Chemical and Physical Properties of an Hepatic Membrane Protein that Specifically Binds Asialoglycoproteins

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The state of aggregation found in water-soluble preparations of an hepatic membrane protein responsible for the clearance of serum asialoglycoproteins has been shown to result from the self-associating properties of a single oligomeric protein. The smallest functional unit identifiable in aqueous solution possessed an estimated molecular weight of 500,000 with each of the successive components increasing in size by an equal amount to form an oligomeric series bearing an integral ratio of 1:2:3:4:5. The tendency towards self-association was promptly and completely reversed by the addition of Triton X-100 with the concomitant appearance of a single component. Extensive treatment with sodium dodecyl sulfate permitted the identification and isolation of two subunits with estimated molecular weights of 48,000 and 40,000, respectively. Amino acid and carbohydrate analyses revealed both subunits to be glycoproteins with a closely similar, but not identical, composition.

In a previous paper, the isolation and purification of a binding protein from rabbit liver, specific for asialoglycoproteins, was described (1). This preparation was characterized as a lipid-free sialoglycoprotein whose binding activity was dependent upon the integrity of the terminal sialic acid residues. However, the aggregated state of the final, water-soluble preparation precluded any meaningful estimate of purity. The present study, undertaken in an attempt to resolve this problem provides evidence for the homogeneity of the isolated material and demonstrates the aggregated state to result from the interaction of a self-associating oligomeric protein which is reversibly converted to a single species in the presence of 1% Triton X-100.

MATERIALS AND METHODS

Proteins used as standards in gel electrophoresis and gel filtration were from the following sources: α-chymotrypsinogen, ovalbumin, and β-galactosidase (Escherichia coli) from Worthington Biochemical Corp.; catalase (beet root) and aldolase (rabbit muscle) from Boehringer Mannheim GmbH; transferrin (human) and ferritin (horse spleen) from Miles Laboratories, Inc.; pepsin (crystallized three times) from Calbiochem; fumarase (heart muscle) and bovine serum albumin from Armour Pharmaceutical Co. Ceruloplasmin was prepared from Cohn's Fraction IV of human plasma by a method described previously (1). Ferguson plots (5) for the present protein were obtained from gels of varying total acrylamide concentrations (% T) but with a constant ratio of methylenebisacrylamide to acrylamide (1:2) using the proper dilution of a stock solution containing 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide/100 ml.

Electrophoresis was performed using a multiphasic buffer system, i.e., System A (pH 9.45) as described by Rodbard and Chrambach (4). Ferguson plots for the present protein were obtained from gels of varying total acrylamide concentrations (% T) but with a constant ratio of methylenebisacrylamide to acrylamide (1:2) using the proper dilution of a stock solution containing 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide/100 ml.

Electrophoresis was carried out in gels (0.6 × 7 cm) for 2 to 2.5 hours at 4°C. Mobilities were determined relative to bromphenol blue. The following proteins were selected as the reference standards (molecular weight given in parentheses): bovine serum albumin monomer (67,000), transferrin (90,000), bovine serum albumin dimer (134,000),...
ceruloplasmn (151,000), ferritin (443,000), and \( \beta \)-galactosidase (515,000).

Electrophoresis in gels containing 0.1% Triton X-100 was carried out with the same buffer system. However, before electrophoresis, the solution of binding protein was made 1.0% in Triton X-100 and incubated for 1 hour at room temperature.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described by Weber and Osborn (6). The following proteins were selected as the reference standards (molecular weight given in parentheses): chymotrypsinogen (25,700), pepsin (35,000), aldolase (40,000), ovalbumin (45,000), furamase (49,000), IgG light chain (23,500), IgG heavy chain (50,000), and porcine thryoglobulin (660,000). Mobilities were determined relative to Pyronin Y. Ferguson plots employed to check anomalous migration were obtained from gels prepared with the same acrylamide gel stock solution described above.

Binding protein was denatured and reduced extensively before electrophoresis by incubating the sample at 48° in 0.1% sodium phosphate, pH 7.0, containing 1.0% sodium dodecyl sulfate for 24 to 72 hours, at which point the solution was made 10% with respect to \( \beta \)-mercaptoethanol and the incubation was continued for an additional 3 hours at 48°. Electrophoresis was carried out for 5 to 6 hours at room temperature.

