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Discoidin, a Developmentally Regulated Carbohydrate-Binding Protein from *Dictyostelium discoideum*. Purification and Characterization†

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ABSTRACT: A carbohydrate-binding protein from the cellular slime mold, *Dictyostelium discoideum*, previously shown to be synthesized as these cells become cohesive, was purified by affinity chromatography. The protein was assayed by agglutination of formalinized erythrocytes, which is selectively blocked by sugars with a galactose configuration. The protein bound quantitatively to a column of Sepharose 4B and was quantitatively eluted with D-galactose. A minor contaminant was removed either by isoelectric focusing or by adsorption of the active protein to formalinized erythrocytes and elution with D-galactose. Purified agglutinin had a molecular weight of 100,000 ± 2000 determined by sedimentation equilibrium in the presence of galactose. In the absence of galactose the protein tended to aggregate. Subunit molecular weight measured under dissociating conditions by sedimentation equilibrium was 28,000 ± 2000. Determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the subunit molecular weight was 26,000 ± 1000. The molecular weight data suggest that the native molecule is a tetramer. A single symmetrical peak of agglutination activity with an $s_{20,w}$ of 5.1 S was determined by sucrose density gradient centrifugation. The protein is rich in aspartic and glutamic acids and 3-hydroxyamino acids. Its isoelectric point was 6.10 determined in the presence of galactose. The protein contained no hexosamine and no detectable neutral sugar. The role of this developmentally regulated agglutinin in the differentiation of *Dictyostelium discoideum* is considered.

The life cycle of the cellular slime mold, *Dictyostelium discoideum*, is divided into two distinct phases (Bonner, 1967; Gerisch, 1968): the first, a single-cell vegetative state in which cells grow and multiply; and a second social stage characterized by cellular aggregation, intercellular adhesion, and further differentiation culminating in fruiting body formation and sporulation. Recently, we have reported on the presence of a developmentally regulated carbohydrate-binding protein from *Dictyostelium discoideum* (Rosen *et al.*, 1973), whose synthesis closely parallels the development of cell cohesiveness. This "lectin-like" protein agglutinates formalinized sheep erythrocytes and agglutination is specifically inhibited by sugars with a galactose configuration. A striking 400-fold increase (Rosen, 1972) in specific agglutination activity is observed in a 12-hr period after cells are deprived of food, a signal which initiates cellular aggregation. Preliminary evidence was presented indicating that this factor is present at the cell surface of cohesive slime mold cells. These observations, although circumstantial, suggest that this agglutinin may mediate intercellular adhesion in this organism (Rosen *et al.*, 1973). In this paper, we describe the purification and characterization of "discoidin,"[1] a protein from *Dictyostelium discoideum*.

Experimental Procedures

Materials. Chemicals used throughout this investigation were the best available commercially.

Cell Culture. Dictyostelium discoideum, strain A3 cells, a mutant derived from the NC-4 strain, were grown in axenic culture (Loomis, 1971) to cell densitites of 7×10^6 cells/ml. Growth-phase cells of this mutant line contain high levels of agglutination factor (Rosen *et al.*, 1973). The cell density is critical since densities substantially higher or lower than those used here have much less extractable factor.

Preparation of Extract. Slime mold cells were harvested by certrifugation at 1500 rpm in a Sorvall RC-2 refrigerated centrifuge and washed three times with cold water. The cells were resuspended at room temperature at a concentration of 10^6–10^7 cells/ml in a 0.015 M Tris–0.075 M NaCl–0.075 M KI buffer containing 1 mM EDTA (pH 7.3); this buffer (Takeuchi and Yabuno, 1970) will be subsequently referred to as ECT[2] buffer. Cells were either homogenized in a Potter-Elvejhem

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[1] The authors propose the name "discoidin" for this developmentally regulated carbohydrate-binding protein from *Dictyostelium discoideum*.

[2] Abbreviations used are: ECT, EDTA-calcium-free salt-Tris buffer; ECT-galatose, ECT buffer containing 0.3 M D-galactose; PBS, phosphate-buffered saline.

glass homogenizer or were ultrasonically disrupted with a Bronwill Biosonik Sonifier. Homogenates were centrifuged at 50,000 rpm (150,000*g*) for 45 min in a Beckman Model L preparative ultracentrifuge. The soluble supernatant was then shaken vigorously at 4° for 1 hr. A precipitate which formed was removed by centrifugation at 15,000 rpm in an RC-2 refrigerated Sorvall centrifuge for 15 min. The resultant supernatant, which contained all the agglutination activity of the initial extract, was used for further purification.

