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CIRCULAR DICHROISM OF LIMULIN: *LIMULUS POLYPHEMUS* LECTIN

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1. Introduction

The lectin of *Limulus polyphemus* hemolymph has been isolated by preparative ultracentrifugation and starch gel electrophoresis [1,2] and by DEAE–Sephadex and gel filtration [3]. With these techniques, the purification factor was in the range 20–80. The circular dichroism of this purified material has been reported [2,4]. However, more recently it was shown that though the above material was homogeneous with respect to molecular weight, polyacrylamide gel electrophoresis and immunoelectrophoresis, it was quite heterogeneous with respect to crossed immunoelectrophoresis. A pure lectin was obtained by affinity chromatography on immobilized bovine submaxillary mucin [5–7]. The lectin (Limulin) was specifically eluted with a Ca^{2+}-free buffer and the purification factor was 33 000 [6]. The present study of circular dichroism of Limulin was undertaken to assess:

(i) The nature and the extent of secondary structure in the protein.
(ii) The existence of a correlation between the Ca^{2+}-binding capacity of the lectin and the conformation of the protein.

2. Materials and methods

Frozen hemolymph from *L. polyphemus* was obtained from the Marine Biological Laboratory, Woods Hole, MA. Limulin was prepared according to [6]. The active material obtained after the chromatography on DEAE–Sephadex column was applied to a column of bovine submaxillary mucin substituted Sepharose in a buffer containing $CaCl_2$. A large part of material passed through the column and was devoid of agglutinating activity (Limulin I). Some impurities were eluted by increasing the ionic strength (Limulin II), and the pure lectin was eluted with a Ca^{2+}-free buffer (Limulin III).

Protein solutions were adjusted to the desired conditions by exhaustive dialysis at 4°C against the appropriate buffer: Ca^{2+}-free buffer, 0.1 M NaCl, 0.05 M Tris–HCl, pH 8.5; buffer containing Ca^{2+}, 0.1 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris–HCl, pH 8.5.

Circular dichroism (CD) spectra were drawn using a Marck III dicrograph (Jobin-Yvon). Cells of 1 mm and 10 mm pathlength for the range 200–260 nm and 250–320 nm were used, respectively. The protein concentrations were from 0.1–0.3 mg/ml.

The mean residue molecular ellipticity (θ) was calculated taking 38 000 as the molar extinction coefficient of Limulin I and of Limulin III, and 255 as the number of amino acids/monomer (A.-C.R., unpublished results). The helical and β structure content of Limulin III were calculated according to the method in [8] but with set of reference spectra obtained using 9 proteins: myoglobin, ribonuclease, lysozyme, α-chymotrypsin, papain, subtilisin, concanavalin A, insulin, lactate dehydrogenase (J.-C.M., unpublished results).

3. Results and discussion

3.1. *The CD spectrum of Limulin I*

The CD spectrum obtained from the non-agglutinating fraction (Limulin I) in the presence of Ca^{2+} is illustrated in fig.1. The far ultraviolet part of this spectrum shows a negative maximum at 212 nm

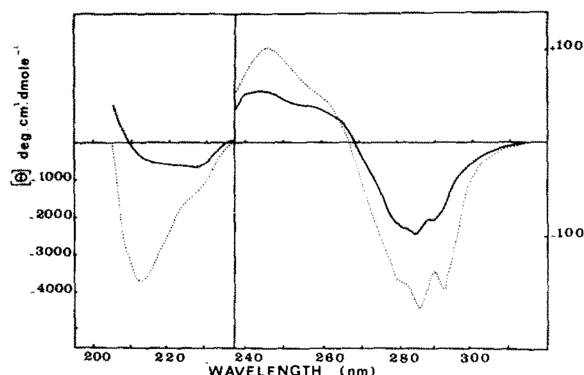

Fig.1. The far and near ultraviolet CD spectra of Limulin I and the pure lectin Limulin III, at pH 8.5, 0.01 M $CaCl_2$, 0.1 M NaCl, 0.05 M Tris–HCl. (· · · · · ·) Limulin I; (———) Limulin III.

and a shoulder at 225–230 nm. The value of the intensity at the maximum is low (≃ 3700 deg.cm^2.dmol^{-1}). The shape of this spectrum is very similar to that in [4], but the intensities in our spectra are slightly larger. The near ultraviolet spectrum shows maxima at 292 nm, at 286 nm and a shoulder at 280 nm; there is also a positive maximum at 246 nm with a shoulder near 260 nm. This part of the spectrum is also very similar to that in [4]. It corresponds to the aromatic amino acid residues and also to the disulfide bridges. Contribution of the disulfide chromophore seems likely because of the presence of a signal at wavelength higher than 300 nm. However because this material was shown to be a mixture of proteins [6,7], we did not go further into the analysis of these data.