Protein bands were stained with Coomassie brilliant blue G 250 in 12.5% trichloroacetic acid (7) or fast green in 7.5% acetic acid (8). Before staining, Triton X-100 or sodium dodecyl sulfate was eluted by incubating the gels for 18 hours with 10% acetic acid in 50% ethanol or plots employed to check anomalous migration were obtained from gels prepared with the same acrylamide gel stock solution described above.

Preparative Disc Gel Electrophoresis—The binding protein oligomers were isolated from the same size gels and buffer system as described above for analytical gel electrophoresis. The gels were frozen at -70° after electrophoresis and cut into several segments with a razor blade so that each contained a single oligomeric component located by comparison with a stained gel as marker. The gel segments were crushed with a glass homogenizer and extracted with 10 mM Tris-Cl buffer at pH 8.3 for 3 days at 4°. Approximately 100 \( \mu \)g of the binding protein was applied to each gel; recovery of protein was about 80%.

Preparative gel electrophoresis in sodium dodecyl sulfate was carried out in 10% acrylamide; other conditions were as described for the analytical run. The binding protein was labeled with Fluorescamine essentially as described by Flagand et al. (9) except that 0.1% sodium dodecyl sulfate was added to the reaction mixture to dissociate the binding protein. The sample applied to a single gel contained 0.2 \( \mu \)g of Fluorescamine-labeled protein and 100 \( \mu \)g of unlabelled protein. The electrophoretic mobility of the subunits was not altered significantly by the dye. Following electrophoresis, the appropriate fluorescent areas were cut out from the gel, homogenized, and extracted with 0.1% sodium dodecyl sulfate for 3 days at room temperature. The recovery of protein was about 80%.

Isoelectric Focusing—Isoelectric focusing was performed with an LKB apparatus, model 8101, according to the directions of Vesterberg and Svensson (10). Ampholine, pH range 3 to 10, was added at a concentration of 1.0% and Triton X-100 at a concentration of 0.1%. About 500 \( \mu \)g of the binding protein which had been incubated in 0.5 mM Tris, pH 7.8, and 0.25% Triton X-100 for 30 min at 4°, was added to the center of the column. Electrophoresis was carried out at 200 volts for 3 days with an initial current of 3.7 mA. After focusing, 2.0-mI fractions were collected and aliquots of each fraction were used for binding activity and protein determination.

Sugar Analysis—Sialic acid was determined by the thio-Atarbituric acid method of Warren (11) after hydrolysis of the subunits of binding protein in 0.1 N H\(_2\)SO\(_4\) for 1 hour at 60°. Mannose, galactose, and glucosamine were separated and estimated by gas-liquid chromatography of butaneboronic acid esters of hexitols and glucosaminol according to the method of Eisenberg (12). The sample (0.2 to 0.5 \( \mu \)g of protein), containing an internal standard of 50 \( \mu \)mol of norleucine, were placed in 1 ml of constant boiling HCl and sealed in glass tubes under reduced pressure. Hydrolysis was carried out for 24 or 48 hours at 110°. Analytical analyses were performed with a Beckman GC 65 gas chromatograph.

Amino Acid Analysis—Samples for amino acid analysis were prepared by the method of Moore and Stein (13). The samples (0.1 to 0.2 \( \mu \)g of protein), containing an internal standard of 50 \( \mu \)mol of glutamic acid, were hydrolyzed in 0.15 ml of 1 N HCl at 100° for 6 hours in a sealed tube. Analyses were performed with a Beckman GC 65 gas chromatograph.

Protein Determination—Protein was determined by the micro method of Lowry et al. (14) with cysteine bovine serum albumin as a standard. A minor modification was introduced to remove the interference from Triton X-100 as described previously (1).