Erythrocyte Agglutination Assay. Agglutination activity in slime mold extracts (Rosen *et al.*, 1973) was assayed with formalinized sheep erythrocytes as follows. (1) Fresh erythrocytes were collected in Alsever's solution and were formalinized as described (Butler, 1963). (2) A soluble slime mold extract was prepared as described, and a twofold dilution series of slime mold supernatant was made in 0.15 M NaCl, and 0.5 ml of each dilution was mixed with 2 ml of 0.075 M NaCl–0.075 M Na_2HPO_4 (pH 6.4) (PBS). To this, 0.5 ml of a 2.5% suspension of formalinized sheep erythrocytes was added. The mixture was incubated at 25° for 10 min and centrifuged at 500*g* for 5 min. Cells were resuspended in 1 ml of 1% normal rabbit serum (Gibco Laboratories) in 0.15 M NaCl, recentrifuged, and resuspended in 0.5 ml of normal rabbit serum. Bovine serum albumin, 0.6 mg/ml in 0.15 M NaCl, can be substituted for the serum. (3) The agglutination of treated erythrocytes was measured in Microtiter "V" Plates (Cooke Engineering). Then 25 μl of each cell suspension was added to each of triplicate wells. The plate was shaken, covered with transparent tape, and left at room temperature for at least 90 min before agglutination patterns were evaluated. (4) The agglutination end point was taken as the first dilution of the extract at which erythrocytes settled into a clearly circumscribed dot at the bottom of the plate well. The agglutination activity was defined as the reciprocal of this end point dilution and specific agglutination activity was defined as agglutination activity per milligram of protein (Lowry *et al.*, 1951). As discussed in detail (Rosen *et al.*, 1973) the accuracy of this agglutination assay is limited as a result of the twofold dilution procedure.

Affinity Purification of Agglutination Factor on Sepharose 4B. Columns with 10–1000-ml bed volumes of Sepharose 4B (Pharmacia) were preequilibrated at 4° with ECT buffer. The capacity of the columns to quantitatively adsorb agglutination activity was determined in preliminary experiments. Washing with 3 column volumes of ECT buffer and elution with ECT buffer containing 0.3 M galactose are described in the Results section.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in a discontinuous sodium dodecyl sulfate–polyacrylamide system (Laemmli, 1970) with a 3% acrylamide stacking gel and a 7.5% separating gel. Stacking and separating gels were 1 and 10 cm, respectively. Samples were run in a buffer consisting of 25 mM Tris, 192 mM glycine, and 0.1% (w/v) sodium dodecyl sulfate (pH 8.4). Samples were prepared prior to electrophoresis in solubilizer containing 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride at an approximate protein concentration of 1 mg/ml. To assure complete solubilization, samples were heated at 100° for 5 min immediately before application to gels. Cytochrome *c*, 5 μg, was routinely added to samples as a reference protein. No tracking dye was necessary since the protein and electrophoretic fronts were clearly visible. Electrophoresis was carried out in a Canalco apparatus at a constant current of 3.5 mA/gel. Running time under these conditions was 3 hr.

Following electrophoresis, gels were stained for protein with Coomassie Blue (Fairbanks *et al.*, 1971). Stained gels were scanned at 550 nm on a Gilford Model 2400-S spectrophotometer with a Model 2410 linear transport accessory.

Molecular weight standards (Schwarz/Mann) were prepared in the solubilizing buffer. The relative migration of protein standards to agglutinin was calculated relative to cytochrome *c* run in parallel gels. A standard calibration curve of the log of the molecular weight *vs.* relative mobility was constructed (Shapiro *et al.*, 1967). The following protein standards were used: human γ globulin (mol wt 160,000, 80,000 in the presence of 2-mercaptoethanol); bovine albumin (mol wt 67,000); ovalbumin (mol wt 45,000); chymotrypsinogen (mol wt 25,000); and cytochrome *c* (mol wt 12,400).

Carbohydrate Staining of Acrylamide Gels. The location of glycoproteins on acrylamide gels was determined using a modification of the Schiff periodate procedure (Glossmann and Neville, 1971). Human transferrin (Sigma) which contains 6% carbohydrate, only 4% of which is Schiff positive, was employed as a standard to determine sensitivity of the staining procedure. The reported carbohydrate composition of commercial transferrin was confirmed by phenol–sulfuric acid analysis. Schiff periodate stained gels were scanned at 550 nm and then stained with Coomassie Blue.

Sedimentation Equilibrium. A molecular weight analysis of purified agglutination factor was carried out by equilibrium sedimentation. A Beckman Model E analytical ultracentrifuge with Rayleigh interference optics was used for all experiments. Samples of purified agglutination factor at a concentration of 0.5, 1, and 2 mg/ml of protein in ECT–galactose buffer were dialyzed against appropriate running solvent and run at 16,000 rpm and 25°. Subunit weight average molecular weights were determined at protein concentrations of 0.5 and 1 mg/ml at 34,000 rpm and 25° in the presence of 6 M guanidinium chloride and 0.1 M 2-mercaptoethanol. Fringe positions were measured employing an automated microcomparator (Carlisle *et al.*, 1974). The partial specific volume of 0.735 was used for subunit and native molecular weights. Data were analyzed using a computer program developed by Dr. Dennis E. Roark. The weight-average molecular weight was determined by the method of Yphantis (Yphantis, 1964; Roark and Yphantis, 1969).

