Protein–Ligand Interaction. A Calorimetric Study of the Interaction of Oligosaccharides and Hen Ovalbumin Glycopeptides with Concanavalin A†

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ABSTRACT: A calorimetric study is reported concerning the interaction between concanavalin A (Con A) and some oligosaccharides and glycopeptides hydrolyzed from hen ovalbumin. The measurements were carried out in acetate buffer, pH 4.5, where, by far, the prevailing form of the protein is the dimeric one [Kalb, A. J., & Lustig, A. (1968) *Biochim. Biophys. Acta 168*, 366; Dani, M., Manca, F., & Rialdi, G. (1981) *Biochim. Biophys. Acta 667*, 108]. The calorimetric technique allows the direct determination of the binding enthalpy ΔH°_{B}, the evaluation of the apparent association constant K'_{B}, and then the evaluation of the apparent free energy and entropy, $\Delta G^{\circ'}_{B}$ and $\Delta S^{\circ'}_{B}$. Three groups of data have been collected in the present study. The first one concerns the interaction between concanavalin A and some mono- and disaccharides [methyl α-glucopyranoside (αMGlup), methyl α-mannopyranoside (αMManp), D-maltose, D-trehalose, and D-cellobiose]. The analysis of the data indicates that in these cases there are small favorable entropic and enthalpic contributions to the affinity. The stoichiometry of the reaction is 2 mol of ligand/mol of Con A dimer, the sites resulting being equivalent and noninteracting. Melezitose, the only trisaccharide studied, shows a different behavior: its affinity for Con A is higher as compared to the other oligosaccharides containing α-glucosyl residues and closer to that of methyl α-mannopyranoside. However, the stoichiometry is different, namely, 1 mol of ligand/dimer of Con A. The third group of ligands studied is formed by three derivatives of some branched mannans. The values of the corresponding K'_{B} and the other thermodynamic parameters indicate the formation of strong complexes, with a stoichiometry of 1 mol of ligand/dimer of Con A. The analysis of the thermodynamic parameters obtained allows formulation of a new hypothesis on the mechanism of the association process of glycoconjugates to this lectin; namely, the surface of Con A can adapt to a "polyfunctional" branched saccharide or glycoconjugate having at least three sugar rings with two terminal α-linked manno- or glucopyranoside rings. In the absence of this structure of the ligand, only the part of the active site operates, which can bind α-manno- and α-glucopyranosyl derivatives. The main basis of our hypothesis, which excludes the presence of an extended site, is based on the anticooperative effect promoted on the site of the second subunit of Con A dimer by the adsorption of the ligand on the site of the first subunit.

Biological recognition and adhesion processes often involve the formation of saccharide–protein complexes. To understand the selectivity and origin of the association energy, it is important to know the nature of the forces controlling the saccharide–protein interaction. One of the best characterized carbohydrate-mediated recognitions of the cell surface receptor is the association between lectins and the carbohydrate moiety of the glycolipids and glycoproteins present on the cell membrane. Some simple specific sugars can inhibit this interaction, because they can compete for the combination sites on the lectin.

Among the lectins, concanavalin A (Con A), isolated from jack beans, is one of the best known [cf. Lis and Sharon (1973)]. It has been shown to exist in solution at pH 5.8 or lower as a dimer and at pH 7 or higher as a tetramer [cf. Kalb and Lustig (1968)]. Structural determinations indicate a molecular weight of 25 500 for each subunit [cf. Edmundson et al. (1971) and Wang et al. (1971)]. It has been crystallized, and the sequence of each subunit [cf. Edelman et al. (1972) and Hardman et al. (1972)] and the three-dimensional structures of the monomer and tetramer were solved by X-ray crystallographic studies at high resolution [cf. Becker et al. (1975), Cunningham et al. (1975), Reeke et al. (1975), and Wang et al. (1975)].

