Isolation and Characterization of the Major Mannose-Binding Protein in Chicken Serum†

Ke-Yi Wang, Theresa B. Kuhlenschmidt, and Y. C. Lee*

Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

Received February 6, 1985

ABSTRACT: Mannose-binding activity is abundantly present in chicken serum. The major mannose-binding protein has been isolated from chicken serum by affinity chromatography and gel filtration. The protein consists of two subunits of 75 000 and 26 500 daltons. Unlike hepatic lectins or other mannose-binding proteins, this protein does not require calcium for binding mannose-containing glycoconjugates. The chicken serum mannose-binding protein is immunochemically distinct from the chicken hepatic lectin and rabbit serum mannose-binding protein.

Since a mannose-specific (Man-specific)[1] binding protein was first discovered in alveolar macrophages (Stahl et al., 1978), Man-binding proteins have been found in a number of sources. Man-binding activity was reported to be present in mammalian livers (Kozutsumi et al., 1980). Kozutsumi et al. (1981) later noted the Man-binding activity in rabbit serum and found the mammalian Man-binding proteins from the liver and the serum to be quite similar with respect to sugar-binding specificity, subunit molecular weights, and requirement of calcium for binding. They also cross-reacted immunochemically. The Man/GlcNAc-specific binding protein isolated from rat liver (Townsend & Stahl, 1980) was first thought to localize in nonparenchymal cells but has subsequently been postulated to be derived from the hepatocytes (Brownell et al., 1984). Similar activities have been reported in human serum (Colley & Baenziger, 1983; Kawasaki et al., 1983) and rat serum (Colley & Baenziger, 1983).

During our studies of the chicken hepatic lectin (Kuhlenschmidt & Lee, 1980, 1984; Kuhlenschmidt et al., 1982), we noticed the presence of Man-binding activity in the chicken serum (Kuhlenschmidt & Lee, 1982). In view of the different sugar specificities between lectins of avian (GlcNAc) and mammalian (Gal/GalNAc) livers, the presence of a similar Man-binding activity in both the chicken and mammalian sera is extremely interesting and merits in-depth study. In this report, we describe a convenient method for purification of the major protein of Man-binding activity. Some of its properties are characterized and compared with the mammalian counterparts and with chicken hepatic lectin.

Materials and Methods

Materials. The following materials were obtained from the indicated sources: frozen chicken serum (nonsterile, trace hemolyzed) (Pel-Freez, Rogers, AK); rabbit IgG, β-glucuronidase, ferritin, Sepharose 4B, and Triton X-100 (Sigma Chemical Co.); BSA (Pentex, fraction V) (Miles Laboratories, Inc.); Bio-Gel A 1.5m, SDS, acrylamide, and protein standards for gel electrophoresis (Bio-Rad); Sephacryl S-300 (Pharmacia, Piscataway, NJ); agarose LE and gel bond films (Marine Colloids, FMC, Rockland, ME); Parafilm (American Can Co., Greenwich, CT). Mannans from yeast variants (Ballou & Raschke, 1974) are a generous gift from Dr. C. E. Ballou of the University of California at Berkeley.

BSA-based neoglycoproteins of amidino type (Lee et al., 1976) and cluster ligands (Lee, 1978) were prepared according to the published methods. Man_2Lys and Man_3Lys_2 were prepared by coupling the *p*-nitrophenyl ester of carboxymethyl 1-thio-α-D-mannopyranoside with L-Lys or L-Lys-L-Lys in dimethyl sulfoxide. The details of preparation will be described elsewhere.

Antiserum against purified chicken serum Man-binding protein (CS-II; see Results) was produced in rabbits. Initially, 74 μg of CS-II in complete Freund's adjuvant was injected subcutaneously. One week later, an additional 50 μg of CS-II was injected, and 6 weeks after the second injection, 50 mL of blood was collected from each animal. The blood was allowed to clot at 37 °C for 1 h followed by standing overnight in the cold, and the serum was obtained after centrifugation. Guinea pig antiserum against rabbit serum Man-binding protein was a gift of Drs. T. Kawaski and I. Yamashina (Kyoto University, Japan).

An affinity column for the isolation of Man-binding protein was prepared by first coupling BSA to CNBr-activated Sepharose 4B and then attaching Man derivatives by amidino linkage according to the method described earlier (Kuhlenschmidt & Lee, 1984). Coupling of about 20–30 mol of Man on each mole of BSA (ca. 5 mg of BSA/mL of Sepharose gel) was usually attained.

Methods. The standard binding assay was based on the modified version (Kuhlenschmidt & Lee, 1984) of the ammonium sulfate precipitation method (Hudgin et al., 1974)

† Supported by U.S. Public Health Service Research Grants AM9970 and CA21901 and by Training Grant GM07231 (to T.B.K.). Contribution No. 1288 from the McCollum-Pratt Institute.

* Correspondence should be addressed to this author at the Department of Biology, The Johns Hopkins University.

[1] Abbreviations: BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; Man_n-BSA, a bovine albumin derivative which contains *n* residues of mannose linked to the protein by amidino linkage (Lee et al., 1976); Man_2Lys, L-lysine modified with carboxymethyl 1-thio-α-D-mannopyranoside at both amino groups by amide bond formation; Man_3Lys_2, L-Lys-L-Lys modified analogously at all three amino groups; Man, mannose; Fuc, fucose; Glc, glucose; Gal, galactose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; I_{50}, concentration of inhibitor that causes 50% inhibition; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); AHT glycosides, glycosides of [2-(6-aminohexanamido)]tris(hydroxymethyl)methane.

