M NaCl at pH 6.1 for a few minutes and then dried, the amide I band exhibits an appreciable change in shape and maximum frequency (see Fig. 4). The change in frequency from 1631 cm^{-1} to 1650 cm^{-1} is indicative of a loss in β-pleated sheet amide conformation. The altered concanavalin A film binds very little dextran.

Discussion

Conformation and orientation of the polypeptide chains

From the correlation of the infrared spectra of films of synthetic polypeptides with their X-ray and electron diffraction patterns, it has been shown that an amide I band at approx. 1630 cm^{-1} corresponds to β-pleated sheet amide conformations [28]. For α-helix and random-coil conformations the frequency is approx. 1650 cm^{-1}. These results appear to be valid for the few proteins which have been investigated by both infrared and diffraction methods. For the concanavalin A monolayers in this study the amide I peak was at 1631 $\pm$ 3 cm^{-1} for film pressures between 8 and 20 dynes/cm, at pH values varying from 5.5 to 9.3 and with 5.4 M urea treatment of the protein. The absorption on the high-frequency side of the peak resulting in the asymmetry of this band (see Fig. 4a and b) is probably due to the weak 1680 cm^{-1} band characteristic of antiparallel β-pleated sheets [16] and that at approx. 1650 cm^{-1} due to the contribution by non-β structures. Consequently, concanavalin A in monolayers maintains the predominant β-pleated sheet configuration evident in both its solution and crystal phases. Only when the monolayers coating the Ge plate are exposed to aqueous does the amide I peak appear at 1650 cm^{-1}, indicating a loss of most of the β-structure. An explanation for this effect cannot be given although β to α-helix transitions in synthetic polypeptides have been generated by solvents which are strong hydrogen bond breakers like *m*-cresol [29].

The average orientation of the polypeptide chains in these films was determined from measuring their spectra for incident light polarized first in the incident plane (H) and then perpendicular to it (V). A typical example of such spectra is shown in Fig. 2. For an absorption band the ratio of the absorbances for the two polarizations, namely, $A_V/A_H = D$, is related to the angle, θ, which its transition moment makes with the normal to the surface by the equation,

$\cot^2\theta = (1 - 0.92\ D)/2.46\ D$, for an isotropic distribution of moments about the normal [20]. Within the experimental uncertainties of these measurements, the directions of both the N-H and C=O stretching moments were found to be parallel to the surface for the films studied ($D \simeq 1$ giving $\theta \approx 90°$). This implies that the polypeptide chains, on the average, lie parallel to the surface. For the absorption at 3300 cm^{-1} due to the OH groups in dextran bound to the concanavalin A monolayers, a value for D of 0.41 was found. This dichroic ratio is indicative of a random distribution of transition moments which would be expected for the bound dextran.

Saccharide binding

The results of the studies on the binding of dextran to compressed monolayers of concanavalin A are summarized in Table I. This binding did not affect the shape of the amide I and II bands, indicating the absence of any strong perturbations of the average amide configurations. This is in agreement with the results for monosaccharide binding from CD studies in solution [9], although both NMR [8] and X-ray studies [10] showed that monosaccharide binding produces conformation changes near the binding site in the solution and crystalline phases, respectively.

