Carbohydrate Research, 29 (1973) 265–270
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Note

Carbohydrate-induced conformational change in concanavalin A

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(Received February 20th, 1973; accepted for publication, March 9th, 1973)

Carbohydrate-binding proteins occur widely in the plant kingdom. These proteins, commonly derived from seeds, are called phytohemagglutinins or lectins (reviewed by Sharon and Lis[1]). It has become increasingly apparent that lectins can serve as probes for carbohydrate structure and function. The jack-bean lectin, namely, concanavalin A (con A), has received considerable attention because of its diversity of important properties. For example, con A is a potent mitogen[2], restores contact inhibition to transformed tissue-culture cells[3], induces marked histamine susceptibility in mice[4], precipitates polysaccharides and glycoproteins[5], and agglutinates several cell-types[6]. Recently, Edelman *et al.*[7] and Hardman and Ainsworth[8] determined the tertiary structure of con A.

Several authors have presented preliminary evidence for a saccharide-induced, conformational change in con A. Pflumm *et al.*[9] and McCubbin *et al.*[10] showed that con A exhibited changes in its near-ultraviolet circular dichroism spectrum in the presence of carbohydrate ligand. Hassing and Goldstein[11] found that small concentrations of ligand give rise to changes in the u v. absorption spectra of solutions of con A. Akedo *et al.*[12] discovered that certain sugars change the isoelectric point of con A. Additional evidence for ligand-induced, conformational changes is now presented; it is shown that ligand protects con A against hydrolysis by pronase and against heat aggregation. In addition, when con A is tagged with the "reporter" 2-(bromomethyl)-4-nitrophenol, the ligand induces a visible difference-spectrum.

EXPERIMENTAL

Concanavalin A was prepared from finely ground jack-beans (Sigma Chemical Company, St. Louis, Mo.) by the Sephadex adsorption method of Agrawal and Goldstein[13]. The concentrations of solutions of con A were determined by measuring the u.v. absorption at 280 nm, assuming that $E_{1\%,1cm} = 11.4$ (see ref. 14).

Pronase digestion of con A was performed in 0.05M phosphate–0.15M sodium chloride buffer (pH 7.5). Solutions of pronase were prepared by filtering a suspension

(10 mg/ml) of pronase (Calbiochem, B grade) through a 0.2 μm polycarbonate filter. The filtrate was adjusted to 5 mg of protein per ml with buffer. Proteolysis was conducted at room temperature (24 ±2°) at a final wt. ratio of 10 mg of pronase protein per 60 mg of con A in a total volume of 30 ml. Aliquots (2 ml) were withdrawn at intervals, and each was mixed with an equal volume of 10% trichloroacetic acid (TCA). The TCA–protein suspension was incubated for 18 h at 0°, and centrifuged, and the absorbance at 280 nm of the supernatant liquor was measured, using 5% TCA solution as a blank. An identical solution of con A and pronase was treated in parallel, except that 20 μmoles of methyl α-D-mannopyranoside per ml were present.

The thermal aggregation of con A was studied by incubating solutions of the agglutinin at 60°, and determining the resulting opacities at 600 nm in 0.5-in., rounded cuvettes. A Bausch and Lomb Spectronic 20 colorimeter was used for all turbidimetric measurements. Temperatures were maintained to within ±0.5° by means of a circulating-water bath (Tamson Model PBC-4). Glass marbles were placed on top of the cuvettes to prevent evaporation.

The 2-hydroxy-5-nitrobenzyl derivative of con A was prepared by a modification of the method of Horton and Koshland[15]. Aliquots (50 μl) of 2-(bromomethyl)-4-nitrophenol (100 mg in 1.0 ml of dry acetone) were added at 15-min intervals to 10 ml of con A (16.4 mg/ml) in 1.0M sodium chloride (pH 5.6). After each addition of the alkyl bromide, the pH was adjusted to 5.6 with 1.0M potassium hydroxide. Fifteen minutes after the addition of the fourth aliquot, the suspension of modified con A was centrifuged for 10 min at 12,000 *g* (to remove insoluble reaction-products). The clear solution of protein was passed through a column of Bio-Gel P-6, pre-equilibrated and eluted with 0.05M phosphate–1.0M sodium chloride (pH 7.2). The extent of modification of tryptophan was determined by diluting an aliquot of the substituted con A with 6.0M potassium hydroxide to pH 12–13 and determining the absorbance at 410 nm, assuming a molar absorptivity of 18,000 M^{-1} cm^{-1} (see ref. 15). A molecular weight of 108,000 for con A was assumed in all calculations[16,17].

