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ISOLATION AND CHARACTERIZATION OF A MANNAN-BINDING PROTEIN FROM RABBIT LIVER

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SUMMARY: A membrane protein which binds mannan has been isolated from rabbit liver by affinity chromatography. Upon polyacrylamide gel electrophoresis, a single major band was recovered with an estimated molecular weight of 31,000. When assayed as inhibitors, N-acetylmannosamine, N-acetylglucosamine, and mannose were potent inhibitors of mannan binding; N-acetylgalactosamine and mannose-6-phosphate were inert. Glycoproteins with terminal N-acetylglucosamine and/or mannose residues which are cleared rapidly from the circulation by the liver were the most potent inhibitors. On the basis of these results, it is proposed that the mannan-binding protein is the principle mediating the hepatic uptake of glycoproteins with terminal N-acetylglucosamine and/or mannose residues.

The role of prosthetic oligosaccharides as regulatory determinants for the catabolism of circulating glycoproteins has received much attention. Besides the well characterized mammalian hepatic receptor responsible for asialoglycoprotein catabolism (1 - 3) and the avian receptor for N-acetylglucosamine terminated glycoprotiens (4), alternative modes for the rapid clearance of glycoproteins from mammalian circulation have been described. These include systems for clearance of N-acetylglucosamine- and mannose-terminated glycoproteins such as agalacto-orosomucoid, ahexosamino-orosomucoid and RNase B [EC 2.7.7.16] (5 - 10). However, whether, or to what extent, these observations reflect the presence of specific receptors has not been resolved. In the course of studies on a presumptive binding protein for mannose-terminated glycoproteins, we have observed a remarkable binding of mannan by rabbit liver preparations. Subsequent studies have resulted in isolation of a membrane protein which recognizes glycoproteins with terminal N-acetylglucosamine and mannose residues.

MATERIALS AND METHODS

$Na^{125}I$, carrier-free, in diluted NaOH was obtained from the Radiochemical Center, Amersham, England. Mannan (430 μg) was iodinated with 1 mCi $Na^{125}I$, by a modification of the procedure of Greenwood *et al.* (11). The iodinated mannan, purified by passage through a column of Sephadex G-25, was recovered with specific activities ranging from 0.3 to 0.5 μCi per μg.

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Orosomucoid was a gift of the American National Red Cross Laboratory, Bethesda, MD, U.S.A. Asialo-orosomucoid, agalacto-orosomucoid and ahexosamino-orosomucoid were prepared as described previously (4). Hog kidney α-mannosidase [EC 3.2.1.24] purified according to the procedure of Okumura and Yamashina (12) had a specific activity of 7 units per mg protein. Rat preputial gland β-glucuronidase [EC 3.2.1.31], kindly provided by Dr. K. Kato, Kyushu University, Japan, had a specific activity of 104 units per mg protein.

The yeast used, Saccharomyces cerevisiae, was a commercially available Baker's yeast purchased from the Oriental Yeast Co., Tokyo, Japan. Mannan prepared from this yeast as described in ref. (13) comprised approximately 3 % of protein. The following materials were kindly provided by Dr. S. Suzuki of Tohoku College of Pharmacy, Japan: A mixture of a series of linear oligosaccharides with α1→6 linked mannose from S. cerevisiae mannan (14); (Manα1→6Man)$_n$ with an estimated molecular weight of 8,000, obtained by α-mannosidase (Arthrobacter GJM-1) digestion of S. cerevisiae mannan (15); Manα1→2Manα1→2Man, obtained from Candida albicans A-207 mannan (16).

Oligosaccharides, Manα1→6Man, Manα1→6Manα1→6Man, and Manα1→6Manα1→6Man α1→6Man were isolated by gel filtration of a mixture of manno-oligosaccharides with α1→6 linkage on the column of Bio-gel P-2. Manα1→3Manα1→2Manα1→2Man was isolated from S. cerevisiae mannan according to the procedure of Lee and Ballou (13).

Preparation of affinity resins of Sepharose 4B-asialo-orosomucoid and Sepharose 4B-mannan was carried out essentially as described in ref. (2). Asialo-orosomucoid (100 mg) or mannan (30 mg) was coupled to 60-ml aliquots of Sepharose 4B which had been activated with cyanogen bromide (17).