Sucrose density gradient experiments performed (Martin and Ames, 1961) using linear gradients of sucrose, 5–25%, were made in PBS (pH 7.3). Samples were centrifuged in a Beckman Model L centrifuge with a swinging bucket rotor for 5–20 hr. Gradients were uniformly sampled by piercing the bottoms of tubes and collecting drops. Purified alcohol dehydrogenase, bovine serum albumin, and ovalbumin were employed as standards.

Amino Acid Analysis. Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer following hydrolysis in 6 N HCl *in vacuo* for 24–48 hr. Peak areas on chromatograms were integrated and compositions were calculated by means of a computer program developed in the laboratory of Dr. Eugene A. Davidson. Normalized peak areas were obtained by averaging data from three standard runs. Standards were run weekly or whenever fresh ninhydrin was run. Appropriate corrections were made for the loss of threonine and serine during the hydrolytic procedure.

Isoelectric Focusing. Experiments were performed in accord with instructions provided in the manual for the LKB 8101 isoelectric focusing column (LKB, Bromma, Sweden). One milligram of agglutinin was equilibrated with ECT–galactose buffer and mixed into a sucrose gradient containing 0.3 M galac-

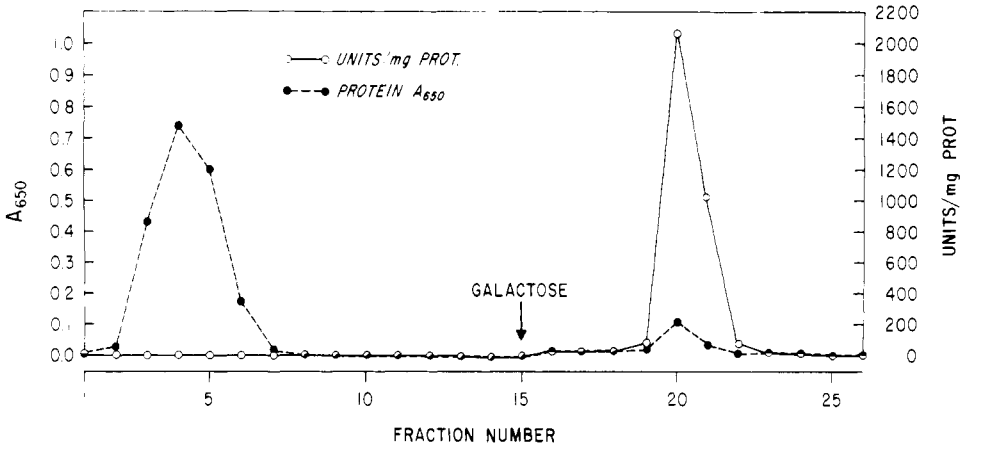

FIGURE 1: Chromatographic profile resulting from the purification of slime mold agglutinin, employing a column of Sepharose 4B as an affinity absorbant: (●- - -●) protein absorbance at 650 nm; (O–O) specific agglutination activity; the arrow indicates the addition of galactose to the ECT buffer as an eluent.

tose. In other experiments, galactose was omitted from the gradient. Ampholine carrier (pH 3–11) was used initially and ampholine carrier (pH 5–7) was used in all subsequent experiments at a concentration of 1%. Focusing was performed for 60 hr at 4°.

Hexosamine Analysis. Glucosamine and galactosamine analyses were performed on a Beckman Model 120C amino acid analyzer following hydrolysis *in vacuo* in 6 N HCl for 12–24 hr. Pure glucosamine, galactosamine, and mannosamine were employed as standards.

Neutral Sugar Analysis. Analysis of neutral monosaccharides was determined by the phenol-sulfuric procedure (Dubois *et al.*, 1956). A mixture of galactose, glucose, and mannose (2:1:1) (w/w) served as a standard. Absorbance was measured at 490 nm with a Beckman Kintrac VII recording spectrophotometer.

