Circular dichroism and fluorescence investigations on *Vicia faba* lectin–saccharide binding

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Vicia faba lectin contained 40–57 % β -conformation, 4–23 % α -conformation along with random coil at pH 7.2 depending upon the analytical methods used. The percentage of β -conformation increased with the addition of *N*-acetyl-D-glucosamine or methyl α -D-mannopyranoside. The structural transitions of *V. faba* lectin were affected by alkali at pH 9.6 and 10.6. Binding constants and free energy changes for the interaction between *V. faba* lectin and *N*-acetyl-D-glucosamine and methyl α -D-mannopyranoside were estimated at pH 7.2 using the c.d. and fluorescence methods.

INTRODUCTION

Vicia faba seed lectin has been purified by affinity chromatography on different grades of Sephadex (Goldstein & Poretz, 1986). Allen *et al.* (1978) used 3-*O*-methylglucosamine attached to CH-Sepharose for the purification of the seed lectin. Recently Datta *et al.* (1984a) reported the isolation of *V. faba* seed lectin on a chitin column. The interactions between purified *V. faba* lectin and ligands were studied by fluorescence quenching titration (Datta *et al.*, 1984b, 1987) and spectrophotometric methods (Datta *et al.*, 1984c, 1986). Circular dichroism probe and fluorescence measurements are useful modern techniques for investigating the interactions between lectins and ligands (Lis & Sharon, 1984). In the present paper binding of saccharides to purified *V. faba* lectin has been studied by using the fluorescence method and c.d. spectra. C.d. has been utilized to investigate the conformation of *V. faba* lectin molecule, and to measure the conformational transition caused by pH change and by specific sugars.

MATERIALS AND METHODS

N-Acetyl-D-glucosamine and methyl α -D-mannopyranoside were obtained from Sigma Chemical Co. Reagents and chemicals used were of analytical grade or the best quality available. *V. faba* lectin was purified using chitin as an affinity matrix (Datta *et al.*, 1984a). The protein concentrations were determined from its $A_{1\text{cm}}^{1\%}$ value of 7.22 at 280 nm in 0.15 M-phosphate-buffered saline, pH 7.2 (PBS) containing 0.1 mM-CaCl₂, -MgCl₂ and -MnCl₂ (Datta *et al.*, 1984a). For pH studies, 0.15 M-acetate buffers, pH 4.5–6.5 with intervals of 0.5 unit, 0.15 M-phosphate buffers, pH 7.2 and pH 7.5–9.5 with intervals of 0.5 unit, and 0.15 M-glycine buffers, pH 10, pH 10.6 and pH 11.6, were used.

C.d. readings were taken on a Jasco J20A recording spectropolarimeter. Spectra were measured at a protein concentration of 2.0 mg/ml (0.1 cm cell) in the region above and below 250 nm. The data are expressed in terms of mean residue ellipticities, $[\theta]_{\text{m.r.w.}}$ in degrees·cm²·dmol⁻¹. A value of 110 was taken as the

mean residue weight of *V. faba* lectin. All readings were taken at room temperature (25 ± 2 °C). An aliquot (20 μ l) of the *V. faba* lectin (40 μ g in PBS) was directly mixed separately with 80 μ l of phosphate buffer, pH 7.2, or glycine buffer, pH 10.0 and pH 11.6, yielding the final pH of 7.2, 9.5 and 10.6 respectively. In the other set of experiments, 20 μ l of *V. faba* lectin was directly mixed separately with a known concentration of saccharides, 0.010 M-*N*-acetyl-D-glucosamine and 0.018 M-methyl α -D-mannopyranoside in 80 μ l of phosphate buffer, pH 7.2. In the blank, 20 μ l of PBS was directly mixed with 80 μ l of buffer of pH 7.2, 10.0 and 11.6 for making the final pH 7.2, 9.5 and 10.6 respectively, or with a known concentration of saccharides in 80 μ l of buffer, pH 7.2. The sample and blanks were incubated for 2 h before analysis. Calculation of the secondary structure of the *V. faba* lectin was performed by the methods of Chen *et al.* (1974) and Provencher & Glockner (1981).

