Assay and Characterization of Carbohydrate Binding by the Lectin Discoidin I Immobilized on Nitrocellulose†

Russell E. Kohnken and Edward A. Berger*

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Received December 2, 1986; Revised Manuscript Received February 23, 1987

ABSTRACT: Discoidin I is the most abundant galactose binding lectin produced by the cellular slime mold *Dictyostelium discoideum* and has been implicated in cell–substratum adhesion. We have developed an assay of carbohydrate binding activity utilizing binding of ^{125}I-asialofetuin to discoidin I, or to other lectins, immobilized on nitrocellulose. Among the proteins examined, only lectins exhibited the ability to bind asialofetuin. Specificity of asialofetuin binding was demonstrated by competition with monosaccharides, which inhibited binding consistent with the known sugar specificity of the lectins examined. Experiments with fetuin and derivatives differing in their oligosaccharide structure indicated a requirement for terminal galactosyl residues for probe binding to discoidin I. We have used this assay to characterize the carbohydrate binding behavior of discoidin I. The extent of asialofetuin binding to discoidin I was dependent on the concentrations of both lectin and ligand. Interpretation of equilibrium binding data suggested that, under saturating conditions, 1 mol of oligosaccharide was bound per mole of discoidin I monomer. Furthermore, discoidin I in solution and discoidin I on nitrocellulose were equally effective at competing for soluble asialofetuin, suggesting that immobilization had no effect on the carbohydrate binding behavior of discoidin I. Binding was strongly inhibited by ethylenediaminetetraacetic acid; both Ca^{2+} and Mn^{2+} could overcome that inhibition, but Mg^{2+} could not. Preincubation of discoidin I at 60 °C stimulated asialofetuin binding 2-fold by increasing the affinity, while preincubation at higher temperatures resulted in a complete loss of activity. A time course of digestion with trypsin showed that a slightly smaller fragment of discoidin I retained carbohydrate binding activity but that fragments approximately half the size of intact discoidin I did not. Chemical modification of discoidin I suggested that oxidation-sensitive, sulfhydryl, and possibly carboxylate side chains are important for carbohydrate binding. Protection by *N*-acetylgalactosamine indicated that some of those residues may be at or near the carbohydrate binding site, suggesting approaches with which to identify that site.

Discoidin I is a galactose binding lectin synthesized by the cellular slime mold *Dictyostelium discoideum* during starvation-induced development (Simpson et al., 1974). It is a tetrameric protein composed of four identical subunits (Simpson et al., 1974) of relative molecular weight (M_r)[1] 28 100 (Poole et al., 1981). Unlike a number of lectins, a function for discoidin I in vivo has been proposed. Discoidin I contains a tetrapeptide sequence homologous to the cell binding site of fibronectin (Pierschbacher et al., 1985), and a cell surface receptor on *Dictyostelium* cells has been identified which appears to interact with that domain of discoidin I (Gabius et al., 1985). On the basis of experiments demonstrating inhibition of cell–substratum adhesion by both synthetic peptides identical with the fibronectin-like domain of discoidin I and specific monovalent antibodies directed against discoidin I, it has been suggested that discoidin I mediates the cell–substratum adhesion which is required for orderly cellular aggregation during development of this organism (Springer et al., 1984). It is not clear whether carbohydrate binding is required for this function, although packaging of discoidin I into multilamellar bodies and subsequent externalization do appear to involve carbohydrate binding (Barondes et al., 1985; Cooper et al., 1986). Unlike the cell binding site, the domain of discoidin I involved in carbohydrate binding has yet to be localized.

In order to facilitate the identification of the carbohydrate binding site of discoidin I, we have examined in detail the carbohydrate binding behavior of this lectin. On the basis of hemagglutination (Frazier et al., 1975), binding to fixed *Dictyostelium* cells (Bartles & Frazier, 1980), and binding of lactosyl-conjugated bovine serum albumin (Cooper et al., 1983), some characteristics of carbohydrate binding by discoidin I have been described. These include sugar specificity (Frazier et al., 1975), metal dependence (Alexander et al., 1983), and sensitivity to a component extracted from *Dictyostelium* cells with chloroform/methanol (Bartles et al., 1979). In this paper, we describe an assay which measures binding of radioiodinated asialofetuin (ASF) to lectins immobilized on nitrocellulose. This assay is sensitive, simple, and quantitative and has allowed us to characterize further the carbohydrate binding behavior of discoidin I. With this assay, we have identified several reagents which appear to interact with or modify discoidin I at or near its carbohydrate binding site, thereby suggesting strategies for localization of that site.

† This work was supported by NIH Grants GM 25588, GM 30986, and CA 12708.

* Correspondence should be addressed to this author.

[1] Abbreviations: ASF, asialofetuin; RCA 60, *Ricinus communis* agglutinin, type I; RCA 120, *Ricinus communis* agglutinin, type II; Con A, concanavalin A; GalNAc, *N*-acetylgalactosamine; SDS, sodium dodecyl sulfate; M_r, relative molecular weight; SD, standard deviation about the mean; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PBS/Az, 20 mM potassium phosphate, pH 7.2, 150 mM NaCl, and 1 mM NaN_3; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

0006-2960/87/0426-3949$01.50/0

Materials and Methods

Preparation of Discoidin I. *Dictyostelium discoideum* strain Ax3 was grown axenically in medium described by Loomis (1971) and starved in suspension for 5–7 h prior to lysis by freezing/thawing and sonication. Discoidin I was extracted from lysed cells and purified by affinity chromatography on Sepharose 4B according to Frazier et al. (1975). As it was eluted from the Sepharose column with 400 mM galactose, the first peak was generally greater than 95% discoidin I, with the remainder consisting of discoidin II (see Figure 6A, lane 7, for an SDS gel profile of a typical preparation of discoidin I). No further purification was performed, and aliquots were stored at −70 °C at greater than 1 mg/mL protein concentration in 400 mM galactose and PBS/Az. Aliquots were frozen and thawed no more than twice for experiments, though additional cycles appeared to have little effect on ASF binding activity (data not shown).

Preparation of Radioiodinated ASF. ASF was purchased from Sigma or prepared by desialylation of fetuin by acid hydrolysis (Spiro & Bhoyroo, 1974). Iodination was performed by using Chloramine T (Greenwood et al., 1963) with $Na^{125}I$ (carrier free) from Amersham. The reaction was terminated after 10 min with sodium metabisulfite. Free iodine was removed by gel filtration on Bio-Gel P-6, and the recovery of protein and the specific activity were estimated by precipitation with trichloroacetic acid. Recovery of radioactivity precipitable by trichloroacetic acid was typically 75–95%, and the specific activity varied from 3×10^4 to 6×10^5 cpm/pmol of ASF. ^{125}I-ASF was stored in aliquots at −70 °C and subjected to no more than one freeze/thaw cycle.

Binding of ASF to Immobilized Protein. In most experiments, discoidin I (or other proteins) at 1 mg/mL was spotted by using a Hamilton syringe onto dry squares of nitrocellulose (9 × 9 mm) and allowed to air dry for 15–30 min. The squares were then blocked in PBS/Az supplemented with 3 mg/mL casein and 400 mM galactose for 20–30 min. The squares were rinsed briefly in PBS/Az and blotted to drain excess liquid. Incubation of these squares with ASF was routinely performed in 12-well Costar tissue culture plates which were pretreated with 3 mg/mL casein and 1 mM *p*-aminobenzoic acid in PBS/Az for 2 h prior to assay and then rinsed thoroughly with water. A typical incubation contained six squares of nitrocellulose, spotted with 0, 1, and 3, μg of protein (in duplicate for a total of 8 μg of protein per incubation), and 1–3 pmol of ^{125}I-ASF in 1 mL of 1 mM $CaCl_2$ and 1 mM *p*-aminobenzoic acid in PBS/Az. Incubation was allowed to proceed overnight on a shaker at 21 °C and terminated by aspiration of the incubation medium followed by five 5-min washes with 3 mg/mL casein in PBS/Az. Bound radioactivity to each square was detected with a Beckman γ counter. Blank binding was defined as the amount of ^{125}I-ASF bound to nitrocellulose squares that were not spotted with any protein. Assays were occasionally performed in larger volumes with multiple spots of protein on one larger sheet of nitrocellulose, which was cut into smaller squares at the end of the assay for quantitation.

