Biochimica et Biophysica Acta, 439 (1976) 160–166
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BBA 37369

CHEMICAL MODIFICATION STUDIES ON THE α-D-GALACTOPYRANOSYL BINDING LECTIN FROM THE SEEDS OF *BANDEIRAEA SIMPLICIFOLIA*

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(Received January 14th, 1976)

SUMMARY

The role of amino, carboxyl and sulfhydryl groups in the binding of carbohydrate ligands to the α-D-galactopyranosyl binding lectin from *Bandeiraea simplicifolia* has been evaluated using specific chemical modification techniques. The results indicate that carboxyl groups participate in the carbohydrate binding phenomenon, whereas free amino and sulfhydryl groups do not appear to be directly involved.

INTRODUCTION

The α-D-galactopyranosyl binding lectin isolated from *Bandeiraea simplicifolia* seeds is a glycoprotein composed of four similar subunits (M_r = 28 500) [1]. Each of the subunits has one carbohydrate binding site [2], and contains a divalent metal (e.g. Ca(II)) which is necessary for carbohydrate binding activity [1]. The lectin has been shown to contain 30–40% β-structure as the major ordered secondary structure [3]. This structure is largely insensitive to changes in pH, binding of sugar or removal of the intrinsic calcium ions [3]. The lectin precipitates polysaccharides and glycoproteins containing α-D-galactopyranosyl end-groups [1].

Inasmuch as lectins bear a relationship to immunoglobulins in their immunochemical behavior, we considered it to be of some interest to gain an understanding of the molecular mechanisms underlying carbohydrate-protein interactions in lectin systems. An approach to this problem is via functional group-specific modification of aminoacyl residues in the protein. This approach has earlier been exploited for concanavalin A [4–6] and we now report similar studies on the *B. simplicifolia* lectin.

MATERIALS AND METHODS

B. simplicifolia lectin was isolated as described earlier [1]. Carbohydrates were

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Phosphate-buffered saline —0.01 M sodium phosphate, 0.15 M sodium chloride, 0.1 mM calcium chloride, pH 7.2; the glycine hydrochloride— glycine methyl ester hydrochloride; the carbodiimide hydrochloride-1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride.

purchased from Pfanstiehl Laboratories, Illinois, U.S.A. Methyl methanethiolsulfonate was a generous gift from Dr. G. Kenyon, University of California, San Francisco, California, U.S.A. [^{14}C]Glycine methyl ester hydrochloride (spec. activity $1.6 \cdot 10^6$ dpm/mmol) was prepared as described earlier [4]. All other chemicals were of highest quality available.

The capability of native and various chemically modified lectin samples to interact with guaran was assayed by the quantitative precipitin technique [1, 7], using a total volume of 500 μl. Inhibition of quantitative precipitation was performed by the addition of increasing amounts of inhibitor to an equivalence mixture of lectin and guaran in a total volume of 500 μl. Nitrogen in digested samples was determined by a modification [1] of the ninhydrin procedure of Rosen [8]. For the immunization experiments, two adult New Zealand white rabbits were challenged with the lectin homogenized in Freund's complete adjuvant (Difco Laboratories, Michigan, U.S.A.) at a concentration of 0.8 mg/ml. All other methods for raising antisera and assay by quantitative precipitin curves have been described [9]. Ouchterlony plates were prepared as described earlier [10].

Modification of carboxyl groups was performed essentially as described [4]. Parallel reactions were performed in the absence and presence of methyl α-D-galactopyranoside (0.2 M). In typical experiments, a solution of lectin (5.0 ml; 9 mg/ml) in 0.15 M sodium chloride was adjusted to pH 5.75 after addition of the ^{14}C-labeled glycine hydrochloride (190 mg). To this solution was added 0.5 M 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride (0.3 ml) and the reaction mixture was stirred at room temperature. It was unnecessary to add acid to maintain the pH at approximately 5.75. After 2 h the reaction was quenched by addition of 1 M sodium acetate buffer (pH 5.5) and the samples were exhaustively dialyzed against phosphate buffered saline. Aliquots of the samples were counted in a Searle Isocap/300 liquid scintillation counter using a scintillation fluid containing 2 parts toluene containing 8.3 g/l "PPO" and 0.25 g/l "POPOP" (New England Nuclear, Massachusetts, U.S.A.) and 1 part Triton X-100.

Amino acid analyses were performed on samples (≈ 10 μmol) hydrolyzed for 22 h in 6 M hydrochloric acid at 110 °C. The analyses were carried out on a Beckman Spinco Amino Acid Analyzer, Model 120 B, with the assistance of Dr. C. H. Williams, Jr., and Mr. Keith Wilkinson, of this University.