Results

Purification. Agglutination activity could be partially purified by shaking the soluble extract at 4° for 1 hr. The shaking denatured and precipitated half of the soluble protein without detectable loss of activity in the residual supernatant. The agglutination activity in the supernatant obtained after shaking could be quantitatively bound to a column of Sepharose 4B which is composed of a linear polymer of alternating units of D-galactose and 3,6-anhydro-L-galactose. In a typical small scale purification (Figure 1) 10 ml of soluble extract from 1×10^8 cells was applied to a column containing a 10-ml bed volume of Sepharose 4B preequilibrated at 4° with ECT buffer. The extract was applied at 2 ml/hr and, after standing for an additional hour, the column was washed with 3 column volumes of ECT buffer. About 95% of the protein of the extract was eluted from the column and contained no detectable agglutination activity (Figure 1). Quantitative displacement of agglutination activity from Sepharose 4B was achieved by eluting with 3 column volumes of ECT buffer containing 0.3 M D-galactose (Figure 1). Agglutination activity and the associated protein were eluted as a single symmetrical peak. This step led to a 20-fold increase in specific agglutination activity compared to the shaken soluble extract which was applied and a 40-fold increase with respect to the unshaken soluble extract. The procedure was subsequently scaled up with the use of Sepharose 4B columns containing as much as 1-l. bed volumes and purification of up to 0.5 l. of soluble extract gave a chromatographic profile identical with that shown in Figure 1. In all cases, the agglutination activity present in the initial extract was quantitatively recovered from the column. *N*-Acetyl-D-galactosamine could be substituted for D-galactose to elute the agglutination activity but 0.3 M D-glucose or mannose was ineffective.

Stability of Purified Agglutination Factor. The relative stability of agglutination activity under various conditions is summarized in Table I. The presence of 0.3 M galactose enhanced the stability of agglutination activity. Agglutinin in ECT–galactose buffer, quick frozen in liquid N_2, could be stored frozen without loss of activity for an indefinite period of time.

Acrylamide Gel Electrophoresis. Sodium dodecyl sulfate–acrylamide electrophoresis of up to 400 μg of protein pooled from Sepharose 4B revealed a major component as well as a minor component which migrated more rapidly (Figure 2, insert) and represented 5–7% of the applied protein (Figure 2) in pooled fractions. The subunit average molecular weights in sodium dodecyl sulfate of the slow (major) and fast (minor) components were 26,000 ± 1000 and 24,000 ± 1000 daltons, respectively. Heavily overloaded sodium dodecyl sulfate gels of 7.5, 10, and 12.5% acrylamide revealed only two components.

TABLE I: Stability of Purified Agglutination Activity.[a]

Sample	Units of Agglutination Act./ml after 3 weeks[b]	% Retention of Agglutination Act.
ECT–galactose buffer, quick frozen	2048	100
ECT–galactose buffer, 25°	2048	100
ECT–galactose buffer, + 10^{-3} M sodium azide, 4°	2048	100
ECT–galactose buffer, 4°	1024	50
ECT buffer without EDTA, 4°	512	25
ECT buffer, 4°	256	12.5
Water, 4°	128	6.25

[a] Samples were assayed and stored as described, then reassayed 3 weeks later. [b] Initial agglutination activity was 2048 units/ml.

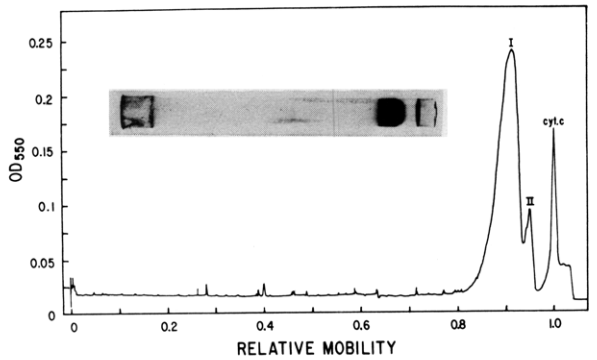

FIGURE 2: Insert: sodium dodecyl sulfate gel electrophoresis of purified agglutinin. When sodium dodecyl sulfate gels are overloaded with samples purified on Sepharose 4B, a single minor fast-moving contaminant is observed in addition to the major polypeptide subunit of the agglutinin. Figure: a typical spectrophotometric scan of a sodium dodecyl sulfate gel resulting from electrophoresis of purified agglutinin. Peak I is the major polypeptide subunit of the agglutinin and peak II represents a minor lower molecular weight contaminant which migrates slightly ahead of the major band. No additional protein bands were observed on scans of overloaded gels. Cytochrome *c* was added to the sample as a marker.

Independent determination of molecular weights on 12.5% acrylamide slabs in sodium dodecyl sulfate gave identical subunit molecular weight values.

In samples which were not completely solubilized prior to electrophoresis in sodium dodecyl sulfate, undissociated protein in the molecular weight range of 75,000–110,000 daltons could be seen in addition to low molecular weight components. Quantitative dissociation into polypeptide subunits was achieved by heating or by addition of 8 M urea to the sample and gel (Figure 3). The fast-moving component is present in both gels. The minor component may be a subunit of a second carbohydrate-binding protein which was copurified on Sepharose 4B, a specific or nonspecific receptor molecule tightly bound to multivalent agglutinin and likewise copurified, or a degradation product of agglutinin subunits.

Localization of Activity. When individual fractions collected from Sepharose 4B columns were electrophoresed in sodium dodecyl sulfate, the slow moving component was present in a higher proportion in earlier fractions than the fast moving component.