Modification Reaction Conditions. (*A*) *EDC.* Discoidin I at 60 μg/mL in 50 mM potassium phosphate, pH 5.7, was reacted with 10 mM EDC, prepared fresh in the same buffer, with or without 50 mM GalNAc in a reaction volume of 250 μL. After the reaction proceeded for 60 min at 21 °C in the dark, 20 mM lysine and 200 mM galactose in PBS/Az were added; 0, 1, 2, and 3 μg of discoidin I from these reactions was spotted onto nitrocellulose with a Hybridot apparatus from Bethesda Research Labs and assayed as described above.

(*B*) *Trinitrobenzenesulfonate.* Discoidin I at 60 μg/mL in 50 mM potassium phosphate, pH 7.0, was reacted with 1 mg/mL trinitrobenzenesulfonate, prepared fresh in the same buffer, with or without 50 mM GalNAc, in a reaction volume of 250 μL. The reaction was performed and terminated exactly as described for EDC.

(*C*) *Fluorescein Isothiocyanate.* Reaction of discoidin I with fluorescein isothiocyanate was performed exactly as described for EDC except that 1 mg/mL fluorescein isothiocyanate was the modifying reagent, and the pH of the reaction was 7.0. Fluorescein isothiocyanate was prepared fresh at 25 mg/mL in dimethyl sulfoxide. A comparable amount of dimethyl sulfoxide was added to those assays which did not receive any fluorescein isothiocyanate.

(*D*) *N-Ethylmaleimide.* Reaction of discoidin I with *N*-ethylmaleimide was performed exactly as described for EDC except that 1 mM *N*-ethylmaleimide was the modifying reagent, and the pH of the reaction was 6.5. *N*-Ethylmaleimide was prepared fresh in the reaction buffer. The reaction was terminated with cysteine instead of lysine.

(*E*) *Diethyl Pyrocarbonate.* Reaction of discoidin I with diethyl pyrocarbonate was performed exactly as described for EDC except that 5 mM diethyl pyrocarbonate was the modifying reagent, and the pH of the reaction was 8.0. Diethyl pyrocarbonate was prepared fresh in the reaction buffer. The reaction was terminated with imidazole instead of lysine.

(*F*) *KI plus Chloramine T.* Reaction of discoidin I with KI and Chloramine T was performed exactly as described for EDC except that 1 mM KI and 60 μg/mL Chloramine T were the modifying reagents, and the pH of the reaction was 7.0. Both KI and Chloramine T were prepared fresh in the reaction buffer. Reactions were terminated by the addition of 80 μg/mL sodium metabisulfite.

(*G*) *Chloramine T.* Reaction of discoidin I with Chloramine T was performed exactly as described for EDC except that 60 μg/mL Chloramine T was the modifying reagent, and the pH of the reaction was 7.0. Chloramine T was prepared fresh in the reaction buffer. Reaction was terminated by the addition of 80 μg/mL sodium metabisulfite.

SDS Gel Electrophoresis. Electrophoresis was performed by using a 15% resolving gel and a 7.5% stacking gel according to Laemmli (1970). The gels were stained with Coomassie blue and scanned with a Kontes fiber optic scanner interfaced with a Hewlett-Packard 3393A integrator.

Miscellaneous. Protein concentration was determined according to Lowry et al. (1951) using BSA as a standard. ASF was periodate treated according to Bergey et al. (1986).

Materials. GalNAc was obtained from Calbiochem, EDC from Aldrich, and nitrocellulose from Schleicher & Schuell (BA 85) or Gelman (Biotrace NT). Gel electrophoresis reagents were obtained from Bio-Rad. All other reagents, unless otherwise designated, were obtained from Sigma.

Results

Protein and Sugar Specificity of ASF Binding. It has been reported that ASF is a potent inhibitor of hemagglutination by discoidin I (Bartles et al., 1979), suggesting that it interacts with discoidin I at the carbohydrate binding site. We utilized that characteristic, along with the protein-immobilizing capability of nitrocellulose, to develop an assay of carbohydrate binding activity. In order to assess whether binding of ASF was a reflection of carbohydrate binding by the immobilized protein, we examined binding to a variety of proteins (Table I). The non-lectin proteins, bovine serum albumin, hemoglobin, and gelatin, bound only minute amounts of ASF, whereas each of the lectins shown exhibited substantial levels

Table I: Protein Specificity of ASF Binding[a]

protein	ASF bound (fmol/μg of protein)
non-lectins	
bovine serum albumin	0.4
hemoglobin	0.1
gelatin	0.0
lectins	
discoidin I	39
discoidin II	12
Con A	14
RCA 60	63

[a] ASF binding was assayed as described under Materials and Methods. ASF concentration was 2.0 nM at 1.7×10^5 cpm/pmol; 3 μg of protein was applied to nitrocellulose in duplicate in each case, and average binding to duplicate blank pieces of nitrocellulose was subtracted.

Table II: Effects of Blocking Proteins on ASF Binding by Discoidin I[a]

blocking protein	total binding	blank binding	discoidin I binding	signal-to-noise ratio
casein	130	13	117	9.0
bovine serum albumin	459	207	252	1.2
hemoglobin	335	91	244	2.7
gelatin	1029	762	267	0.4

[a] Nitrocellulose was blocked with each of the above proteins after discoidin I was applied at 3 μg per square in duplicate. ASF concentration was 2.1 nM at 7.5×10^4 cpm/pmol. ASF binding is expressed as femtomoles of ASF per nitrocellulose square. Total binding is ASF binding to squares spotted with discoidin I; blank binding is ASF binding to squares not spotted with discoidin I; discoidin I binding is the difference between the previous two measurements; signal-to-noise ratio is the ratio of discoidin I binding to blank binding.

of binding. Discoidin II is another endogenous galactose binding lectin of *Dictyostelium* which is immunologically and structurally related to discoidin I (Berger & Armant, 1982) but has slightly different sugar specificity (Frazier et al., 1975; Madley et al., 1981) and developmental characteristics (Cooper & Barondes, 1984). These assays were performed using casein as an agent to block background binding to nitrocellulose. In Table II, the effects of various blocking proteins on ASF binding by discoidin I are presented. Total and blank ASF binding varied considerably depending on which protein was used as the blocking agent. Casein exhibited by far the lowest blank binding of ASF. ASF binding attributable to discoidin I (total binding minus blank binding) was clearly detectable against blank binding and approximately 50% lower when casein was used than when other proteins were the blocking agents. This may be due to the carbohydrate content of casein (Baker et al., 1963), making it a possible competitor. Nevertheless, the signal-to-noise ratio was much greater for casein, so that we routinely used it as the blocking agent in the experiments described below, except where indicated.