The binding assay was carried out essentially according to "Assay A" of ref. (2). The standard incubation mixture contained, in a final volume of 0.5 ml, the following components: Tris-HCl at pH 7.8, 25 μmol; NaCl, 500 μmol; $CaCl_2$, 20 μmol; Triton X-100, 0.1 % (W/V); bovine serum albumin, 0.6 % (W/V); ^{125}I-mannan, 300 ng; purified binding protein, 0.2 to 2.0 μg or an equivalent amount of crude preparation. The mixture was incubated for 15 min at room temperature and the labeled complex was precipitated by the addition of an equal volume of cold saturated ammonium sulfate that had been adjusted to pH 7.8 with solid Tris. After 10 min, at 0°, the precipitate was collected by filtration, washed and counted.

The isolation of a mannan-binding protein from rabbit livers was carried out as follows. Frozen livers (150 g) were homogenized with cold acetone in a Waring blendor and the homogenate was filtered under reduced pressure and allowed to dry in air. The acetone powder was stirred for 30 min at 4° in 500 ml of 0.2 M NaCl and centrifuged at 12,000 g for 15 min. The residual pellet was suspended by blending in 400 ml of extracting buffer consisting of 0.01 M Tris-HCl, pH 7.8, 0.4 M KCl and 2 % Triton X-100. After stirring for 30 min at 4°, the crude extract was obtained by centrifugation. The pellet was suspended in the same buffer and extraction was repeated three more times. The combined crude extract (1.6 l) was made 0.02 M in $CaCl_2$ and applied to the Sepharose 4B-mannan column (60 ml) which had been equilibrated with a loading buffer consisting of 0.01 M Tris-HCl, pH 7.8, 0.02 M $CaCl_2$, 1.25 M NaCl and 0.5 % Triton X-100. The column was washed with several additional bed volumes of this buffer prior to elution of the binding protein with an eluting buffer consisting of 0.02 M Tris-HCl, pH 7.8, 1.25 M NaCl, 2 mM EDTA and 0.5 % Triton X-100. The eluate (crude mannan-binding-protein fractions) was made 0.02 M in $CaCl_2$ and applied to the Sepharose 4B-asialo-orosomucoid column which had been equilibrated with the loading buffer to remove asialo-glycoprotein-binding protein. The fractions which passed through the column were pooled and then adsorbed onto a second, smaller affinity column of the Sepharose 4B-mannan. The column was washed with the loading buffer and the binding protein was eluted as described above except that the Triton X-100 concentration of the eluting buffer was reduced to 0.05 %. The major portion of the binding activity was recovered

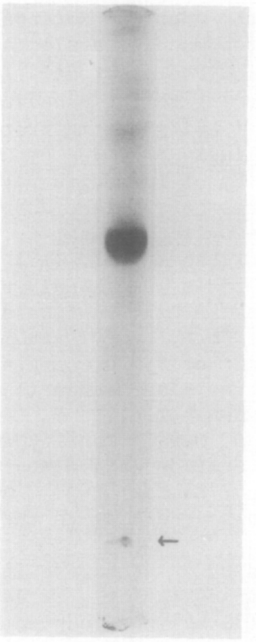

Fig. 1. Polyacrylamide gel electrophoresis of the isolated binding protein in the presence of sodium dodecyl sulfate. The binding protein (13 μg) freed from detergent by ethanol precipitation was subjected to electrophoresis in 10 % polyacrylamide gel according to the procedure of Weber and Osborn (27). Protein bands were stained with Coomassie Brilliant Blue G-250 in 12.5 % trichloroacetic acid. The arrow denotes the migration of marker dye, Pyronine Y.

in the second bed volume of the eluting buffer. The binding protein specific for asialo-glycoproteins was prepared simultaneously from the pass-through fractions of the first Sepharose 4B-mannan column as described in ref. (3).

Protein was determined by the method of Lowry et al. (18).

