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# INVERSE RELATIONSHIP OF PROTEIN CONCENTRATION AND BINDING ACTIVITY OF ALPHA-GALACTOSIDE RECEPTORS FROM SUGARCANE MEMBRANES

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## Summary

1. The binding activity of purified α-galactoside receptor proteins from a number of plant species decreases when the protein concentration is increased from 2 ng/ml to 100 μg/ml.

2. The apparent loss of binding activity at high protein concentrations corresponds to the formation of high molecular weight multimers.

3. Raffinose and melibiose cause a ligand-dependent increase in binding activity and a corresponding decrease in the relative abundance of multimers at any given protein concentration.

4. The self-inhibition of binding activity at high protein concentrations arises from a competition between ligand binding by oligomers and self-association of these oligomers into multimeric species which have little or no binding activity.

## Introduction

α-Galactoside receptors have been isolated from membrane fractions of numerous plants and are similar but not identical subunit proteins [1]. These receptors may be involved in membrane transport of sugars [2]. In addition, the receptor from clones of sugarcane susceptible to *Helminthosporium sacchari*, an incitant of eyespot disease, binds a galactosidic toxin produced by this pathogen and triggers subsequent events in membranes which lead to ionic imbalance and eventual lysis of sensitive cells [3]. Purified receptors behave anomalously in that binding activity at higher protein concentrations (100 μg/ml) is much less than activity at lower concentrations (4 μg/ml) [4]. This report summarizes further analysis of this seemingly paradoxical situation.

## Experimental Procedure

Receptor protein was isolated from sugarcane, clone 51NG97, via affinity chromatography on melibiose-Sepharose columns after extraction of membrane fractions with 1.0 M sodium trichloroacetate in 50 mM Tris-HCl, pH 7 [1]. Sizing was performed on Bio-Gel A1.5 (0.9 × 95 cm) and Sephadex G-200 (0.9 × 95 cm) in 10 mM Tris-HCl, pH 7. Protein standards were obtained from Boehringer-Mannheim. Binding activity was measured via equilibrium dialysis using membranes with an exclusion limit of molecular weight 6000 to 8000 [1]. [$^{14}$C]Helminthosporoside and [$^{14}$C]raffinose were prepared as previously reported [1]. [$^{3}$H]Raffinose was synthesized by mixing raffinose (2 mM) and galactose oxidase (Worthington, 61 units) for 4 min in 1 ml of 0.1 M sodium phosphate, pH 7.4, at 25°C. The mixture was then boiled for 1 min and allowed to cool at which time small crystals of $NaB^{3}H_4$ (320 mCi/mmol) were added. After 2 min, 40 μmol $NaBH_4$ was added to ensure complete reduction of aldehydes. The reaction mixture was then injected into a high-pressure liquid chromatograph containing a Waters carbohydrate column. The solvent was acetonitrile/water (78 : 22, v/v), flow rate 0.9 ml/min. Fractions corresponding to the elution time of raffinose (approx. 30 min) were pooled. Purity of the tritiated product was ascertained by subjection to chromatography against raffinose on Whatman 541 filter paper in butanol/acetic acid/water (4 : 1 : 5, v/v) and propan-2-ol/methylethyl ketone/acetic acid/water (10 : 5 : 2 : 4, v/v). Protein concentrations were measured using a differential absorption technique at 215 and 225 nm [5].

## Results

*Receptor binding activity.* The [$^{3}$H]raffinose binding activity at a receptor concentration of 50 ng/ml was 30 times greater than the activity at 1 μg/ml (Fig. 1). This inverse pattern of binding activity versus protein concentration was also observed with [$^{14}$C]helminthosporoside and [$^{14}$C]raffinose in the presence of receptors from varieties of tobacco and mint as well as other clones