Difference-spectral measurements were performed by adjusting the baseline on a Cary Model 15 spectrophotometer with identical solutions (3.0 ml) of tryptophan-modified con A, at 3.45 mg/ml in 0.05M phosphate–1.0M sodium chloride (pH 7.2). After adjustment of the baseline, 100 μl of 0.5M methyl α-D-mannopyranoside was added to the cuvettes in the sample beam, and 100 μl of buffer was added to cuvettes in the reference beam. The spectrum was then scanned.

RESULTS AND DISCUSSION

When con A was incubated with pronase in the presence of methyl α-D-mannopyranoside, a high-affinity ligand for the protein[18], the rate of proteolytic digestion was markedly lowered (see Fig. 1). In the absence of ligand, 54% of the con A was rendered soluble in TCA by pronase after four hours, whereas under the same

conditions, but in the presence of methyl α-D-mannopyranoside, only 26% of the agglutinin was rendered TCA-soluble.

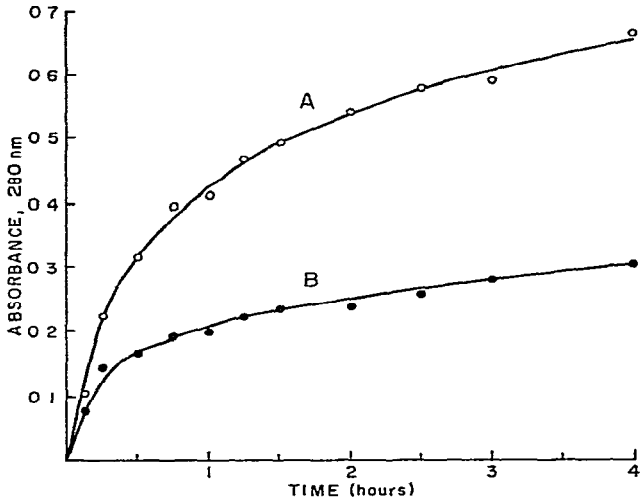

Fig. 1. Pronase hydrolysis of concanavalin A in the absence (A) and presence (B) of methyl α-D-mannopyranoside. (Details are given in the text.)

Frequently, globular proteins aggregate when heated to high temperatures in aqueous solution. Doyle *et al.*[19] showed that exposure to temperatures above 50° prevents the precipitation of glycogen by con A. It may be seen from Fig. 2 that

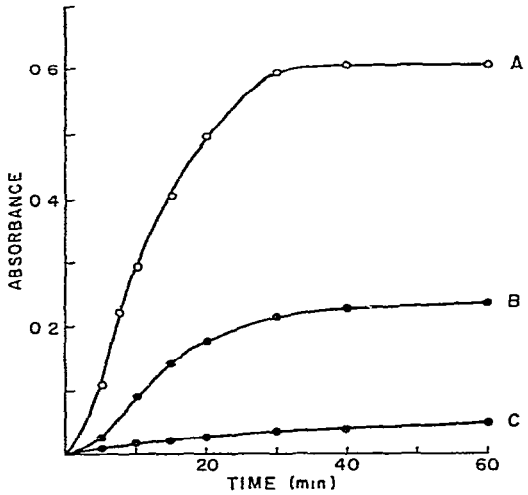

Fig. 2. Thermal aggregation profile of concanavalin A in the absence and presence of ligands. [A, Control of concanavalin A (2.0 mg); B, concanavalin A plus 100 μmoles of methyl α-D-mannopyranoside; C, concanavalin A plus 500 μmoles of methyl α-D-mannopyranoside. The final volumes were 4.0 ml.]

methyl α-D-mannopyranoside markedly lessens the extent of aggregation of con A at 60°. The heat-aggregated protein did not bind glycogen, indicating that the protein was denatured. Dialysis against phosphate, acetate, or Tris hydrochloride buffers did not restore the activity.

The foregoing observations are interpreted in terms of a ligand-induced change in the conformation of con A. Such sugars as D-galactose, which do not interact[18] with con A, do not protect the protein either from pronase digestion or thermal aggregation. The fact that a ligand protected against the low proteolytic specificity of pronase argues for a conformational change in con A upon binding to a sugar. At saturation, con A binds only two carbohydrate ligands[16,17]. Thus, it is doubtful that masking of the carbohydrate binding-site by ligand would result in such a marked change in the susceptibility of con A to the action of pronase. This might be possible were proteolytic enzymes of restricted specificity employed as probes for conformational changes. Markus *et al.*[20] showed that Methyl Orange increases the resistance of human serum albumin to attack by chymotrypsin, trypsin, papain, subtilisin, and pronase. Similarly, they[20] found that the chymotryptic hydrolysis of rabbit antibodies and Fab fragments was lowered in the presence of specific hapten. It was suggested by Markus *et al.*[20] that ligand stabilizes a particular conformational state of the protein, thereby limiting the number of protease-susceptible peptide bonds.