RESULTS AND DISCUSSION

From 150 g of frozen livers, approximately 1 mg of purified binding protein was recovered with a specific activity of 50 to 60 ng mannan bound per μg protein, a figure closely comparable to that shown previously for galactose-specific hepatic binding protein (2, 3).

Upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the isolated binding protein gave rise to a major band with a few slower moving minor constituents, as illustrated in Fig. 1. Approximate molecular weight of the major band was estimated to be 31,000.

In an attempt to determine the specificity of the binding protein, a number of manno-oligosaccharides and modified mannans were assayed as inhibitors of the binding of ^{125}I-mannan to the isolated binding protein. Mannan digested with

Table 1

Inhibitory activity of manno-oligosaccharides

Compound	Amount to give 50 % inhibition (mM)
Mannose	41
Manα1→2Manα1→2Man	11
Manα1→3Manα1→2Manα1→2Man	10
Manα1→6Man	10
Manα1→6Manα1→6Man	4
Manα1→6Manα1→6Manα1→6Man	4
$(Man\alpha1\rightarrow6Man)_n$ MW 8,000	0.04

pronase and purified by gel filtration on Sephadex G-100 retained full inhibitory activity. In contrast, mannan subjected to Smith degradation (19) lost its inhibitory activity almost completely. These findings imply the specificity of the binding protein to be directed towards the oligosaccharide moiety rather than towards the polypeptide moiety of mannan.

The results summarized in Table 1 revealed that α1→6 linked mannose was a more potent inhibitor than α1→2 or α1→3 linked mannose under conditions where the molecular sizes of the oligosaccharides were comparable. Inhibitory activity was shown to vary directly with the size of the oligosaccharide chain.

Inhibition of binding by various simple sugars is given in Table 2. Whereas, mannose was, as expected, a good inhibitor, N-acetylglucosamine, which is a minor component of mannan, and N-acetylmannosamine, which is not normally present in mannan, were the most potent inhibitors tested. Since mannose, N-acetylmannosamine and N-acetylglucosamine share a common three-dimensional structure, except at C-2, these results suggest that these sugars may share a common site on the binding protein which is blind to the C-2 position. Mannose-6-phosphate, which has been postulated to be a recognition component on lysosomal enzymes for pinocytosis in human fibroblasts (20 - 22), was devoid of inhibitory potency under the conditions tested. It is noteworthy that the asialo-glycoprotein-binding protein isolated from the same livers showed a distinctly different spectrum of inhibition by simple sugars.

Table 3 presents data on the inhibitory activities of various glycoproteins on the binding of mannan to the binding protein. Among the derivatives of oroso-

Table 2

Inhibitory activity of various sugars

Compound	Inhibition of binding	
	Mannan-BP[a)]	ASGP-BP[b)]
30 mM		
N-Acetylmannosamine	70	---
N-Acetylglucosamine	49	1
Mannose	40	4
L-Fucose	26	37
2-Deoxyglucose	16	7
Glucose	15	10
Galactose	13	64
Lactose	5	81
Glucosamine	0	5
Galactosamine	0	69
N-Acetylgalactosamine	0	83
Mannosamine	0	---
2 mM		
Mannose-6-phosphate	0	---
Glucose-6-phosphate	0	---

a) 1.0 μg of purified mannan-binding protein was used.

b) 8.2 μg of purified asialo-glycoprotein-binding protein was used.

mucoid, agalacto-orosomucoid was the strongest inhibitor and ahexosamino-orosomucoid was the next, being consistent with the potent inhibitory activities of N-acetylglucosamine and mannose, respectively. More provocative were the greater inhibitions displayed by lysosomal α-mannosidase and preputial gland β-glucuronidase. These glycosidases, which are glycoproteins with carbohydrate moieties consisting exclusively of mannose and N-acetylglucosamine residues (12, 23), as are ovalbumin adn RNase B (24), have been shown to be rapidly cleared from the circulation and to be taken up by the liver following intravenous injection into rat (25).