The four subunits have essentially identical sequences and very similar, highly coiled and tilted conformations rich in β-sheets. Each of them has an active site for the binding of oligosaccharides and glycoconjugates. The probable binding area has been hypothesized to be on the surface of each polypeptide chain not far from Ca^{2+} and Mn^{2+} sites [cf. Hardman et al. (1979)]. The topography of the active site in solution has been studied through reaction with several monosaccharides that were tested also as inhibitors of the interaction between Con A and some glycoproteins [cf. Goldstein et al. (1975), Yariv et al. (1968), So and Goldstein (1968), Portez and Goldstein (1970), and Dani et al. (1981)]. Very recently calorimetric [cf. Munske et al. (1984)] and turbidimetric studies [cf. Colonna et al. (1985)] on complex oligosaccharides have been proposed that can be compared with preceding studies on glycopeptides from immunoglobulins [cf. Kornfeld and Ferris (1975) and Dani et al. (1982)] or other sources (Young & Leon, 1974; Ohyama et al., 1985).

Goldstein and co-workers outlined that oligosaccharides α(1→3), α(1→4), and α(1→6) linked, containing a nonre-

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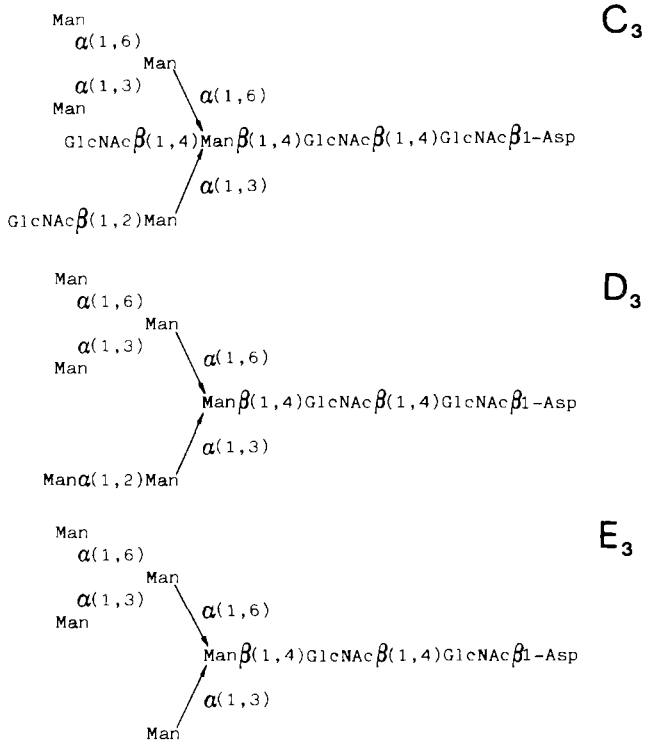


FIGURE 1: Structures of hen ovalbumin glycopeptides used in this study.

ducing terminal mannose or glucose ring, have an affinity comparable to that of corresponding monosaccharides [cf. Goldstein et al. (1965, 1973)]. That leads to the conclusion that there are no extended binding sites for this kind of saccharide. On the other hand, the (1→2)-enchained mannans [cf. So and Goldstein (1968), Goldstein et al. (1974), and Brewer and Brown (1979)] as well as branched glycopeptides [cf. Young and Leon (1974), Kornfeld and Ferris (1975), and Ohyama et al. (1985)] show an enhanced affinity at increasing molecular weight. That leads to two hypotheses: the existence of an extended binding site on the Con A surface [useful for (1→2)-mannans and -glucans] or of a statistical effect due to the number of nonreducing terminal residues of manno- and glucopyranosides presented by the interacting oligosaccharide or glycopeptide [cf. Brewer and Brown (1979)].