Using the reaction conditions described above, it was shown in a separate experiment that methyl α-D-mannopyranoside (0.2 M) did not "protect" the lectin from modification. It was reported previously that under the conditions used, there was no significant modification of tyrosyl residues [4].

Acetylation of the protein was performed essentially as outlined in refs. 6 and 11. In a typical experiment, a solution of lectin (3.5 ml; 10.4 mg/ml) in 4 M sodium acetate was cooled in an ice-bath. To the rapidly stirred solution was added six portions (10 μl) of acetic anhydride over a one hour period. The reaction mixture was then exhaustively dialyzed against phosphate buffered saline. The recovery of soluble protein was approximately 75% as determined by the microbiuret assay [12]. The extent of modification of amino-groups was determined by the ninhydrin procedure [8] using L-leucine as standard and by the trinitrobenzenesulfonic acid assay [13] using native lectin as the standard. The amount of *O*-acetylation was determined by the hydroxamate method [14] using ethyl acetate as standard as described earlier [6].

Circular dichroism spectra of the acetylated lectin in sodium phosphate buffer (0.01 M; pH 7.2) were recorded using the equipment and procedures described [3].

Attempted inhibition of precipitation by mercuric chloride was performed at 23 °C by addition of the salt solution to incubation mixtures of lectin (100 μg) and guaran (50 μg) in phosphate buffered saline to final mercuric chloride concentrations of 0.2, 1.0 and 5.0 mM.

Attempted inhibition of precipitation by reaction with methyl methane thiolsulfonate was performed similarly by addition of methyl methane thiosulfonate to the lectin (140 μg) to methyl methane thiolsulfonate concentrations up to 8 moles per mole sulfhydryl group. This reaction mixture (in phosphate buffered saline) was incubated for two hours at 37 °C before addition of guaran (50 μg).

Demetalized lectin [1] (170 μg) was reacted for 2 h at 37 °C with one molar equivalent of methyl methane thiolsulfonate prior to addition of calcium chloride (final concentration 1 mM). No difference was discerned in the capacity of the untreated samples to precipitate guaran (50 μg).

RESULTS AND DISCUSSION

Carboxyl groups in proteins can be transformed to amides in high yield using a water-soluble carbodiimide (e.g. the carbodiimide hydrochloride) and a suitable amine, (e.g. the glycine hydrochloride) [15]. The reaction is usually performed at pH 4.75. However, it appeared reasonable to assume that any involvement of carboxyl groups in the binding of α-D-galactopyranosyl groups to the *B. simplicifolia* lectin would be most clearly discernable if the modification reaction was performed at a higher pH inasmuch as this lectin loses its polysaccharide precipitating ability below pH 5 [1].

Two lectin samples were reacted under identical conditions at pH 5.75 with the ^{14}C-labeled glycine hydrochloride and the carbodiimide hydrochloride in the absence and presence of methyl α-D-galactopyranoside. After two hours the reaction was quenched and the samples dialyzed free of reagents. The incorporation of [^{14}C]glycine methyl ester was determined by counting of ^{14}C and by amino acid analysis. The former procedure showed the incorporation of about 8 and 9 glycine methyl ester residues per mole lectin subunit in the presence and absence of methyl α-D-galactopyranoside, respectively. The corresponding figures found by amino acid analysis were 9 and 10, respectively. The ability of methyl α-D-galactopyranoside to protect the lectin from modification was further assessed by quantitative precipitin studies (Fig. 1). The "protected" sample showed about 80% of the precipitating power of the native lectin with guaran, whereas the corresponding figure for the "unprotected" lectin was approximately 20%. Both precipitin reactions could be inhibited by methyl α-D-galactopyranoside. Methyl α-D-mannopyranoside, a non-binding glycoside, failed to protect the lectin from modification with the glycine hydrochloride and the carbodiimide hydrochloride. Thus, a specifically bound carbohydrate ligand protected the lectin from loss of activity, as well as incorporation of glycine methyl ester residues.

Rabbit antiserum raised to the native lectin showed a strong cross reaction with both the "protected" and "unprotected" lectin. As seen in Fig. 2, "spur" formation is most pronounced between the "unprotected" protein and the native protein.

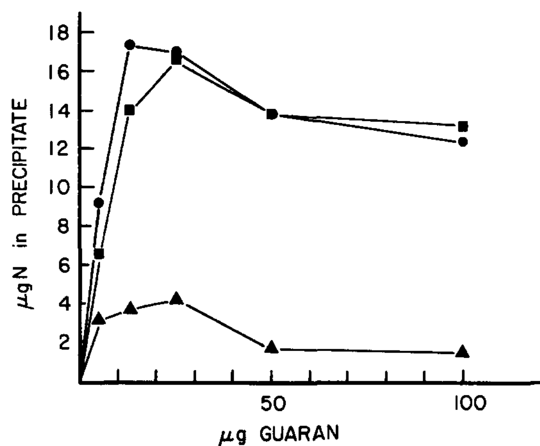

Fig. 1. Precipitation of guaran by native and modified *B. simplicifolia* lectin. ●, native lectin (14.5 μgN); ■, carboxyl-modified, "protected" lectin (20.7 μgN); ▲, modified, "unprotected" lectin (17.9 μgN). N, nitrogen content of lectin.

