COOPERATIVITY IN CONCANAVALIN A-BINDING TO THYMOCYTE MEMBRANES: CORRELATION WITH POLYMERIZATION OF A RECEPTOR GLYCOPROTEIN

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Received February 23,1976

SUMMARY

At low concanavalin A-concentrations, binding of this lectin to isolated thymocyte microsomal membranes exhibits positive cooperativity, with a Hill coefficient of 1.75. Treatment of the membranes with low levels of glutaraldehyde leads to the selective cross-linking of a 55,000 D glycoprotein, a known concanavalin A receptor. A parallel increase in lectin-binding and a concomitant shift to high-affinity, non-cooperative binding occurs.

INTRODUCTION

Few studies have dealt in depth with processes involved in concanavalin A binding to cell surfaces. Indeed, virtually all work on lectin-membrane interactions assumes that the membrane receptors correspond closely to the "hapten sugars" which can block or reverse lectin membrane interactions. It is established, however, that the association constant for the reaction of concanavalin A with "hapten sugars" (1), is 100-fold smaller than the values observed for the interaction of the lectin with adipocytes (2). Moreover spontaneous dissociation of the concanavalin A-adipocyte complex, as well as exchange of bound ^{125}I-labelled concanavalin A with unlabelled lectin, are vanishingly slow, although addition of "hapten sugar" fosters very rapid breakdown of the complex (2). Finally, the binding curve for adipocytes has a sigmoid shape indicating heterogeneous binding sites and suggesting cooperative interaction between receptor sites (2). The binding of concanavalin A to human lymphocytes is also complex (3), with three saturation plateaux.

We have examined the interaction of concanavalin A with thymocyte membranes (4) and have found that mitogenic levels of the lectin enhance the turnover of

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a 55,000 D membrane glycoprotein and induce its release into the culture medium. We have purified this protein from Triton X-100 extracts of thymocyte membranes by affinity chromatography and have demonstrated that it could exist in mono- and dimeric forms (5). We now show that concanavalin A-binding to thymocyte membranes is positively cooperative at low lectin concentrations and that this cooperativity depends upon the state of the 55,000 D glycoprotein.

MATERIALS AND METHODS

All chemicals used were of highest purity grade available. Concanavalin A (3 x recrystallized) was purchased from Miles Laboratories (Elkhart, Ind.). Lactoperoxidase Boehringer (Mannheim, Germany) in 2.5 M$(NH_4)_2SO_4$ was freed of $(NH_4)_2SO_4$ by dialysis against the buffer used for labelling. Carrier-free Na [^{125}I] (17Ci/mg), in 0.1 N NaOH was obtained from New England Nuclear, (Cambridge, Mass.). HEPES (4-hydroxy-methyl)-1-piperazinylethane-2-sulfate), α-methyl-D-glucopyranoside and Coomassie blue were purchased from Sigma (St. Louis, Mo.), acrylamide, N,N'-methylenebisacrylamide, N,N'-diallyltartardiamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate from Bio Rad Laboratories (Richmond, Cal.), and Millipore filters (type GS; pore size 0.22 μm) from Millipore Corp. (Bedford, Mass.). ^{125}I-activity was counted in a Packard Autogamma Spectrometer.

Microsomal membranes were isolated from the thymocytes of white New Zealand rabbits (female, 2 months old) as in (4,5,6). For glutaraldehyde treatment washed microsomal membranes were suspended in 0.001 M HEPES, pH 7.5, to a protein concentration of 1 mg/ml. The membranes were treated at glutaraldehyde concentrations from 0.0016 - 0.0256 M for 30 min. at 20°C. The samples were diluted 20-fold and collected by pelleting at $1.2 \cdot 10^7$ g·min. For studies of concanavalin A binding the membranes were resuspended in the original volume of HEPES, 0.001 M, 0.0002 M $CaCl_2$, 0.0002 M $MnCl_2$, pH 7.5.

