BINDING OF CONCANAVALIN A-SEPHAROSE TO GLYCOPROTEINS OF LIVER MICROSOMAL MEMBRANES

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1. Introduction

Many biological membranes contain glycoproteins of mostly unknown structure and function, and the use of plant lectins seems to be extremely valuable in the study of these membrane components. One of them, concanavalin A (Con A), is known to interact with polysaccharides having unsubstituted α-D-glucopyranosyl, α-D-mannopyranosyl, or β-D-fructofuranosyl residues [1]. Previous investigators analyzed the interaction of Con A with whole cells and isolated fractions mainly by using agglutination and ferritin labeling techniques [2–6]. It has been suggested that microsomal membranes also contain glycoproteins, even after removal of adsorbed and luminal proteins [7–9]. In order to elucidate the role of these glycoproteins in the structure of microsomal membranes, it appeared mandatory to study their possible interactions with specific lectins, first of all Con A. Sepharose-bound Con A was used, which after binding to membrane components can be separated in a single step by centrifugation.

2. Materials and methods

Adult male albino rats, weighing 180–200 g, were used. The animals were starved for 20 hr before killing. Radioactive precursors were injected into the portal vein under Nembutal (6 mg/100 g rat) anesthesia. After a fixed time of incorporation of label, the rats were killed by decapitation.

Mitochondrial outer and inner membranes were isolated as earlier [10], and the final fractions were washed twice and suspended in 0.3 M lactose. Total microsomes were separated according to Eriksson [11]. Rough and smooth microsomes were prepared as previously [12]. The rough membranes were further fractionated into a ribosome-rich (rough I) and a ribosome-poor (rough III) fraction, and the smooth membranes were subdivided into a smooth I group (which are aggregated by 7 mM Mg^{2+}) and a smooth II group (which are not aggregated by divalent cations) [11].

Golgi vesicles were prepared by the method of Ehrenreich et al. [13]. Livers from starved rats were homogenized in 0.25 M sucrose (2 g/10 ml) with 4 strokes in a Teflon-glass homogenizer at 800 rpm. The isolated microsomal fraction in 0.25 M sucrose was mixed with an equal volume of 2.1 M sucrose in an SW 27 tube. This was layered with 1.15 M sucrose. Finally the tube was filled up with 0.25 M sucrose followed by centrifugation at 23 000 rpm in a Spinco–Beckman ultracentrifuge for 3 hr. The microsomal and Golgi fractions were washed in Tris–water–Tris [14, 15] and suspended in 0.3 M lactose.

Membrane phospholipids (PLP) were extracted with chloroform–ethanol 1:1, and phosphorus was measured [16].

One ml Con A-Sepharose was washed three times with 4 ml of 0.3 M lactose. In the non-inhibited case the pellet was suspended in 0.3 M lactose to a final volume of 2 ml, and in the inhibited case 1 ml of α-methylmannoside (αMM, 100 mg/ml, which was

found to give sufficient inhibition) and 0.3 M lactose was added to give 2 ml. These two mixtures were referred to as 'slurry' and 'αMM-slurry', respectively. The two slurry tubes were gently shaken for 15 min before use. The incubation medium contained 1 ml of slurry (or αMM-slurry), membranes and, in the appropriate case, detergents. 0.3 M lactose was added to a final volume of 2 ml. Incubation was performed at 20°C for 15 min under gentle shaking. At the end of the incubation, the medium was spun down at 100 *g* for 1 min, and the pellet was washed three times with centrifugation in 5 ml of a washing solution containing 0.3 M lactose, detergent of the concentration used, and, in the inhibited cases, αMM (final concentration: 25 mg/ml). The pellet was transferred to a scintillation vial, and the radioactivity was determined in Bray's solution [17].

The lipid and the protein phases of microsomes were separated by extraction with chloroform–methanol 2:1 at room temperature. The chloroform phase was evaporated to dryness, and the lipid was dissolved in 0.5% Na-deoxycholate (DOC). The proteins were solubilized in 1% sodium lauryl sulphate.

