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Fluorescence studies on concanavalin-A

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Abstract. Using the lectin-concanavalin-A, the tryptophan fluorescence as a function of pH was studied. The pH dependent, fluorescence intensity changes were significantly higher when excited at 305 nm, than when irradiated at 280nm. Only one tryptophanyl per monomer of concanavalin-A was available for oxidation by N-bromosuccinimide in the dimeric form at pH 4·9; no tryptophanyl could be oxidised in the demetallised dimer (pH 3·0) and native tetramer (pH 7·0). Based on this fluorescence data and the already known crystal structure data, it appears that tryptophanyl 88 in concanavalin-A may be selectively excited by 305 nm radiation.

Keywords. Concanavalin-A; fluorescence; selective excitation; 305 nm.

Introduction

Concanavalin-A (ConA) is a lectin and a metalloprotein associated with a Ca^{2+} and a Mn^{2+} ion. It is a powerful agglutinating agent and binds specifically to α-D-manno- and glucopyranosides and is the first lectin whose three dimensional crystal structure was determined (Reeke *et al*., 1975). Physicochemical studies revealed that ConA can exist, depending on pH, as a dimer, a tetramer (Kalb and Lustig, 1968; Mckenzie *et al*., 1972) and also as a demetallised dimer in two different forms (Brewer *et al*., 1983); under physiological conditions, ConA is tetrameric. The important role of aromatic residues in maintaining the tertiary and quaternary structure and also in the biological activity of proteins is now recognised (Sabesan and Harper, 1980). A recent study on ConA using radiotracer technique, reported that tryptophanyl and possibly tyrosyl residues are involved in the carbohydrate binding (Moore and Mudher, 1979). In this context, it is of interest to study the environment of tryptophanyl (Trp) residues using fluorescence as a probe. It was shown recently (Kuramitsu *et al*., 1978; Rao *et al*., 1981) that it is possible to selectively excite only one of the six Trp residues (Trp 108) in lysozyme by using 305 nm as excitation wavelength, instead of the usual 280 nm excitation. The present report deals with an investigation of the fluorescence of ConA excited at 305 nm and also at 280 nm, as a function of pH, and also in the presence of N-bromosuccinimide, which can oxidise Trp to a non-fluorescent oxindole compound.

Abbreviations used: ConA, Concanavalin-A; Trp, tryptophanyl; NBS, N-bromosuccinimide; OD, optical density; Tyr, tyrosine.

Materials and methods

ConA (Sigma Chemical Co., St. Louis, Missouri, USA highly purified) free of sugar and carbohydrates prepared from Jack beans by the method of Olson and Leiner (1967) was used. ConA free of cleaved fragments was prepared by the method of Cunnigham *et al*. (1972) by treating the lectin with ammonium bicarbonate. N-Bromosuccinimide (NBS), urea and other chemicals used for making buffers are all of analytical grade. NBS was recrystallised from 90% ethanol.

The buffer systems used were: pH < 3·0: Gly-HCl; pH 3·8–5·4: CH_3COONa-CH_3COOH; pH 6·0–8·5: KH_2PO_4–Na_2HPO_4 (Diem and Lentner, 1971). An ionic strength of 0·3 was maintained in all the systems by the addition of NaCl. Stock buffer solutions were filtered through a G-3 sintered glass filter, and protein solutions were filtered through filters of 0·45 μm pore size (Maxflow).

Optical density (OD) measurements were made with a Zeiss spectrophotometer, PMQ3, at room temperature. The number of tryptophans (n) oxidised were calculated (Spande and Witkop 1967), using the equation:

$$n = \frac{\Delta OD_{280}}{OD_{280}} \times \frac{1{\cdot}31\,\varepsilon}{5500} \tag{1}$$

where n is number of Trps oxidised per mol of ConA and ε is molar extinction coefficient of ConA at 280 nm.

The concentration of the protein solutions were determined assuming a $\varepsilon_{280}^{1\%}$ of 12·4 at pHs below 7·0, and 13·7 at pHs above 7·0 (Kalb and Lustig, 1968; Yariv *et al*., 1968); the protein concentration in the present study was $\simeq 10^{-6}$ M

Fluorescence spectra were recorded with a Jasco FP-550 grating spectrofluorimeter at room temperature using two different excitation wavelengths, 280 nm and 305 nm. The intensity of emission (I_F) with 305 nm excitation was weak compared to that with 280 nm excitation. The fluorescence intensity I_F at the emission maximum at both the excitation wavelengths (280 and 305 nm) was found to be linear in the concentration range used in the present study.

NBS solutions (1 mm) were added in small aliquots (25 or 50 μl) to ConA solutions and the fluorescence spectra were recorded 30 sec after the addition; I_F was corrected for dilution.

pH measurements were done with a Radiometer model pHM 26 pH meter.