That ASF binding reflects association of the ligand with the carbohydrate binding sites of the lectins was further examined in a number of ways. In the experiments shown in Table III, we tested competition by a variety of simple sugars. It can be seen that the ability of monosaccharides to compete against ASF binding corresponded to the known sugar specificities of several lectins. Thus, binding by Con A was most sensitive to methyl α-mannoside and glucose derivatives, but not galactose derivatives (Poretz & Goldstein, 1970; Beppu et al., 1975). Conversely, ASF binding by discoidin I was most sensitive to galactose derivatives (Frazier et al., 1975), and only galactose inhibited binding to RCA 120 (Nicolson & Blaustein, 1972). Finally, we tested the effects of modification of the carbohydrate content of the ligand. Experiments showed that radioiodinated fetuin exhibited much lower binding than

Table III: Monosaccharide Competition of ASF Binding to Lectins[a]

	ASF binding (% control)		
100 mM sugar	discoidin I	Con A	RCA 120
none	100	100	100
galactose	9	82	21
GalNAc	1	98	122
glucose	84	6	89
N-acetylglucosamine	101	10	102
methyl α-mannoside	101	0	107

[a] Three micrograms of each lectin was spotted in duplicate, and ASF binding to blank nitrocellulose was subtracted. ASF concentration was 1.0 nM at 5.5×10^5 cpm/pmol.

ASF to discoidin I (data not shown). Furthermore, periodate treatment greatly reduced the ability of unlabeled ASF to compete against labeled ASF for binding to discoidin I (data not shown). Taken together, the results in this section indicate the dependence of ASF binding both on the carbohydrate binding capability and specificity of the immobilized protein and on the carbohydrate structure of ASF.

Time and Concentration Dependence of ASF Binding. Many interactions of simple sugars with lectins occur with a low affinity and exhibit a rapid dissociation (Lis & Sharon, 1986). Multivalency of either ligand, lectin, or both tends to stabilize the interaction but increases the time to reach equilibrium. We examined ASF binding to discoidin I over time and the stability of that binding to subsequent washes. For all three amounts of discoidin I shown in Figure 1A, binding was half-maximal within 30 min and nearly at equilibrium by 3 h. We therefore routinely performed our assays with overnight incubations. It can also be seen here that ASF binding was proportional in this concentration range to the amount of discoidin I spotted. Retention of bound ASF during repeated washings is shown in Figure 1B. Representative squares of nitrocellulose spotted either with 2 μg of discoidin I or with no protein after either one wash or five washes are shown as insets. While blank binding decreased 75% during the first three washes to a plateau (usually less that 0.5% of the total radioactivity in the assay), total binding decreased gradually, so that less than 20% of the total binding was lost during the washing steps. Taken together, these results indicate that the ASF binding detected in the standard assay was representative of binding at equilibrium.

The effects on equilibrium binding of varying ASF and discoidin I concentrations were examined in the experiments shown in Figure 2. When analyzed according to the method of Scatchard (1949), ASF binding in the presence of varying ASF concentrations indicated a saturable, homogeneous population of binding sites on discoidin I (Figure 2A). With the use of a nonlinear curve-fitting program (Munson & Rodbard, 1980), K_D was estimated at 126 and 144 nM for 1 and 3 μg of discoidin I spotted, respectively. The total number of binding sites was 2.9 and 8.3 pmol for 1 and 3 μg of discoidin I, respectively, again demonstrating linearity of binding with the amount of protein spotted. Since this experiment was performed with constant ^{125}I-ASF concentration and varying unlabeled ASF concentration, the validity of these calculations is based on the assumption that the labeled and unlabeled ligands have identical binding characteristics. This was tested directly by comparing ASF binding over a concentration range of 1–25 nM ASF. Regardless of whether ASF was solely ^{125}I-ASF or a mixture with unlabeled ASF, binding was comparable (data not shown).

When the amount of discoidin I spotted was varied (Figure 2B), the data again indicated a saturable homogeneous pop-

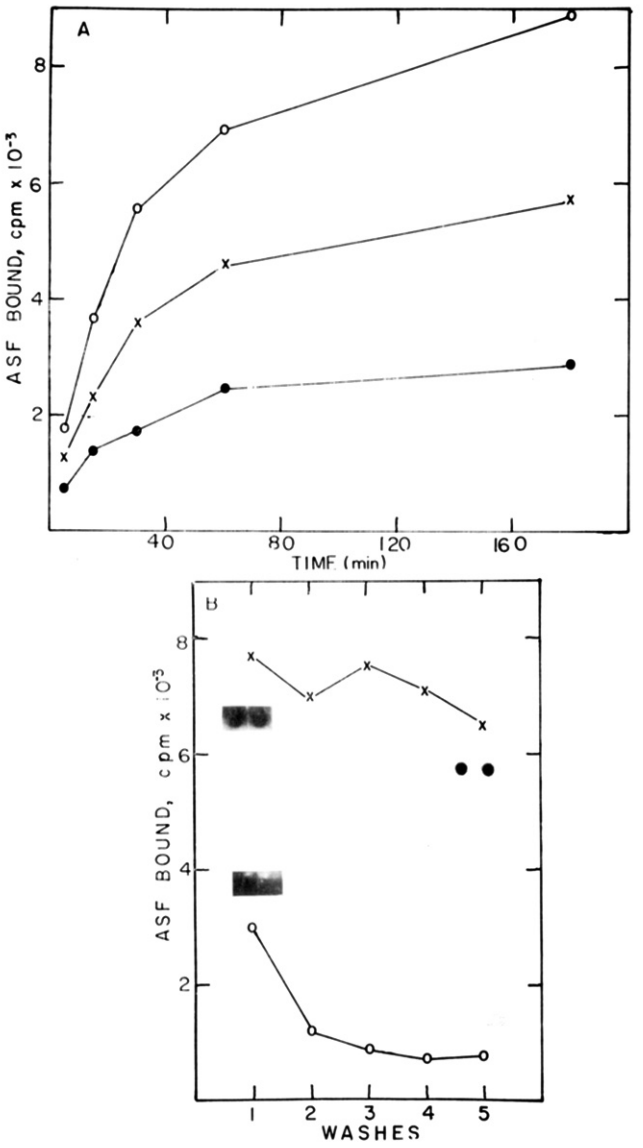

FIGURE 1: Association and dissociation of discoidin I and ASF. (A) ASF binding was assayed as described under Materials and Methods except that the incubations were terminated at the indicated times. ASF concentration was 1.3 nM at 3.8×10^5 cpm/pmol; 0, 1, 2, and 3 μg of discoidin I were spotted, and the binding to discoidin I minus the binding to the blank nitrocellulose is plotted: (●) 1 μg; (×) 2 μg; (○) 3 μg. (B) ASF binding was assayed as described under Materials and Methods except that the nitrocellulose squares were washed for the indicated number of times at 5 min per wash. ASF concentration was the same as in (A). Data from two different experiments were averaged: (○) ASF binding to blank nitrocellulose; (×) total ASF binding to nitrocellulose spotted with 2 μg of discoidin I. Representative duplicate squares of nitrocellulose are shown as insets directly above or beneath the corresponding points on the curves.