In an attempt to clarify these contrasting aspects, we extended the calorimetric studies by examining the binding behavior of a series of mono-, di-, and trisaccharides and some glycopeptides, hydrolyzed from hen egg ovalbumin [cf. Atkinson et al. (1981)]. The structures of the glycopeptides studied are reported in Figure 1. Calorimetry is a powerful tool for investigating these association processes; in fact, it permits not only detection of the thermal effects associated with the binding reactions but also evaluation of the binding constants and the stoichiometry of the reaction. In our opinion the data reported in this paper, particularly the analysis of the stoichiometry and of the enthalpic and entropic contributions to ΔG°_{B}, outline a new interesting feature of the binding of the glycoconjugates to Con A. The interaction with the simple trisaccharide melezitose and with branched glycopeptides can be explained by admitting a capability of the surface of Con A (surrounding the mannose binding site) to adapt to the structure of these "polyfunctional" ligands.

Materials and Methods

Preparation of Con A. Concanavalin A (Sigma product) was dissolved in 0.1 M HCl, dialyzed once for 3 h against 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7, 3 times against twice distilled water, and once in 0.1 M HCl to dissolve the precipitate, and then dialyzed in 10^{-3} M acetate buffer, pH 4.5, containing 10 M^{-3} $CaCl_2$ and 10 M^{-3} $MnCl_2$. These conditions assure that the aggregation state of the protein is populated quite exclusively by the dimers. That excludes changes in reciprocal relations between the two subunits during the mixing processes, due to variation of the quaternary structure upon the dilution. The molecular weight of the metal-containing monomer was assumed to be 27000. On this basis the concentration was determined by UV absorbance at 280 nm, assuming $E^{\%}_{1cm} = 11.4$ [cf. Brewer et al. (1973) and Doyle et al. (1975)].

Saccharides. All the mono-, di-, and trisaccharides employed were crystalline Sigma products, and the concentrations were determined by weighing the dry product.

Glycopeptides. The glycopeptides employed were obtained from hen ovalbumin according to the procedure already reported in the literature [cf. Atkinson et al. (1981)].

Microcalorimetry. The thermal effects associated with the binding reactions were detected by a Batch LKB microcalorimeter equipped with a modified microtitrator system, at 25 °C. The heat of dilution of the saccharides and glycopeptides was instrumentally subtracted by adding the ligand to both the measure and reference cells, while the heat of dilution of Con A, independently measured, was found to be negligible in the concentration range explored, thus confirming no variations in the aggregation state.

Calculation of Thermodynamic Parameters. The experimental heat recorded during the mixing of the two reagents is related to the heat of binding ΔH(bin) and to the heats of dilution of the protein and ligand $\Delta H(\text{dil})_P$ and $\Delta H(\text{dil})_L$, respectively:

$$\Delta H(\text{mix}) = -Q(\text{exp}) = \Delta H(\text{bin}) + \Delta H(\text{dil})_P + \Delta H(\text{dil})_L \quad (1)$$

As said before, the heat of dilution of the protein was negligible and the heat of dilution of the ligand was instrumentally subtracted. The value of ΔH(bin) is proportional to the quantity of the complex formed:

$$\Delta H(\text{bin}) = ([PL]/[P_0])\Delta H^{\circ}_{B} \quad (2)$$

where ΔH°_{B} is the standard enthalpy of complexation for every independent site and [PL] and [P_0] are the concentrations of the complex and protein, respectively. Expressing ΔH(bin) as a function of the concentration of free ligand [L], of the saturation enthalpy ΔH(max), and of the apparent binding constant K'_B, we obtain

$$\Delta H(\text{bin}) = K'_B[L]\Delta H(\text{max})/(1 + K'_B[L]) \quad (3)$$

or, in linear form

$$1/\Delta H(\text{bin}) = 1/\Delta H(\text{max}) + 1/\Delta H(\text{max})[L]K'_B \quad (4)$$

For each value of ΔH(bin), the concentration of the free ligand is given by

$$[L] = [L_0] - n\Delta H(\text{bin})[P_0]/\Delta H(\text{max}) \quad (5)$$

By imposition of a value to n, the number of sites, ΔH(bin), and K'_B can be determined through an iterative least-squares method. The linearity of $1/\Delta H$(bin) vs. 1/[L] proves the reliability of the value assigned to n.