Con A–Sepharose (concanavalin A covalently bound to Sepharose 4 B, 8 mg Con A/ml gel sediment) was bought from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Radioactive precursors were delivered by the Radiochemical Centre, Amersham, England. They were: glycerol-2-^{3}H (500 mCi/mmol), DL-leucin-1-^{14}C (3.6 mCi/mmol), D-mannose-1-^{3}H (3.3 Ci/mmol), and D-glucosamine-6-^{3}H (10 Ci/mmol).

3. Results and discussion

Washed microsomal membranes, prelabeled in vivo with glucosamine-^{3}H and solubilized with DOC, interact with Con A–Sepharose (table 1). This binding, which corresponds to about 25% of total microsomal radioactivity, is inhibited to a very large extent by αMM. The specificity for a bound mannose is also demonstrated by the absence of binding to Sepharose-4 B.

In these experiments the amount of radioactivity bound is directly proportional to the amount of membrane material (fig. 1). We used concentrations in this linear range.

Intact microsomal vesicles exhibit only a 5% binding of total radioactivity (table 2), indicating only a limited number of binding sites available on the surface. In order to expose all terminal mannosyl groups, different detergents were tested for solubilization. Lithium-diiodosalicylate (LIS), a chaotropic agent, DOC, an ionic, and Triton X-100, a non-ionic detergent, all gave a maximal binding of radioactive material to Con A–Sepharose in concentration which completely solubilized the membranes. Since LIS is a strongly denaturing agent and Triton X-100 appears to give a higher non-inhibitable binding, DOC was chosen as solubilizer in a final concentration of 0.5%.

In order to determine the nature and composition of the bound material, different precursors of sugar, protein, and lipid were injected in vivo (table 3). Similarly to glucosamine-^{3}H, as much as 28% of the total microsomal radioactivity could be recovered in the Con A-Sepharose pellet after mannose-^{3}H admini-

Table 1
Binding of DOC-solubilized microsomal membrane components to Con A-Sepharose

Medium	Cpm in pellet
No Sepharose	0*
Sepharose-4B	523
Sepharose-4B + αMM	675
Con A-Sepharose	10 156
Con A-Sepharose + αMM	594

* No pellet.

Microsomes were prepared from rats which were injected 60 min before decapitation with 0.2 mCi glucosamine-^{3}H, through the portal vein. The microsomes were washed with Tris–water–Tris and resuspended in 0.3 M lactose. 1 ml washed slurry (see Materials and methods) and 0.1 ml 10% deoxycholate (DOC) was mixed with 0.4 ml 0.3 M lactose and 0.5 ml microsomes (from 0.25 g liver). This gives a final DOC concentration of 0.5%. Incubation was performed at 20°C for 15 min under gentle shaking. At the end of incubation the solution was centrifuged at 100 *g* for 1 min and the pellet was washed 3 times with 0.3 M lactose–0.5% DOC. The pellet was transferred to a scintillation vial containing 10 ml Bray solution and the radioactivity was determined. In the appropriate case, α-methyl-mannoside (αMM) was incorporated into the slurry at least 15 min before use. The total radioactivity of the solubilized microsomes (0.5 ml) was 39 500 cpm.

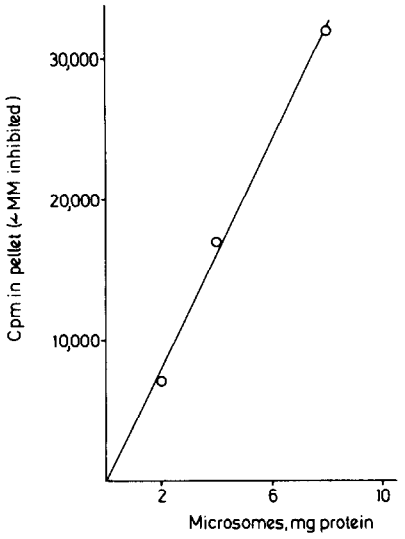

Fig. 1. Relationship between microsomal concentration and amount of radioactivity bound by Con A–Sepharose. Experimental details as in table 1. Values on the ordinate give that part of the Con A–Sepharose bound radioactivity which is specifically inhibited by αMM.