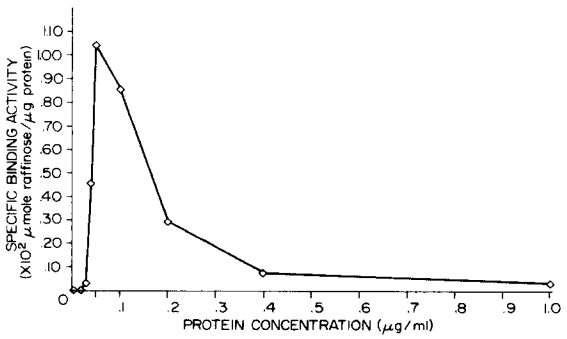


**Fig. 1. Raffinose-binding activity of receptor protein from sugarcane clone 51NG97 as a function of protein concentration. Receptors (2—500 ng) were mixed with [$^{3}$H]raffinose ($8.5 \cdot 10^{-3}$ μmol, 2μCi/μmol) in 0.5 ml of 1 mM Tris-HCl, pH 7.0, and subjected to equilibrium dialysis for 8 h. Counting time was 10 min.**

of sugarcane. Because these receptors are subunit proteins [1], we tested dependence of activity upon protein concentration in the presence of either a nonsubunit protein or subunit receptors from other plants.

When bovine serum albumin, a nonsubunit protein, was used to augment protein concentrations at a constant concentration (50 ng/ml) of the receptor from sugarcane clone 51NG97, binding activity was constant up to total protein concentrations of 1.5 μg/ml. Bovine serum albumin by itself had no α-galactoside binding activity. Conversely, when α-galactoside receptors from two clones of sugarcane (51NG97 and H50-7209) were mixed in equal proportions and monitored for binding activity over a range of protein concentrations, a 30-fold loss in activity occurred as the protein concentration was raised from 50 ng/ml to 1 μg/ml. A similar result was obtained by mixing α-galactoside receptors from mint and clone 51NG97 of sugarcane.

*Physical nature of the receptors.* Purified receptors from sugarcane, mint and tobacco yielded single bands after electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) [1]. However, these preparations gave a polydisperse elution profile after column chromatography on Bio-Gel A1.5 (Fig. 2). This polydispersity could be caused by the presence of contaminating proteins and/or an integral series of molecular weight species produced by self-association of a single type of protein [7]. To distinguish between these two possibilities, fractions corresponding to peak 1 (Fig. 2) were pooled, concentrated, desalted and subjected to SDS-polyacrylamide gel electrophoresis. The fractions corresponding to combined peaks 2, 3 and 4 were treated likewise. Both preparations showed a single band corresponding to a molecular weight of 104 000, identical to that of receptor preparations which had not been chromatographed on Bio-Gel A1.5. Also, at equal protein concentrations (4 μg/ml), the binding activity of each preparation was identical to that of unchromatographed receptors (approx. $8 \cdot 10^{-3}$ μg helminthosporoside/μg protein).

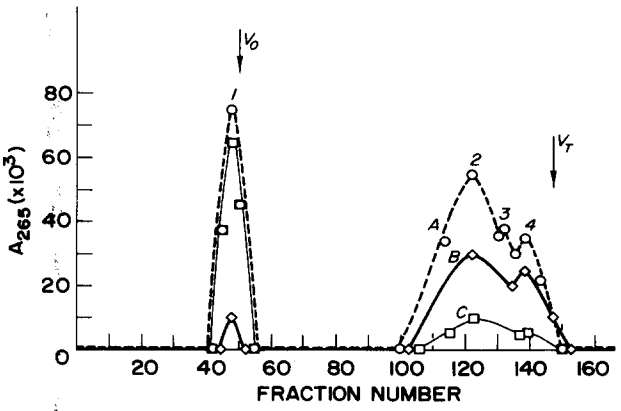


**Fig. 2. Depiction of elution profiles of receptor protein from sugarcane clone 51NG97 (27 μg/0.5 ml) on Bio-Gel A1.5 (95 × 0.9 cm). Eluant: 10 mM Tris-HCl, pH 7.0. Fraction size: 0.4 ml. A (○- - - - -○) represents the initial pass of the receptor preparation. Fractions 100 to 150 were combined, concentrated, and desalted, then repassed on the column. The resultant elution profile is represented by B (◇———◇). Likewise, fractions 42 and 55 were combined and rechromatographed after which the elution profile C (□———□) was obtained. Column effluents were monitored with a Gilson IF-UV Flowmeter equipped with a 265 nm interference filter. Because baselines were uneven, representative points were measured and used to generate the graphs.**