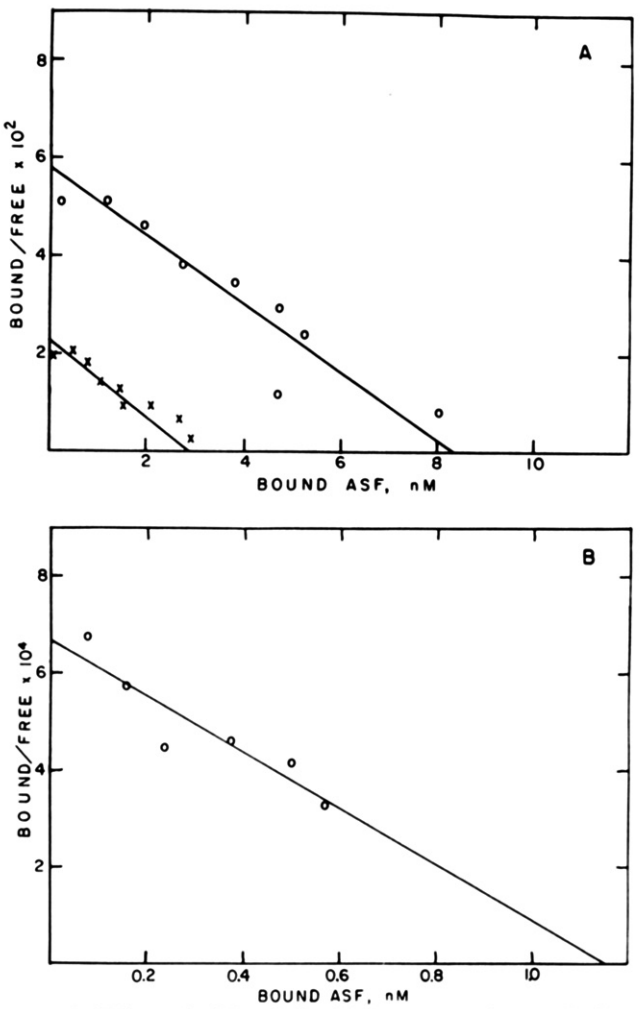

FIGURE 2: Effects of ASF and discoidin I concentrations on binding activity. (A) ASF concentration was varied from 2.16 to 1000 nM by inclusion of 2.16 nM ^{125}I-ASF (89 000 cpm/pmol) and varying concentrations of unlabeled ASF; 1 and 3 μg of discoidin I were spotted. Binding was assayed as described under Materials and Methods. The data were analyzed by using the nonlinear curve-fitting program LIGAND (Munson & Rodbard, 1980) as adapted for Applesoft. Background or nonspecific binding was determined by this program to be less than 0.012 pmol of ^{125}I-ASF (0.6% of total) and was subtracted out prior to plotting the data: (×) 1 μg of discoidin I; (○) 3 μg of discoidin I. The lines were generated by the program as the best fit for specific binding to discoidin I. (B) The amount of discoidin I spotted onto 15 × 15 mm size squares of nitrocellulose was varied from 3 to 45 μg. ASF concentration was 0.95 nM at 8.9×10^4 cpm/pmol. Otherwise, binding was assayed as described under Materials and Methods. Data were analyzed by an unweighted linear regression.

ulation of binding sites. The x intercept approximated the concentration of ASF in the assay, suggesting that all of the ligand was available for binding. However, the K_D determined in this experiment was approximately 1730 nM, which differs markedly from the values obtained in the experiment shown in Figure 2A. The estimate from Figure 2B assumes that each discoidin I monomer applied was able to bind one molecule of ASF. However, in Figure 2A, only 0.08 mol of ASF was bound/mol of discoidin I spotted under saturating conditions (2.8 pmol of ASF bound/μg of discoidin I divided by 36 pmol of discoidin I monomer/μg). If the data in Figure 2B are recalculated assuming that the available discoidin I concentration was only 8% of the total amount applied, then the K_D estimate becomes 134 nM, in excellent agreement with that determined from the data in Figure 2A.

There are several factors which could contribute to the apparent low stoichiometry of ASF bound to discoidin I in the assay. The first is the multivalency of ASF, which has six oligosaccharides with terminal galactosyl residues per molecule (Spiro & Bhoyroo, 1974; Spiro, 1964). Thus, each mole of ASF has 6 mol of potential binding ligands. Second, of the discoidin I applied to nitrocellulose, only 70% remained bound throughout the course of the assay, as ascertained by experiments using ^{125}I-discoidin I (data not shown). Finally, as seen in Figure 1B, about 20% of ASF binding was lost during washing. These factors can increase the calculated number of carbohydrate binding sites more than 10-fold. Assuming these corrections are valid, approximately 0.82 mol of oligosaccharide is bound at saturation per mole of discoidin I monomer, suggestive of one carbohydrate binding site per discoidin I monomer. However, this value must be taken as

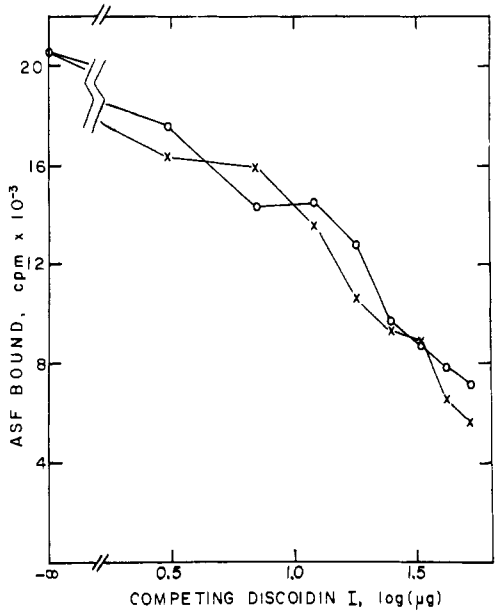

FIGURE 3: Competition for ASF binding by discoidin I in solution and immobilized on nitrocellulose. ASF binding was assayed as described under Materials and Methods with the following modifications. Three pieces of nitrocellulose were included in each assay: one 9 × 9 mm square spotted with 0 μg of discoidin I for blank binding; one 9 × 9 mm square spotted with 3 μg of discoidin I to measure ASF binding; and one 15 × 15 mm square spotted with varying amounts of discoidin I for competition. All squares were blocked with hemoglobin rather than casein to minimize the presence of any other potential competitors. ASF concentration was 3.11 nM at 2.6×10^4 cpm/pmol. Blank binding in this case (using hemoglobin) was approximately 3.4×10^3 cpm and was subtracted to give the values shown. For competition by discoidin I on nitrocellulose, the large square of nitrocellulose was spotted with varying amounts of discoidin I from 3 to 52 μg. For competition by soluble discoidin I, the large square was left unspotted, and the discoidin I was added to the assay in solution at 3–52 μg/assay. ASF binding in the presence of the competing discoidin I is plotted against the log (competing discoidin I concentration): (○) competing discoidin I on nitrocellulose; (×) competing discoidin I in solution.

a preliminary estimate since it is unclear whether steric constraints would allow all six carbohydrate chains of a given ASF molecule to simultaneously occupy carbohydrate binding sites on discoidin I.

Discoidin I Immobilized on Nitrocellulose Behaves as Discoidin I in Solution. In order to test whether immobilization alters the ability of discoidin I to bind ASF, we performed the experiment shown in Figure 3. This experiment compared the ability of discoidin I, either present in solution or immobilized on nitrocellulose, to compete for ASF against separate samples of immobilized discoidin I. A constant amount of discoidin I immobilized on nitrocellulose was incubated with a constant amount of ASF and varying amounts of additional discoidin I, either present in solution or spotted on a separate piece of nitrocellulose. Competition by the additional discoidin I is plotted as the amount of ASF bound to the constant discoidin I vs. the log of the concentration of the additional discoidin I. Over the concentration range examined (up to 52 μg of discoidin I), discoidin I in solution and discoidin I on nitrocellulose showed comparable ability to compete for ASF. These data suggest that discoidin I on nitrocellulose bound ASF in a manner which was indistinguishable from that of discoidin I in solution.