^{125}I-labelling of concanavalin A was by lactoperoxidase-catalyzed iodination at 25°C in 1.0 ml, 0.001 M HEPES, 0.0002 M $CaCl_2$ and 0.0002 M $MnCl_2$, pH 7.5, 10^{-6} M KI, containing 5 mg lectin protein, 0.1 mg lactoperoxidase, 10 μl butylated hydroxytoluene (100 mg/100 ml H_2O), 20 μl-Na [^{125}I] (0.02 mCi). Iodination was initiated by adding 20 μl of 0.003% H_2O_2 in 0.02 M Tris-HCl, pH 7.5, and was maintained by three further additions of 10 μl H_2O_2 at 1 min. intervals. Excess ^{125}I was removed by dialysis of the reaction mixture for 36 hrs. (total) against 3 x 1000 ml 0.001 M HEPES, 0.0002 M $CaCl_2$ and 0.0002 M $MnCl_2$, containing 0.005 M KI, and 1 x against the same buffer without KI. The labelling procedure gave a specific activity of 2-4 Ci/mole lectin (tetramer). Also, [^{125}I]-concanavalin A had the same mitogenic activity as unlabelled lectin - namely a 20-fold increase of [^{3}H]-thymidine incorporation by thymocytes after 24 h exposure to 5 μg/ml of concanavalin A.

Binding of [^{125}I]-concanavalin A to microsomal membranes was determined by a filtration technique. We used Millipore filter-membranes to collect microsomal membranes, having first demonstrated that these filters retain more than 95% of ^{125}I-labelled membranes. To prevent filter obstruction we used no more than 20 μg membrane protein per assay. To minimize non-specific binding of [^{125}I]-concanavalin A to the filters, these were first treated with a solution containing 2 mg of concanavalin A (unlabelled) in 4 ml 0.001 M HEPES, 0.0002 M CaCl, 0.0002 M $MnCl_2$, pH 7.5. Microsomal membranes, incubated at a concentration of 125 μg/ml with [^{125}I]-concanavalin A, were then mixed into 5 ml 0.001 M HEPES, 0.0002 M $CaCl_2$ 0.0002 M $MnCl_2$, pH 7.5, containing 20 μg concanavalin A/ml and collected by filtration under suction on the Millipore filters. The filters were washed with additional 10 ml of 0.001 M HEPES, 0.0002 M $CaCl_2$, pH 8.5, containing 20 μg concanavalin A/ml. In this way we obtain full recovery of microsomal membranes on the filters and reduce the amount of non-specifically bound [^{125}I]-concanavalin A by 90 to 97%. To com-

pensate for residual, non-specific concanavalin A-adsorption, we measured binding of the labelled lectin to filters under conditions equivalent to those used for membrane binding. We find that,under extreme conditions, at 200 µg concanavalin A/ml at a membrane concentration of 125 µg/ml, 60% of the radioactivity is retained by the filters. However, without membranes, not more than 10% of the lectin is retained under these conditions. The maximal contamination of the retained material by non-specifically adsorbed concanavalin A is therefore ∿ 6%. In the range of lectin levels of interest here (≤ 75 µg/ml) contamination is < 3%. To determine to what extent concanavalin A binding can be inhibited by the presence of α-methyl-D-glucopyranoside we followed the procedure in (7). The microsomal membranes were incubated in 0.001 M HEPES, 0.0002 M $CaCl_2$, 0.0002 M $MnCl_2$, pH 7.5, containing 0.2 M α-methyl-D-glucoside present. To construct binding curves, the amount of concanavalin A bound in presence of 0.2 M "hapten sugar" is then subtracted from the amount bound without "hapten sugar".