Cross-linking—Dimethylsuberimidate was synthesized from suberodinitre (Aldrich Chemical Co.) by the method of Davies and Stark (15). Cross-linking (amidation) of proteins was carried out in 0.2 M triethanolamine hydrochloride, pH 8.5, in the presence of 0.1% Triton X-100. Dimethylsuberimidate and the binding protein solution which had been dialyzed overnight against the same buffer to remove Trit ion, were mixed to give 0.1 to 0.6 mg/ml of protein and 0.5 to 12 mg/ml of dimethylsuberimidate in a final volume of 0.05 to 2.0 ml, and the reaction mixture was left at room temperature for 3 hours. The proteins were then denatured for 1 hour at 48° in 1% sodium dodecyl sulfate and 10% \( \beta \)-mercaptoethanol. Polyacrylamide gel electrophoresis was performed in 5.0% or 3.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

RESULTS

Column Chromatography—Previous attempts to purify the water-soluble binding protein by ion exchange chromatography on DEAE cellulose, CM cellulose, and hydroxylapatite had been uniformly unsuccessful (1). However, upon addition of Triton X-100 to the DEAE-cellulose chromatographic system, initial evidence suggesting homogeneity of the binding protein was obtained (Fig. 1). A single component was eluted with sodium chloride at a concentration between 0.1 and 0.15 M. The specific activity was invariant throughout the peak; all values fell within the range of 40 to 52 ng of \( ^{125}\text{I} \)-asialo-orosomucoid bound/\( \mu \)g of protein. Recoveries of binding activity and protein were 91 and 92%, respectively.

In view of earlier results with gel chromatography on Sepharose 6B, in which the aeous binding protein was recovered as a continuum of high molecular weight species extending from 6 x 10\(^3\) to greater than 4 x 10\(^6\) (1), the effect of Triton X-100 was examined. In the presence of this detergent, the binding protein was eluted as a single, symmetrical peak consistent with homogeneity in regard to molecular size (Fig. 2). Here, too, the specific activity was constant throughout the peak in the range of 37 to 46 ng of asialo-orosomucoid bound/\( \mu \)g of protein. This peak was divided into three pools as indicated in the figure and each pool was subjected to amino acid analysis and to polyacrylamide disc gel electrophoresis in the

![Fig. 1. DEAE-cellulose chromatography of the binding protein in the presence of Triton X-100. An aqueous solution of the binding protein (500 \( \mu \)g), made 1% in Triton X-100, was stored at 4° for 30 min after which it was diluted with buffer to give a final concentration of 10 mM Tris-Cl, pH 7.8, and 0.25% Triton X-100. This material was charged onto a column of DEAE-cellulose (0.9 x 8 cm) previously equilibrated with the same buffer. Chromatography was carried out in the cold at a flow rate of 10 ml/hour. After washing with 1 column volume of buffer, the binding protein was eluted with 100 ml of a linear gradient ranging from 0 to 0.5 M NaCl in the same buffer. Fractions of 1.6 ml were collected and aliquots were assayed for binding activity and protein. Specific activity (\( \square \)) is expressed as the nanograms of \(^{125}\text{I} \)-asialo-orosomucoid bound per \( \mu \)g of protein.](http://www.jbc.org/)
activity attended this procedure and the final recovery of isoelectric point at pH 4.7. However, considerable loss of binding activity was only 11%.

0.1% Triton X-100 gave a single, symmetrical peak with an electrophoresis of the aqueous binding protein resulted in the reversibly to asialo-orosomucoid in response to the addition, or removal, of calcium ion.

The amino acid composition of the three fractions was identical, within experimental error (Table I). Upon disc gel electrophoresis in sodium dodecyl sulfate, each of the fractions revealed staining patterns which were indistinguishable from each other and comparable to that seen in Fig. 2A.

On the basis of the above evidence, it seemed reasonable to presume that the isolated binding protein was free from major contamination with inert protein. In order to confirm the absence of the latter, the binding protein was subjected to Sepharose 4B gel chromatography under a variety of experimental conditions designed to cause characteristic and reproducible changes in binding behavior.