Results

In Figure 2 the values of ΔH(bin), expressed in kilojoules per mole of Con A dimer, are reported as a function of the ratio between the total concentration of the oligosaccharides

Table I: Thermodynamic Parameters per Site and Number of Binding Sites, n, per Con A Dimer for Concanavalin A–Oligosaccharide or –Glycopeptide Systems at 25 °C, in 10^{-3} M Acetate Buffer, pH 4.5

system	n	K'_B[a,b]	$-\Delta H^\circ_B$[b,c]	$-\Delta G^{\circ\prime}_B$[c,d]	$T\Delta S^{\circ\prime}_B$[c,e]
Con A–αMGluP	2	$(2.5 \pm 0.4) \times 10^3$	18 ± 1	19.4 ± 0.4	1.4 ± 1.4
Con A–αMManP	2	$(2.8 \pm 0.6) \times 10^4$	23.8 ± 0.5	25.4 ± 0.6	1.6 ± 1.1
Con A–maltose	2	$(3.5 \pm 0.6) \times 10^3$	14 ± 1	20.2 ± 0.4	6.2 ± 1.4
Con A–cellobiose			0		
Con A–trehalose	2	$(7.7 \pm 1.3) \times 10^3$	12.2 ± 0.6	22.2 ± 0.5	10.0 ± 1.1
Con A–melezitose	1	$(1.0 \pm 0.3) \times 10^4$	31 ± 2	22.8 ± 0.8	−8.2 ± 2.8
Con A–C_3	1	$(7.3 \pm 3.6) \times 10^4$	46 ± 2	28 ± 1	−18 ± 3
Con A–D_3	1	$(1.7 \pm 0.7) \times 10^5$	61 ± 4	30 ± 1	−31 ± 5
Con A–E_3	1	$(3.1 \pm 1.7) \times 10^6$	79.0 ± 0.5	37 ± 2	−42.0 ± 2.5

[a] Units: liters per mole. [b] Listed errors represent the standard deviations as obtained by fitting the data to eq 4. [c] Units: kilojoules per mole. [d] Listed errors are half the range of $\Delta G^{\circ\prime}_B$ calculated from the upper and lower error in K'_B. [e] Listed errors are the sum of the errors on $\Delta G^{\circ\prime}_B$ and ΔH°_B.

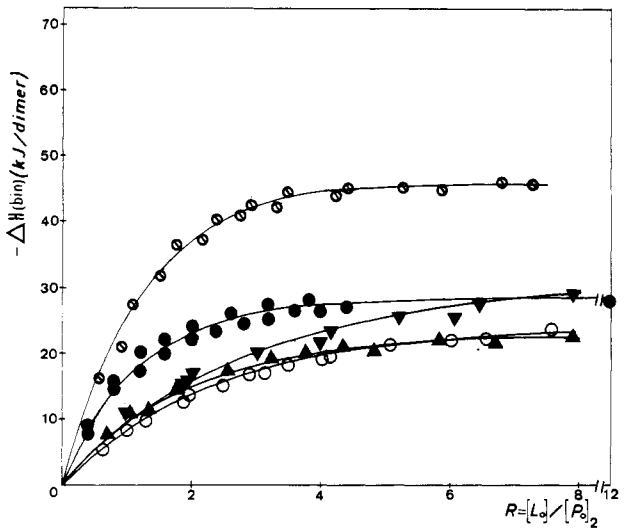


FIGURE 2: Calorimetric titration of Con A with methyl α-glucopyranoside (▼), methyl α-mannopyranoside (⊘), D-maltose (○), D-trehalose (▲), and melezitose (●) at 25 °C, acetate buffer 10^{-3} M, pH 4.5. For each system the concentration of Con A dimer was kept constant, ranging from 2.59×10^{-4} to 2.42×10^{-4} M.