At pH 6.1, methyl α-D-mannopyranoside displaced most of the bound dextran or inhibited its binding to concanavalin A. In contrast, D-galactose either had no effect or appeared to produce an increase in the absorbance at 3300 cm^{-1} which is indicative of an increase in dextran binding. A possible explanation for this peculiar result which also occurred at pH 6.8 (Hehre, E.G., personal communication) is that the long strands of M_r 46 · 10^6 dextran molecules bound to the monolayer may be trapping a sufficiently large number of monosaccharides to contribute to the O-H absorption at 3300 cm^{-1}. To explain this additional absorbance in the D-galactose experiments assuming this sugar has no effect on dextran binding to concanavalin A (percent specificity is zero) requires each bound dextran to trap approx. 45 · 10^3 D-galactose molecules. This translates into one trapped monosaccharide molecule per five glucose molecules on each dextran. A consideration of this mechanism, which would be equally operative in the methyl α-D-mannopyranoside experiments, would increase the percent specificity of this saccharide from 49 to 60% and from 59 to 77% for the two experiments at pH 6.1 with the addition of divalents and from 100 to 125% at pH 6.8 (see Table I). Since the specificity of the binding depends on the difference between the percentages for methyl α-D-mannopyranoside and D-galactose, respectively, it can be seen from Table I that these questionable adjustments for trapping have very little effect on these differences. Although the results for the D-galactose experiments at pH 6.1 (without divalents) and pH 7.5 did not give negative values for the percent specificity, there does not appear to be any reasons for not invoking monosaccharide trapping in these cases as well. For these, trapping would increase the percent specificities approx. 15% for both the methyl α-D-mannopyranoside and D-galactose interactions. The amount of bound dextran and the percent specificity for concanavalin A monolayers at pH 6.1 and at a pressure of 16 dynes/cm (linear region) were the same as at 26 dynes/cm. This indicates that the presumed initiation of a bilayer at the higher pressure does not affect the saccharide-binding site.

Increasing the pH of the subphase decreased the dextran binding by a factor of approx. 2.5 at pH values of 6.8 and 7.5. At pH 9.3 the binding of the polysaccharide was eliminated for film pressures of 19 dynes/cm (linear) and 24 dynes/cm. This behavior differs somewhat from the effect of pH on the precipitation of dextran by concanavalin A in solution [27]. In the latter, the precipitation was maximum between 6 and 7.5 and then dropped-off sharply to a very small value at 9.4. In addition, for concanavalin A monolayers, it appears that at pH 7.5 the binding of the monosaccharides has a nonspecific character, since both methyl α-D-mannopyranoside and D-galactose equally inhibited the binding of dextran. Adding 1 mM Ca^{2+} and Mn^{2+} to the subphase at pH 7.5, however, restored both the binding of dextran as well as the saccharide specificity to approximately the values at pH 6.1. This would seem to indicate that increasing the pH above 6.8 starts to remove Mn^{2+} and Ca^{2+} from the S1 and S2 sites on concanavalin A, respectively. It has been shown in solution [30], that removal of these ions causes a sufficient change in the conformation of the sugar-binding site to prevent saccharide binding. This behavior also seems to be present in concanavalin A monolayers. Consistent with this interpretation was a 70% reduction in the binding of dextran at pH 6.1 to monolayers of apo-concanavalin A relative to the native molecule. Addition of 1 mM Ca^{2+} and Mn^{2+} to the subphase restored the binding to its normal value. The loss of dextran binding at pH 9.3 may be attributed to both the loss of Ca^{2+} and Mn^{2+} and to the change in the amide conformation. The latter has been observed in solution by CD [9,25,26] and is also indicated by the change in shape of the amide I band in the present study (see Fig. 4). Since a suitable buffer could not be found which would allow the addition of these divalents at this pH, it was not possible to determine the relative importance of these effects.

Effects of additional protein denaturation on dextran binding

Concanavalin A monolayers formed from a 0.27 M urea-spreading solution (following dilution of 5.4 M urea solution) at pH 7.5 and compressed to approx. 24 dynes/cm bound dextran very weakly. Since the binding was considerably smaller than that measured at pH 7.5 without urea, the urea must be responsible for the additional loss. This is not unexpected since it had been previously shown [26] that urea-treated concanavalin A suffers a loss of its hemagglutination capability. In addition, this treatment had been shown [26] to change the CD spectra in the amide region; in the present study, the shapes of the amide I and II bands were unaffected. However, the absorbances of the bands were increased by 50% with respect to the untreated protein. Since the coated Ge surface also became partially hydrophilic, this indicates that the urea-treated concanavalin A appears to form a substantial bilayer at the air/aqueous interface at film pressures corresponding to the bending of the isotherm.