Metal Dependence of ASF Binding. It has been reported (Alexander et al., 1983) that sugar binding by discoidin I exhibits a divalent cation dependence. In the experiments summarized in Figure 4A, it was observed that EDTA at 1

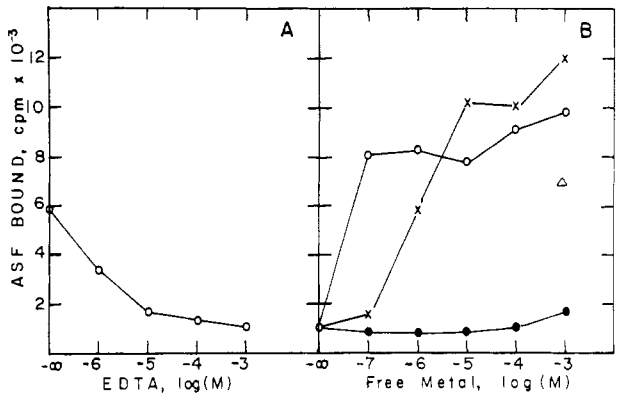

FIGURE 4: Metal ion dependence of ASF binding. ASF binding was assayed as described under Materials and Methods except that, after blocking but before the assay, the nitrocellulose was washed with 1 mM EDTA and 200 mM galactose in PBS/Az for 2 min, rinsed with PBS/Az, blotted, and then assayed. ASF concentration was 1 nM at 4.5×10^5 cpm/pmol; 1 and 3 μg of discoidin I were spotted, and the averages of these from two experiments are plotted as cpm bound per microgram of discoidin I. Binding obtained from blank pieces of nitrocellulose has been subtracted to give the data shown. (A) The concentration of EDTA was varied in the assay. (B) The concentration of divalent cations was varied in the presence of 1 mM EDTA in the assay. Free cation concentration was calculated by using K_A(Ca-EDTA) = 2.846×10^7 M^{-1}, K_A(Mg-EDTA) = 3.583×10^5 M^{-1}, and K_A(Mn-EDTA) = 8.022×10^{10} M^{-1} (Bartfai, 1979). (×) Ca^{2+}; (○) Mn^{2+}; (●) Mg^{2+}; (△) control containing 1 mM Ca^{2+} but which had never been exposed to EDTA.

μM in the assay inhibited ASF binding by almost 50% and that 1 mM inhibited 80%. The ability of divalent cations in the presence of 1 mM EDTA to restore ASF binding is shown in Figure 4B. While Ca^{2+} and Mn^{2+} were optimally effective at free concentrations of 10 and 0.1 μM, respectively, Mg^{2+} was ineffective up to 1 mM. For both Mn^{2+} and Ca^{2+}, ASF binding was greater than a control which had never been exposed to EDTA, but which did contain 1 mM Ca^{2+} (no EDTA in the assay). This may indicate a tightly bound, but relatively ineffective, cation present with discoidin I in our preparations, or some other inhibitory factor which can be removed by EDTA. Our results differ from those of Alexander et al. (1983) in that we observed that Mn^{2+} was more effective and Mg^{2+} was less effective than they described. While our assay measures direct binding of a carbohydrate-containing ligand, their assay measured binding and subsequent elution of discoidin I from Sepharose 4B. Thus, the observed differences may reflect differences in the assays employed.

Temperature-Dependent Stimulation of Discoidin I. We examined the thermal stability of discoidin I by preincubation at various temperatures for 5 min prior to immobilization and assay. The effects on ASF binding are presented in Figure 5A. Surprisingly, preincubation at temperatures up to 60 °C stimulated subsequent ASF binding up to 2-fold. Greater temperatures dramatically inactivated discoidin I, in agreement with a previous report (Bartles et al., 1979). In order to determine whether this stimulation occurred via a change in binding affinity or capacity we examined the ASF concentration dependence of binding to discoidin I which was pretreated at 55 vs. 21 °C (Figure 5B). When analyzed according to the method of Scatchard (1949), these data indicated that thermal stimulation lowered the K_D (from 96 to 49 nM in this experiment) with little or no effect on the binding capacity of discoidin I.

Proteolytic Digestion of Discoidin I. Discoidin I was subjected to several fragmentation procedures prior to immobilization in order to determine which, if any, are potentially useful for localization of the carbohydrate binding site. In-

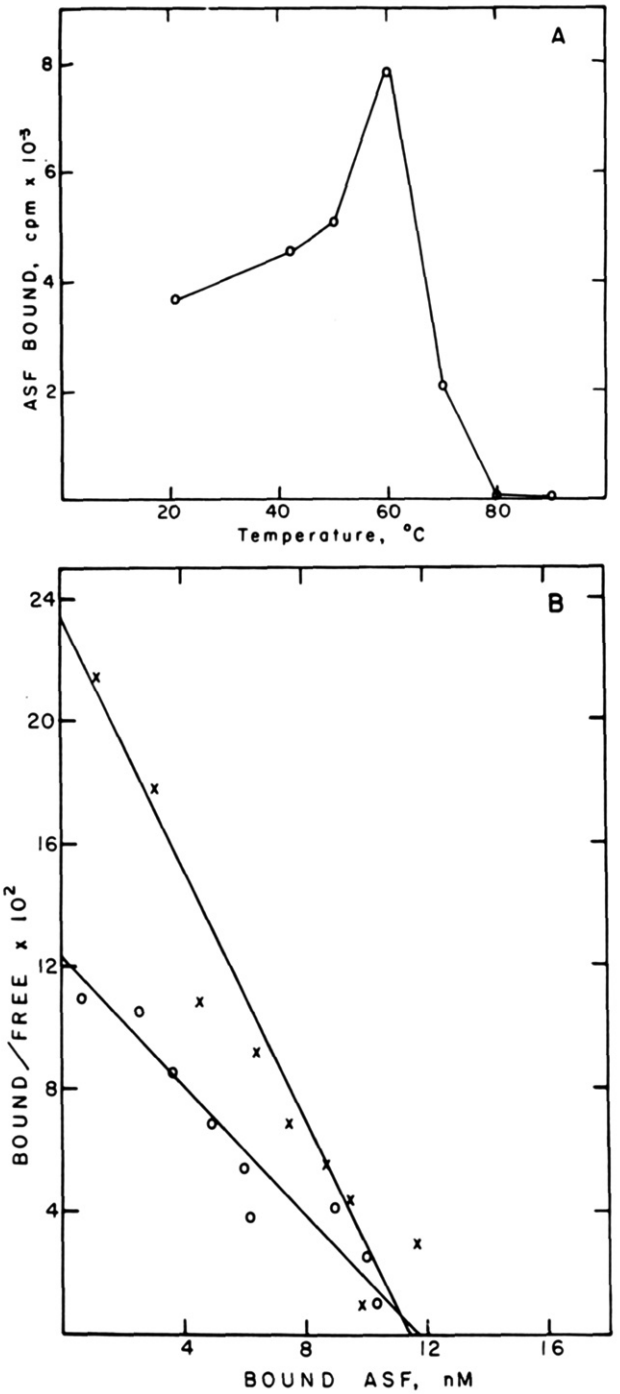

FIGURE 5: Effects of preincubation at different temperatures on ASF binding. ASF binding was assayed as described under Materials and Methods. (A) 1 mg/mL discoidin I was preincubated for 5 min in 200 mM galactose and PBS/Az at the indicated temperatures prior to being spotted on nitrocellulose. ASF concentration was 2.3 nM at 7.2 × 10^4 cpm/pmol; 1 and 3 μg of discoidin I were spotted, and the average binding per microgram of discoidin I is plotted (blank ASF binding has been subtracted). (B) ASF binding was performed exactly as in Figure 2A except that one set was performed with discoidin I preincubated at 21 °C (○) and the other with discoidin I preincubated at 55 °C (×). Data were again analyzed and fitted as described in the legend to Figure 2A.