3.2. *The CD spectrum of Limulin III*

The CD spectrum of the agglutinating fraction corresponding to the lectin, Limulin III, is shown in fig.1. In the far ultraviolet, the spectrum has a very low intensity between 210 nm and 235 nm with a maximum near 225 nm. It is difficult to explain why the spectrum is of such a low intensity (probably one of the lowest reported for a protein, up to now). We tried to calculate the fraction of the various conformations involved in the secondary structure of the protein, using a method similar to that in [8,9]. Unfortunately, it was not possible to obtain a good fit; this is not unexpected since the amplitude of this spectrum is smaller than the difference usually obtained between experimental and calculated spectra using this type of method. Nevertheless, it is clear that this spectrum does not correspond to a protein with any appreciable amount of α-helix (<10%) or β structure. It has been generally thought that lectins contained a large amount of β structure, as in *Helix pomatia* agglutinin [10] in concanavalin A [11,12], in *Pisum sativum* lectin [13], in *Ricinus communis* lectin [14], in *Dolichos biflorus* and *Robinia pseudoacacia* lectins [15] and in *Bandeirea simplicifolia* lectin [6]. Wheat germ agglutinin (WGA) is a special case with only 10% of β structure and a negative trough at 206 nm [17]. Despite the difficulty in the analysis of the data, the particular shape of the CD spectrum of Limulin III cannot be explained by either a large number of S–S bridges (as it could be with WGA) because Limulin III contains only few S–S bridges [18], or by the contribution of the aromatic residues since their content is quite similar to that of many other proteins. The percentage of carbohydrate in Limulin is very low (about 3%) and it seems very unlikely that their contribution in this wavelength range could be responsible for the low ellipticity of the protein.

The CD spectrum in the near ultraviolet wavelength range has a shape similar to that of Limulin I but with a lower intensity. A small contribution of the disulfide chromophore seems likely as in the case of Limulin I.

3.3. *The effect of freezing on the CD spectrum of Limulin III*

Figure 2 shows the effect of freezing on the circular dichroism of the protein. The negative band became narrower with a maximum at 228 nm, the CD is positive for wavelengths higher than 218 nm. This clearly demonstrated that upon freezing Limulin, there was an irreversible change of the conformation of the protein. However, the agglutinating activity was only slightly lowered after one freezing and one thawing cycle.

3.4. *The effect of Ca^{2+} on the conformation of Limulin III*

It has been shown that the lack of calcium in the Limulin sample led to drastic changes of its biological properties [1]: the agglutinating activity and the

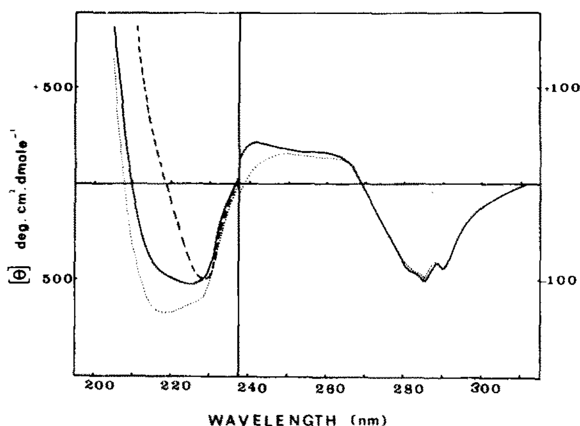

Fig.2. The far and near ultraviolet CD spectra of the pure Limulin III, at pH 8.5, 0.1 M NaCl, 0.05 M Tris–HCl. (———) In the presence of 0.01 M $CaCl_2$, freshly prepared; (· · · · ·) in the absence of Ca^{2+} ions, freshly prepared; (- - - -) after thawing a frozen solution (0.01 M $CaCl_2$).

adsorption to an immobilized mucin was abolished. Therefore, it was of interest to compare the CD spectra of the Limulin in the presence and absence of calcium. The absence of calcium induced an increase in the ellipticity in the far ultraviolet wavelength range. However even with this increase the CD signal remained very small; the maximum ellipticity being about 700 deg.cm^2.dmol^{-1} at 217 nm. The aromatic part of the CD spectrum does not show any noticeable change. These experiments demonstrated that Ca^{2+} induced a conformational change of the protein, in which the environment of the aromatic amino acid did not vary substantially. Therefore, the conformational change induced by withdrawing Ca^{2+} was very limited. In the opposite, the conformational change induced by binding of Ca^{2+} to concanavalin A affected the electronic environment of tyrosine and tryptophan residues [19].

4. Conclusion

Limulin I and Limulin III exhibit large differences in their circular dichroism spectra, in the 200–260 nm range. The spectrum obtained with the native pure lectin Limulin III, has a very low intensity and indicated the absence of ordered conformation (α-helix and β-structure). The conformation is found to be sensitive to freezing. Removal of Ca^{2+} ions does not greatly disturb the environment of the aromatic amino acids, but induces an appreciable change in the far ultraviolet CD, and therefore a partial conformational change of the protein. This conformational change could explain the lack of agglutinating activity in the absence of calcium.

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