The results of three separate experimental conditions are summarized in Fig. 1. Upon incubation of the binding protein in the presence of 111H-asialo-orosomucoid and 50 mM calcium chloride, a rapidly moving peak was identified as a complex between the radioactive ligand and the binding protein (O—O). Omission of calcium from the elution buffer resulted in the disappearance of this peak and the emergence of a slower moving component (●—●) identical with that of the uncomplexed protein shown in Fig. 2. Finally, in the absence of asialo-orosomucoid but in the presence of calcium, the binding protein was recovered as a broad, shallow peak with evidence of anomalous retention to the Sepharose column (●—●).

Despite some mutual overlapping of the three elution areas, it may be concluded from these results that essentially all of the protein present possessed the unique property of binding reversibly to asialo-orosomucoid in response to the addition, or removal, of calcium ions.

Isoelectric focusing of the binding protein in the presence of 0.1% Triton X-100 gave a single, symmetrical peak with an isoelectric point at pH 4.7. However, considerable loss of activity attended this procedure and the final recovery of binding activity was only 11%.

Analytical Gel Electrophoresis—In a conventional system at pH 9.45 and a total acrylamide concentration of 3%, disc gel electrophoresis of the aqueous binding protein resulted in the appearance of multiple components (Fig. 4A). Upon modification of the assay conditions to the extent that the binding protein was made 0.1% in Triton X-100 prior to electrophoresis, the larger molecular weight species, which exhibited a limited capacity to enter the gel, disappeared concomitant with the appearance of a new, fast moving peak; the major discernible bands of intermediate size were unaffected (Fig. 4B). Upon increasing the total acrylamide concentration from 3% (Fig. 4B) to 4% (Fig. 4C), an overall decrease was seen in the rate of band migration with no change in the relative position of the individual bands. Utilizing this property for a more detailed examination of the relationship among the multiple constituents, a total of seven electrophoretic analyses were carried out in which the total acrylamide concentration was varied from 3 to 6% in order to generate the data for the Ferguson plot shown in Fig. 5. Each band yielded a straight line with a correlation coefficient of 0.996 to 0.999 and was extrapolated to a common point at a 2% gel concentration. The latter observation is the predicted behavior for proteins bearing an identical charge at a given pH but varying in their molecular size and is suggestive of an homologous series of a single, oligomeric protein (16).

To characterize these bands more fully, an estimate was made of their molecular size by taking advantage of the linear relationship between the square root of the retardation coefficient (Ks, the slope of log Rs versus gel concentration) and the cube root of the molecular weight (17). The linear regression of
Fig. 4. Disc gel electrophoresis of the binding protein and its subunits. A, an aqueous solution of the binding protein (30 μg) was applied onto a 3% acrylamide gel. Electrophoresis was carried out using a multiphasic buffer system “System A” (pH 9.45) for 2.5 hours at 4°C. Protein bands were stained with Coomassie brilliant blue G-250 in 12.5% trichloroacetic acid. See “Methods” for details. B, a 0.1% Triton X-100 solution of the binding protein (40 μg) was applied onto a 3% acrylamide gel. Electrophoresis and staining were carried out as described in A. C, a 0.1% Triton X-100 solution of the binding protein (40 μg) was applied onto a 4% acrylamide gel. Electrophoresis and staining were carried out as described in A. D, a 0.1% Triton X-100 solution of the binding protein (20 μg), made 1.0% in Triton X-100 and stored at room temperature for 1 hour, was applied to a 4% acrylamide gel containing 0.1% Triton X-100. Electrophoresis and staining were carried out as described in A except that the gel was soaked in 10% acetic acid in 50% ethanol overnight prior to staining. E, an aqueous solution of the binding protein (13 μg) was incubated in 1.0% sodium dodecyl sulfate/0.5 mM EDTA/0.01 M sodium phosphate, pH 7.0, for 3 days at 48°C. The solution was then made 10% in β-mercaptoethanol and 4 M in urea. Incubation was continued for an additional 3 hours at 48°C before applying the sample to a 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. Electrophoresis was carried out at room temperature for 6 hours according to the method of Weber and Osborn (60). The gel was stained with 1% fast green in 7.5% acetic acid after soaking in 12.5% trichloroacetic acid overnight. F, subunit A (7 μg) was incubated in 1% sodium dodecyl sulfate, 0.01 M sodium phosphate, pH 7.0 for 24 hours. The sample was made 10% in β-mercaptoethanol, incubated 1 additional hour at 48°C and applied to a 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. Electrophoresis was carried out as in E. Protein was stained with Coomassie brilliant blue G-250 in 12.5% trichloroacetic acid. G, subunit B (14 μg) was incubated and analyzed by electrophoresis as described in F.