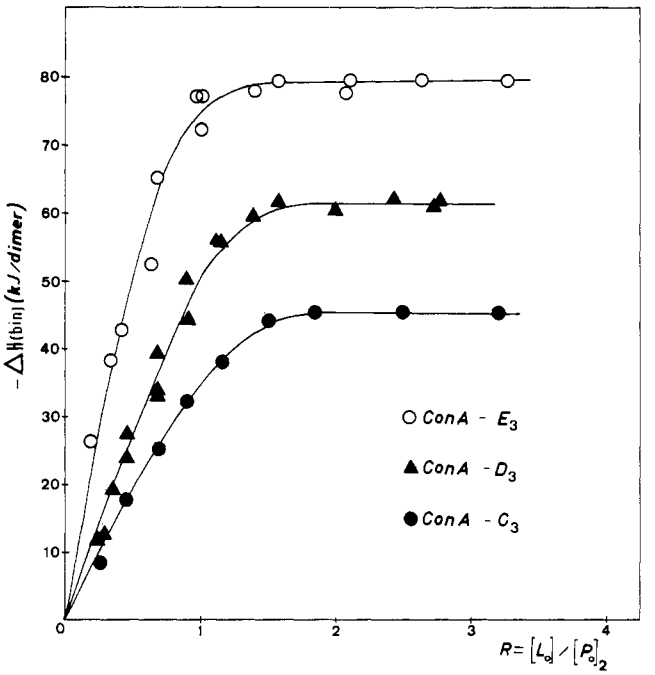


FIGURE 3: Calorimetric titration of Con A with glycopeptides E_3 (○), D_3 (▲), and C_3 (●). Experimental conditions as in Figure 2.

employed $[L_0]$ and that of Con A dimer, $[P_0]_2$. The curves drawn through the points were constructed from the ΔH(bin) values, calculated from eq 3, by using the values for K'_B and ΔH(max) obtained from the iterative least squares, once the number of binding sites per Con A dimer was imposed. In Table I the number of sites, the ΔH°_B per site, and the K'_B values are reported for the monosaccharide derivatives αMGluP and αMManP for the disaccharides maltose, cellobiose, and trehalose and for the trisaccharide melezitose. The free energy $\Delta G^{\circ\prime}_B$ and the entropy $T\Delta S^{\circ\prime}_B$ per site are also reported.

In Figure 3 the ΔH(bin) values are reported relative to the three glycopeptides employed. The different trend of these curves is to be noted, as respect to those obtained for the oligosaccharides. After a steep raise, the curves reach an invariant value, thus indicating the formation of strong complexes. In these cases, the ΔH(max) values can be obtained directly from the plot and a first indication of the stoichiometry of the reaction is obtained from the intersection of the linear parts of the curves. As can be seen, this intersection falls around a ratio equal to unity, thus indicating that only 1 mol of glycopeptide binds per mole of Con A dimer. Moreover, with $n = 2$ per Con A dimer, the iterative least-squares procedure did not give significant results, while the experimental data were well fitted with $n = 1$.

In Table I, the thermodynamic parameters relative to the glycopeptides named C_3, D_3, and E_3 [cf. Atkinson et al. (1981)] are also reported. As can be seen, the three glycopeptides differentiate distinctly one from the other for the binding parameters. Both the enthalpies and entropies of binding are negative, and their absolute values decrease along the series $E_3 > D_3 > C_3$. Glycopeptide E_3 is the one characterized by the highest ΔH°_B and K'_B, while glycopeptide C_3 binds with the smallest ΔH°_B and K'_B.

Discussion

The thermodynamic parameters reported in Table I indicate αMManP as the best inhibitor of Con A among the oligosaccharides studied. The value of the binding constant is the highest and in agreement with other values reported in the literature, obtained from calorimetry [cf. Dani et al. (1981) and Munske et al. (1984)] and other techniques (So & Goldstein, 1968). For the disaccharides maltose and trehalose, the thermal effects and the binding constants are close to those obtained for the interaction with αMGluP, and this is an indication that, in these cases, the association occurs through only one glucopyranosyl ring. Cellobiose, the β(1→4)-isomer of maltose, does not react with Con A, thus repeating the behavior of other β-isomers. Studies on the mechanism of binding of mono- and oligosaccharides to Con A by proton magnetic relaxation dispersion suggested that Con A–carbohydrate interaction can be explained in terms of the existence of a single saccharide binding site. The greater affinity of melezitose, and other oligosaccharides containing an (1→2)-mannose unit, was hypothesized to be due to an increase in the probability of binding associated with the presence of

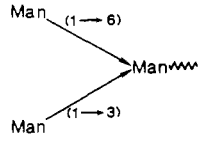
more than one residue acting as a ligand in the oligosaccharide chain (Brewer & Brown, 1979). However, the possibility of an extended binding site on the protein was not completely excluded by the authors.