Densitometry of various fractions electrophoresed in sodium dodecyl sulfate and stained with Coomassie Blue indicated that the fractions with a higher ratio of slow component to fast component had higher specific agglutination activity.

Erythrocyte Adsorption. The conclusive assignment of agglutination activity to a single component on sodium dodecyl sulfate gels was accomplished using formalinized erythrocytes as a specific affinity adsorbant. The Sepharose fraction employed had nearly equal concentrations of slow (I) and fast moving (II) components (Figure 4A). A sample was incubated with sheep erythrocytes and centrifuged and an aliquot of supernatant assayed for agglutination activity and electrophoresed (Figure 4B). Supernatants obtained after washing the erythrocytes with saline (Figure 4C) and with saline containing 0.3 M galactose (Figure 4D) were also assayed for agglutination activity and electrophoresed. The gel patterns (Figure 4) clearly demonstrate that erythrocytes have preferentially and quantitatively adsorbed out the slow moving band I and all agglutination activity leaving only fast moving component II in the incubation supernatant. The saline wash contained a trace of inactive slow moving component I, whereas washing the erythrocytes with 0.3 M galactose led to the release of most of the adsorbed slow moving component (I), and its associated agglutination activity from the erythrocyte surface.

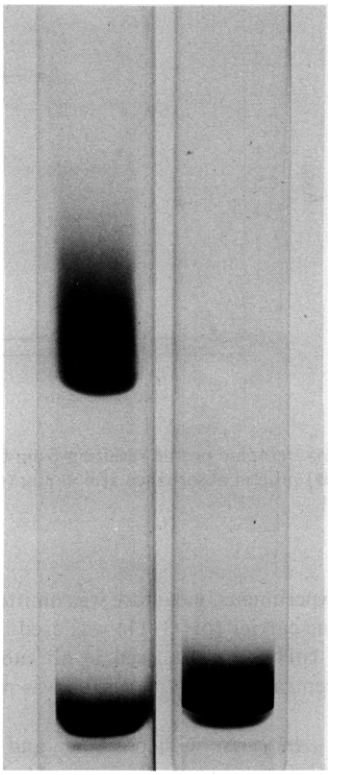

FIGURE 3: Sodium dodecyl sulfate gel patterns of partially dissociated (a) and completely dissociated (b) samples of purified agglutinin. The slow moving high molecular weight material seen in a could be completely dissociated into the polypeptide sununit seen in b by heating to 100° prior to electrophoresis or by addition of 8 M urea. The minor fast moving contaminant is visible in both gels.

This experiment demonstrates conclusively that agglutination activity as measured with sheep erythrocytes is clearly associated with the slow moving band (I) seen on sodium dodecyl sulfate–acrylamide gels. Thus, the sheep erythrocyte serves as a more specific adsorbant for purification of slime mold agglutinin than Sepharose, although recovery of adsorbed agglutination activity from cells is not complete.

Schiff Staining of Acrylamide Gels. Since several plant lectins are reported to contain covalently bound carbohydrate (Lis and Sharon, 1973), periodic acid Schiff staining of gels was undertaken to evaluate the presence of carbohydrate in specific components within sodium dodecyl sulfate gels. A staining procedure (Glossmann and Neville, 1971) was specifically chosen to eliminate the artifactual staining of non-carbohydrate-containing materials. The procedure allows for elution of all protein-bound and free sodium dodecyl sulfate from gels, maintaining an acid pH throughout the procedure to avoid nonspecific acid–base staining of proteins. Glycoprotein controls without periodate treatment were run concurrently. Human transferrin served as a glycoprotein standard. Schiff staining of acrylamide and sodium dodecyl sulfate–acrylamide gels containing 50–500 μg of purified agglutinin demonstrated that covalently bound Schiff positive carbohydrate made up less than 0.25% of the native or sodium dodecyl sulfate dissociated protein. This value was determined by comparison of the negative Schiff staining on a sodium dodecyl sulfate gel known to contain 500 μg of "discoidin" to the lowest concentration of purified transferrin, of known carbohydrate content, on a series of sodium dodecyl sulfate gels which exhibited detectable Schiff

