Photoactivatable Glycopeptide Reagents for Site-specific Labeling of Lectins*

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Photoactivatable, iodinated glycopeptides bearing oligosaccharides of defined structure have been synthesized for use as lectin binding site-specific agents. Two such glycopeptides have been examined utilizing concanavalin A, RCA1, (Ricinus communis agglutinin), RCAII (R. communis toxin), and the Gal/GalNAc-specific lectin from human and rat hepatocytes. Covalent incorporation upon photoactivation only occurs with a glycopeptide which is specifically bound by the lectin and is inhibited only by appropriate haptenic monosaccharides. The efficiency of covalent coupling is on the order of 2%. Half-maximal covalent incorporation occurs at a concentration of photoactivatable glycopeptide which corresponds to the previously determined association constant for lectin binding in each case. Covalent incorporation of glycopeptide is accompanied by a decrease in the relative mobility of the lectin upon sodium dodecyl sulfate polyacrylamide gel electrophoresis equivalent to that expected for addition of an oligosaccharide moiety. The high degree of specificity and the relatively high affinity of these photoactivatable glycopeptides makes them promising agents for the examination of lectins such as the Gal/GalNAc-specific receptor present on mammalian hepatocytes.

Affinity labeling of receptor binding sites has been utilized to examine a number of biological receptor systems (1, 2). Although affinity probes for site-specific labeling of lectins have been constructed with monosaccharides, these reagents have suffered from relatively poor affinities and thereby limited or undefined specificity (3, 4). Since both plant and mammalian lectins have extended binding sites which bind oligosaccharides with greater affinity and specificity than monosaccharides (5–11), affinity probes prepared from oligosaccharides should display considerably greater specificity than monosaccharide-based reagents. The capacity for site-specific labeling of mammalian lectins, such as the hepatic Gal/GalNAc-specific lectin which mediates the endocytosis of glycopeptides and glycoproteins, would provide a useful tool for examining the molecular events involved in endocytosis as well as a means for mapping the oligosaccharide binding sites of lectins. We have therefore developed methods for preparing 125I-labeled glycopeptides bearing photoactivatable azide derivatization and have characterized these derivatives using lectins of differing specificity. These photoactivatable glycopeptide probes have in each case proved to be highly specific at both high and low concentrations with a 1 to 2% efficiency of photocrosslinking of four different lectins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Con A (crystallized three times) was from ICN Pharmaceuticals, Cleveland, OH. RCA1, and RCAII, and the Gal/GalNAc-specific lectin from human and rat liver were prepared as described previously (7, 8). Succinimidyl-3-(4-hydroxyphenyl)propionate was from ICN Pharmaceuticals. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide was from Sigma, and 1,3,4,5-tetrachloro-3,5-(diphenylyl)carbocyanine iodide (Lissamine-Gen) from Pierce. The preparation of glycopeptides from ovalbumin, bovine IgG, and fetuin has been described (6). N-Succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, N-succinimidyl 3-(4-hydroxyphenyl)-1,3-dithiopropionate, and N-hydroxysuccinimidyl-4-azido-benzoate was purchased from Pierce.