Fluorescence spectra of *V. faba* lectin were measured in an Aminco–Bowman spectrophotofluorimeter (Datta *et al.*, 1987). The *V. faba* lectin (1 mg in 1 ml of PBS) was buffered in each case with 2 ml of buffer solution (pH 4.5–9.5), and then an aliquot (1.5 ml) was taken from these solutions and mixed with 1.5 ml of buffer of different pH separately to make the final pH 5.0–9.0. In the other set of experiments, *V. faba* lectin, 1 mg/ml in PBS, was buffered in each case with 2 ml of buffer solution (pH 4.5–9.5) and then an aliquot of 1.5 ml was taken and mixed with 1.5 ml of solutions of different pH containing known concentrations of 0.018 M-*N*-acetyl-D-glucosamine or 0.021 M-methyl α -D-mannopyranoside to make the final pH 5.0–9.0. In the blanks, 3.0 ml of buffer solution of pH 5.0–9.0 or 3.0 ml of buffer solution of pH 5.0–9.0 containing the same concentrations of saccharides as in the experimental system were used respectively. The samples were incubated for 2 h before analysis. Excitation wavelength was 295 nm. The two fluorescence peaks were observed at 340 nm and 380 nm in presence of lectin. On addition of *N*-acetyl-D-glucosamine and methyl α -D-mannopyranoside only the 380 nm peak was responsible. Change of the fluorescence intensity at 380 nm on addition of *N*-acetyl-D-glucosamine or methyl α -D-mannopyranoside was used to

Abbreviation used: PBS, phosphate-buffered saline.

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estimate the binding constants between *N*-acetyl-D-glucosamine and *V. faba* lectin, and methyl α -D-mannopyranoside and *V. faba* lectin respectively. All readings were performed at room temperature ($25 \pm 2^\circ\text{C}$). The binding constants for lectin-saccharide interaction were calculated by using the following equation (Yoshii & Ishiyama, 1983):

$$K_a = LS/(L_0 - LS) \cdot (S_0 - LS)$$

Here LS is the molar concentration of the *V. faba* lectin-saccharide complex, and L_0 and S_0 are the total molar concentrations of the *V. faba* lectin and saccharide respectively. The quantity $(S_0 - LS)$ was approximated to S_0 , since the amount of the saccharides bound was negligible as compared with the total saccharide concentration. As the change in the c.d. or fluorescence intensity is assumed to be proportional to the amount of complex LS and the lectin molar concentration L_0 , these terms can be replaced by $\Delta\theta$ (or ΔF) and $\Delta\theta_{\max}$ (or $\Delta F_{\max}$) respectively. In this case, the binding constant is estimated by a Scatchard plot of $\Delta\theta$ versus $\Delta\theta/S_0$ on the basis of the modified equation:

$$\Delta\theta = -1/K_a \cdot \Delta\theta/S_0 + \Delta\theta_{\max}$$

RESULTS AND DISCUSSION

V. faba lectin showed a negative band centred at 212 nm and a positive band at 235 nm (Fig. 1). About 51 % β conformation of the lectin molecular at 219 nm was calculated according to Chen *et al.* (1974), taking the value of -6890 degrees $\cdot\text{cm}^2 \cdot\text{dmol}^{-1}$ for the β conformation as standard. The *V. faba* lectin molecule revealed about 57 % β conformation at 216 nm, using the standard value of -9210 degrees $\cdot\text{cm}^2 \cdot\text{dmol}^{-1}$ (Chen *et al.*, 1974). Thomas *et al.* (1979), and Shimazaki *et al.* (1975) previously estimated the β conformation of soybean agglutinin and *Ricinus communis* agglutinins using the

standard values of Chen *et al.* (1974). An α conformation of 21–23 % of the *V. faba* lectin was determined according to the method of Alder *et al.* (1973). Matsumoto *et al.* (1983) reported earlier that *V. faba* lectin contained 20 % α -helix according to Chen *et al.* (1974). The rest of the polypeptide chain backbone apparently is the aperiodic loop and turn conformation, as has been found in many proteins (Jirgensons, 1973).

These estimates of the various conformations for reasons outlined elsewhere (Jirgensons, 1973; Madison & Schellman, 1972), are of a low degree of precision, e.g. it is known that the optical activity of the pleated sheet depends on the length and width of the sheet (Woody, 1969). Moreover, the sheets are often twisted, and the helices are often distorted. The optical activity of the aperiodic bends and kinks is not well investigated, although it is now generally accepted that their conformations should not be equated with the random flight flexible chains.