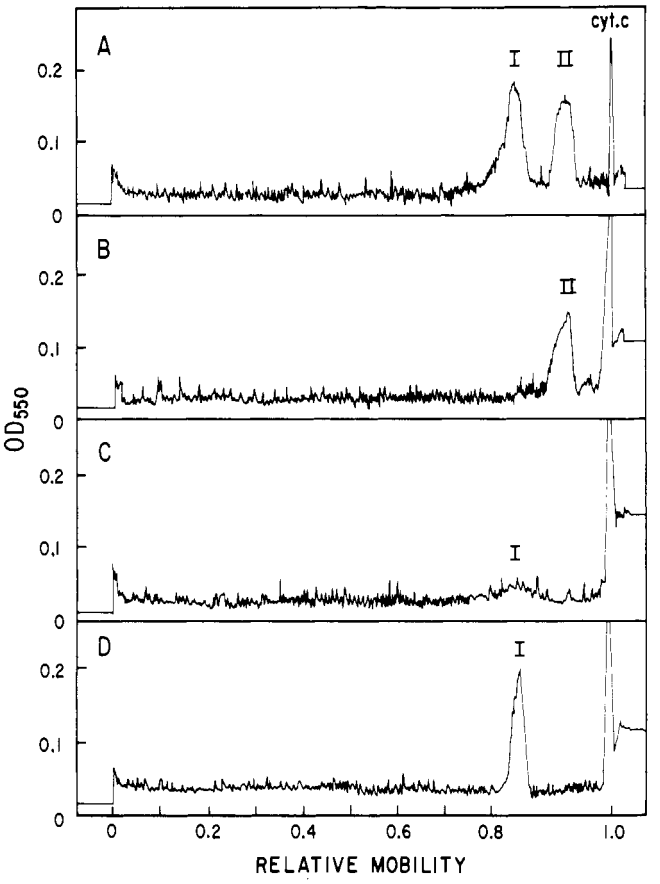

FIGURE 4: Spectrophotometric sodium dodecyl sulfate gel scans of agglutinin from Sepharose which was adsorbed to and specifically eluted from sheep erythrocytes. A Sepharose fraction is seen in A which contains nearly equal amounts of polypeptide subunit I and fast moving contaminant II. Gel scan B represents a sample of A which was incubated with sheep erythrocytes and the resulting supernatant electrophoresed. The profile and absence of agglutination activity in the supernatant clearly demonstrate that the agglutinin (I) was quantitatively adsorbed to the erythrocyte surface leaving behind the contaminant (II). The material seen in C was obtained by washing the erythrocyte pellet with saline and subsequent electrophoresis of the wash supernatant. A trace of polypeptide subunit I is detectable on this gel although the wash was free of detectable agglutination activity. Finally, when the erythrocyte pellet is washed with galactose-containing saline, recovery of the agglutinin subunit I and agglutination activity was achieved.

positive staining. The results indicated that the two components were not glycoproteins. All Schiff-stained gels were subsequently stained with Coomassie Blue, to assure that the protein had indeed entered the gels.

Density Gradient Centrifugation. To determine an $s_{20,w}$ value and an approximate molecular weight of native agglutinin, samples of crude and purified extracts were centrifuged in sucrose density gradients, fractionated, and assayed for agglutination activity. Agglutination activity sedimented as a single symmetrical peak which contained essentially all applied activity. Sedimentation of activity was linear with time with the gradients employed. An $s_{20,w}$ value of 5.1 S for the agglutinin was calculated from the ratio of distances migrated from the meniscus as compared to standards. Assuming identical partial specific volume for agglutinin and standards, we estimate the molecular weight of the agglutination factor in crude extracts as approximately 89,000 daltons.

Sedimentation Equilibrium. Sedimentation equilibrium analysis of active agglutination factor indicated a high degree of monodispersity in ECT-galactose buffer. In addition to a homogeneous principal species, there was evidence of a smaller minor component representing less than 5% of the sedimenting protein. Data were analyzed employing computer programs developed by Dr. Dennis E. Roark and the weight average molecular weight of the agglutinin was calculated to be 100,000 ± 2000. Molecular weight analysis in the absence of galatose indicated an indefinite and nonstoichiometric self-association of protein. No attempt was made to analyze the monomer-oligomer interactions (Roark and Yphantis, 1969) as they were considered nonstoichiometric aggregates which appeared to lack discrete heterogeneity.

Subunit average molecular weights were determined by sedimentation equilibrium in the presence of 6 M guanidinium chloride and 0.1 M 2-mercaptoethanol. The molecular weight of the subunit was 28,000 ± 2000. A two-species plot (Roark and Yphantis, 1969) of these data showed that the principal component had an average molecular weight of approximately 28,000 ± 2000. These data are entirely consistent with a monomer-tetramer model with a stoichiometry of approximately 3.8. A monomer-trimer model, also conceivable, is highly unlikely after analysis of all existing data. Although the data support a tetramer model, they do not distinguish between an α_4 or $\alpha_2\beta_2$ type subunit structure.

Amino Acid Composition. The amino acid composition of purified agglutinin is summarized in Table II. A notable feature is the high aspartic acid composition, 15.2%. Aspartic and glutamic acids together represent greater than 23% of the residue composition of the protein. The electrophoretic behavior of the protein suggests that the proportion of amide is low. In addition, the 3-hydroxyamino acids, serine, and threonine comprise greater than 17% of the total composition. Aromatic and sulfur-containing amino acids are present in the agglutinin.