For dodecylsulfate polyacrylamide gel electrophoresis the membranes solubilized in 1% dodecylsulfate/40 mM dithiothreitol were separated as in (4-6,8) using 7% acrylamide, cross-linked with 3.75% N,N'-diallyltartardiamide containing 1% sodium dodecylsulfate. The electrophoresis buffer was Tris/acetate/EDTA (0.04 M/0.02 M/ 0.002 M), pH 7.4. Coomassie brilliant blue staining for proteins, reaction with the periodate-Schiff reagent and photometric scanning were as in (8). For the periodate-Schiff reaction we used 6% acrylamide gels, cross-linked with 2.5% N,N'-methylenebisacrylamide. Protein components are numbered and their integrated Coomassie staining quantified as in (4).

Protein was assayed by the ninhydrin method (9) or fluorimetrically (10) using bovine serum albumin as standard. To determine the protein concentration of glutaraldehyde-treated membranes the protein fluorescence in 0.1% dodecylsulfate was compared with the fluorescence of untreated proteins.

RESULTS AND DISCUSSION

Figure 1 shows the variation of concanavalin A-binding to native and glutaraldehyde-treated membranes as a function of lectin concentration (125 µg membrane protein/ml). In both cases binding is complete within 15 min at 25°C. The native membranes exhibit a complex binding curve with inflection points at 10, 75 and 200 µg concanavalin A/ml. The saturation level is at $4.8 \cdot 10^{15}$ molecules of tetrameric lectin per mg membrane protein. The sigmoid character of the curve at < 75 µg lectin/ml suggests positively cooperative lectin binding, probably superimposed upon a saturable, low-affinity process.

An entirely different binding sequence is obtained after membranes have been treated with 0.0064 M glutaraldehyde (Fig. 1). The multiphasic character of the curve in Figure 1 disappears, binding at low lectin levels increases and saturation occurs at $6.0 \cdot 10^{15}$ molecules of tetrameric lectin/mg membrane protein.

Figure 2 represents the data of Figure 1 (low lectin levels) in the form of a double logarithmic Hill plot (11). Here y is the fractional binding-saturation, full-saturation corresponding to the plateau values in Figure 1. The analysis re-

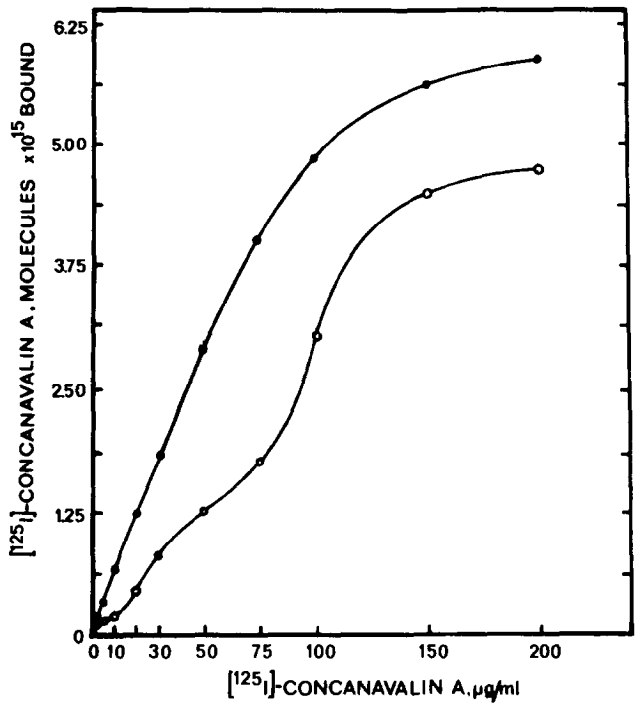

Figure 1. Binding of concanavalin A to native and glutaraldehyde-treated microsomal membranes. The abscissa gives the concentration of concanavalin A, the ordinate the number of molecules of tetrameric [^{125}I]-concanavalin A bound per mg membrane protein: 0——0 native membranes; ●——● glutaraldehyde (0.0064 M)-treated membranes. Each point is the mean of four independent binding experiments assayed in duplicate. Range is ± 12% at concanavalin A levels > 100 µg/ml and < 5% at lower levels.

veals that: (a) concanavalin A-binding to native membranes is positively cooperative, with a Hill coefficient of 1.75 at $y \sim 0.1$; (b) binding to glutaraldehyde-treated membranes is non-cooperative, with a Hill coefficient of 0.87 at $y > 0.1$. The asymmetry of the curves indicates that more than one class of binding site contributes to the values of full saturation in Figure 1.