A very unexpected result was the effect of immersing the Ge plate coated with a concanavalin A monolayer into water or 0.15 M NaCl at pH 6.1. This procedure produced a change in the amide I band, shifting its maximum from 1631 cm^{-1} to 1650 cm^{-1} (see Fig. 4), indicating the loss of a sizeable portion of the β-pleated sheet conformation. Although the explanation for this change cannot be given, β to α-helix conformation changes have been observed in syn-

thetic polypeptides treated with solvents which are capable of breaking interchain hydrogen bonds [29]. Accampanying this change in conformation was the inability of the modified concanavalin A film to bind dextran. Therefore, it appears that the β-polypeptide structure is a requirement for the viability of the carbohydrate-binding site.

Concluding remarks

The principal result of this study is that monolayers of concanavalin A spread on a saline subphase interact with the polysaccharide, dextran B-1355, and the monosaccharides, methyl α-D-mannopyranoside and D-galactose, in a similar manner as in solution. These interactions can be summarized as follows: (a) concanavalin A monolayers at pH 6.1 strongly bind dextran, methyl α-D-mannopyranoside displaces most of the dextran while D-galactose displaces very little, if any. The monolayers bind methyl α-D-mannopyranoside but not D-galactose at the carbohydrate-binding site of concanavalin A as indicated by the inhibition of dextran binding by the former but not the latter. Consequently, the specific binding site for saccharides on concanavalin A is intact in the monolayer phase. Apparent deviations from ideal specificity may be due to the nonspecific trapping of the monosaccharides by the bound dextran. (b) Increasing the pH from 6.1 causes a 60% reduction in dextran binding at values of 6.8 and 7.5 and a complete loss at pH 9.3. This behavior is also in qualitative agreement with results in solution. (c) Treating concanavalin A with a concentrated urea solution prior to forming monolayers also greatly reduces its binding of dextran; this result parallels a loss in the hemagglutination capability of urea-treated concanavalin A solutions. (d) Monolayers of apo-concanavalin A at pH 6.1 weakly bind dextran; addition of 1 mM Ca^{2+} and Mn^{2+} to the subphase restores the dextran binding. As a consequence of the concanavalin A-sugar interactions just enumerated, it appears that a monolayer of concanavalin A spread on saline is a valid 'model system' for the study of these interactions.

Since the peak of amide I absorption is at approx. 1631 cm^{-1}, the polypeptide chains are principally in the β-pleated sheet conformation in the concanavalin A monolayers, This is in agreement with the observations for both the solution and crystalline phases of concanavalin A. The dichroic amide spectra indicates that the chains are folded parallel to the air/aqueous interface. The binding of dextran and methyl α-D-mannopyranoside does not appreciably alter the amide spectra. The binding of methyl α-D-mannopyranoside to concanavalin A in the crystalline phase caused large shifts of atomic positions near the binding site as well as a disruption of some of the β-structure [10]. Either these effects do not occur in monolayers, or more likely, these changes are too localized to be observed in this study.

Two treatments of the monolayers were found to alter the amide I absorption. Increasing the pH to 9.3 produced a change in the amide I band shape without altering the frequency of its maximum with the amide II shifting to higher frequencies. This indicates a perturbation of the average amide conformation without changing the predominance of the β-pleated sheet configuration. Inserting the coated Ge plate into aqueous for a few minutes shifted the

absorption maximum to approx. 1650 cm^{-1} indicating a change in amide conformation to either α-helix or random coil or a combination of these. Correlated with both treatments and their effect on perturbing the amide configuration was a loss in the capability of concanavalin A to bind dextran. This indicates that a disruption of the average amide conformation produced a structural change in the carbohydrate-binding site.

Acknowledgements

The author wishes to thank Drs. W. Ganz and C.F. Brewer from this institution for many helpful discussions. In addition, Dr. I. Listowsky kindly measured the CD spectra of several samples.

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