After repassage of isolated protein fractions on Bio-Gel A1.5, peak 1 protein and protein of peaks 2, 3 and 4 yielded a $V_0$ peak as well as the later peaks (Fig. 2). Peaks 1, 2, 3 and 4 on Bio-Gel A1.5 corresponded, respectively, to $M_r$ values of greater than $1.5 \cdot 10^6$, 133 000, 67 000 and less than 45 000.

The relative abundance of oligomers, those proteins represented by peaks 2, 3 and 4 on Bio-Gel A1.5, was inversely related to protein concentration and ionic strength (Table II). Likewise, addition of 2 mM melibiose, an α-galactoside, caused a 5-fold increase in the relative abundance of oligomers in solution (Table I).

To resolve further the composition of peak 4 from Bio-Gel A1.5 (Fig. 2) and to ascertain which of the oligomers increased after treatment with melibiose, purified receptors were chromatographed on Sephadex G-200 in low ionic strength buffer (2 mM Tris-HCl) with or without 1 mM melibiose. In both instances, peaks were observed at $V_0$ and elution volumes corresponding to $M_r$ values of 140 000, 65 000 and 16 000 (Fig. 3). Melibiose, however, caused a 2-fold increase in the magnitude of the peak for the protein of $M_r$ 65 000 (Fig. 3).

*Effects of ligands and ionic strength on binding.* In addition to increasing the relative abundance of oligomers in solution, ligands had a stimulatory effect on binding activity. At any particular concentration of receptor, increasing the concentration of raffinose in the assay solution caused an increase in specific binding activity (Fig. 4). The stimulatory effect was greater at 1 μg/ml of receptor than at 50 ng/ml where oligomers are in greater abundance (Fig. 4, Table II).

Finally, binding activity was studied as a function of ionic strength and protein concentration in relation to the effects of these parameters on the physical form of receptors in solution (Table II). Helminthosporoside-binding activity at a low protein concentration (0.1 μg/ml) was obliterated at high ionic strengths (Table II). This observation is related to a decrease in relative abundance of oligomers at high ionic strengths (Table I). At a higher protein concentration

TABLE I

RELATIVE ABUNDANCE OF MULTIMERS AND OLIGOMERS AT DIFFERENT CONCENTRATIONS OF PROTEIN, MELIBIOSE AND BUFFER

Receptor protein at a given concentration was equilibrated in the appropriate buffer for 2 h at 25°C, then chromatographed over Bio-Gel A1.5 (0.9 × 95 cm) in the presence of that buffer. The elution profile was monitored at 220 nm with a Gilson Holochrome Spectrophotometer equipped with 40-μl flow cells. The multimer:oligomer ratio was calculated by triangulating the areas under the peaks.

| Protein (μg/ml) | Tris-HCl, pH 7.0 (mM) | Multimer:oligomer |
|---|---|---|
| 35.0 | 10 | 5.7 |
| 5.4 | 10 | 0.3 |
| 1.7 | 10 | 0.1 |
| 1.0 | 10 | 0.08 |
| 2.0 | 25 | 9.0 |
| 2.0 | 10 | 0.3 |
| 2.0 | 1 | 0.05 |
| 2.3 | 10 | 0.3 |
| 2.3 | 10 + 1 mM melibiose | 0.06 |

TABLE II

HELMINTHOSPOROSIDE-BINDING ACTIVITY OF RECEPTOR PROTEIN FROM SUGARCANE CLONE 51NG97 AS A FUNCTION OF IONIC STRENGTH

Toxin (5 μg, $1.0 \cdot 10^{-5}$ Ci/μg) and receptor (0.05 or 1.7 μg) were mixed in 0.5 ml of different concentrations of Tris-HCl, pH 7.0, then equilibrium dialysis was performed as noted in Materials and Methods. After 8 h, contents of each half-cell were added to 5 ml Aquasol and counted for 100 min.