To clarify the effect of glutaraldehyde-treatment on lectin-binding, we compared normal with treated membranes by dodecylsulfate polyacrylamide gel electrophoresis. The high molecular weight proteins and glycoproteins (Fig. 3), 1-5.1, derive primarily from plasma membrane and bands 6-10 from endoplasmic reticulum (4). Increasing levels of glutaraldehyde (0.0016 M to 0.0256 M) progres-

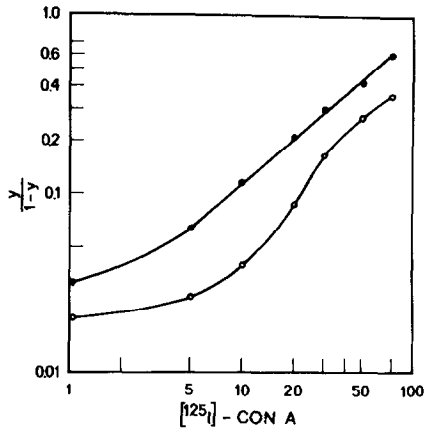

Figure 2. Hill plot of data in Figure 1, showing cooperativity of concanavalin A binding in native membranes (0——0) and lack of cooperativity in membranes treated with 0.0064 M glutaraldehyde (●——●). Concanavalin A concentration in μg/ml.

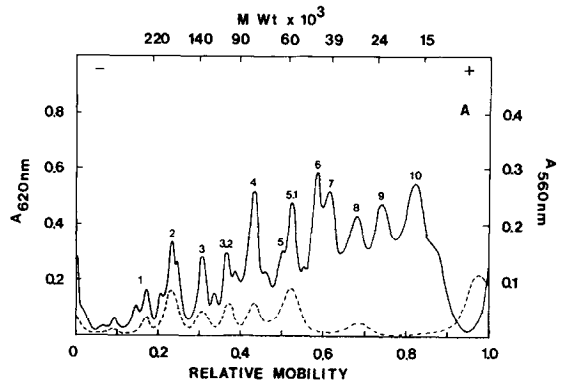

Figure 3. Dodecylsulfate polyacrylamide gel electropherograms of microsomal membranes from rabbit thymocytes. The abscissa gives the relative mobility and the molecular weights, respectively, the ordinate the absorbance at 620 nm for Coomassie blue (——) and at 560 nm for periodate-Schiff staining (– – –). The proteins and glycoproteins are numbered from 1-10 according to decreasing molecular weight.

sively modify the electrophoretic pattern. Component 5.1, the 55,000 D, concanavalin A-reactive glycoprotein (5) is particularly susceptible, even at low glutaraldehyde levels. This is apparent from Figure 4 which shows a sharp decrease, at low concentrations of cross-linker, in $[I_{5.1}/I_6]$, the integrated staining intensity (4) of component 5.1 relative to that of component 6. Indeed with 0.0064 M glutaralde-