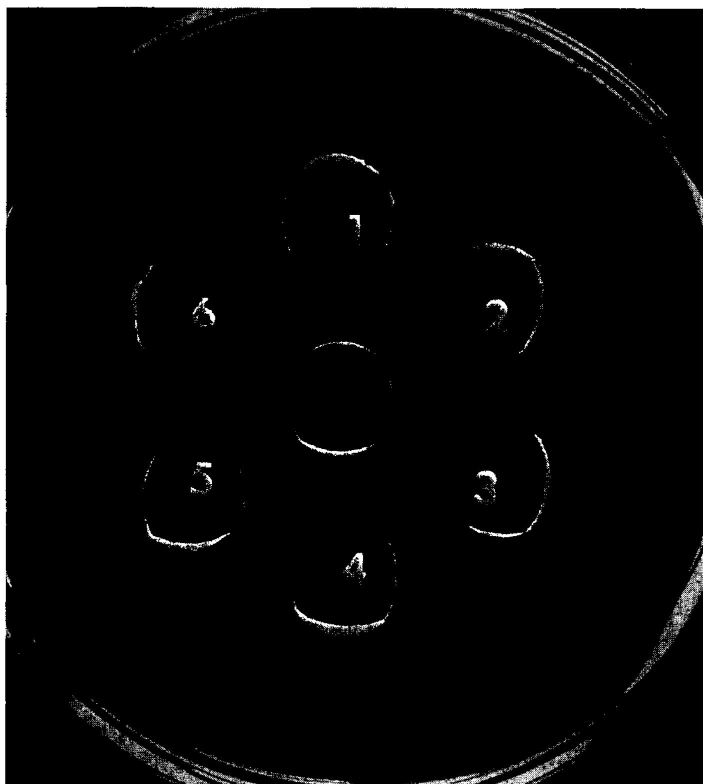

Fig. 2. Ouchterlony double diffusion in agar. Center well, antiserum to native lectin; Well 1, native lectin; Well 2, carboxyl-modified "unprotected" lectin; Well 3, carboxyl-modified "protected" lectin; Well 4, native lectin; Well 5, saline control; Well 6, acetylated lectin. All lectin samples were 0.1 mg/ml in phosphate buffered saline.

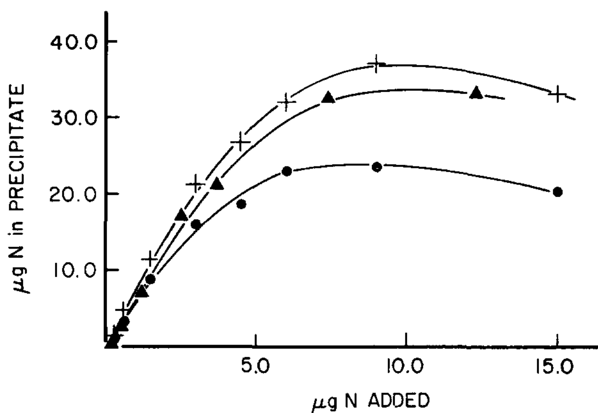

Fig. 3. Quantitative precipitin curves of antiserum (200 μl) to native lectin with native and modified lectins. +, native lectin; ▲, carboxyl-modified, "protected" lectin; ●, carboxyl-modified, "unprotected" lectin. N, nitrogen content of lectin.

These immunochemical reactions were further investigated by the quantitative precipitin technique. As shown in Fig. 3, there are significant differences in the reactivities of the two modified proteins with antiserum to the native lectin. The amidation with glycine methyl ester may mask certain antigenic determinants in the native protein since a smaller amount of precipitate is formed with the modified samples. Furthermore, the strongly bound galactoside ligand can obviously protect against some modification of determinants since the amount of precipitate formed with this sample is about 90% of that of native protein, whereas the corresponding figure for the "unprotected" protein is only 65%. The data also suggests that carboxyl functions may play a particularly important role as part of an antigenic determinant.

Taken together the experiments reported above indicate that carboxyl-groups are importantly involved in the specific carbohydrate binding capability of the *B. simplicifolia* lectin.