Table 2
Effect of various detergents on the binding of microsomal membrane components to Con A–Sepharose

Detergent %		Cpm in pellet −αMM	Cpm in pellet +αMM	αMM Inhibition % cpm of total
None		4324	1309	5
LIS	0.08	5306	1195	7
	0.2	11 736	1834	16
	0.4	16 047	1867	24
DOC	0.1	13 299	2256	18
	0.25	15 620	2695	21
	0.5	18 644	2062	27
Triton	0.05	9238	1974	12
	0.1	16 688	4814	20
	0.2	19 763	6374	22

Lithium diiodosalicylate (LIS), deoxycholate (DOC), and Triton X-100 were preincubated with the slurry in the indicated concentrations. Each sample contained 1 mg protein per ml.

αMM Inhibition = $\frac{(-\alpha MM) - (+\alpha MM)}{\text{total radioactivity}} \cdot 100$. Total radioactivity was 60 400 cpm. In other respects, experimental conditions as in table 1.

Table 3
Con A–Sepharose binding of microsomal components labeled with different precursors

Label	Cpm in pellet −αMM	Cpm in pellet +αMM	αMM Inhibition % cpm of total
Glucosamine-^{3}H	24 596	4849	24
Mannose-^{3}H	2653	491	28
Leucine-^{3}H	5559	1140	20
Glycerol-^{3}H	1673	374	13

Rats were injected 60 min before decapitation with 200 μCi glucosamine-^{3}H, 200 μCi mannose-^{3}H, 38 μCi leucine-^{3}H, or 50 μCi glycerol-^{3}H.

αMM Inhibition = $\frac{(-\alpha MM) - (+\alpha MM)}{\text{total radioactivity}} \cdot 100$.

stration. Surprisingly, 20% of the total leucine-^{3}H and 13% of the glycerol-^{3}H label were also bound. Since microsomes were washed by the Tris–water–Tris procedure, which is known to remove all the non-incorporated radioactivity, luminal and adsorbed proteins, the results indicate a membranous oligosaccharide–lipid–protein complex in the bound material.

The αMM-inhibited binding of intact outer and inner mitochondrial membranes occurs to a limited extent, since 2–3% of total membrane-associated radioactivity appeared in the pellet (table 4). The results are similar among the various type of microsomal as well as Golgi membranes. A sizable amount of radioactivity of in vivo prelabeled mitochondrial inner and outer membranes, however, attach to Con A–Sepharose when incubated in 0.5% DOC. Also, labeled components of Golgi membranes exhibit binding to a similar degree. All the microsomal subfractions isolated in these experiments interact with the lectin to a relatively high extent. The ribosome-rich rough vesicles (rough I) stand out, having 34% of their total radioactivity associated with the pellet. Isolated ribosomes do not contain glycoprotein and do not incorporate glucosamine-^{3}H in vivo, and consequently the high binding with rough I microsomes can be regarded as an inherent membrane property.

In this investigation, the label incorporated into the appropriate sugar moiety was used as marker for the chemical interaction. If, however, the incorporation

Table 4
Binding of components from various cytoplasmic membranes to Con A–Sepharose

Fraction		0.5% DOC-solubilized membranes			Intact membranes
		−αMM	+αMM	αMM inhibition	αMM inhibition
		cpm in pellet		% cpm of total	% cpm of total
Mitochondrial membranes	Outer	163	80	10	2
	Inner	935	232	14	3
Microsomes	rough I	13 600	2600	34	4
	rough III	8680	1517	20	3
	smooth I	7063	856	18	4
	smooth II	6904	945	16	4
Golgi membranes		1291	208	16	2

Subfractionations as in Materials and methods. Experimental details as in table 1. 0.5% DOC is added where indicated. αMM Inhibition = $\frac{(-\alpha MM) - (+\alpha MM)}{\text{total radioactivity}} \cdot 100$.