**Synthesis of Glycopeptide Probes**—All glycopeptides were subjected to descending paper chromatography in butanol:ethanol:water (4:1:1) for 48 h prior to derivatization. Sialic acid was removed from complex oligosaccharides by treatment with 2 N acetic acid at 100 °C for 15 min. From 1 to 10 μmol of purified glycopeptide was derivatized with succinimidyl-3-(4-hydroxyphenyl)propionate in 0.1 M borate buffer, pH 8.5. The succinimidyl-3-(4-hydroxyphenyl)propionate was first dissolved in dioxane and then added to the glycopeptide in 4 equal portions over 2 h such that the dioxane ranged from 10 to 40% of the reaction volume and a total 10-fold molar excess of derivatizing agent over glycopeptide was utilized. The derivatized glycopeptide was resolubilized with gel filtration on Bio-Gel P-4 in 0.1 M ammonium bicarbonate, pH 8.5. The buffer was removed by repeated lyophilization from the water. The carboxyl group of the glycopeptide was then derivatized with 1.0 M ethylenediamine, pH 4.8, by addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and incubation for 16 h. The glycopeptide product was iodinated utilizing Iodo-Gen labeling efficiency of 125I-glycopeptide and 125I-labeled glycopeptide was iodinated utilizing Iodo-Gen and 1 mCi of 125I/10 to 20 μmol of glycopeptide (12). The product was separated from free 125I and reagent by gel filtration on Sephadex G-10 in water. The 125I-glycopeptide product was then derivatized with a succinimidyl, photoactivatable agent in the dark in 100 μl of 0.1 M borate buffer, pH 8.0, by addition of a 100-fold excess of the succinimidyl reagent in dioxane. After 15 min, the product was separated from reagent by gel filtration over a 1-ml column of Sephadex G-25 in a foil-wrapped tuberculin syringe in semidarkness. The product was taken up in a known volume and aliquots utilized as described for the individual experiments below.

**Photoactivation**—Following incubation of the glycopeptide probe with the lectin under examination in the dark, activation was accomplished with 3 KaKo Auto 201 electronic flash units from which the plastic covers were removed to expose the xenon flash tube at a distance of 3 to 5 cm from the reaction tube (6 × 50 mm or 10 × 75 mm borosilicate glass tube). It was determined in control experiments that exposure to five flashes was sufficient to consume in excess of 90% of the activatable probe.

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† The abbreviations used are: Con A, concanavalin A; RCA1; RCAII, the agglutinin of R. communis, and toxins M, 60,000, respectively, prepared from R. communis beans; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Preparation for Gel Electrophoresis—Following photo activation, reactions were brought to a final concentration of 12% trichloroacetic acid at 4 °C. The precipitated proteins were collected by centrifugation in an Eppendorf Microfuge for 15 min at 4 °C. The pellets were washed twice with 12% trichloroacetic acid and then with −20 °C ethanol. The samples were then subjected to SDS-PAGE as described by Laemmli (13).

RESULTS

Synthesis of Photoactivatable Glycopeptides—A number of different schemes were tried for the synthesis of photoactivatable, iodinated glycopeptides. Iodination following introduction of both the hydroxyphenyl group and the arylazide as well as coupling of the succinimide ester of the iodinated hydroxyphenyl moiety after addition of the arylazide proved unsatisfactory. The approach outlined in Fig. 1 has proved facile and reproducible in yield of material. The initial introduction of the hydroxyphenyl derivative onto the NH₂ terminus permits all further purification procedures to be monitored and quantitated on the basis of the absorbance at 280 nm. Conversion of the remaining carboxyl function to an amino function with ethylenediamine permits the introduction of photoactivatable moieties by means of succinimidal derivatives which react rapidly and proceed to completion at low concentrations. This is of particular importance when preparing small amounts of the iodinated derivative which must be synthesized immediately before use. These probes can be radiolabeled with ¹²⁵I to specific activities of greater than 50,000 cpm/pmol. Therefore, even at the relatively low efficiencies frequently observed for covalent coupling during photoactivation one can expect to incorporate considerable radiolabel activity. In addition, as has been demonstrated by Kiehm and Ji (14), the use of flash units with xenon flash tubes assures complete activation and a short half-life for the activated agent while avoiding significant damage to proteins or intact cells.