(Kₐ) against (molecular weight) plotted in Fig. 6 is based upon a series of proteins with known molecular weights in the range of 70,000 to 500,000. The solid line in this figure was calculated by the method of least squares; the dotted line represents an extrapolation.

Interpolation of the (Kₐ) values for the six oligomeric bands on this standard curve yielded the following estimates of molecular weights: 2.7 × 10⁴ (Band 1); 5.0 × 10⁴ (Band 2); 9.7 × 10⁴ (Band 3); 14.1 × 10⁴ (Band 4); 18.2 × 10⁴ (Band 5); and 24.6 × 10⁴ (Band 6). It is obvious that the values for the higher homologues must be considered an approximation. However, it is reasonable to note that each of the values obtained, when expressed as a ratio to the molecular weight of Band 2 as unity, bears an approximate integral relation expressed by the series 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and suggests that the higher members of the series gain in size by the incremental addition of a 500,000 molecular weight unit.
of Triton X-100 in the gel was significantly less than the value gel eluted, 1.7 ng of binding protein. Furthermore, knowing the volume of molecular weight of 2.7 x 10^5 for the binding protein in Band 1, no more than 0.7 nmol of Triton X-100. Based on an estimated detergent, the sensitivity of the procedure could have detected this experiment was repeated with [3H]Triton X-100 (0.225 binding protein with detergent prior to electrophoresis. When significance of Band 1 resulting from brief incubation of the molecular weight in the presence of this detergent.

This negative result prompted further investigation of the significance of Band 1 resulting from brief incubation of the binding protein with detergent prior to electrophoresis. When this experiment was repeated with [3H]Triton X-100 (0.225 mCi/g) and Band 1 was eluted, no radioactivity was detected in the eluate containing 1.7 ng of binding protein. Due to the relatively low specific activity of the available radioactive solution was readily demonstrated by the fact that separate elution of Bands 1 to 4, followed by re-electrophoresis, as in Fig. 4B, yielded a complete spectrum. The specific activity of each component was unchanged from that of the intact, starting material. The effect of detergent in reversing the tendency of the binding protein to self-associate is shown in Fig. 4D. Here the concentration of Triton X-100 was maintained at 0.1% by inclusion in the gel with the resultant formation of a single, fast moving component migrating slightly slower than Band 1 of Fig. 4C.

In view of the recently reported successful use of suberimdate polymerization (18) to determine the molecular weight of a membrane-bound receptor for acetylcholine in the presence of Triton X-100, this technique was utilized in an attempt to estimate the molecular weight of the single binding component shown in Fig. 4D. The conditions used, described under "Methods," led to the tentative recognition of several fused subunits (see "Discussion") but failed to provide satisfactory evidence for the presence of a predominant species indicative of the molecular weight in the presence of this detergent. Consequently, a valid estimate of the size of the binding protein in the presence of 0.1% Triton X-100 could not be made.

This negative result prompted further investigation of the significance of Band 1 resulting from brief incubation of the binding protein with detergent prior to electrophoresis. When this experiment was repeated with [3H]Triton X-100 (0.225 mCi/g) and Band 1 was eluted, no radioactivity was detected in the eluate containing 1.7 ng of binding protein. Due to the relatively low specific activity of the available radioactive detergent, the sensitivity of the procedure could have detected no more than 0.7 nmol of Triton X-100. Based on an estimated molecular weight of 2.7 x 10^5 for the binding protein in Band 1, it can be calculated that less than 10 nmol of detergent were bound/nmol of protein. Furthermore, knowing the volume of gel eluted, 140 µl, it could be concluded that the concentration of Triton X-100 in the gel was significantly less than the value of 3 x 10^-4 M, reported for the critical micelle concentration (19). Consequently, it would seem reasonable to infer that nonmicellar species of this detergent may interact effectively with the binding protein.