Results

The fluorescence spectra of ConA were recorded as a function of p H . The wavelength of maximum emission, λ (max, em), was found to be constant at 340 nm over the pH range 1·5 to 8·5, when irradiated with 305 nm, but λ (max, em) when excited at 280 nm was red shifted by 5nm, from 333–338 nm, as the pH was raised to 7·0 (figure 1A). The pH dependence profile of fluorescence intensity (I_F) at 335 nm (with 280 nm excitation) and at 340 nm (with 305 nm excitation), shown in figure 1B, has a maximum around pH 7·0. As the solution was rendered neutral, the I_F was enhanced by about 30%, when excited by 280 nm, but as much as a 90% increase was observed in fluorescence emission with

305 nm excitation (figure 1 A). Above pH 7·0, in the alkaline region, a diminished fluorescence is a common feature of the two sets of data.

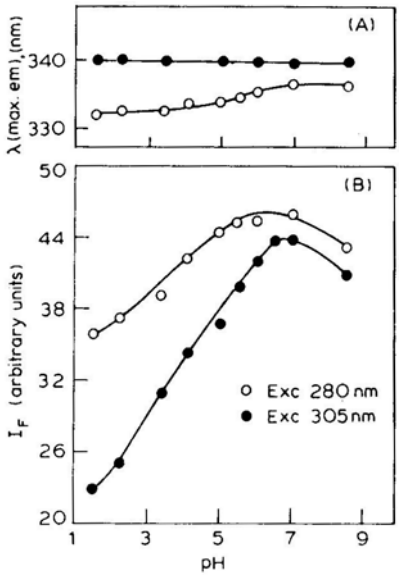

Figure 1. λ(max, em), (A) and fluorescence intensity; I_F (B) of ConA observed as a function of pH using 280 nm excitation (○)- and 305 nm excitation (●).

Oxidation of tryptophanyl residues using NBS

The course of oxidation of Trps by NBS in ConA was followed by the measurement of optical density at 280 nm as well as of fluorescence intensity as a function of added NBS at three pHs; the number of tryptophans oxidised in ConA was calculated using eq. (1) and the values obtained are tabulated in table 1. No tryptophan was available for oxidation, even after the addition of large amount of NBS, in demetallised dimer of ConA (pH 3·0) and also in the tetramer (pH 7·0). In the dimeric ConA (pH 4·0), however, two Trps out of the eight present were oxidised on addition of 12 mols of NBS per mol of ConA dimer; this oxidation, however, remarkably destabilized the protein, and turbidity was found to set in.

Table 1. Number of Trp of Con-A oxidised by NBS.

		Number of Trp oxidised/dimer	
pH	ConA form	No urea	8 M urea
3·0	Demetallised dimer	0	8
4·9	Dimer	2	6
7·0	Tetramer	0	4

In view of the optical density data, NB S oxidation of dimeric ConA was followed by fluorescence only at pH 4·9. The decrease in fluorescence emission at 340 nm, as a function of mols of NBS added is depicted in figure 2A. There was an initial loss in fluorescence till 12 mols of NB S were added and thereafter I_F remained constant; the optical density data indicated that only two Trp residues per dimer were oxidised. However, the relative decrease in I_F in the 305 nm excited emission spectra is markedly higher ($\simeq$ 70%), than that influorescence excited at 280 nm ($\simeq$ 35%). Again the λ (max, em) with 280 nm excitation blue shifted by 10 nm (333–323 nm) on addition of NBS (figure 2B); there was, however, no change in the emission maximum (340 nm) of the spectrum excited at 305 nm.

Oxidation of Trp residues in 8 *M urea*

NBS oxidation of Trp residues in ConA was also studied in the presence of 8 M urea at three different pHs. The total number of Trps oxidised were calculated to be 6 and 4 per dimer, respectively in dimeric (pH 4·9) and tetrameric (pH 7·0) forms, while all the 8 Trps were oxidised in the demetallised dimer (pH 3·0) (table 1). On oxidation of Trps of ConA in 8 M urea solutions, the relative loss in fluorescence excited at 305 nm was similar to that observed at 280 nm excitation.

Discussion

Each monomeric ConA contains four Trp at positions 40, 88,109, and 182, and seven tyrosines (Tyr) at 12, 22, 54, 67, 77, 100 and 176. Even so, the fluorescence behaviour of ConA, as any other class-B protein, is considered as that due to its Trp residues (Cowgill, 1963) because the quantum efficiency of Tyr is much less than that of Trp in proteins (Chen, 1967; Longworth, 1971). The increase in fluorescence intensity observed (figure 1B) as pH was raised from 1·5 to 7·0 is associated mainly with the ionisation of side chain carboxyl groups of aspartyl/ and glutamyl residues in ConA; carboxyl groups which are proximate to Trp are known to quench fluorescence and their ionisation removes the quenching (White, 1959; Gally and Edelman, 1961). The relatively larger pH- dependent increase in fluorescence intensity observed with 305 nm excitation, compared to that excited at 280 nm (figure 1A), suggests that tryptophans excited at 305 nm are in an environment different from most of the Trp excited at 280 nm (Kronman, 1976; Kuramitsu *et al*., 1978; Rao *et al*., 1981). Pelly and Horowitz (1976) have shown from fluorescence quenching studies of ConA by iodide ions that two Trps are near the molecular surface. Actually, analysis of three dimensional crystal structure data of ConA (Reeke *et al*., 1975) clearly indicates that out of the four Trps, only two (at 88 and 182) are on the molecular surface, and, further, one of them (Trp 88) makes van der Waals contacts with two aspartyl residues, Asps 136 and 139. It appears then that Trp 88 is the most likely residue that is selectively excited by 305 nm radiation.