0006-2960/85/0424-5932$01.50/0

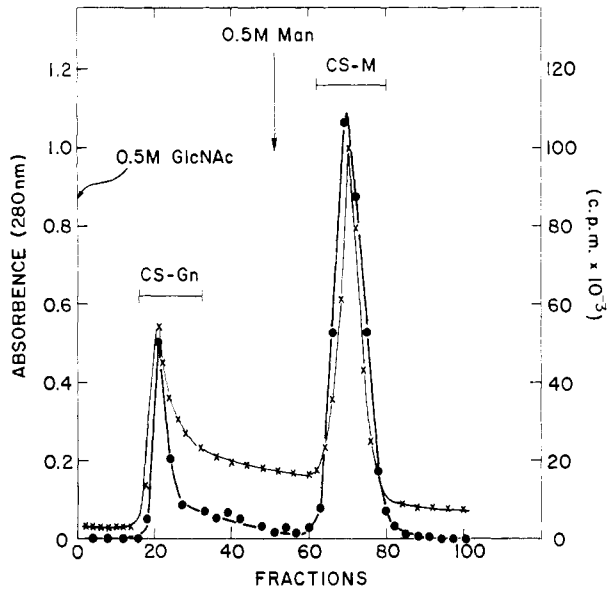

FIGURE 1: Elution of chicken serum Man-binding protein from Man-BSA–Sepharose. Chicken serum (1000 mL) was directly applied to a column (3.6 × 10.5 cm) of Man-BSA–Sepharose 4B. The column was washed with buffer I until there was no absorbance at 280 nm in the effluent and then eluted with 0.5 M GlcNAc followed by 0.5 M Man as shown. The flow rate was 72 mL/h, and 4.4-mL fractions were collected. The binding activity (●) and A_{280nm} (×) were measured as described under Methods. The fractions eluted with GlcNAc and Man are designated CS-Gn and CS-M, respectively. The high base line of A_{280nm} in the eluate when GlcNAc or Man was used is caused by the UV-absorbing impurities in the sugars.

in which ^{125}I-Man$_{43}$-BSA was used as labeled ligand. The labeled ligand (usually 1 μg) was shaken with the binding protein sample for 15 min at 25 °C. The mixture was placed in an ice bath and mixed with cold saturated ammonium sulfate, pH 7.8, to a final concentration of 50% saturation. After 10 min, the precipitate was collected on a Whatman GF/C or 934-AH filter disk, and the precipitate was washed 3 times with 40% saturated ammonium sulfate containing 0.1% BSA. The filter disk was air-dried and wrapped in a Parafilm sheet before its radioactivity was measured. One unit of activity was defined as that quantity of binding protein which binds 1 ng of the labeled ligand, and the specific binding activity was defined as binding units per microgram of protein.

SDS–PAGE was carried out as described by Laemmli (1970). Protein samples were boiled for 5 min in 63 mM Tris-HCl buffer (pH 6.3) containing 0.3% SDS, 10% glycerol, 5% mercaptoethanol, and 0.01% bromophenol blue. The denatured protein samples were electrophoresed in a slab gel made of a 10% or 5% separating gel and a 5% stacking gel. The gels were stained with Coomassie Blue G-250 for visualization of protein bands. Agarose gels for double immunodiffusion (Ouchterlony & Nilsson, 1973) were prepared by pouring 9 mL of hot 1% (w/v) agarose in phosphate-buffered saline including 0.1% Triton X-100 on a 5 × 7.5 cm glass plate.

Protein concentration was determined by a microbiuret method (Zamenof, 1957). Iodination of ligands was by the chloramine-T method (Greenwood et al., 1963) as modified by Kuhlenschmidt & Lee (1984).

RESULTS

Affinity Purification of Man-Binding Protein in Chicken Serum Protein. Chicken serum (1000 mL) was directly applied to the Man-BSA–Sepharose column (3.6 × 11 cm) equilibrated in buffer I (10 mM HEPES, 1 M NaCl, and 20 mM $CaCl_2$, pH 7.8). The column was washed with buffer I until the A_{280nm} of the effluent became negligible. The column

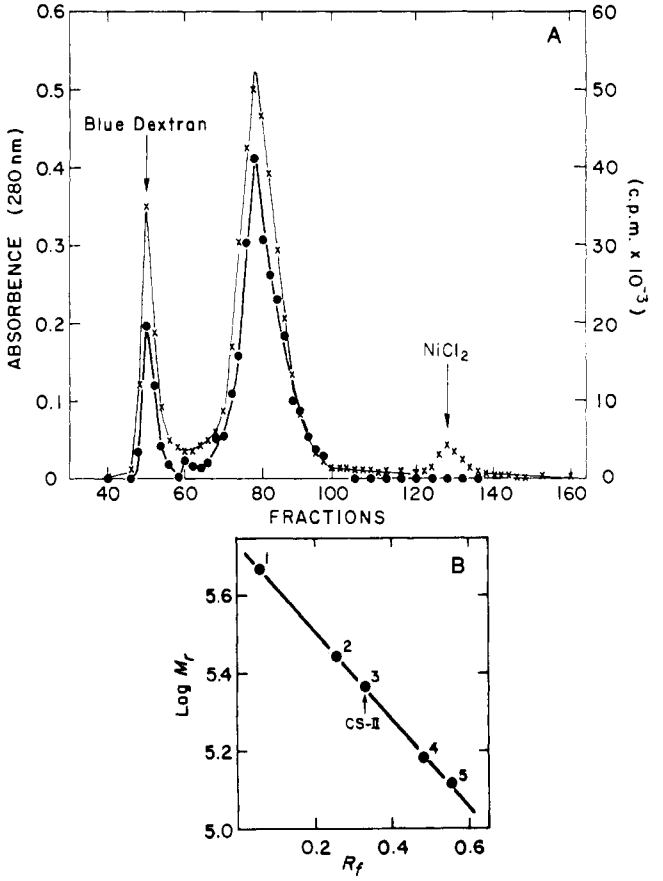

FIGURE 2: Gel filtration of CS-M on an agarose column. (A) The affinity-purified fraction, CS-M, was subjected to gel filtration on a column (1.5 × 146 cm). The column was eluted with buffer II at a rate of 22 mL/h, collecting 2.2-mL fractions. The absorbance at 280 nm (×) and Man-BSA binding activity (●) were measured. The arrows indicate the positions of the void volume (V_0) and total inclusion volume (V_i), respectively. Blue dextran and $NiCl_2$ were used for measurement of these volumes, because of their coloration. (B) Plot of the elution position of protein standards vs. molecular weight: (1) ferritin (M_r 470 000), (2) β-glucuronidase (M_r 280 000), (3) half-ferritin (M_r 235 000), (4) rabbit IgG (M_r 150 000), and (5) BSA dimer (M_r 130 000). $R_f = (V_e - V_0)/(V_i - V_e)$, where V_0 is the void volume of the column (measured with dextran), V_e is the elution volume of the sample, and V_i is the inclusion volume (measured with D-glucose).