| Buffer concentration (mM) | Specific binding activity (μg toxin/μg protein) | |
|---|---|---|
| | Protein concentration | |
| | 0.10 μg/ml | 3.4 μg/ml |
| 1 | 15.5 | 1.0 |
| 5 | 11.3 | 0.7 |
| 10 | 5.1 | 0.5 |
| 25 | 2.6 | 0.2 |
| 50 | 0.2 | 0.2 |
| 100 | 0.2 | 0.2 |

(3.4 μg/ml) where multimers would be in greater abundance (Table I), increasing the ionic strength of the assay buffer caused a decrease in binding activity similar to that at lower receptor concentration but of lesser magnitude (Table II).

## Discussion

Membrane proteins, when substantially purified of normal membrane constituents, often behave as amphipathic molecules in aqueous buffers [6,7], i.e., they obtain an equilibrium between monomeric, oligomeric and micellar forms in response in hydrophobic forces. α-Galactoside receptors from plant mem-

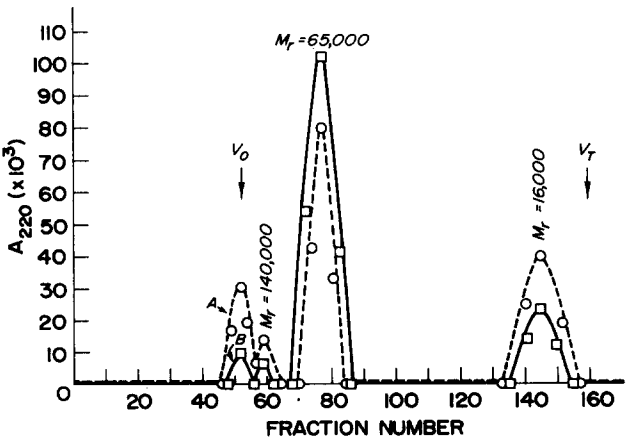


**Fig. 3. Depiction of elution profile on Sephadex G-200 (0.9 × 95 cm) of receptor protein from sugarcane clone 51NG97. Fraction size 0.4 ml. Profile A was obtained after incubating 1 μg protein 0.5 ml of 1 mM Tris-HCl, pH 7.0, for 1 h at 25°C. Profile B was obtained after incubating 1 μg receptor in 0.5 ml of 1 mM Tris-HCl, pH 7.0, containing 1 mM melibiose for 1 h at 25°C prior to chromatography. The eluant was 1 mM Tris-HCl, pH 7.0. Column effluent was monitored with a Gilson Holochrome set at 220 nm. The graphs were generated as noted in Fig. 2.**

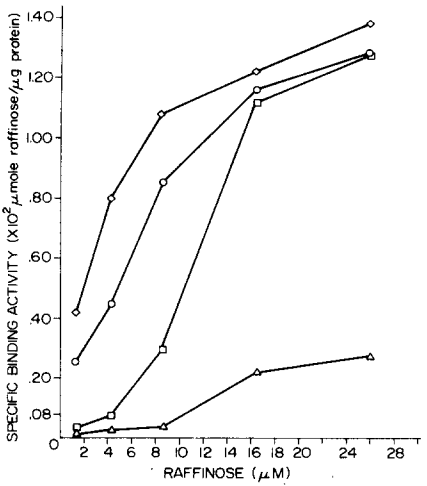


**Fig. 4. Raffinose-binding activity of receptor protein from sugarcane clone 51NG97 as a function of ligand and protein concentrations. [$^3$H]Raffinose (1.4—26.0 μmol) was mixed with purified receptor (25 ng to 1.0 μg) in 0.5 ml of 1 mM Tris-HCl, pH 7.0, and subjected to equilibrium dialysis. Counting time was 10 min. Protein concentrations: ◇———◇, 0.05 μg/ml; ○———○, 0.1 μg/ml; □———□, 0.2 μg/ml; △———△, 1.0 μg/ml.**