The decrease in fluorescence intensity above pH 7 in the alkaline region can be ascribed to the ionisation of tyrosyls; the phenoxide moieties act as ‘energy sinks’ for energy transfer from Trp to Tyr (Edelhoch *et al*., 1967; Cowgill, 1963). It is to be noted that conformational changes that occur on changing pH may also affect the fluorescence intensity and λ (max, em) (Miller and Nwokedi, 1975).

The wavelength of emission maximum of Trp unlike of tyrosyls depends very much on the locale of the residues and λ (max, em) could be as low as 330 nm for Trp in the interior of the protein and as high as 350 nm for Trp at or near the molecular surface (Teale, 1960). In the present study, the λ (max, em) for 280 nm excitation was 333 nm at pH 1·5, and this was red shifted by about 5 nm on changing the pH to 7·0 and it is significant that over the pH range 1·5–8·5, the emission maximum of 305 nm excited fluorescence was unaltered at 340 nm.

NBS oxidation studies are expected to throw light on the relative exposure of tryptophanyl residues in a protein for, NBS readily oxidises those Trp at or near the molecular surface (Spande and Witkop, 1967). In fact, the extent of reaction of a protein with NBS in the presence of a denaturant, say 8 M urea, gives an estimate of the extent of denaturation (Hassing and Goldstein, 1972). Fluorescence intensity, as expected, decreased on addition of NBS at pH 4·9, and again, the decrease is significantly higher in the case of 305 nm excitation than with 280 nm irradiation (figure 2A). The decrease in I_F with 280 nm excitation was also accompanied by a blue shift in λ (max, em) (figure 2B) suggesting that a Trp near the molecular surface was oxidised.

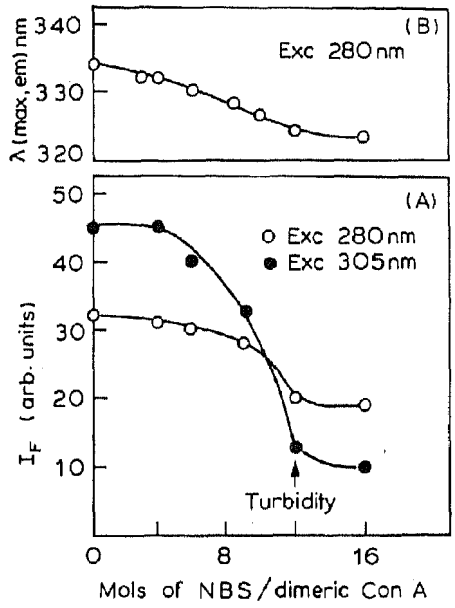

Figure 2. Changes in fluorescence intensity, I_F (A) and λ (max, em), (B) of ConA observed using 280 nm excitation (○) and 305 nm excitation (●) on addition of NBS.

The instability induced in the system leading to aggregation and turbidity, when one Trp per monomer of the dimeric ConA (pH 4·9) was oxidised, indicates clearly that this Trp has an important role in maintaining the tertiary and quaternary structure of ConA; aggregated ConA loses its biological activity (Hassing and Goldstein, 1972). The fact that Trp 88 is involved in monomer–monomer interactions in the formation of dimer (Reeke *et al*., 1975) supports our suggestion of this residue to be the one selectively excited by 305 nm radiation.

ConA in the demetallised form is reported to be more easily denatured by 8 M urea than the native ConA containing metal ions (Agarwal and Goldstein, 1968) and further

this denaturation is irreversible (Pflumm and Beychok, 1974). Miller and Nwokedi (1975) showed that denaturation of ConA by urea is a two stage process; a low concentration of urea causes an increase in fluorescence intensity and at higher concentration (> 4M), quenching of fluorescence accompanied by a red shift in the λ (max, em) was observed. The present studies on ConA in the presence of 8 M urea showed that all the Trp residues are exposed and available for oxidation by NBS only at pH 3·0 in the demetallised dimer (table 1) and the fluorescence emission maximum (350 nm) shifts to that of tyrosyl residues (305 nm) as a result of oxindole formation. The unfolding of the lectin by 8 M urea at p Hs 4·9 and 7·0 was found to be incomplete as evidenced by the fact that some Trp were still inaccessible for oxidation.

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