was then first eluted with 220 mL of 0.5 M GlcNAc in buffer I followed by 220 mL of 0.5 M Man in buffer I. As shown in Figure 1, two peaks containing Man-binding activity, coincidental with the protein peaks, were observed. The fractions marked CS-Gn and CS-M were pooled separately and concentrated with Amicon ultrafiltration using a XM-50 membrane. The concentrated fraction was diluted with buffer II (10 mM HEPES, 1 M NaCl, and 5 mM EDTA, pH 7.8) and concentrated again by ultrafiltration as described. The dilution–concentration cycle was repeated several times to ensure complete removal of sugars. The affinity column was rejuvenated after several runs by washing with 3 M NaSCN and equilibrated in the elution buffer.

Gel Filtration of CS-M. The CS-M fraction was further purified by gel filtration through a column of Bio-Gel A1.5m (1.5 × 146 cm) equilibrated in buffer II. As shown in Figure 2, two peaks of Man-binding activity were obtained (CS-I and CS-II). From a calibration curve obtained by chromatographing reference proteins under the same conditions, it was estimated that the apparent molecular weight of CS-I is >500 000 and that of CS-II 235 000 (Figure 2B). More than 90% of the applied protein was recovered in this step, and treatment of the column with NaSCN did not elute any more

Table I: Purification of Chicken Serum Binding Protein

stage of purification	protein[a] (mg)	act.[b] ($\times 10^{-6}$ units)	sp act. (units/μg)	% yield
serum	40600	3.8	0.096	100
Man-BSA-Sepharose				
CS-Gn	15.6	0.64	40.5	16.5
CS-M	25.8	1.9	74.0	49.2
Bio-Gel A1.5m				
CS-I	5.5	0.23	41.2	5.8
CS-II	19.6	1.8	90.1	45.6

[a] Protein determination by microbiuret (Zamenof, 1957). [b] Activity measured with ^{125}I-Man$_{43}$-BSA. One nanogram of ^{125}I-Man$_{43}$-BSA bound is defined as 1 unit of activity.

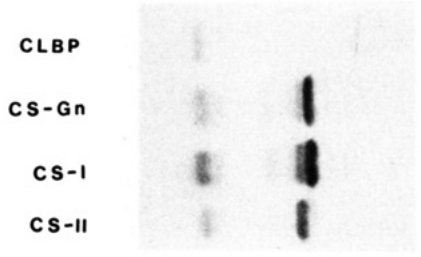

FIGURE 3: SDS-PAGE of chicken serum Man-binding proteins. Protein samples were boiled for 5 min in the presence of mercaptoethanol and subjected to electrophoresis (Laemmli, 1970) using a 10% separating gel until the tracking dye (bromophenol blue) reached the bottom of the gel (ca. 7 h). The gel was fixed and stained with Coomassie Brilliant Blue (Fairbanks et al., 1971). CLBP is chicken liver GlcNAc-binding protein. Reference proteins were electrophoresed and stained under the same conditions (results not shown). The proteins used are phosphorylase B (M_r 93 000), BSA (M_r 67 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), and Kunitz soybean trypsin inhibitor (M_r 21 000).

protein from the column. Table I summarizes the steps of purification and the yield after each step.

Initially, Sephacryl S-300 was used for gel filtration of the affinity-purified fraction (Kuhlenschmidt, 1983). However, the recovery of Man-binding protein was less than 50%, and frequently, a later peak (near the total inclusion volume) with some binding activity was observed (CS-III). In some experiments, after the emergence of CS-I and CS-II peaks, the Sephacryl S-300 column was treated with 3 M NaSCN to yield a peak designated CS-II′. Gradual removal of NaSCN from this fraction resulted in partial recovery of activity with the specific activity reaching about one-third of that of CS-II.

Gel Electrophoresis of Man-Binding Fractions. Man-binding proteins at various stages of purification were analyzed by SDS-PAGE. Analysis of mercaptoethanol-treated proteins in a 10% separating gel (Figure 3) revealed that CS-I and CS-Gn contained at least four different subunits, while CS-II contained only two major bands (75 000 and 26 500 daltons). Without the mercaptoethanol treatment, CS-II did not enter the 10% separating gel. With a 5% separating gel, CS-II did enter the gel, and from its mobility (similar to that of myosin), its molecular weight was estimated to be ca. 200 000. CS-III, after mercaptoethanol treatment, appeared to be the same as CS-II and CS-II′ under the same conditions of SDS-PAGE.

The chicken hepatic GlcNAc-binding protein has been shown to consist of a single subunit of 27 000 daltons (Kuhlenschmidt & Lee, 1984), which can be shown by SDS-PAGE without prior treatment with mercaptoethanol. However, the detection of the 26 500-dalton subunit from CS-II required mercaptoethanol treatment.

Quantification of the major subunits in CS-II was carried out by densitometry of the stained gels (with an LKB 2202 Ultroscan laser densitometer). The ratio of the high (75 000) and low (26 500) molecular weight bands remained consistent (ca. 3.5) for many preparations of different degrees of purity, including some which have been rechromatographed on the affinity column. The ratio was also the same for CS-III and CS-II′.