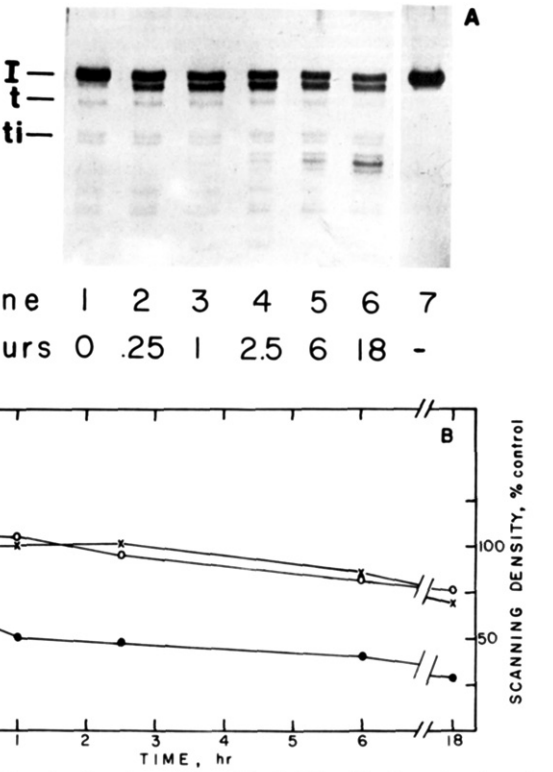

FIGURE 6: Trypsin digestion of discoidin I. Discoidin I was incubated at 2 mg/mL in 200 mM galactose and PBS/Az with or without 0.2 mg/mL trypsin (TPCK treated) at 21 °C. At the indicated times, 25 μL was removed, mixed with 25 μL of 0.2 mg/mL soybean trypsin inhibitor in 200 mM galactose and PBS/Az, and then stored at 4 °C. After the last time point, all samples were spotted on nitrocellulose and assayed as described under Materials and Methods. In addition, aliquots were also subjected to SDS gel electrophoresis, stained, and scanned as described under Materials and Methods. (A) Lanes 1 through 6 present an SDS gel profile of the time course of trypsin digestion of discoidin I. Discoidin I without any additions is shown in lane 7. I, position of intact discoidin I; t, position of intact trypsin; ti, position of intact soybean trypsin inhibitor. (B) A comparison is presented of the ASF binding activity of trypsin-treated discoidin I with the amount of intact discoidin I or the amount of discoidin I plus its largest fragment. (×) ASF binding activity of discoidin I digested with trypsin for the indicated times; (●) integrated scanning density of intact discoidin I vs. time of trypsin digestion; (○) integrated scanning density of the sum of discoidin I and the largest fragment. Scanning data are presented as the percent of the zero time scanning density. ASF binding was assayed as described under Materials and Methods. ASF concentration was 3.4 nM at 2.6 × 10^4 cpm/pmol.

cubation of discoidin I in 70% formic acid for 4 h at 4 °C (standard conditions for CNBr cleavage) resulted in complete loss of ASF binding. We next examined an enzymatic fragmentation procedure. A time course of proteolysis by trypsin is shown in Figure 6. The SDS gel profile in Figure 6A indicates a rapid cleavage to yield a fragment slightly smaller than discoidin I, followed by a much slower degradation into intermediate size fragments (approximately M_r 15000). When ASF binding was measured, only a slow loss of activity was observed (Figure 6B). From a comparison of ASF binding with scanning densities of SDS gel bands, it is clear that binding did not correlate well with the amount of intact discoidin I but did correlate very well with the sum of intact discoidin I and its largest fragment. This suggests that the large fragment retained ASF binding activity. Therefore, either the amino and/or the carboxy terminus of discoidin I is dispensable for carbohydrate binding. In addition, smaller fragments of discoidin I apparently lacked carbohydrate binding activity, suggesting either cleavage within the active site or loss of conformational structure necessary for binding.

Chemical Modification of Discoidin I. Reagents that modify specific amino acid side chains in proteins have proven useful to study and identify active sites. We examined the sensitivity of ASF binding by discoidin I to a number of modification reagents, and the results are summarized in Table IV. In each case, discoidin I was pretreated for 1 h at 21 °C as described under Materials and Methods prior to being

Table IV: Effects of Chemical Modification Reagents on ASF Binding[a]

modification reagent	ASF binding (% control) (±SD) +reagent, −GalNAc	+reagent, +GalNAc	−reagent, −GalNAc
Chloramine T (n = 2)	1 ± 1*	100 ± 34	92 ± 7
KI[b] (n = 6)	10 ± 13*	26 ± 47*	34 ± 53*
N-ethylmaleimide (n = 5)	66 ± 27*	120 ± 44	108 ± 22
EDC (n = 6)	76 ± 19	96 ± 19	95 ± 12
trinitrobenzenesulfonate (n = 3)	91 ± 36	130 ± 48	103 ± 7
fluorescein isothiocyanate (n = 2)	84 ± 7	80 ± 18	79 ± 6
diethyl pyrocarbonate (n = 2)	86 ± 39	106 ± 14	94 ± 17

[a] Discoidin I was modified, as described under Materials and Methods, prior to being spotted on nitrocellulose. Control binding in each case (defined as 100%) represents the ASF binding observed when discoidin I was exposed to the conditions of the modification reaction, in the presence of GalNAc and in the absence of the modification reagent. The number of experiments is indicated for each reagent. In those cases where there were only two experiments performed, the range is indicated rather than the standard deviation. Data were analyzed by analysis of variance, and differences which were significant ($P < 0.05$) by F test, and between paired means by the least significant difference test, are indicated by an asterisk. [b] All of these permutations, including those designated "−reagent", contained Chloramine T; in this case, reagent refers only to KI.

spotted on nitrocellulose. ASF binding activity was normalized in each experiment such that binding after incubation of discoidin I in the absence of the modifying reagent and in the presence of 50 mM GalNAc was defined as 100%. This allows the effects of modification and any protection by GalNAc to be evaluated more readily. In the first series of experiments, we examined the effects of iodination using Chloramine T. Since this protocol exposes the protein to strong oxidizing conditions as well as to modification of specific amino acid residues (tyrosine), we designed these experiments to discriminate between the oxidative effects of Chloramine T and the effects of iodination. As shown in the first row, ASF binding was almost completely abolished by Chloramine T; however, inclusion of GalNAc during the incubation prevented that inhibition. This suggests an oxidation-sensitive amino acid at or near the carbohydrate binding site. When we next examined the effect of KI in the presence of Chloramine T, we observed that ASF binding was again significantly inhibited but that the inhibition was no longer prevented by the inclusion of GalNAc. This suggests that the detrimental iodination occurring with KI and Chloramine T was not restricted to the carbohydrate binding site. This conclusion supports earlier findings (E. A. Berger, unpublished data) that lactoperoxidase-catalyzed iodination of discoidin I strongly inhibited its hemagglutination activity, whereas exposure to the same reaction conditions in the absence of KI had no effect. These results are also consistent with problems reported in the use of ^{125}I-labeled discoidin I prepared by oxidative means for functional assays (Bartles & Frazier, 1980). The sulfhydryl-modifying reagent N-ethylmaleimide also inhibited ASF binding significantly, and as with Chloramine T, GalNAc offered protection. EDC, which activates carboxyl groups to react with endogenous or exogenous amino groups, followed by lysine, which both quenches unreacted EDC and reacts with any available activated carboxyl groups, gave variable inhibition (15–45% in four of six experiments) which was prevented by inclusion of GalNAc. Neither trinitrobenzenesulfonate nor fluorescein isothiocyanate, both of which modify amino groups, altered ASF binding to any appreciable degree. Likewise, diethyl pyrocarbonate, which reacts with histidine, did not significantly affect discoidin I carbohydrate binding activity. It should be noted that these conditions were not optimized for modification but rather selected to minimize buffer effects on ASF binding. Increasing the extent of these reactions may point to additional residues important to carbohydrate binding. These results suggest that a sulfhydryl group(s) and/or other oxidizable amino acids, and possibly carboxyl residues, are near the carbohydrate binding site and that their modification decreases the ability of discoidin I to bind ASF.