Table 5
Con A–Sepharose-bound radioactivity in rough and smooth microsomes at various time points

	Time, min	Total radioactivity cpm / g liver	cpm / mg PLP	αMM inhibition % Cpm of total
Supernate	35	508 000		1
Total microsomes	35	138 000		20
Rough microsomes	35		23 200	25
Smooth microsomes	35		97 500	14
Supernate	65	228 000		1
Total microsomes	65	76 000		26
Rough microsomes	65		13 240	31
Smooth microsomes	65		68 500	18
Supernate	140	82 000		3
Total microsomes	140	66 000		19
Rough microsomes	140		10 860	22
Smooth microsomes	140		31 800	14

Rough and smooth microsomes were prepared as in [11]. Supernate denotes the non-sedimentable solute after 60 min centrifugation at 105 000 *g*. All incubations were performed in the presence of 0.5% DOC. αMM Inhibition gives that part of the total microsomal radioactivity which specifically binds to Con A–Sepharose.

pattern among subfractions is unequal, this could explain the difference between rough and smooth microsomes in table 4. To exclude this artefact, glucosamine-^{3}H was injected and rough and smooth microsomes were prepared after 35, 65, and 140 min (table 5). It is clear that, in spite of the higher labeling of smooth microsomes on a phospholipid basis, the time course of incorporation and Con A–binding are similar for the two subfractions.

It is established that intramembranous dolichol phosphate serves as sugar-carrying lipid intermediate in glycoprotein synthesis [18]. Fig. 2 demonstrates that after 60 min of in vivo incorporation almost all radioactivity is protein attached, and only 0.5% of label can be recovered in the chloroform phase. Thus, bound material in the pellet has to consist of glycoprotein–lectin complex.

The above experiments demonstrate that the use of Con A–Sepharose in a centrifugation system is a simple and effective method in the study of macromolecular membrane components having terminal α-mannosyl groups. Mitochondrial outer and inner membranes, Golgi vesicles, and microsomal membranes bind only to a very limited extent to Con A–Sepharose, indicating that they lack mannosyl receptors on their outer surfaces. All these membranes possess, however, intramembranous glycoproteins with termi-

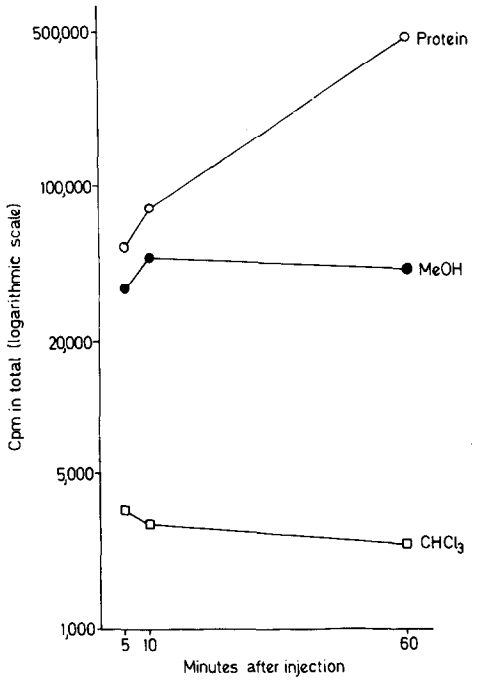

Fig. 2. Distribution of radioactivity in the protein and lipid phases after glucosamine-^{3}H injection. Rats were injected with 200 μCi glucosamine-^{3}H, 5, 10, and 60 min before killing, respectively. Lipids were extracted from the microsomes with chloroform–methanol (see Materials and methods). The radioactivity was measured after partition in the chloroform and methanol phases, and also in the protein precipitate.

nal α-mannose available to concanavalin A, after solubilization with DOC. It should be kept in mind that the inside of the microsomes is topologically equivalent to the outside of the plasma membrane, which is reported to have Con A receptors [19]. Rough microsomes display the highest interaction with Con A–Sepharose among subcellular membranes studied here. Since in these experiments in vivo sugar labeling was used as glycoprotein marker, it is not yet clear whether the observed variations are due to differences in the amount of acceptor sites or differential labeling caused by the nature of the individual oligosaccharides.

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