Subunits—In view of the apparent homogeneity of the binding protein and the estimated molecular weight of the smallest oligomeric component seen on disc gel electrophoresis, attention was turned to a determination of the number and size of the subunits present. The binding protein proved to be remarkably resistant to complete dissociation (1). However, incubation for 3 days at 48° in 1% sodium dodecyl sulfate, followed by an additional 3-hour exposure to 10% β-mercapto-ethanol was sufficient for conversion to two subunits as shown in Fig. 4E. The introduction of additional denaturing stress prior to electrophoresis, such as 0.5 mM EDTA, 6 M urea, or carboxymethylmethylation of the reduced binding protein, produced no further changes.

The molecular weight of the two subunits, 48,000 and 40,000, respectively, was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate on 10% gels as shown in Fig. 7. This procedure is not unequivocal and can give rise to erroneous estimates of molecular weight resulting from the anomalous binding of sodium dodecyl sulfate to proteins because of unusual properties associated with their charge, conformation, or carbohydrate content (20-22). To check these possibilities, the binding protein was dissociated in sodium dodecyl sulfate, as described above, and subjected to electrophoresis at four different acrylamide concentrations (5, 7.5, 10, and 12.5% acrylamide). In the resulting Ferguson plot, the two subunits gave linear regression lines with a correlation coefficient of 0.997 for subunit A and 0.994 for subunit B. The free mobility (intercept at 0% acrylamide) was similar for both subunits and closely comparable to those of a number of protein standards. Again utilizing the relationship of the retardation coefficient calculated from these plots to the molecular weight of a series of standard proteins, values of 47,000 for subunit A and 40,000 for subunit B were estimated. The excellent agreement of the values obtained by both procedures would appear to minimize the possibility of anomalous behavior and to lend credence to the reliability of the molecular weight determination. Additional attempts to cor-
roborate these values by gel filtration in the presence of 6 M guanidine hydrochloride and 10 mM dithiothreitol were unsuccessful. The binding protein, preincubated with these reagents, emerged in the void volume of Bio-Gel A-5m column as evidence of its resistance to these denaturing agents.

Isolation and Analysis of Subunits—Initial attempts to estimate the relative abundance of the two subunits utilized the densitometric tracings illustrated in Fig. 8. Integration of the areas under the two curves revealed the ratio of A:B to be 1:2. In order to isolate the subunits free from contamination with each other and in quantities sufficient for analysis, preparative gel electrophoresis was carried out as described under “Methods.”

The successful resolution of the two subunits is illustrated in Fig. 4, F and G. Approximately 60% of the starting protein was recovered; from 3.6 mg of the intact binding protein, 0.7 mg of subunit A and 1.4 mg of subunit B were obtained.

The results of the amino acid analysis and the carbohydrate composition of each of the two subunits are summarized in Tables I and II, respectively. Only minor differences in the amino acid composition were found; subunit A has a slightly higher content of alanine, isoleucine, and aspartic acid, whereas subunit B is relatively richer in proline and leucine.

That each of the subunits is a glycoprotein consisting of sialic acid, galactose, mannose, and glucosamine is shown by the data in Table II. When calculated on a molar basis, subunit A has a significantly higher carbohydrate content than does subunit B. Whether this reflects a greater average chain size or an increased number of carbohydrate chains cannot be answered on the basis of the available data.

Discussion

In an earlier paper (1) the isolation of an hepatic binding protein specific for asialoglycoproteins was reported. However, the highly aggregated state of the final, water-soluble protein obscured any estimate of the purity of the preparation and imposed severe restrictions on attempts to examine its physical and chemical properties in detail.

Evidence for homogeneity of the isolated binding protein was obtained only after restoration of detergent to the purified, water-soluble protein. In the presence of Triton X-100, under a variety of conditions, the binding protein was recovered as a single, symmetrical peak on DEAE-cellulose (Fig. 1) and Sepharose 4B chromatography (Figs. 2 and 3). More important, however, was the resolution achieved by disc gel electrophoresis. The “aggregated” state in aqueous solution was shown to consist of a series of oligomeric proteins which reverted to a single species in the presence of detergent.