Discussion

A requirement for any assay is that it reliably describes the behavior under study. ASF binding to immobilized discoidin I was designed to measure carbohydrate binding. Several lines of evidence indicate that ASF binding represents association between oligosaccharides on the ligand and carbohydrate binding sites of the lectins. First, of the proteins examined, only those known to be lectins were able to bind ASF. Second, inhibition of ASF binding by simple sugars correlated with the known specificities of each of the lectins. Finally, experiments with modified forms of the ligand indicated that discoidin I only bound ligand which contained terminal galactosyl residues. An important feature of this assay is that immobilization appeared to have no effect on the behavior of discoidin I. This was demonstrated by showing that discoidin I was able to compete for ASF identically whether it was immobilized or in solution. Taken together, these results indicate that the ASF binding assay accurately describes the carbohydrate binding behavior of discoidin I.

ASF binding exhibited a concentration dependence for both ASF and discoidin I which was indicative of a single population of binding sites on discoidin I. When the number of oligosaccharide moieties on ASF, the amount of discoidin I actually immobilized, and the loss of bound ligand during washing are taken into account, the data are consistent with a 1:1 stoichiometry between discoidin I monomer and oligosaccharide chains on the ligand. These results suggest the presence of one carbohydrate binding site per discoidin I monomer. However, this value must be confirmed by more direct approaches, since it is based on the untested assumption that all six carbohydrate side chains of a given ASF molecule are capable of simultaneously associating with discrete carbohydrate binding sites on discoidin I.

Previously, carbohydrate binding by discoidin I had been primarily characterized by hemagglutination (Frazier et al., 1975), by binding of iodinated discoidin I to fixed *Dictyostelium* cells (Bartles & Frazier, 1980), or by binding of iodinated lactosyl bovine serum albumin to discoidin I immobilized in polystyrene wells (Cooper et al., 1983). Our assay is conceptually similar to the polystyrene assay but takes advantage of the large and reproducible binding capacity of nitrocellulose for proteins (Kumar et al., 1985; Schleicher & Schuell Application Update, 1986). The 70% immobilization that we observed is consistent with reports for other proteins (Davis et al., 1984; Hirose et al., 1986) and substantially greater than that reported for immobilization to polystyrene wells (Cantatero et al., 1980; Davis et al., 1984; Pesce et al., 1977). Furthermore, we observed no variation in the assay due to different lots or even different manufacturers of nitrocellulose (data not shown).

A broad range of assays has been used to characterize other lectins including fluorescence and ultraviolet difference spectroscopies (Dean & Homer, 1973; Neurohr et al., 1980), equilibrium dialysis (Zentz et al., 1978), nuclear magnetic resonance (Dahlquist & Raftery, 1969; Brewer et al., 1973), immobilization in poly(vinyl chloride) wells (Lesniak & Liu, 1984), and precipitation of lectin–ligand complexes (Carter & Sharon, 1977; Etzler, 1977). We propose that the assay

described here can be a valuable complement to these assays. While we routinely used 1–3 μg of protein in our assays, binding could be reliably measured at 100 ng of lectin, comparable to or greater than the sensitivity described for other assays (Lehrman et al., 1986). The nitrocellulose assay is easy to perform, inexpensive, quantitative, and potentially applicable to a great number of lectins having an affinity for the oligosaccharide chains of asialofetuin or other suitable glycoproteins.

Using this assay, we have obtained information about the carbohydrate binding site of discoidin I and identified approaches to characterize further and identify that site. Digestion with trypsin suggested that active fragments of discoidin I can be obtained and that the amino- and/or carboxy-terminal ends of discoidin I are unnecessary for carbohydrate binding. In addition, the loss of activity from smaller fragments, though still approximately half the size of intact discoidin I, indicated either cleavage within the carbohydrate binding domain, loss of conformation necessary for binding in a fragment containing the binding site, or the existence of a binding domain which includes residues that are not in close proximity in the amino acid sequence. This latter possibility has been observed in the carbohydrate binding sites of a number of proteins (Quiocho, 1986). If this is the case for discoidin I, then identification of residues involved in carbohydrate binding might be more easily obtained by using site-specific reagents. Modifications by *N*-ethylmaleimide, Chloramine T, and EDC have suggested that sulfhydryl group(s), other oxidizable amino acids, and possibly carboxyl residues are involved in ASF binding. Protection by GalNAc further suggests that these residues may be at or near the active site. Alternatively, any or all of these modifications could occur at an amino acid residue which acts allosterically to inhibit ASF binding, but this possibility also requires that active-site occupancy by GalNAc allosterically prevents modification of those amino acids. Tryptophan, tyrosine, and charged amino acids appear to be common constituents of carbohydrate binding sites (Quiocho, 1986). β-Galactose-specific lectins often require a reducing environment, suggesting the presence of oxidizable residues in their function (Lis & Sharon, 1986; Levi & Teichberg, 1981). Thus, modification of discoidin I by these reagents not only suggests the similarity of discoidin I to other carbohydrate binding proteins but also offers a tool to identify amino acids involved in that site. Since GalNAc appears to protect amino acids required for ASF binding from modification, the technique of differential modification using both labeled and unlabeled reagents should be useful (Brooker & Slayman, 1983).

Finally, we have used this assay in the development of other parallel approaches to identification of the carbohydrate binding site. One of those is an immunological approach. We have found that polyclonal antibodies to discoidin I block ASF binding (data not shown), raising the potential utility of site-specific monoclonal antibodies. We have also prepared a photoactivatable derivative of galactose, *N*-(4-azidosalicyl)-2-amino-2-deoxy-D-galactosamine, which blocks ASF binding as effectively as GalNAc and interacts with discoidin I in a manner consistent with true photoaffinity labeling of the carbohydrate binding site (R. E. Kohnken and E. A. Berger, unpublished results). Thus, development of the ASF binding assay has provided information about the carbohydrate binding behavior of discoidin I and has indicated a possible role for a number of amino acid residues in ASF binding which may be at or near the active site. In addition, a number of specific and independent methods have been developed which should enable localization of the carbohydrate binding site of discoidin I.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Louise Ohrn. We also thank Dr. Christine Collins and Dr. Donna Bozzone for critical reading of the manuscript.

Registry No. GalNAc, 1811-31-0; Con A, 11028-71-0; Ca, 7440-70-2; Mn, 7439-96-5; nitrocellulose, 9004-70-0; galactose, 59-23-4; glucose, 50-99-7; *N*-acetylglucosamine, 7512-17-6; methyl α-mannoside, 27939-30-6.

REFERENCES

Alexander, S., Cibulsky, A. M., & Lerner, R. A. (1983) *Differentiation (Berlin) 24*, 209–212.

Baker, B. E., Huang, F. Y. Y., & Harington, C. R. (1963) *Biochem. Biophys. Res. Commun. 13*, 227–230.

Barondes, S. H., Haywood-Reid, P. L., & Cooper, D. N. W. (1985) *J. Cell Biol. 100*, 1825–1833.

Bartfai, T. (1979) *Adv. Cyclic Nucleotide Res. 10*, 219–242.

Bartles, J. R., & Frazier, W. A. (1980) *J. Biol. Chem. 255*, 30–38.

Bartles, J. R., Pardos, B. T., & Frazier, W. A. (1979) *J. Biol. Chem. 254*, 3156–3159.

Beppu, M., Terao, T., & Osawa, T. (1975) *J. Biochem. (Tokyo) 78*, 1013–1019.

Berger, E. A., & Armant, D. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2162–2166.