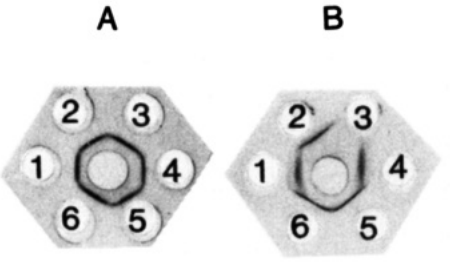

FIGURE 4: Double immunodiffusion of binding proteins and antisera to the chicken liver and serum binding protein. (A) The center well contained 40 μL of rabbit antisera to chicken serum binding protein (CS-II). The peripheral wells contained ca. 4 μg each of (1) CS-II from the Bio-Gel A1.5m column, (2 and 6) CS-II from the Sephacryl S-300 column, (3) CS-III from the sephacryl S-300 column, (4) a mixture of CS-II and CS-II′, (5) CS-II′, and (6) CS-II. (B) The center well contained 40 μL of rabbit antiserum against CS-II. The peripheral wells contained ca. 4 μg each of the following: (1) CS-Gn; (2) CS-I; (3) chicken hepatic binding protein; (4) CS-Gn; (5) CS-II′; and (6) CS-II.

Immunochemical Properties of Serum Man-Binding Proteins. The immunochemical properties of various Man-binding protein samples were compared by double immunodiffusion (Ouchterlony & Nilsson, 1973) as shown in Figure 4. The results can be summarized as follows:

(1) Antiserum against CS-II strongly reacted with CS-II from Bio-Gel A1.5m, with CS-II from Sephacryl S-300, and also with CS-III and CS-II′, showing complete fusion of precipitin lines (Figures 4A). Thus, these preparations are identical by this immunochemical criterion.

(2) Chicken hepatic GlcNAc-specific lectin showed no reaction toward the anti-CS-II serum. Conversely, antiserum against the hepatic lectin did not react with CS-II (Kuhlenschmidt, 1983).

(3) The antiserum against CS-II reacted with CS-Gn and CS-I, but the precipitin lines are not completely fused (Figure 4B). This is also in agreement with the results from rocket immunoelectrophoresis (not shown) in which two rockets were detected from CS-Gn and CS-I. Thus, these fractions contain at least two components cross-reacting with the anti-CS-II serum.

(4) CS-II did not react with anti-rabbit mannan-binding protein IgG from guinea pig. Conversely, the antiserum against CS-II was not reactive toward the mannan-binding protein from rabbit (T. Kawasaki, personal communication).

Conditions for Ligand Binding. Optimal conditions for binding were established for CS-II, using ^{125}I-Man$_{43}$-BSA as labeled ligand as follows:

(1) Effect of pH on Binding and Stability of the Binding Protein. The effect of pH on binding was investigated by using the standard binding assay conditions in which only the pH was varied over the range of 4–9 (Figure 5). The binding is effective over a remarkably wide range, virtually unaffected by pH between the values of 5.5 and 9.0. Although the binding was less efficient below pH 5, this could be partly due to slow inactivation of the binding protein itself, and partly due to weaker intrinsic binding (see below).

The stability of the binding protein (117 μg) to different pH conditions for 24 h at 4 °C or for 120 min at 37 °C. The solution was then adjusted to pH 7.8 to conduct the standard binding assay. The pH-stability curve (Figure 6) showed the same tendency as the pH-binding activity curve (Figure 5).

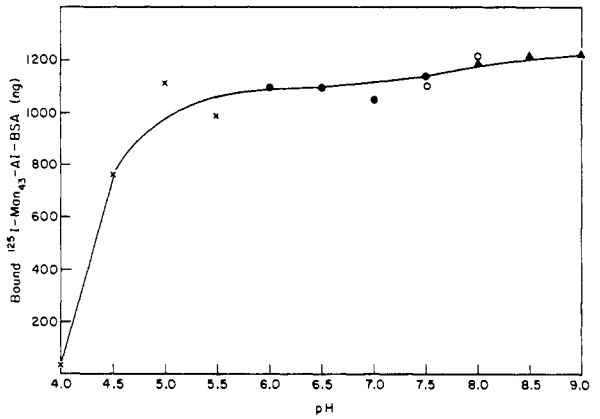

FIGURE 5: pH dependence of Man_{43}-BSA binding to CS-II. CS-II (2.4 μg) and 1 ng of ^{125}I-Man_{43}-BSA were incubated under standard assay conditions in buffer A containing 50 mM sodium acetate (×), PIPES (●), HEPES (○), or Tris (△) at the indicated pH. Specifically bound labeled ligand was measured after precipitation in ammonium sulfate at the indicated pH.

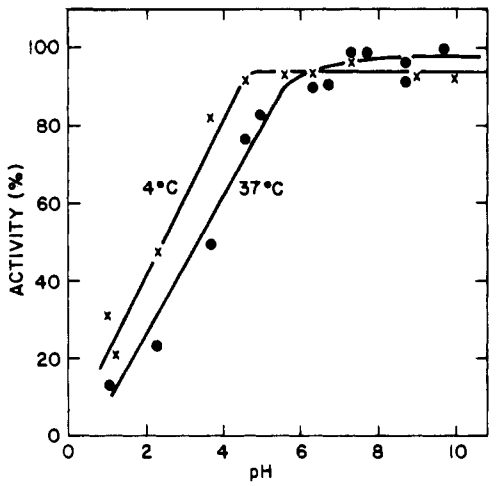

FIGURE 6: Stability of CS-II under different pH conditions. CS-II (117 μg/mL) in various buffers of the indicated pHs (see Figure 6) was incubated either at 4 °C for 24 h (×) or at 37 °C for 2 h (●). Portions (20 μL) of the incubation mixture were taken and diluted with the standard binding assay buffer (380 μL) and incubated under standard conditions to determine binding activity.

Although this may explain, in part, the diminished activity observed at the lower pH range, the dramatically low binding activity at pH <4 cannot be due to denaturation of the binding protein.

(*2*) *Requirement of Divalent Cations for Binding.* To a series of standard binding assay mixture, increasing amounts of either $CaCl_2$ or EDTA were added. The results indicate that the binding not only is independent of additional calcium but also is impervious to deprivation of divalent cations in general. Even when CS-II was dialyzed against buffer II (containing 5 mM EDTA), its binding activity was not impaired. This is the most salient difference between this Man-binding protein and the other Man-binding proteins, or chicken hepatic GlcNAc lectin.

(*3*) *Ammonium Sulfate Precipitation of the Man-Binding Protein.* Complete precipitation of the binding complex was attained with 45–50% saturation in the cold, while Man_{43}-BSA by itself did not precipitate at this level of saturation. With 45–50% ammonium sulfate, precipitation of the binding complex was complete within 5 min.