Analysis of the multiple bands by means of the Ferguson plot permitted the assignment of approximate molecular weights to the individually resolved components. The molecular weight of the smallest unit seen in the absence of detergent was 500,000 with each of the successive components increasing in size by an equal amount to form an oligomeric series bearing an integral ratio of 1:2:3:4:5.

It should be emphasized that the assignment of even approximate molecular weights by the techniques employed here is subject to considerable uncertainty. Foremost among the experimental problems is the absence of well characterized and appropriate standards with molecular weights in excess of 500,000. Second, the migration of proteins is, to a very considerable extent, a function of their three dimensional structure; little or nothing is known about the conformational state of the oligomers described here. Third, the contribution of trace amounts of residual Triton X-100, even if too small to be detected, can be neither ignored nor easily evaluated. However, despite these reservations, there appears to be an internal consistency with regard to the relative size distribution of the individual components comprising the aggregated state of the aqueous binding protein.

Table I

Amino acid composition of binding protein and its subunits

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Binding protein</th>
<th>Subunit</th>
<th>Subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.7</td>
<td>4.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.5</td>
<td>3.7</td>
<td>3.8</td>
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<td>Arginine</td>
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<td>5.9</td>
<td>6.1</td>
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<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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<td>4.3</td>
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<tr>
<td>Serine</td>
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<td>7.6</td>
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<tr>
<td>Glutamic acid</td>
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<td>12.5</td>
<td>12.8</td>
</tr>
<tr>
<td>Proline</td>
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</tr>
<tr>
<td>Glycine</td>
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<td>9.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.3</td>
<td>6.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Half cystine</td>
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<td>3.7</td>
</tr>
<tr>
<td>Valine</td>
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<td>7.1</td>
<td>6.7</td>
</tr>
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<td>Methionine</td>
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</tr>
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<td>Isoleucine</td>
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<td>1.5</td>
</tr>
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</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
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</table>

*The roman numerals refer to the pooled fractions shown in Fig. 3. Hydrolysis was carried out at 110°C for 24 hours.

*The letters refer to the two subunits illustrated in Fig. 4, F and G, respectively. Hydrolysis was carried out at 110°C for 48 hours.

Table II

Carbohydrate content of subunits A and B

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Subunit A</th>
<th>Subunit B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/100 mg protein</td>
<td>mol/mol A-</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>20.6</td>
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</tr>
<tr>
<td>Galactose</td>
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</tr>
<tr>
<td>Mannose</td>
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<td>10.7</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>21.3</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Calculation based on a molecular weight of 48,000.

* Calculation based on a molecular weight of 40,000.

Fig. 8. Densitometric tracing of the binding protein subunits. Conditions for the preparation and disc gel electrophoresis of the binding protein subunits are given in the legend to Fig. 4E. Scanning of the gels was carried out on a Zeiss densitometer at 642 nm.
A rationalization in accord with all of the experimental data is presented in Fig. 9. Detergent exposure of the binding protein to sodium dodecyl sulfate, in either the presence or the absence of Triton X-100, yielded two subunits of molecular weight 48,000 and 40,000, respectively, which occurred in a relative abundance of 1:2. As reported earlier (1), and confirmed in this study, less drastic treatment with sodium dodecyl sulfate gave rise to incomplete subunit formation. Estimates of the molecular weight of the additional species seen under these conditions are now interpretable in terms of the hypothetical scheme outlined in Fig. 9. Similarly, suberimidate polymerization of the single species seen in the presence of 0.1% Triton X-100 gave rise to several components whose estimated molecular weights permitted the assignment of the structures shown in brackets. Unfortunately, this technique failed to yield a single definitive component and no valid estimate of the molecular weight of the binding protein in 0.1% Triton X-100 can be made.

From the data presented here, it would seem reasonable to conclude that the rabbit liver binding protein has been isolated in a form which is largely free from inert protein and that the tendency towards self-association in aqueous solution is a reflection of a general attraction which exists between analogous hydrophobic regions of the same molecule that is common to many proteins of membranous origin.

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