For improvement of our results, we are using Provencher & Glockner's equation (1981) because Chen's equation (1974) occasionally gives a high value for α -helix content in proteins with little or no α -helix. Therefore, we used the method of Provencher & Glockner (1981) when *V. faba* lectin showed 4–10 % α -conformation and 40–46 % β -conformation by using a computer analysis program. Matsumoto *et al.* (1983) showed that *V. faba* lectin contains 2 % α -helix and 38 % β -sheet structure. The values are in good agreement with the secondary structure of concanavalin A (2 % α -helix and 36 % β -sheet) confirmed by an X-ray study, because the similarity of the c.d. spectrum of *V. faba* lectin and that of concanavalin A suggests that the secondary structure of *V. faba* lectin is similar to that of concanavalin A (Matsumoto *et al.*, 1983). It is interesting that the lectin concanavalin A is known to have about 50–60 % of the β conformation, as found by X-ray structural analysis (Shimazaki *et al.*, 1975). The values calculated for the β - and α -conformational values of *V. faba* lectin must be taken with some reservation due to the uncertainty in quantification of this conformation by c.d. A predominance of β -conformation in several lectin molecules had been shown utilizing c.d. (Pflum *et al.*, 1971; Pere *et al.*, 1975; Bures *et al.*, 1972; Shimazaki *et al.*, 1975; Lönngren *et al.*, 1976; Herrmann *et al.*, 1978). The positive band at 271.5 nm was probably due to phenylalanine, while the band at 285 nm may be attributed to tyrosine, these deductions being made on the basis of studies by Matsumoto *et al.* (1980). The two saccharides *N*-acetyl-D-glucosamine and methyl α -D-mannopyranoside influence the c.d. spectrum of *V. faba* lectin in the u.v. spectral zone. In the presence of *N*-acetyl-D-glucosamine, the *V. faba* lectin contained 85–95 % β -conformation, whereas methyl α -D-mannopyranoside induced 65–75 % β -conformation in the protein molecule (Fig. 1). These estimates were made according to Chen *et al.* (1974). Computer analysis according to the method of Provencher & Glockner (1981) showed 70–75 % β -conformation in presence of *N*-acetyl-D-glucosamine and 58–65 % β -conformation in presence of methyl α -D-mannoside. This showed that the interaction of lectin with *N*-acetyl-D-glucosamine was more substantial than that with methyl α -D-mannopyranoside. The presence of free amino groups and carboxyl groups is essential for complex formation by the *V. faba* lectin (Datta *et al.*, 1984b). Therefore the

Fig. 1. C.d. spectra of *V. faba* lectin at pH 7.2 and 25°C

—, *V. faba* lectin; ----, in the presence of 7 mM-*N*-acetyl-D-glucosamine; - · - · -, in the presence of 7 mM-methyl α -D-mannoside; · · · ·, *V. faba* lectin at pH 9.6; - - - - - , *V. faba* lectin at pH 10.6.

Fig. 2. Scatchard plots of $\Delta\theta$ versus $\Delta\theta/S_0$ by the c.d. method at pH 7.2 and 25 °C

●, *N*-acetyl-D-glucosamine; ▲, methyl α -D-mannoside.

Fig. 3. Fluorescence spectra of *V. faba* lectin at pH 7.2 and 25 °C

●, In the absence of any saccharides; ■, in the presence of 7 mM-*N*-acetyl-D-glucosamine; ▲, in the presence of 7 mM-methyl α -D-mannoside.

changes in the u.v. region possibly indicate that the saccharide had some effect on both free amino groups and carboxyl chromophores. The flexibility in the binding site was perhaps hindered because of blocking of binding residues by the saccharide. However, the possibility remains that the weak aromatic transitions can gain or lose intensity through vibronic vicinal interactions that do not affect the backbone peptide chromophore (Thomas *et al.*, 1979). The binding constants (K_b) and standard free energy changes (ΔG^0) for interaction of *N*-acetyl-D-glucosamine or methyl α -D-glucosamine or methyl α -D-mannopyranoside to *V. faba* lectin were estimated by Scatchard plots obtained from the c.d. intensity values (Fig. 2, Table 1).