The *B. simplicifolia* lectin was acetylated employing acetic anhydride. This procedure results in acetylation of free amino functions (e.g. ε-amino groups of lysyl residues) as well as the hydroxyl groups of tyrosine, threonine and serine; free sulfhydryl groups may also be modified. According to the ninhydrin [8] and trinitrobenzenesulfonic acid [13] assays, the degree of modification of free amino groups was 77 and 76%, respectively. This corresponds to modification of about 8 of the 10 lysyl residues in each lectin subunit. The alkaline hydroxamate procedure [14] indicated that about 2 moles of hydroxy and thio amino acids had been modified per mole lectin subunit. The circular dichroism spectra of the acetylated protein were largely unchanged in both the far and near ultraviolet regions. This experiment shows that virtually no perturbation of the secondary or tertiary structure resulted from the acetylation reaction. On double diffusion in agar against antiserum to native protein (Fig. 2), no spur (i.e. cross-reactivity) with the native protein was discernable. This result suggests that amino-functions do not play an important role in the antigenic determinants of this protein. Interestingly, similar results were found in the case of acetylated concanavalin A [6].

The carbohydrate binding capacity of the acetylated lectin was investigated by the quantitative precipitin reaction with guaran. This polysaccharide precipitated 94% of the modified lectin, a value close to that found for the native material. The precipitation was specifically inhibited by methyl α-D-galactopyranoside and D-galactose at concentrations of 0.66 mM and 1.8 mM, respectively, for 50% inhibition. These results indicate that free amino functions apparently are not involved in the carbohydrate binding phenomenon or in the stabilization of the carbohydrate binding region of the lectin. Since modification also occurred on hydroxy amino acyl residues, these also might not be directly involved in the carbohydrate binding. However, since the degree of modification on these residues was low, the involvement of these residues is less firmly established.

Each of the four subunits of the *B. simplicifolia* lectin contain one free sulfhydryl group [1] and it was implied that these groups might be necessary for the lectin to display carbohydrate binding activity [1].

The alkylating reagent methyl methane thiosulfonate has been shown to be a potent agent for sulfhydryl group modification [16]. However, this reagent failed to react with the lectin (as monitored by precipitin reactions) even when an eightfold molar excess over sulfhydryl groups was used. Demetalized *B. simplicifolia* lectin [1] also resisted modification by methyl methane thiosulfonate. Addition of Ca(II) to demetalized lectin that had been incubated with methyl methane thiosulfonate resulted in complete restoration of carbohydrate-binding activity. Under the same conditions, the activity of the *Phaseolus lunatus* lectin, a further sulfhydryl containing protein [17–19], was completely abolished (Murphy, L. and Goldstein, I. J., unpublished results).

When mercuric chloride, in concentrations up to 5 mM, was included in the incubation mixtures of lectin and guar gum, no inhibition of precipitation was observed. Preliminary experiments indicated that the lectin also could not be alkylated using iodoacetamide unless it was first denatured using high concentrations of urea or guanidine hydrochloride.

These results indicate that the lectin's sulfhydryl groups are not readily modified and consequently are probably "buried" in the interior of the protein molecule. This is in accordance with earlier results of titrations of the lectin with 5,5'-dithiobis-(2-nitrobenzoic acid). Without denaturant (urea or sodium dodecyl sulfate) only 50–75% of the sulfhydryl functions were titrable [1]. However, the earlier finding of hemagglutination inhibition [1] by action of mercuric ions is difficult to explain and could be due to an effect on the red blood cell membrane rather than on the lectin.

Inasmuch as none of the reagents reported above modify the sulfhydryl groups, it is a reasonable hypothesis that free sulfhydryl groups are not directly involved in the carbohydrate or metal binding sites of the lectin. An alternate possibility of a conformational change on ligand binding thereby exposing the binding groups can be discarded from earlier studies [3] which indicated that no significant change in conformation takes place on metal or carbohydrate binding.

It is noteworthy that the results obtained in this study closely parallel those found in similar studies of concanavalin A [4, 6] and wheat germ agglutinin [20]. The carbohydrate binding capacity of both these lectins is greatly reduced on modification of carboxyl groups via carbodiimide mediated amidation. In the case of concanavalin A a strongly bound ligand, methyl α-D-mannopyranoside can also protect against

modification. The involvement of carboxyl groups in the carbohydrate binding phenomenon of con A has been confirmed by independent methods [21]. Con A can be exhaustively acetylated with only moderate loss of biological activity, i.e. polysaccharide precipitating power [6] and mitogenic capability [22]. Wheat germ agglutinin, on the other hand, tolerates acetylation of amino groups only and loses most of its agglutinating capacity on *O*-acetylation [20].

ACKNOWLEDGEMENTS

The authors are indebted to Dr. J. Shafer of this University for valuable discussions. This work was supported by National Institute of Health Grant AM 10171. A travel grant to one of us (Jörgen Lönngren) from the Swedish-American Foundation is gratefully acknowledged.

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