Demonstration of Specificity—In order to demonstrate that the glycopeptide derivatives we have described would in fact be highly specific affinity-labeling agents, a number of different approaches were taken. First, two prototype glycopeptide derivatives were prepared which have different oligosaccharide structures. One of these was a high mannose-type oligosaccharide isolated from ovalbumin, GP-V; and the other was a complex oligosaccharide prepared from fetuin, Fet-Glm-I, which have the structures shown in Fig. 2. Thus, affinity labeling of lectins which differ in their oligosaccharide specificity utilizing probes of differing oligosaccharide structure could be compared. For example, GP-V is bound tightly by Con A, whereas Fet-Glm-I is not (6); the converse is true for RCAI and RCAII which are prepared from castor beans (7). In Fig. 3, it can be seen that the GP-V derivative is able to label Con A in the presence or absence of lactose (Lanes 2 and 3, respectively), whereas labeling is completely inhibited by α-methylmannoside (Lane 1). In contrast, no specifically inhibitable label is incorporated under these conditions when utilizing Fet-Glm-I (Lanes 4, 5, and 6, respectively). It should also be noted that the Con A which has been affinity-labeled displays a slower mobility on SDS-PAGE as compared to unlabeled Con A, equivalent to the shift in molecular weight expected from addition of a carbohydrate moiety. A second, less highly labeled band of radioactivity with a slower mobility is also present in Lanes 2 and 3, and incorporation into this material is inhibited by α-methylmannoside (Lane 7). Coomassie blue staining also reveals the presence of a small amount of protein of slower mobility than the major protein band in this commercial preparation of Con A. This material presumably accounts for the affinity-labeled band of slower mobility. We do not currently know what the relationship of this material is to the major protein band; however, it may be that this preparation consists predominantly of the naturally

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\text{GP-V} \quad \text{Fet-Glm-I}
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Fig. 2. Structures of glycopeptides utilized as photoactivatable probes. The sugars are: G, N-acetylglucosamine; M, mannose; g, galactose. GP-V was prepared from ovalbumin and Fet-Glm-I was from fetuin.
were labeled by the GP-V derivative (Fig. 3). Con A, lZ'I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 5, RCAI, 125I-Fet-Glm-I, 0.2 m lactose; Lane 7, RCAI, 125I-GP-V, 0.2 m a-methylmannoside; Lane 9, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 10, RCAII, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 11, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 12, RCAII, 125I-Fet-Glm-I, Lane 13, Stds, 125I-GP-V, 0.2 m a-methylmannoside; Lane 14, Stds, 125I-GP-V, 0.2 m lactose; Lane 15, Stds, 125I-GP-V, Lane 16, Stds, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 17, Stds, 125I-Fet-Glm-I, 0.2 m lactose; Lane 18, Stds, 125I-Fet-Glm-I, 0.2 m lactose; Lane 12, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 6, Con A, 125I-Fet-Glm-I.

FIG. 3. Autoradiograph of SDS-PAGE of affinity-labeled Con A. Con A (10 μg) was incubated with either 125I-Fet-Glm-I (5.9 × 10⁶ cpm) or 125I-GP-V (1.4 × 10⁶ cpm) in the dark. Photoactivation was done in 200 μl of 50 mM NaPO₄, pH 7.4, with 0.2 m lactose or 0.2 m a-methylmannoside as inhibitors when indicated. The arrows indicate the position of the native Con A as determined by staining with Coomassie blue. Lane 1, Con A, 125I-GP-V, 0.2 m a-methylmannoside; Lane 2, Con A, 125I-GP-V, 0.2 m lactose; Lane 3, Con A, 125I-GP-V; Lane 4, Con A, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 5, Con A, 125I-Fet-Glm-I, 0.2 m lactose; Lane 6, Con A, 125I-Fet-Glm-I.

occuring proteolytic fragment of Con A described by Cunningham et al. (14α) which still retains binding activity.