TABLE II: Amino Acid and Amino Sugar Composition of Agglutination Factor from *Dictyostelium discoideum*.[a]

Amino Component	Residues/1000 Residues of Amino Compds
Amino Acids	
Lysine	33.9
Histidine	20.7
Arginine	47.2
Aspartic acid	151.7
Threonine[b]	95.7
Serine[b]	81.9
Glutamic acid	81.3
Proline	51.1
Glycine	96.6
Alanine	71.7
Half-cysteine[c]	15.5
Valine	75.7
Methionine	1.4
Isoleucine	43.8
Leucine	54.5
Tyrosine	36.7
Phenylalanine	40.7
Amino Sugars	
Glucosamine	0.00 (<0.10)
Galactosamine	0.00 (<0.10)

[a] Amino acid and amino sugar analysis of purified slime mold agglutinin. [b] Value corrected for destruction due to 24-hr hydrolysis. [c] Determined as cysteic acid.

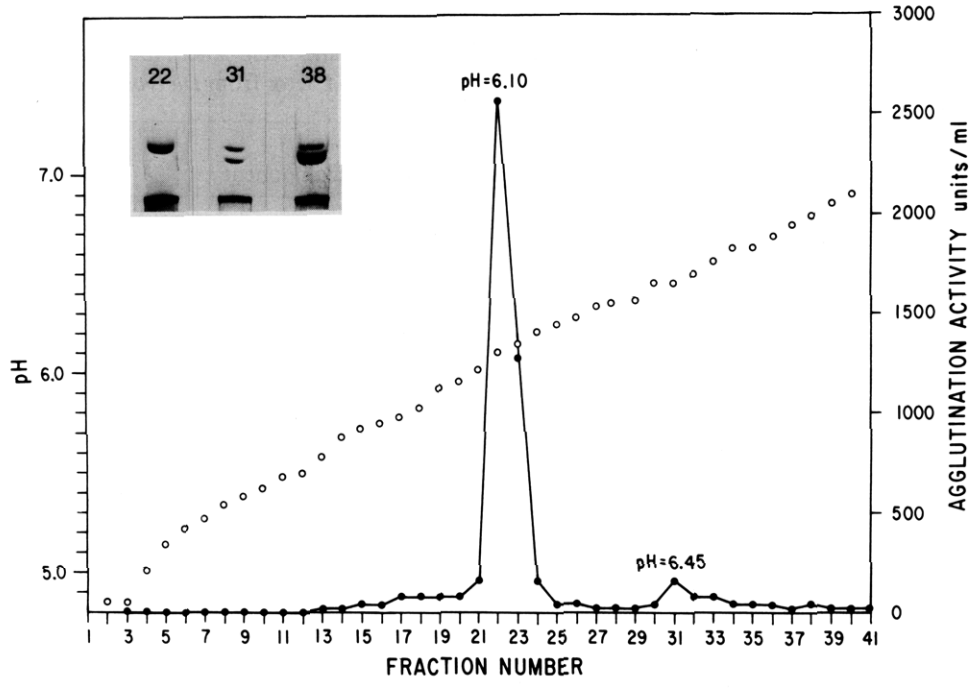

FIGURE 5: Isoelectric focusing on an ampholite–sucrose gradient, pH 5.0–7.0. A major symmetrical peak of agglutination activity occurs at fraction 22 at pH 6.10. Proteins from 0.5 ml of fraction 22, 1.0 ml of fraction 21, and 1.5 ml of fraction 38 were dialyzed, concentrated, and electrophoresed in sodium dodecyl sulfate–acrylamide gels (inserts). The bottom band on the acrylamide gels is the cytochrome *c* marker.

Generally, they are poorly represented or absent in glycoproteins and protein polysaccharides. The composition is similar to the galactose-binding proteins from *Ricinus communis* which have been recently characterized (Nicolson *et al.*, 1974). Tryptophan was identified on spectra obtained with a Cary 15 recording spectrophotometer but quantitation was not achieved due to partial solubility of the lyophilized sample. The overall amino acid composition is generally representative of those reported for many lectins from plant sources (Lis and Sharon, 1973).

Amino Sugar Analysis. Hydrolysates of agglutination factor (2–8 mg) were examined on the amino acid analyzer for glucosamine, galactosamine, or mannosamine; none were present. Significant loss of hexosamine should not occur under these conditions which ensure maximum liberation and appropriate corrections were made for hydrolytic destruction. Based on a computer-calculated molecular weight of 107,575 daltons per 1000 residues of amino acid, the protein contains less than one residue of hexosamine per 1000 residues of amino compound or less than 0.16 mol % total hexosamine. The absence of hexosamine was confirmed by paper chromatography of hydrolysates.