Interestingly, there was less labeled ligand–lectin complex precipitated with 60% ammonium sulfate than with 45%. Presumably, the higher concentration of ammonium sulfate resulted in reversal of binding or inactivation of the binding protein.

Table II: Displacement of Bound Labeled Ligand by Unlabeled Ligand[a]

unlabeled ligand added (μg)	% displacement	unlabeled ligand added (μg)	% displacement
100	100[b]	0.1	78.9
1	100	0.01	59.1

[a] After 10 ng of ^{125}I-Man_{43}-BSA was incubated with 0.5 μg of CS-II for 15 min at 25 °C (the conditions under which all ^{125}I-Man_{43}-BSA's were bound and precipitable with ammonium sulfate), unlabeled ligand was added to the mixture. After 5-min incubation at 25 °C, the labeled ligand which could not be precipitated with the same concentration of ammonium sulfate was presumed to be free. [b] Incubation for only 1 min.

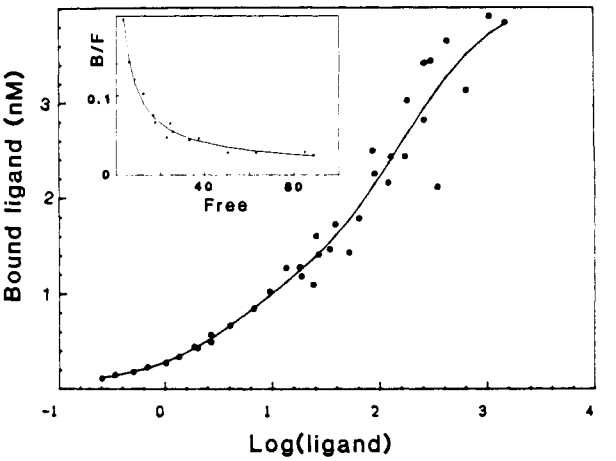

FIGURE 7: Equilibrium binding of ^{125}I-Man_{43}-BSA CS-II. CS-II (350 ng) and various amounts of the labeled ligand were incubated at 25 °C for 15 min, and the specifically bound ^{125}I-Man_{43}-BSA was determined as described. The binding data were fit to one-, two-, or three-site models using LIGAND. The solid line represents the fitted curve (three-site model), and the experimental points are shown. The best-fit binding parameters from the analyses are summarized in Table III. A partial plot of bound/free vs. total is shown in the inset.

Physical Parameters of Binding. Binding of Man_{43}-BSA to CS-II was very fast, binding being complete within 5 min at 25 °C (plus a 10-min incubation at 0 °C in 50% saturated ammonium sulfate). The bound ligand is also much more readily dissociable from the chicken serum Man-binding protein (Table II) than the hepatic GlcNAc-binding protein. Various amounts of the labeled ligand were incubated at 25 °C in the standard binding assay mixture containing 350 ng of the binding protein, and the binding complex was precipitated with ammonium sulfate as described. The data for the precipitated labeled ligand (bound ligand vs. total ligand as shown in Figure 7) were analyzed with the nonlinear regression program LIGAND (Munson & Rodbard, 1980) implemented on a VAX 11/780 computer (Engineering Computing Facility, The Johns Hopkins University). The results of this analysis are shown in Table III. The *F* test comparing the goodness of fit for various models indicates that a three-site model is somewhat better than a two-site model, which in turn is better than a one-site model. The stoichiometry of ^{125}I-Man_{43}-BSA binding was 0.9 mol bound/mol (assuming M_r 200 000) of binding protein for the two-site model and 1.20 mol/mol for the three-site model.

Sugar-Binding Specificity. The sugar-binding specificity of the Man-binding protein was studied in detail with synthetic and natural glycoconjugates possessing exposed Man residues using the inhibition techniques described for chicken hepatic lectin studies (Kuhlenschmidt & Lee, 1984). Various con-

Table III: Binding Parameters[a] of Man_{43}-BSA to Chicken Serum Mannose-Binding Protein

parameters[b]	model		
	one site	two site	three site
K_1 (nM)	3.3 (±16%)	0.76 (±29%)	0.08 (±217%)
K_2 (nM)		50 (±33%)	3.9 (±65%)
K_3 (nM)			172 (±85%)
R_1 (mol/mol)	0.48 (+8%)	0.17 (±17%)	0.038 (±36%)
R_2 (mol/mol)		0.73 (±37%)	0.30 (±25%)
R_3 (mol/mol)			0.87 (±91%)
total R (mol/mol)	0.48	0.90	1.20
F test[c]	23.86 (P = 0)	4.28 (P = 0.023)	1

[a] The data of Figure 7 were analyzed by using LIGAND, and the best-fit parameters for different models are listed. [b] K, dissociation constant for ligand-binding protein complex; R, moles of ^{125}I-Man_{43}-BSA bound per mole of binding protein, assuming M_r 200 000 for Man-binding protein. [c] Defined as $F = [(ss1 - ss2)/(df1 - df2)]/(ss2/df2)$ where ss stands for the residual sum of squares and df stands for the degrees of freedom. Numerals 1 and 2 stand for models 1 and 2.