The data reported here imply that the mannan-binding protein isolated in the present study is the principle mediating the hepatic uptake of glycoproteins with terminal N-acetylglucosamine and/or mannose residues. While this work was in

Table 3

Inhibitory activity of glycoproteins

Glycoprotein	Amount to give 50 % inhibition (mg/ml)	(μM)
Orosomucoid	24	550
Asialo-orosomucoid	8.0	200
Agalacto-orosomucoid	0.33	9
Ahexosamino-orosomucoid	1.70	52
Ovalbumin	3.30	77
α-Mannosidase	0.12	1
β-Glucuronidase	0.38	1

progress, further evidence supporting the presumptive role of the mannan-binding protein was provided by Achord et al. (10). They demonstrated the cross inhibition of clearance of agalacto-orosomucoid and mannan from rat plasma.

The physiological role of the mannan-binding protein is currently uncertain. It may participate in the regulation of serum glycoprotein homeostasis in conjunction with asialo-glycoprotein-binding protein. However, in the presence of an efficient mechanism maintaining near zero levels of circulating asialo-glycoproteins, the direct precursors of ligands appropriate for the mannan-binding pro tein, the role of the mannan-binding protein may be auxiliary. The rapid clearance of circulating lysosomal enzymes with carbohydrate constituents rich in mannose and N-acetylglucosamine raises the possibility that the mannan-binding protein may play a significant role in the packaging mechanism suggested by Neufeld et al. (26).

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REFERENCES

1. Ashwell, G., and Morell, A. G. (1974) Adv. Enzymol. 41, 99-128.
2. Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J., and Morell, A. G. (1974) J. Biol. Chem. 249, 5536-5543.
3. Kawasaki, T., and Ashwell, G. (1976) J. Biol. Chem. 251, 1296-1302.
4. Kawasaki, T., and Ashwell, G. (1977) J. Biol. Chem. 252, 6536-6543.

5. Stockert, R. J., Morell, A. G., and Scheinberg, I. H. (1976) Biochem. Biophys. Res. Commun. 68, 988-993.
6. Stahl, P., Schlesinger, P. H., Rodman, J. S., and Doebber, T. (1976) Nature 264, 86-88.
7. Winkelhake, J. L., and Nicolson, G. L. (1976) J. Biol. Chem. 251, 1074-1080.
8. Baynes, J. W., and Wold, F. (1976) J. Biol. Chem. 251, 6016-6024.
9. Achord, D. T., Brot, F. E., Bell, C. E., and Sly, W. S. (1977) Fed. Proc. 36, 653.
10. Achord, D. T., Brot, F. E., and Sly, W. S. (1977) Biochem. Biophys. Res. Commun. 77, 409-415.
11. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) Biochem. J. 89, 114-123.
12. Okumura, T., and Yamashina, I. (1973) J. Biochem. 73, 131-138.
13. Lee, Y-C., and Ballou, C. E. (1965) Biochemistry 4, 257-264.
14. Peat, S., Whelan, W. J., and Edwards, T. E. (1961) J. Chem. Soc. 29-34.
15. Jones, G. E., and Ballou, C. E. (1969) J. Biol. Chem. 244, 1052-1059.
16. Sunayama, H. (1970) Japan. J. Microbiol. 14, 27-39.
17. Cuatrecasas, P., and Anfinsen, C. B. (1971) Methods Enzymol. 22, 345-378.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
19. Spiro, R. G. (1967) Methods Enzymol. 8, 26-52.
20. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. 74, 2026-2030.
21. Kaplan, A., Fischer, D., Achord, D. T., and Sly, W. S. (1977) J. Clin. Invest. 60, 1088-1093.
22. Sando, G. N., and Neufeld, E. F. (1977) Cell 12, 619-627.
23. Himeno, M., Ohhara, H., Arakawa, Y., and Kato, K. (1975) J. Biochem. 77, 427-438.
24. Kornfeld, R., and Kornfeld, S. (1976) Ann. Rev. Biochem. 45, 217-237.
25. Stahl, P., Six, H., Rodman, J. S., Schlesinger, P., Tulsiani, D. R. P., and Touster, O. (1976) Proc. Natl. Acad. Sci. 73, 4045-4049.
26. Neufeld, E. F., Sando, G. N., Garvin, A. J., and Rome, L. H. (1977) J. Supramol. Structure 6, 95-101.
27. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.