branes apparently are behaving as amphipaths. They exhibit a polydisperse pattern during column chromatography (Figs. 2 and 3). Because of the electrophoretic and kinetic identity of the different fractions from the A1.5 column, this polydispersity appears to represent dynamic populations of molecular weight species of the same protein rather than the presence of contaminating proteins. The estimates of molecular weights (Figs. 2 and 3) are indicative of a molecular weight series including monomer, tetramer, octomer and multimer. The relative abundance of the different molecular weight species is very sensitive to protein concentration and ionic strength. At high protein concentrations or high ionic strengths, the multimeric or micellar forms predominate (Table II).

A useful feature of α-galactoside receptors is that they retain activity after purification. Thus, ligand binding reflects the functional state of receptors which can be correlated to the physical state in solution. Under conditions in which multimers predominate (high protein concentration and/or high ionic strength), binding activity is inhibited (Fig. 1, Table II). The inverse relationships between protein concentration and binding activity is not a simple one. High concentrations of a nonsubunit, hydrophobic protein such as bovine serum albumin do not affect the binding activity of receptors from sugarcane. However, augmenting the concentration of sugarcane receptors by receptors of membrane origin from other clones of sugarcane or mint causes a marked decrease in binding activity, presumably because these receptors are comprised of amphipathic subunits which can participate in the formation of multimers.

Based on these results an abbreviated model of the behavior of receptors in solution can be illustrated as follows:

Multimer ↔ Oligomers ↔ Monomers
↕
Oligomer-Ligand

Thus, elevated protein concentrations would favor the formation of multimers because of the tendency toward micelles of high concentrations of amphipaths [8]. Likewise, high ionic strengths strongly favor hydrophobic bonds between proteins and increase the abundance of multimers. The effects of either high protein concentration or high ionic strength are reduced in the presence of ligand. Melibiose causes a substantial increase in the relative abundance of oligomers, in particular the postulated tetramer (Table I, Fig. 3). At any particular concentration of receptor, increasing the concentration of raffinose causes an increase in specific binding activity (Fig. 4). Below a critical protein concentration (approx. 50 ng/ml), binding activity decreases (Fig. 1). This could represent a situation in which the concentration of monomers in solution is insufficient to associate into oligomers, the active form of these receptors.

The 'oligomers' in the above illustration are most likely tetrameric and octomeric forms of the receptors (Fig. 3). The evidence from G-200 chromatography indicates that the active binding species is the tetramer. Furthermore, when receptors are isolated using a detergent instead of a chaotrope, the tetramer is the only species observed and it had $K_d$ values for raffinose and helminthosporoside nearly the same as that of trichloroacetate-purified receptors [1,9]. Evaluation of the binding data using procedures outlined by King and Altman [10] predicts that the most important parameters which determine the extent of binding are the rate of dissociation of the multimer and the rates of association of ligand with tetrameric and dimeric species. The lack of a dimeric species during column chromatography in the presence of melibiose (Fig. 3) argues against the involvement of the dimer as a binding species. Unfortunately, at present, the high binding ratios at low protein concentrations prevent a more critical analysis of possible models of binding.

It is possible that the receptors are glycoproteins and, at high protein concentrations, the inhibition of ligand binding is caused by receptors binding to galactosyl moieties on other receptors. The importance of this idea is minimized, however, because at low ionic strengths and high protein concentrations, oligomeric forms predominate and ligand binding is substantial (Table II). Also, after treatment of receptors with 2-chloroethanol, only oligomers are detectable and ligand binding is maximal [1].

A more likely explanation for the observed self-inhibition is that these receptors are amphipathic and, in aqueous buffers, hydrophobic forces promote self-association of oligomers into inactive multimers and compete with ligand binding which stabilizes oligomers.

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