The c.d. spectra of *V. faba* lectin in the pH range 7.2–10.6 are shown in Fig. 1. The protein contained 40–57% β -conformation, 4–23% α -conformation together with random coil at pH 7.2. At pH 9.6, peaks were lower than at pH 7.2. An elevated c.d. peak at 235 nm was observed at pH 10.6. Two positive peaks were observed at 238 nm and at 272 nm at pH 7.2. The positive peak at 240 nm and a negative peak at 270 nm appeared at pH 9.6; similar phenomena were observed at 235 nm and at 285 nm at pH 10.6. The ionization of tyrosine and phenylalanine residues causes a great change in the c.d. spectrum at pH higher than 7.0 (Jirgensons, 1980). *V. faba* lectin contains a higher amount of acidic amino acids (Datta *et al.*, 1984a). The acidic amino acids acquire negative charges in alkaline conditions; thus a charge repulsion amongst acidic amino acid side chains might alter the helical structure of *V. faba* lectin molecule at pH 9.6 and 10.6.

The fluorescence peak at 340 nm was due to tryptophan only. In Fig. 3, the small area of the peak may be due to the small amount of tryptophan present in the lectin

Fig. 4. Scatchard plots of $\Delta F/S_0$ by the fluorescence method at pH 7.2 and 25 °C

●, *N*-acetyl-D-glucosamine; ▲, methyl α -D-mannoside.

Table 1. Binding constants and standard free energy changes for *N*-acetyl-D-glucosamine and methyl α -D-mannopyranoside at 25 °C, pH 7.2

These K_a values were estimated from the Scatchard plots.

Saccharide	Method	$10^4 \times$ Association constant (K_a) (M^{-1})	Free energy ($-\Delta G^0$) (kJ/mol)
<i>N</i> -Acetyl-D-glucosamine	C.d.	1.06 ± 0.02	22.98 ± 0.08
	Fluorescence	1.63 ± 0.02	24.03 ± 0.03
Methyl α -D-mannopyranoside	C.d.	0.54 ± 0.01	21.30 ± 0.06
	Fluorescence	1.21 ± 0.01	23.29 ± 0.01

Fig. 5. pH-dependence of free energy changes for the binding of *N*-acetyl-D-glucosamine (●) and methyl α -D-mannopyranoside (▲) to *V. faba* lectin at 25 °C

(Datta *et al.*, 1984a). The peak at 380 nm was due to the presence of higher amounts of acidic groups in *V. faba* lectin. The shift of fluorescence towards 400 nm might be due to a higher acidic amino acid content and the relative arrangements of the amino acids in the molecule with the tryptophan side chains being stacked around some regular structure, allowing the side chains to interact (Fasman *et al.*, 1966). It was reported earlier (Datta *et al.*, 1984b) that carboxyl and amino groups are responsible for sugar binding in *V. faba* lectin. The fluorescence intensity at 380 nm considerably increased in the presence of *N*-acetyl-D-glucosamine, whereas a small change was found on the addition of methyl α -D-mannopyranoside (Fig. 3).

The fluorescence of *V. faba* lectin, being considerably enhanced by the addition of *N*-acetyl-D-glucopyranoside or methyl α -D-mannopyranoside, was used to estimate the binding constants for the interactions of saccharides with the lectin. The Scatchard plots obtained from fluorescence intensity values at pH 7.2 were used for the estimation of binding constants (K_a) and standard

free energy change (ΔG^0) for *V. faba* lectin-saccharide interaction (Fig. 4, Table 1).

The value of K_a estimated by the fluorescence permitted us to determine the pH-dependence of ΔG^0 and to ascertain whether certain ionizable groups participated in the binding reaction or not. ΔG^0 values were estimated from the $\log K_a$ values over the pH range 5.0–9.0 (Fig. 5).

The maximum value for ΔG^0 was obtained at pH 7.2. The optimum reactivity of *V. faba* lectin was found to be in the pH region 7.0–7.5 (Datta *et al.*, 1984a). The integrity of the molecular conformation of the lectin was maximum at pH 7.2, which was the optimum pH for its activity. The interaction of the lectin with *N*-acetyl-D-glucosamine or methyl α -D-mannopyranoside conceivably could induce partial protection of the molecule from denaturation at both pH extremes, resulting in lower values of ΔG^0 .

The results obtained from c.d. investigations and fluorescence studies indicated the ability of *N*-acetyl-D-glucosamine or methyl α -D-mannopyranoside to induce organized conformation in the lectin molecule.

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