The lectins are generally regarded as heat-stable proteins. Cifonelli and Smith[21] treated crude jack-bean extracts at 71°, but did not notice any appreciable inactivation of the agglutinin; they used a pH lower than that employed in our studies. Moreover, the crude extract may have contained protective agents. The wheat-germ agglutinin is resistant[22] to prolonged heating at 63°. A D-galactose-binding agglutinin from *Pseudomonas aeruginosa* resists[23] inactivation at 70°. Howe and Barrett[24] isolated a lichen phytohemagglutinin, and showed that it possessed full erythroagglutinating activity after autoclaving. Allan and Crumpton[25] recently found that the lectin from *Phaseolus vulgaris* retained lymphoagglutinating and mitogenic activities following heating to 100° in 1% dodecyl sodium sulfate. These phytohemagglutinins are glycoproteins, whereas con A contains no carbohydrate. As a carbohydrate ligand protected con A from heat denaturation, it is tempting to suggest that carbohydrates may confer thermal resistance to such proteins. A number of glycoproteins, such as ovalbumin, are heat labile, however. It is possible that, on binding of ligand by con A, a particular conformation is stabilized, and that this conformation is more resistant to thermal aggregation. This interpretation would be consistent with the decreased proteolytic digestibility of con A following interaction with ligand (see Fig. 1). So and Goldstein[26] found that the slow, spontaneous, aggregation of con A was retarded by the presence of ligand. Cunningham *et al.*[27] showed that naturally occurring fragments of con A precipitate on incubation overnight at 37° in a buffer of pH 7.9. Incubation in the presence of methyl α-D-mannopyranoside decreased the rate and extent of precipitation[26]. McKenzie *et al.*[28] found that D-glucose stabilizes the time-dependent change in molecular weight of con A

that is observed in the absence of ligand. Simons[29] has recently shown that ligands of ribonuclease induce conformational changes in the protein, and increase its resistance to thermal denaturation. Moreover, Chlumecka *et al.*[30] found that lysyl and arginyl *t*RNA synthetases were protected from heat inactivation at 45° by their substrates.

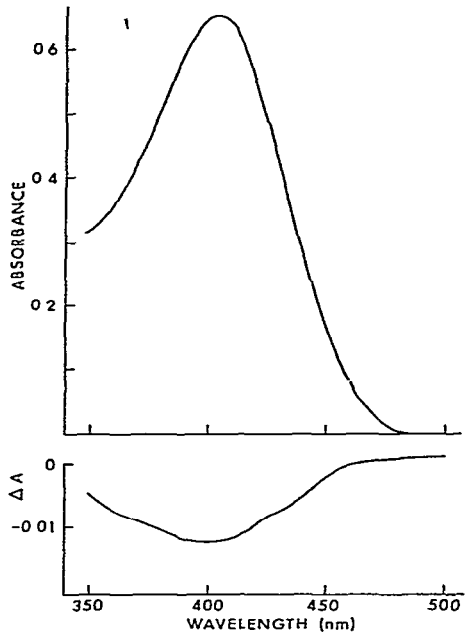

Fig 3. Ligand-induced, difference spectrum in concanavalin A modified with 2-(bromomethyl)-4-nitrophenol. [Top, visible spectrum of the modified protein (1.72 mg/ml); bottom, difference spectrum. The concentration of protein was 3.45 mg/ml. The details are given in the text]

Further support for the hypothesis that ligand induces conformational changes comes from experiments incorporating a specific, "reporter" group in con A. The "reporter" group must be inserted in an area distinct from the ligand binding-site. Con A was treated with 2-(bromomethyl)-4-nitrophenol, and 4.4 moles of tryptophan were modified per mole of protein. The visible spectrum of the con A derivative is shown in Fig. 3 (top). The carbohydrate-binding properties of con A were not detectably altered by this treatment, as the protein retained activity in precipitating glycogen. When ligand was added to the modified con A, a difference spectrum was generated (Fig. 3, bottom) that was characterized by a broad trough having λ_{min} ~400 nm. D-Galactose, which does not interact with con A, did not induce the difference spectrum. This result is best interpreted in terms of a conformational change in con A induced by interaction with ligand.

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