All the oligosaccharides used for this study, with the exception of αMManP, contain nonreducing glucopyranosyl residues, and that could explain the close similarity of the ΔH°_{B} and of the binding constants. According to Brewer and Brown, the higher binding constant of trehalose, as respect to MGluP and maltose, could be due to the presence of two glucopyranosyl rings having free C-3, C-4, and C-6 hydroxyl groups, which have been found to be essential for the interaction with Con A (Goldstein et al., 1965). Maltose, instead, has only one ring possessing these specificities. Moreover, the presence or the absence of possible intramolecular H bonds can contribute to differentiate the maltose isomers and can affect the absolute values of their binding parameters. The similar enthalpies for αMGluP, trehalose, and maltose strengthen the hypothesis of a probabilistic effect contributing, via entropy, to the higher affinity of trehalose.

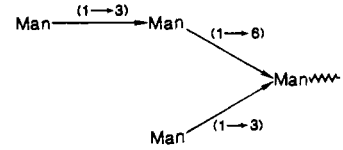
For melezitose, the only trisaccharide studied, a jump is noted in the ΔH°_{B} value, the entropy becoming at the same time negative; that indicates a change in the mechanism of association. The higher value of ΔH°_{B} could be an indication that, in some way, more than one ring reacts with the binding site. However, the most remarkable feature is the apparent inactivation of the site on the second subunit; in fact, the stoichiometry is 1 mol of ligand/mol of Con A dimer.

On the basis of these data, especially of the ΔH°_{B} values for the disaccharides, we think that the hypothesis of an extended, lysozyme-like, binding site can be excluded. Rather, it can be hypothesized that, on the surface of each subunit, only a single saccharide binding site exists. However, in the presence of branched glycoconjugates, a modified form of the binding site can become available. The preexisting site and the newly built part should be at a distance such that a trisaccharide, such as melezitose, is the first molecule that has the minimal length to adapt the two terminal glucopyranosyl rings, anchoring the molecule on the modified surface of Con A monomer. On the contrary, a disaccharide, or a α(1→4), α-(1→6), or α(1→3) linear oligosaccharide, can bind only through one ring because the molecule is too short, or rigid, to promote the formation of a new place that could accommodate a second ring. This second subsite on a subunit of Con A dimer should not preexist nor be "activated" by the absorption of a α-glucosyl or α-mannosyl residue on the first subsite. Rather, a concerted adaptation of the surface of the Con A monomer to the trisaccharide conformation can be hypothesized.

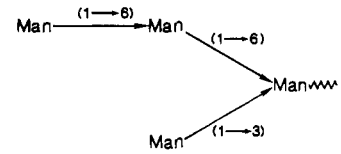
The data for the three glycopeptides studied confirm, in our opinion, the hypothesis of such an interaction mechanism. The binding constants and enthalpies increase along the series $C_3 < D_3 < E_3$, and the entropies, well beyond the cratic contribution, become increasingly unfavorable to the interaction. This could be an evidence of the existence of an increasing number of dipole–dipole and H-bond interactions ruling the association process. A rough inspection of molecular models shows that E_3 has two possibilities to bind to Con A. One is through the terminal unit



and the other through



or



D_3 has the first part in common with E_3, but the second part is one mannose longer, and that is enough to make lower the binding enthalpy and weaker the association constant. C_3 has only the first moiety free of substitution, as for the other two glycopeptides. Therefore, the enhanced binding of E_3 with respect to D_3 is due only to the length of the second unit cited before; namely, a chain formed by four mannose rings seems to have the optimal length for the binding, while a chain of five mannose rings has to distort to permit the adaptation of the terminal mannoses to the protein surface. These results are really in good agreement with those reported by Kornfeld and Ferris (1975) and with the recent data of Ohyama et al. (1985) on the interaction between Con A and several glycopeptides. Quantitative differences in the K'_{B} values, with respect to those reported in the present paper, probably depend on the different techniques and experimental conditions employed.