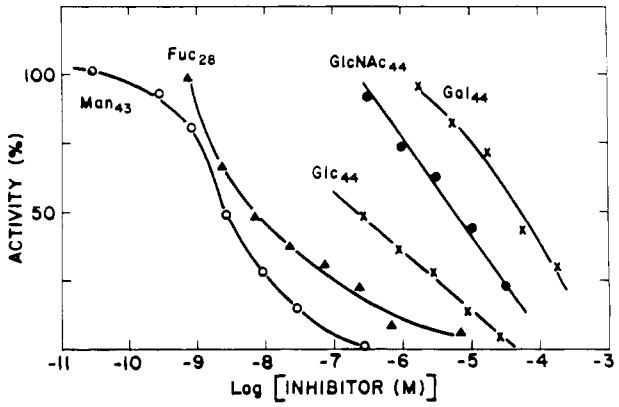

FIGURE 8: Inhibition of ^{125}I-Man_{43}-BSA binding to CS-II. CS-II (0.9 μg, 9 nM), ^{125}I-Man_{43}-BSA (90 pM), and various levels of unlabeled neoglycoproteins were incubated at 25 °C. After 15 min, specifically bound ^{125}I-Man_{43}-BSA was measured. I_{50} values obtained were 2.6 nM (Man_{43}-BSA, ○), 6.3 nM (L-Fuc_{28}-BSA, ▲), 0.2 μM (Glc_{44}-BSA, ×), 6.3 μM ($GlcNAc_{44}$-BSA, ●), and 51 μM (Gal_{44}-BSA, ×), respectively.

centrations of glycoconjugates to be tested were added to the standard incubation mixture containing ^{125}I-Man_{43}-BSA, and the bound labeled ligand was determined by ammonium sulfate precipitation. The results, shown in Figure 8, establish the hierarchy of Man_{43}-BSA = L-Fuc_{28}-BSA > Glc_{44}-BSA > $GlcNAc_{44}$-BSA > Gal_{44}-BSA with the values of 50% inhibition indicated in Table IV.

Simple sugars, as expected, were much weaker inhibitors compared to neoglycoproteins (Table IV). Even the most potent sugars, Man and L-Fuc, still required nearly 0.1 M for 50% inhibition. However, AHT glycosides of Man are more inhibitory than Man itself. This is perhaps due to contribution by the hydrophobic binding of the aglycon in these glycosides.

With the BSA-derived neoglycoproteins, there appears to be a clear dependence of binding affinity on the clustering of sugar residues (Figure 9) as found in the chicken hepatic lectin (Kuhlenschmidt & Lee, 1984) or other similar lectins (Krantz et al., 1976; Schlesinger et al., 1980). Interestingly, low molecular weight ligands, such as Man_{1-3}-AHT, Man_2Lys, and Man_3Lys_2, all showed 50% inhibition of ^{125}I-Man_{43}-BSA at about 2 mM range. These results indicate that clustering of mannosyl residues in these forms is not as effective as those on the neoglycoproteins.

Discussion

Although there is a distinct difference in the sugar-binding specificity of the avian (GlcNAc) and mammalian (Gal) hepatocytes, the major sugar-binding protein in the chicken serum showed the same specificity (Man) as the major mammalian serum sugar-binding protein. The procedure described here enabled us to purify the major Man-binding protein in the chicken serum.

Table IV: Inhibition of Labeled Man_{43}-BSA Binding to CS-II

inhibitor	I_{50}[a] (M)	I_{50}(Man) (M)[b]
Man_{43}-BSA	2.6×10^{-9}	1×10^{-8}
L-Fuc_{28}-BSA	6.3×10^{-9}	
$GlcNAc_{44}$-BSA	6.3×10^{-6}	
Glc_{44}-BSA	2.0×10^{-7}	
Gal_{44}-BSA	5.1×10^{-5}	
$(Man\text{-}6\text{-}P)_{47}$-BSA	$>10^{-6}$	
Man, L-Fuc	~0.1	
GlcNAc, Glc, Gal, ManNAc	>0.1	
Man_2Lys	0.001	
Man_3Lys_2	0.0014	
Man_{1-3}-AHT[c]	0.002	
invertase		7×10^{-6}
mannan (bakers' yeast)		1×10^{-5}
MNN 1[d]		4×10^{-6}
MNN 2[d]		9×10^{-8}
MNN 4[d]		7×10^{-7}

[a] I_{50} values were determined as described under Methods. [b] I_{50} based on the molar concentration of Man. [c] [2-(6-Aminohexanamido)]tris-(hydroxymethyl)methane modified with one to three Man residues (Lee, 1978). [d] Mutant yeast mannans (Ballou & Raschke, 1974).

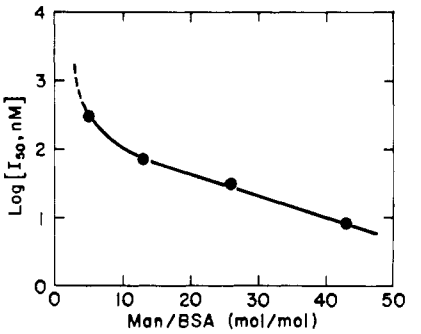

FIGURE 9: Dependency of mannose density on the binding of neoglycoproteins to CS-II. I_{50} values for Man-BSA samples containing different levels of Man binding were determined as described in Figure 8. The log [I_{50}] is plotted against Man/BSA (mol/mol).

By the current scheme of purification, ca. 20 mg of the Man-binding protein can be isolated from 500 mL of chicken serum. If one assumes that a 250-g Leghorn chicken possesses 16 mL of blood (Altman & Dettmer, 1971), then the total Man-binding protein would be more than 650 μg per chicken. From the liver (5–6 g) of the same animal, about 300–500 μg of the GlcNAc-specific lectin can be isolated (Kuhlenschmidt & Lee, 1984). Since the isolation yields for both proteins are roughly equivalent, it is clear that the total amount of Man-binding protein in the chicken serum is comparable to that of the hepatic lectin.

The pure Man-binding protein consists of two subunits of 75 000 and 26 500 daltons, respectively. These subunits can be demonstrated only by a combination treatment of mercaptoethanol and SDS. Without mercaptoethanol, only the 200 000-dalton band was observed. Taking the ratio of the subunits and the apparent molecular weight (200 000) shown by gel electrophoresis and gel filtration, it is reasonable to assume the CS-II is made of two each of the two subunits. Although CS-I possesses Man-binding activity, it does not dissociate into CS-II upon rechromatography on the agrose column. SDS–PAGE in the presence of mercaptoethanol (Figure 3) also reveal that CS-I and CS-Gn are made of subunits different from CS-II.