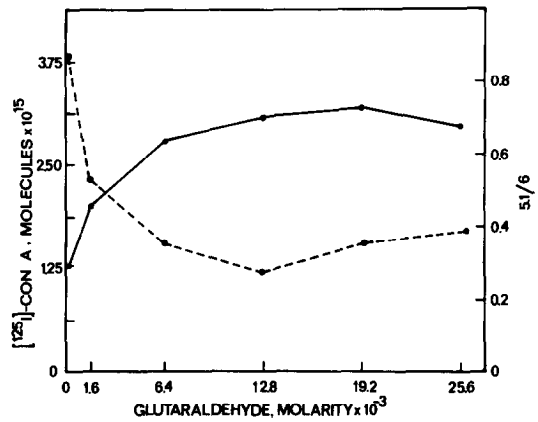

Figure 4. Binding of concanavalin A to microsomal membranes and loss of component 5.1 from the membranes with increasing concentrations of glutaraldehyde. The abscissa gives the glutaraldehyde concentration at which the membranes are treated for 30 min at 25°C, the left ordinate the number of molecules of [^{125}I]-concanavalin A bound per mg protein (●——●). The right ordinate gives the ratio [$I_{5.1}/I_6$] (following Coomassie staining) (O- - -O); it diminishes as 5.1 is cross-linked. The membrane protein concentration was 125 µg/ml. The [^{125}I]-concanavalin A concentration was 50 µg/ml. The results are obtained from three independent experiments, each experiment run in duplicate.

hyde, this protein is reduced by > 60%. We find concomitant, but less marked decreases in components 1 (220,000 D), 2.1-2.3 (∿ 160,000 D), 3 (130,000 D), 3.2 (110,000 D) and 4 (75,000 D) and an increase in staining at the gel top. The selective cross-linking of component 5.1 (Fig. 4) is paralleled by an increase in the amount of concanavalin A bound to the membranes, (c. f. also Figs. 1 and 2).

Concanavalin A-binding to native versus glutaraldehyde-treated membranes provides clues as to the association of the lectin with thymocyte plasma membranes; plasma membrane fragments are the major constituents of microsomal membranes from thymocytes (4) and contain ∿ 80% of 5.1, the concanavalin A-reactive, 55,000 D glycoprotein. The binding curves of native membranes reveal three successive inflections, as also reported for intact lymphocytes (3). Glutaraldehyde-treated membranes show a simpler titration curve with greater binding. All the curves suggest more than one class of binding site.

The Hill plot (11) of binding at low concanavalin A concentrations is diagnostic of positive cooperativity for native membranes. That for treated membranes

reveals non-cooperative, higher-affinity binding at low lectin levels.

Electropherograms of membranes treated with $\leq$ 0.0064 M glutaraldehyde demonstrate selective conversion of the 55,000 D glycoprotein to components of higher molecular weight. This process is 1/2 maximal at $\sim$ 0.0016 M glutaraldehyde and parallels the enhancement of lectin binding by glutaraldehyde treatment (Fig. 4). Also, at the minimal glutaraldehyde concentration required for maximal cross-linking of the 55,000 D glycoprotein, the Hill plot shifts from one characteristic of positively-cooperative binding to one indicating non-cooperative binding.

The cooperative binding in native membranes and its conversion to a high-affinity, non-cooperative process by cross-linking with glutaraldehyde implicates the 55,000 D concanavalin A-reactive glycoprotein: (a) This is the only major membrane protein susceptible to cross-linking at low glutaraldehyde concentrations, suggesting that one or more 55,000 D units are coupled to each other. (b) Earlier studies (5) show that, the purified protein can exist in monomeric, dimeric and possibly tetrameric forms.

Taken together, present and earlier results (4,5) suggest that the 55,000 D glycoprotein is a specific concanavalin A-receptor and that the cooperative binding process derives from polymerization of the receptor protein during binding of a multivalent ligand, concanavalin A. As should be the case, artificial polymerization of the receptor by artificial cross-linking abolishes cooperative binding.

Our work supports the suggestion (12) that concanavalin A can cooperatively modify membrane-enzyme activities through lectin-induced association of enzyme molecules. It is also in accord with the cooperativity model of Changeux et al (13).

ACKNOWLEDGEMENTS

This work was supported by Grant CA-13061 from the U. S. Public Health Service and Contract CB-43922 from the National Cancer Institute. The work was also aided by Award 84759 from American Cancer Society (D.F.H.W.) and by the Max-Planck-Society zur Forderung der Wissenschaften (R. S.-U).

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