Neutral Sugar Analysis. Purified factor, eluted from Sepharose 4B in ECT–galactose buffer, was dialyzed in boiled dialysis tubing against deionized water. A control bag with an equal volume of ECT–galactose buffer was dialyzed in an identical manner in a separate container. The efficiency of dialysis was monitored by addition of 2.5×10^6 cpm per 10 ml of D-[^{14}C]galactose to each bag. Dialyzed samples were lyophilized and assayed by the phenol–sulfuric acid method. After lyophilization, the dialysis blank contained a significant quantity of nondialyzable material which was subjected to carbohydrate analysis in parallel with the agglutination factor. When corrected for the phenol–sulfuric acid positive material present in the dialysis blank, the agglutinin had no detectable neutral sugar within the limits of the assay. These results were consistent with the absence of Schiff periodic acid staining.

Isoelectric Focusing. Isoelectric focusing of purified agglutinin on a gradient of pH 5–7 in the presence of 0.3 M galactose showed a major peak at pH 6.10 which contained almost all the applied agglutination activity (Figure 5). A trace of activity was also present at pH 6.45. Peak fractions were dialyzed and subjected to sodium dodecyl sulfate–acrylamide electrophoresis. The major peak (Figure 5) consisted of a single protein with an R_F value identical with that of the active slow moving 26,000 molecular weight component present in the original sample. The gel pattern of the minor peak (Figure 5), in addition to the 26,000 molecular weight component, contained some 24,000 molecular weight component. Electrophoretic analysis of other fractions within the pH gradient revealed a fraction which was greatly enriched in the 24,000 molecular weight component when compared to a sodium dodecyl sulfate gel of the initial material. In another experiment in which galactose was omitted from the gradient, the observed activity profile, pH 5–7, was identical with that seen in Figure 5. The relative effect of different monosaccharides on the inhibition of agglutination activity (Rosen *et al.*, 1973) was identical for the material in the major, pH 6.1, and minor, pH 6.45, peaks from the ampholite gradient.

Discussion

We have previously reported that *Dictyostelium discoideum* cells synthesize a carbohydrate-binding protein as they become cohesive. The protein has been purified from axenically grown growth-phase cells of *Dictyostelium discoideum* A3 which are very rich in this protein. The protein we have purified from this axenic mutant of NC-4 has previously been shown to be identical with the protein present in differentiated wild type NC-4 cells as determined by the ability of a wide range of monosaccharides to specifically inhibit agglutination of formalinized sheep erythrocytes by proteins from either source (Rosen *et al.*, 1973). Purification was achieved by application of a shaken soluble extract to Sepharose 4B followed by elution with 0.3 M galactose. The protein purified by this procedure was very homogeneous as indicated by ultracentrifugation, polyacrylamide gel electrophoresis, and isoelectric focusing. Approximately 94% of the protein consisted of subunits with a molecular

veight of 26,000 ± 1000. The remaining 6% consisted of a protein with a molecular weight of 24,000 ± 1000. The minor component could be separated from the major component either by isoelectric focusing or by adsorption of the major component to formalinized erythrocytes which did not adsorb the minor component. The major component could then be eluted off the surface of formalized erythrocytes with 0.3 M galactose.

The purified protein has many characteristics like those of the "lectins," a group of carbohydrate binding proteins of unknown function isolated from various plant and animal sources. Like some other lectins (Lis *et al.*, 1966; Hammarström and Kabat, 1969; Galbraith and Goldstein, 1972; Nicolson *et al.*, 1974), the protein from *D. discoideum* is rich in aspartic acid, glutamic acid, and 3-hydroxyamino acids. Unlike many lectins which have been characterized, the one which we have identified is not glycoprotein in nature. In this regard, the protein from *D. discoideum* resembles concanavalin A which has been conclusively shown to lack covalently bound carbohydrate. The protein which we have identified has no detectable acetylhexosaminidase activity (Loomis, 1969), although we cannot exclude the possibility that it possesses other enzymatic activity. The agglutination activity of the protein is not mediated by enzymatic alteration of the erythrocyte surface since it is augmented rather than inhibited at low temperature. In addition, the adsorption of factor to the surface of erythrocytes, necessary for agglutination, is reversible by the addition of specific sugars. The purified protein is a very potent agglutinin since as little as 0.1 μg of protein/ml produces detectable erythrocyte agglutination. Castor bean (Nicolson and Blaustein, 1972) and wax bean agglutinin (Sela *et al.*, 1973) are reported to be active at similar low concentrations when studied with trypsin-treated or transformed cell types. Like some other lectins the protein which we have studied appears to be a tetramer.

In plants, the function of agglutinins is generally unknown. The carbohydrate-binding activity and the striking developmental course which parallels the development of cohesiveness suggest that the factor may interact with a specific carbohydrate receptor on the slime mold cell surface and thus mediate cellular adhesion. However, a conclusive assignment of function for this agglutinin awaits further experiments. In view of its specific carbohydrate-binding activity and striking developmental regulation, this protein, "discoidin," appears likely to play a vital role in the cellular differentiation of this slime mold.

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