The identical experiments were carried out for RCAI and RCAII and are illustrated in Fig. 4. Neither RCAI nor RCAII were labeled by the GP-V derivative (Fig. 5, Lanes 1, 2, 3, 7, 8, and 9). In contrast, the Fet-Glm-I affinity probe labeled both RCAI and RCAII in the presence or absence of a-methylmannoside (Lanes 4, 6, 10, and 12). Affinity labeling was abolished by lactose in both cases (Lanes 5 and 11). As was seen with Con A, the incorporation of the glycopeptide resulted in a shift to slower migration on SDS-PAGE equivalent to the contribution expected for a carbohydrate moiety. Another indication of the specificity of this reagent is the significantly greater extent of labeling of the B subunit as compared to the A subunit (for example, Lane 4). This would be expected since it is the B subunit which contains the oligosaccharide binding site (15, 16). Essentially, no labeling was seen when ovalbumin, chymotrypsinogen, or ribonuclease were exposed to either probe under the same conditions as Con A, as expected since it is the B subunit which contains the oligosaccharide binding site (15, 16).

FIG. 4. Autoradiograph of SDS-PAGE of affinity labeled RCAI and RCAII. The conditions and photoactivatable probes were identical with those described in Fig. 3. Lectins and proteins used were RCAI (24 μg), RCAII (14 μg), ribonuclease A (10 μg), ovalbumin (10 μg), and chymotrypsinogen (10 μg). Lane 1, RCAI, 125I-GP-V, 0.2 m a-methylmannoside; Lane 2, RCAII, 125I-GP-V, 0.2 m lactose; Lane 3, RCAI, 125I-GP-V; Lane 4, RCAI, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 5, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 6, RCAI, 125I-Fet-Glm-I; Lane 7, RCAII, 125I-GP-V, 0.2 m a-methylmannoside; Lane 8, RCAII, 125I-GP-V, 0.2 m lactose; Lane 9, RCAII, 125I-GP-V; Lane 10, RCAII, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 11, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 12, RCAII, 125I-Fet-Glm-I, Lane 13, Stds, 125I-GP-V, 0.2 m a-methylmannoside; Lane 14, Stds, 125I-GP-V, 0.2 m lactose; Lane 15, Stds, 125I-GP-V, Lane 16, Stds, 125I-Fet-Glm-I, 0.2 m a-methylmannoside; Lane 17, Stds, 125I-Fet-Glm-I, 0.2 m lactose; Lane 18, Stds, 125I-Fet-Glm-I, 0.2 m lactose; Lane 12, RCAII, 125I-Fet-Glm-I, 0.2 m lactose; Lane 6, Con A, 125I-Fet-Glm-I.

FIG. 5. Separation of affinity labeled Con A cyanogen bromide fragments on Bio-Gel P-60. A, the profile obtained for 25 μg of Con A treated with cyanogen bromide as described by Waxdal et al. (24). B, the profile obtained for cyanogen bromide-cleaved Con A which has been affinity labeled with GP-V. The column was eluted with 20% formic acid. Approximately equimolar amounts of F-I, F-II, and F-III are obtained.
RCA$_{I}$ and RCA$_{II}$ (Lanes 13 to 18 in Fig. 4) and autoradiograms exposed for the same length of time. Following prolonged exposure radiolabel was detected; however, in these cases the radiolabel corresponded in mobility to the Coomassie blue-stained protein bands and was not subject to inhibition by haptens. The same behavior was noted for lectins with long exposures. During the fixation and staining steps, non-coupled glycopeptide agent is eluted rapidly from the gel. Controls in which previously activated glycopeptides were subjected to SDS-PAGE and autoradiographed without fixation and staining prior to dying the gel did not yield any distinct bands in the region between the stacking gel and the dye front, although there was an increase in the diffuse, background level in the autoradiogram for these lanes. Finally, when other glycopeptides such as those bearing dibranched complex oligosaccharides which could bind to Con A, RCA$_{I}$, and RCA$_{II}$ were examined, the radiolabeled bands corresponded in location to those just described for GP-V with Con A and Fet-Glm-I with RCA$_{I}$ and RCA$_{II}$. Thus, the location of the radiolabel correlates with the lectin being labeled and does not reflect any anomalous mobility of the reagent itself.