The stoichiometry of the reaction between melezitose or the glycopeptides and Con A seems to be 1 mol of glycopeptides/mol of Con A dimer. This is, in our opinion, the main basis for our hypothesis. In fact, this stoichiometry can be explained only by admitting that the binding on a monomer induces a conformational change that varies the interactions between the two protomers. That promotes an anticooperative effect that makes the second protomer unable to bind another molecule of glycopeptide. However, this conformational change, if occurring, cannot be identified calorimetrically. As already pointed out, this interaction mechanism is based on the hypothesis of a greater flexibility of the protein chain as compared to a greater rigidity of the mannan residues of the glycoconjugates and of the same melezitose.

Finally, the presence of more than one branch of three, four, or five rings, suitable for interacting with Con A, makes divalent the glucose or mannose chain, and it is also responsible for the precipitation process which can occur at increasing concentration, ionic strength, etc. In fact, when the precipitation is very extended, a 1:2 stoichiometry of the glycoconjugate to Con A monomer is found (Bhattacharyya et al., 1986).

Registry No. C_3, 74424-56-9; D_3, 39114-02-8; E_3, 38784-68-8; αMGluP, 97-30-3; αMManP, 617-04-9; D-maltose, 69-79-4; D-trehalose, 99-20-7; D-cellobiose, 528-50-7; melezitose, 597-12-6; concanavalin A, 11028-71-0.

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A Novel Progesterone-Induced Messenger RNA in Rabbit and Human Endometria. Cloning and Sequence Analysis of the Complementary DNA†

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ABSTRACT: Complementary DNAs (cDNAs) prepared from messenger RNAs (mRNAs) isolated from endometria of 5 day pregnant rabbits were inserted into the plasmid pBR322. A library of 2400 recombinant plasmid clones was prepared and screened by differential in situ hybridization with cDNAs prepared from mRNAs of rabbits either injected with progesterone or untreated by the hormone. Clones encoding uteroglobin were identified and discarded. Several progesterone-induced and progesterone-repressed clones were identified. One of them corresponded to a relatively frequent mRNA (0.2% of clones in the library) of 2300 nucleotides. The induction of this messenger RNA by progesterone was totally suppressed by the antagonist RU486. This compound displayed a limited agonistic activity when administered alone. A very small increase in mRNA concentration was observed after estradiol administration. The messenger RNA was also found in the liver (where it was constitutively expressed), the ovaries, and the Fallopian tubes of rabbits. A cross-hybridizing messenger RNA was detected in human endometrium during the luteal phase. Sequence analysis showed that the messenger RNA encoded a protein of 370 amino acids with a calculated molecular weight of 40 800. A search in Genbank and National Biomedical Research Foundation data banks showed no identity or marked similarity with previously published DNA or protein sequences.

Physiological and pharmacological studies of progesterone and progestin have, in many cases, used as a model system the important histological changes provoked by these hormones in the rabbit endometrium. These hormonal effects form the basis of the classical tests of Clauberg (Clauberg, 1933) and McGinty (McGinty et al., 1939).

Consequently, it appeared important to try to understand the molecular mechanisms underlying the action of progestins in rabbit endometrium. Initially this was undertaken by analyzing the regulation of uteroglobin synthesis and secretion. Uteroglobin (Beier, 1968) or blastokinin (Krishnan & Daniel, 1967) is the major secretory protein of the progesterone-stimulated endometrium. Its gene has been isolated (Atger et al., 1981; Snead et al., 1981; Suske et al., 1983), and many features of its hormonal regulation have been unraveled

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