During our earlier studies, it was suspected that the hepatic lectin (GlcNAc specific) could be a minor contaminant in the affinity-purified fraction of the serum-binding protein, since it cross-reacted with Man glycoconjugates (Kuhlenschmidt & Lee, 1984; Kuhlenschmidt et al., 1984). The presence of the 26 500-dalton subunit in SDS–PAGE of the serum Man-binding protein could suggest such a contaminant. However, this can be ruled out on the basis of the following argument: (1) The appearance of the 27 000-dalton subunit from the hepatic lectin does not depend on mercaptoethanol treatment, while the 26 500-dalton band was produced only when the protein was treated with mercaptoethanol. This suggests that if the 26 500-dalton subunit were identical with the subunit of the hepatic lectin, at least it is linked to the rest of the molecular species by a disulfide bond rather than by a noncovalent bond. (2) The antibody against the hepatic lectin failed to react with the serum protein, and the antibody against the serum protein did not react with the hepatic lectin. This is evidence against the notion that the hepatic lectin subunit is included in the serum protein by means of a disulfide bond. (3) The purified serum Man-binding protein showed rather weak binding of GlcNAc-BSA (I_{50} = ca. 1 μM). This is far too low to be accounted for by the presence of detectable contamination of chicken hepatic lectin having a corresponding I_{50} value of 1 nM (Kuhlenschmidt & Lee, 1984). (4) Binding by the hepatic lectin is strictly calcium dependent; its activity is rapidly quenched by the addition of chelating agents (EDTA or EGTA), while the serum protein requires no added calcium nor is it susceptible to EDTA treatment.

The chicken serum Man-binding protein also is different from the major Man-binding protein in the mammalian serum and liver. The latter proteins possess only one subunit of 31 000 daltons, and their binding depends absolutely on calcium. The antibody against the rabbit serum Man-binding protein did not react with the chicken serum protein, and the antibody against CS-II also failed to react with the rabbit Man-binding protein.

Analysis of binding data either by nonlinear regression (using LIGAND) or by transformation (Scatchard plot) revealed the heterogeneous nature of binding. Although the three-site model gave a better fit than the two-site model with the data shown, it is best to consider that only as an indication of the heterogeneous mode of binding. This is understandable (Hardy et al., 1985), since a multivalent ligand, ^{125}I-Man_{43}-BSA, was used in the binding study. However, the degree of binding heterogeneity seems greater for this system than other comparable systems of sugar-binding proteins. More detailed studies are necessary to clarify this aspect.

From the data contained in this report, certain stereochemical requirements of this binding protein can be deduced. The efficient inhibition by L-Fuc indicates a situation analogous to the mammalian Man-binding protein (Kawasaki et al., 1983) as well as the hepatic Gal/GalNAc–lectin. Stereochemical similarity between D-Man and L-Fuc has been discussed elsewhere (Lee & Lee, 1982; Kuhlenschmidt & Lee, 1984). Using the same argument, the anomeric configuration probably is not important in the binding. As in the case of Gal/GalNAc–lectin of mammalian liver, the presence of a negative charge on C-6 (e.g., Man-6-P) cannot be tolerated by this binding protein.

As with the hepatic lectins, the chicken Man-binding protein showed a cluster effect, i.e., nearly logarithmic enhancement of binding as the sugar density on BSA is linearly increased (Kuhlenschmidt & Lee, 1984; Lee & Lee, 1982; Connolly et al., 1982). The binding potency of natural mannans differed as much as 100-fold when compared to the total Man concentration. This is perhaps a reflection of the subtle difference in branching patterns of the yeast mannans. Though the cluster effect is also evident in the yeast mannans, none of them was as potent an inhibitor as Man_{43}-BSA. The cluster effect, however, was not evident when the low molecular weight cluster glycosides such as Man_{1-3}AHT, Man_3Lys_2, or Man_2Lys were used as inhibitors for binding of ^{125}I-Man_{43}-BSA. This situation is analogous to the results obtained with Gal_3-AHT when detergent-solubilized rabbit hepatic lectin was used (Connolly et al., 1982).

As in the case for the hepatic lectins and the serum lectins of mammalian species, the biological function of the chicken serum Man-binding protein remains unclear at this time. The serum lectin is present in adult as well as in young chicken (Kuhlenschmidt & Lee, 1982). Our preliminary experiments show that chicken eggs also contain Man-binding activity. Work is currently under way to isolate and purify this protein from chicken eggs.

ACKNOWLEDGMENTS

We express our gratitude to Yoshikazu Nakano and Amy Fogelstrom for their enthusiastic and able assistance. We are grateful to Dr. C. E. Ballou for his gift of the samples of mutant mannans. We also thank Drs. Yamashina and Kawasaki for their gift of the antibody against the rabbit Man-binding protein and Dr. S. Roseman for the antibody against the chicken hepatic lectin.

Registry No. D-Mannose, 3458-28-4.

REFERENCES

Altman, P. L., & Dettmer, D. S. (1971) in *Blood and Other Body Fluids*, p 7, Federation of American Societies for Experimental Biology, Bethesda, MD.

Ballou, C. E., & Raschke, W. C. (1974) *Science (Washington, D.C.) 184*, 127–134.

Brownell, M. D., Colley, K. J., & Baenziger, J. U. (1984) *J. Biol. Chem. 259*, 3925–3932.

Colley, K. J., & Baenziger, J. U. (1983) Abstracts of the Annual Meeting of the Society for Complex Carbohydrates, Lexington, KY, p 41.

Connolly, D. T., Townsend, R. R., Kawaguchi, K., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 939–945.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2617–2624.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.

Hardy, M. R., Townsend, R. R., Parkhurst, S., & Lee, Y. C. (1985) *Biochemistry 24*, 22–28.

Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., & Morell, A. G. (1974) *J. Biol. Chem. 249*, 5536–5543.

Kawasaki, N., Kawasaki, T., & Yamashina, I. (1983) *J. Biochem. (Tokyo) 94*, 937–947.

Kozutsumi, Y., Kawasaki, T., & Yamashina, I. (1980) *Biochem. Biophys. Res. Commun. 95*, 658–664.

Kozutsumi, Y., Kawasaki, T., & Yamashina, I. (1981) *J. Biochem. (Tokyo) 90*, 1799–1807.

Krantz, M. J., Holtzman, N. A., Stowell, C. P., & Lee, Y. C. (1976) *Biochemistry 15*, 3963–3968.