Bergey, E. J., Levine, M. J., Reddy, M. S., Bradway, S. D., & Al-Hashimi, I. (1986) *Biochem. J. 234*, 43–48.

Brewer, C. F., Sternlicht, H., Marcus, D. M., & Grollman, A. P. (1973) *Biochemistry 12*, 4448–4457.

Brooker, R. J., & Slayman, C. W. (1983) *J. Biol. Chem. 258*, 222–226.

Cantatero, L. A., Butler, J. E., & Osborne, J. W. (1980) *Anal. Biochem. 105*, 375–382.

Carter, W. G., & Sharon, N. (1977) *Arch. Biochem. Biophys. 180*, 570–582.

Cooper, D. N. W., & Barondes, S. H. (1984) *Dev. Biol. 105*, 59–70.

Cooper, D. N., Lee, S. C., & Barondes, S. H. (1983) *J. Biol. Chem. 258*, 8745–8750.

Cooper, D. N. W., Haywood-Reid, P. L., Springer, W. R., & Barondes, S. H. (1986) *Dev. Biol. 114*, 416–425.

Dahlquist, F. W., & Raftery, M. A. (1969) *Biochemistry 8*, 713–725.

Davis, J. W., Angel, J. M., & Bowen, J. M. (1984) *J. Immunol. Methods 67*, 271–278.

Dean, B. R., & Homer, R. B. (1973) *Biochim. Biophys. Acta 322*, 141–144.

Etzler, M. E. (1977) *FEBS Lett. 75*, 231–236.

Frazier, W. A., Rosen, S. D., Reitherman, R. W., & Barondes, S. H. (1975) *J. Biol. Chem. 250*, 7714–7721.

Gabius, H. J., Springer, W. R., & Barondes, S. H. (1985) *Cell (Cambridge, Mass.) 42*, 449–456.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–119.

Hirose, N., Krivanek, M., Jackson, R. L., & Cardin, A. D. (1986) *Anal. Biochem. 156*, 320–325.

Kumar, S., Bard, A. H., Samarantay, J. C., Dang, N., & Talwar, G. P. (1985) *J. Immunol. Methods 83*, 125–133.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lehrman, M. A., Haltiwanger, R. S., & Hill, R. L. (1986) *J. Biol. Chem. 261*, 7426–7432.

Lesniak, A. P., & Liu, E. H. (1984) *Anal. Biochem. 142*, 140–147.

Levi, G., & Teichberg, V. I. (1981) *J. Biol. Chem. 256*, 5735–5740.
Lis, H., & Sharon, N. (1986) *Annu. Rev. Biochem. 55*, 35–67.
Loomis, W. F., Jr. (1971) *Exp. Cell Res. 64*, 484–486.
Lowry, O. H., Roseborugh, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Madley, I. C., Herries, D. G., & Hames, B. D. (1981) *Differentiation (Berlin) 20*, 278–282.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Neurohr, K. J., Martin, N. M., & Mantsch, H. H. (1980) *J. Biol. Chem. 255*, 9205–9209.
Nicolson, G. L., & Blaustein, J. (1972) *Biochim. Biophys. Acta 266*, 543–547.
Pesce, A. J., Ford, D. J., Gaizutis, M., & Pollak, V. E. (1977) *Biochim. Biophys. Acta 492*, 399–407.
Pierschbacher, M. D., Hayman, E. G., & Ruoslahti, E. (1985) *J. Cell. Biochem. 28*, 115–126.
Poole, S., Firtel, R. A., Lamar, E., & Rowekamp, W. (1981) *J. Mol. Biol. 153*, 273–290.
Poretz, R. D., & Goldstein, I. V. (1970) *Biochemistry 9*, 2890–2896.
Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287–315.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–667.
Schleicher & Schuell Applications Update (1986) No. 383, Schleicher & Schuell, Keene, NH.
Simpson, D. L., Rosen, S. D., & Barondes, S. H. (1974) *Biochemistry 13*, 3487–3493.
Spiro, R. G. (1964) *J. Biol. Chem. 239*, 567–573.
Spiro, R. G., & Bhoyroo, V. D. (1974) *J. Biol. Chem. 249*, 5704–5717.
Springer, W. R., Cooper, D. N. W., & Barondes, S. H. (1984) *Cell (Cambridge, Mass.) 39*, 557–564.
Zentz, C., Frenoy, J.-P., & Bourrillon, R. (1978) *Biochim. Biophys. Acta 536*, 18–26.

Photoaffinity Labeling of Serum Vitamin D Binding Protein by 3-Deoxy-3-azido-25-hydroxyvitamin D_3†

Rebecca P. Link, Andrzej Kutner,‡ Heinrich K. Schnoes, and H. F. DeLuca*
Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received October 7, 1986; Revised Manuscript Received February 27, 1987

ABSTRACT: 3-Deoxy-3-azido-25-hydroxyvitamin D_3 was covalently incorporated in the 25-hydroxyvitamin D_3 binding site of purified human plasma vitamin D binding protein. Competition experiments showed that 3-deoxy-3-azido-25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3 bind at the same site on the protein. Tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was synthesized from tritiated 25-hydroxyvitamin D_3, retaining the high specific activity of the parent compound. The tritiated azido label bound reversibly to human vitamin D binding protein in the dark and covalently to human vitamin D binding protein after exposure to ultraviolet light. Reversible binding of tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was compared to tritiated 25-hydroxyvitamin D_3 binding to human vitamin D binding protein. Scatchard analysis of the data indicated equivalent maximum density binding sites with a $K_{D,app}$ of 0.21 nM for 25-hydroxyvitamin D_3 and a $K_{D,app}$ of 1.3 nM for the azido derivative. Covalent binding was observed only after exposure to ultraviolet irradiation, with an average of 3% of the reversibly bound label becoming covalently bound to vitamin D binding protein. The covalent binding was reduced 70–80% when 25-hydroxyvitamin D_3 was present, indicating strong covalent binding at the vitamin D binding site of the protein. When tritiated 3-deoxy-3-azido-25-hydroxyvitamin D_3 was incubated with human plasma in the absence and presence of 25-hydroxyvitamin D_3, 12% of the azido derivative was reversibly bound to vitamin D binding protein. After ultraviolet irradiation, four plasma proteins covalently bound the azido label, but vitamin D binding protein was the only protein of the four that was unlabeled in the presence of 25-hydroxyvitamin D_3.

Vitamin D and its hydroxylated metabolites are transported in serum on a specific vitamin D binding protein (DBP).[1] DBP, which is identical with group-specific component (Bouillon et al., 1976; Daiger et al., 1975; Haddad & Walgate, 1976), is an abundant and multifunctional glycoprotein (Haddad, 1984). In addition to binding vitamin D, it can also bind monomers of actin (Cooke et al., 1979; Van Baelen et al., 1980) and associate with membranes of a variety of cell types, including B-lymphocytes (Petrini et al., 1983a,b) and

† This work was supported by Program Project Grant AM-41881 from the National Institutes of Health, NIH Fellowship AM-07194, and the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation.

* Author to whom correspondence should be addressed. No reprints will be available from the authors.
‡ Present address: Institute of Pharmaceutical Industry, Rydygiera 8, 01-793 Warszawa, Poland.

[1] Abbreviations: DBP, vitamin D binding protein; hDBP, human vitamin D binding protein; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2$-D_3, 1,25-dihydroxyvitamin D_3; Az-25-OH-D_3, 3-deoxy-3-azido-25-hydroxyvitamin D_3; BSA, bovine serum albumin; HDL, high-density lipoproteins; EDTA, ethylenediaminetetraacetic acid; HA, hydroxylapatite; Me_2SO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

0006-2960/87/0426-3957$01.50/0