Localization of Affinity Label—Another indication of specificity of affinity label is an important feature required for a truly site-specific affinity label would be incorporation at a definable site which is a part of or adjacent to the binding site itself. Since the amino acid sequence as well as the tertiary structure based on x-ray crystallography are known for Con A (17-23), it is an excellent model lectin to determine whether the affinity label is indeed incorporated in the region which is believed to be the oligosaccharide binding site. We therefore affinity-labeled Con A with GP-V and cleaved the product into three peptides with cyanogen bromide as described (24). The three peptides produced by cyanogen bromide cleavage can be separated by gel filtration on Bio-Gel P-60 as shown in Fig. 5. Greater than 90% of the affinity label was found to be in the fragment F-III (Fig. 5B). In order to establish that this was not due to incomplete degradation of the Con A by the cyanogen bromide, these fragments were examined by SDS-PAGE (data not shown). No intact Con A was present in the F-III fraction and, as was seen with the intact lectin, fragment F-III displayed a slower mobility than the cyanogen bromide fragment and without the affinity label. Thus, the affinity ligand is almost exclusively incorporated into a single peptide fragment. Fragment F-III contains the greatest number of amino acid residues in contact with what is believed to be the carbohydrate binding site of Con A (20) and would therefore be expected to be the most likely site for labeling.

Efficiency of Affinity Labeling—Another question of importance with such reagents is the efficiency of covalent attachment upon photoactivation. Since the binding constants have been established for each of these glycopeptides and their respective lectins, the saturation curve for binding could be compared to that for label incorporation. As can be seen in Fig. 6 for Con A, RCA$_{I}$, and the human hepatic Gal/GalNAc-specific lectin, covalent incorporation of label over an increaseing concentration range of each affinity probe increased in a saturable fashion. The maximal incorporation for each lectin represents some 2% of calculated available binding sites; i.e. at full site occupancy, some 2% of the sites will become covalently occupied by the affinity label. The complete lack of incorporation of glycopeptide probes not bound by the respective lectins even at high concentration is also demonstrated. It is also notable that the concentration of probe resulting in covalent incorporation of half of the maximal amount attained at saturation is in each case essentially equivalent to the $k_d$ previously established for these glycopeptides and their respective lectins.

Site-specific glycopeptide reagents for lectins.

DISCUSSION

Site-specific affinity labels have been utilized for the examination of a number of receptors; however, to date previous attempts to develop site-specific agents for lectins have been confined to derivatives of monosaccharides (3, 4). Although these have proved useful for preparing lectins with differing valencies by blocking one or more sites by covalent modification, the monosaccharide derivatives are bound with relatively poor affinity and exhibit a limited degree of specificity. In addition, none of the monosaccharide derivatives have been prepared in a highly radiolabeled form. Therefore, monosaccharide derivatives did not appear to be good candidates for studies ultimately to be directed at such things as the highly specific membranous lectins which mediate the endocytosis of...
glycopeptides and glycoproteins. Since our studies and those of other laboratories defining the specificity of both plant and mammalian lectins indicate that oligosaccharides are bound with a much greater affinity and degree of specificity than monosaccharides, we proceeded to develop the glycopeptide site-specific agents just described. Utilizing homogeneous lectins of related specificity.

mammalian lectins indicate that oligosaccharides are bound to the site itself. Although the efficiency of covalent incorporation is low, on the order of 2%, the ability to prepare material of high specific activity (as much as 500,000 cpm/pmol) and relatively high affinity indicates that these agents can in fact be utilized to examine lectins at low concentrations such as are seen on cell surfaces. In a preliminary experiment carried out with rat hepatocytes, it appeared that labeling of the Gal/GalNAc-specific lectin was accomplished with relatively little nonspecific labeling. Further studies, however, will be required to definitively establish the identity of the products. These glycopeptide agents should prove highly useful for examination of such lectin receptors and determining the means by which they are able to mediate endocytosis of glycoproteins. These agents may also prove useful for the discovery of new lectins of related specificity.

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