Kuhlenschmidt, M. S., Schmell, E., Slife, C. W., Kuhlenschmidt, T. B., Sieber, F., Lee, Y. C., & Roseman, S. (1982) *J. Biol. Chem. 257*, 3157–3164.

Kuhlenschmidt, T. B. (1983) Ph.D. Dissertation, The Johns Hopkins University, Baltimore, MD.

Kuhlenschmidt, T. B., & Lee, Y. C. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1968.

Kuhlenschmidt, T. B., & Lee, Y. C. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1033.
Kuhlenschmidt, T. B., & Lee, Y. C. (1984) *Biochemistry 23*, 3569–3575.
Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., Roseman, S., & Lee, Y. C. (1984) *Biochemistry 23*, 6437–6444.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, R. T., & Lee, Y. C. (1982) in *The Glycoconjugates* (Horowitz, M. I., Ed.) Vol. IV, Part B, pp 57–83, Academic Press, New York.
Lee, Y. C. (1978) *Carbohydr. Res. 67*, 509–514.
Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Ouchterlony, O., & Nilsson, L. A. (1973) *Handbook of Experimental Immunology* (Weir, C. M., Ed.) Chapter 19, Blackwell, Oxford, U.K.
Schlesinger, P. H., Rodman, J. S., Doebber, T. W., Stahl, P. D., Lee, Y. C., Stowell, C. P., & Kuhlenschmidt, T. B. (1980) *Biochem. J. 192*, 597–606.
Stahl, P. D., Rodman, J. S., Miller, M. J., & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1399–1403.
Townsend, R., & Stahl, P. D. (1980) *Biochem. J. 194*, 209–214.
Zamenof, S. (1957) *Methods Enzymol. 3*, 696–704.

Characterization of a Tyrosine Sulfotransferase in Rat Brain Using Cholecystokinin Derivatives as Acceptors

F. Vargas, O. Frerot, M. Dan Tung Tuong, and J. C. Schwartz*

Unitê de Neurobiologie, Centre Paul Broca de l'INSERM, 2ter Rue d'Alêsia, 75014 Paris, France, and Laboratoire de Physiologie, Universitê Renê-Descartes, 75006 Paris, France

Received April 4, 1985

ABSTRACT: An apparently novel tyrosyl sulfotransferase activity was detected in a crude microsomal fraction from rat cerebral cortex by using 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) as the sulfate donor and various cholecystokinin (CCK) fragments or derivatives as acceptors. Among the latter, the shortest substrate was *tert*-butoxycarbonylaspartyltyrosine (Boc-Asp-Tyr), but the reaction was optimized by increasing the length of the peptide sequence on the C-terminal side up to *tert*-butoxycarbonylcholecystokinin octapeptide (Boc-CCK-8) as well as by the presence of acidic amino acid residues at the N-terminal side. Peptides with an N-terminal Tyr residue (e.g., CCK-7 or enkephalins) were not sulfated. With Boc-CCK-8 the optimum pH was 5.8, and apparent K_M values were 0.14 ± 0.02 mM for the peptide (0.5 μM PAPS) and 0.12 ± 0.01 μM for PAPS (0.25 mM Boc-CCK-8). In the presence of 0.2 mM $MnCl_2$ the V_{max} of the reaction was enhanced without change of apparent affinities of the two substrates. The possible role of this sulfotransferase activity in posttranslational modification of CCK and other secretory proteins is suggested.

Various posttranslational covalent modifications of proteins like phosphorylation of Ser, Thr, or Tyr residues are now recognized as important cell regulation processes and therefore widely studied (Uy & Wold, 1977; Cohen, 1982). In contrast, protein sulfation on Tyr residues was long considered a rare modification and has attracted little attention: the presence of an *O*-sulfate ester group on this amino acid, first detected in fibrinopeptide B (Bettelheim, 1954), was thought to occur only in a few peptides like fibrinogens (Jevons, 1963), gastrin (Gregory et al., 1964), or cholecystokinin (Mutt & Jorpes, 1968). However, recent studies have shown that Tyr-*O*-sulfate residues occur in a large number of secretory proteins, only some of which, like immunoglobin G or fibronectin, are as yet identified (Huttner, 1982; Lee & Huttner, 1983; Baeuerle & Huttner, 1984; Lin & Lippmann, 1985). The role of Tyr sulfation is generally not yet understood, but in the case of cholecystokinin, it appears essential for its recognition by receptors mediating its various hormonal or neuronal actions (Rehfeld, 1981; Dockray, 1982; Morley, 1982). Also, very little is known about the enzyme(s) responsible for this posttranslational modification, which has been described to occur in a cell-free extract from a rat pheochromocytoma with unidentified proteins as acceptors (Lee & Huttner, 1983). Among known sulfotransferases (Mulder et al., 1982) only aryl sulfotransferase IV (EC 2.8.2.1) purified from the cytosol of rat liver has the ability to sulfate Tyr residues (Sekura & Jacoby, 1981; Sekûra et al., 1981). However, this transfer only occurs onto peptides like CCK-7[1] in which the Tyr is in the N-terminal position, indicating that it is presumably not responsible for the sulfation of proteins in which Tyr residues are otherwise located (such as CCK-8 or its precursors).

We describe here the properties of an apparently novel tyrosyl sulfotransferase activity evidenced in a crude microsomal fraction from rat brain, using a simple assay system with synthetic CCK derivatives as acceptors and [^{35}S]PAPS as the sulfate donor.

Materials and Methods

The materials used were obtained from the following sources: nonradioactive PAPS, adenosine 5′-triphosphate, tyrosine methyl ester, 2,6-dichloro-4-nitrophenol, *p*-(chloro-

* Correspondence should be addressed to this author at Centre Paul Broca de l'INSERM, 2ter Rue d'Alêsia, 75014 Paris, France.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CCK, cholecystokinin; CCK-7, cholecystokinin heptapeptide; CCK-8, cholecystokinin octapeptide; CCK-8 (ns), cholecystokinin octapeptide (nonsulfated); PAPS, 3′-phosphoadenosine 5′-phosphosulfate; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/85/0424-5938$01.50/0