Isolation and Order of the Cyanogen Bromide Fragments of Concanavalin A*

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ABSTRACT: Concanavalin A is composed of identical subunits of mol wt 27,000, each of which contains 2 methionyl residues. The three CNBr fragments (F_1, F_2, and F_3) have been isolated from the subunit and characterized. The fragments were ordered by analysis of their NH_2- and COOH-terminal peptides and of methionine-containing peptides obtained from the intact polypeptide chain. Two naturally occurring fragments of concanavalin A (A_1 and A_2) were also reacted with CNBr. Analyses of the cleavage products of these fragments have confirmed the previous assignment of A_1 to the NH_2-terminal portion and of A_2 to the COOH-terminal portion of the concanavalin A molecule.

Concanavalin A (Con A)[1] is a crystallizable protein isolated from the jack bean (Sumner, 1919) that has been used to probe structural changes on the surface of cell membranes (Inbar and Sachs, 1969; Eckhart *et al.*, 1971). Recently, Burger and Noonan (1970) have found that Con A treated with trypsin binds to transformed fibroblasts without agglutinating them and restores their growth pattern to that of normal cells. Con A is also mitogenic for lymphoid cells (Powell and Leon, 1970; Beckert and Sharkey, 1970). The binding properties of Con A are due mainly to its specificity for carbohydrates (Goldstein *et al.*, 1965) but the mechanism of its effects on cells is not known.

Previous structural studies (Olson and Liener, 1967; Wang *et al.*, 1971) have suggested that Con A is composed of identical subunits of molecular weight 27,000. Each polypeptide chain contains 2 methionyl residues and treatment with CNBr (Gross and Witkop, 1962) should therefore yield 3 fragments. These fragments facilitate the determination of the amino acid sequence of Con A. In this communication, we report the isolation and characterization of the three CNBr fragments (F_1, F_2, and F_3) of Con A and four CNBr-cleavage products obtained from the naturally occurring fragments, A_1 and A_2 (Wang *et al.*, 1971). We also provide proof of the order of all of these fragments in the polypeptide chain.

Materials and Methods

The isolation of Con A, the preparation of the intact subunit, and the isolation of the naturally occurring fragments, A_1 and A_2, were carried out as previously described (Wang *et al.*, 1971).

CNBr Cleavage and Isolation of the Fragments. The polypeptide chains were treated with CNBr (Gross and Witkop, 1962) in 70% formic acid as reported by Waxdal *et al.* (1968).

The CNBr fragments from the subunit of Con A were isolated by gel filtration on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) in 20% formic acid. The CNBr fragments of A_1 and A_2 were isolated by gel filtration on Sephadex G-75 (Pharmacia, Uppsala, Sweden) in 1 M propionic acid. In both cases absorbance measurements at 280 nm were used to detect the eluted material.

Tryptic Digestion. The intact subunit and the CNBr fragments were digested with trypsin (L-1-tosylamino-2-phenylethyl chloromethyl ketone treated trypsin, Calbiochem, Los Angeles, Calif.) at pH 7.8 in a pH-Stat or at pH 8.0 in 1% NH_4HCO_3 (substrate concentration, 0.5–2%; trypsin concentration 0.005–0.04%). Digestion was performed at room temperature for 1–18 hr and the solution was then lyophilized.

Peptide Purification. The procedures for peptide purification by gel filtration, ion-exchange chromatography, and high-voltage electrophoresis have been previously described (Cunningham *et al.*, 1968).

Amino Acid Analyses. Samples for amino acid analysis were hydrolyzed in metal-free 6 N HCl at 110° *in vacuo* for 24, 48, or 72 hr. The analyses were performed according to the technique of Spackman *et al.* (1958) as previously described (Edelman *et al.*, 1968). Methionine values were determined by the performic acid oxidation method (Moore, 1963). The reported values for homoserine are the sums of the values for homoserine and homoserine lactone. Tryptophan values were estimated by the spectrophotometric method of Goodwin and Morton (1946).

End-Group Analyses and Amino Acid Sequence Determination. Qualitative identification of NH_2-terminal amino acid residues was performed by the dansyl method of Gray (1967). The dansylamino acids were separated by two-dimensional thin-layer chromatography on polyamide plates (Woods and Wang, 1967; Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970). The COOH-terminal residues of the Con A subunit were released by treatment with carboxypeptidase A (diisopropyl fluorophosphate treated, Worthington, Freehold, N. J.). The COOH-terminal residues of F_3 were determined by sequence analysis of the COOH-terminal tryptic peptide (M. J. Waxdal and G. M. Edelman, 1971, unpublished data). Fragments F_1 and F_2 each contained a single homoserine residue; this residue was assigned to the COOH terminus (Gross and Witkop, 1962). Amino acid sequences were established by the dansyl-Edman procedure (Gray, 1967) as used by Gottlieb *et al.* (1970).

Molecular Weight Determinations. A Spinco Model E ultracentrifuge equipped with interference optics and automatic temperature control was used to determine the molecular weight of the intact polypeptide chain and the CNBr fragments

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[1] The abbreviations used are: Con A, concanavalin A; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Hsr, homoserine; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.

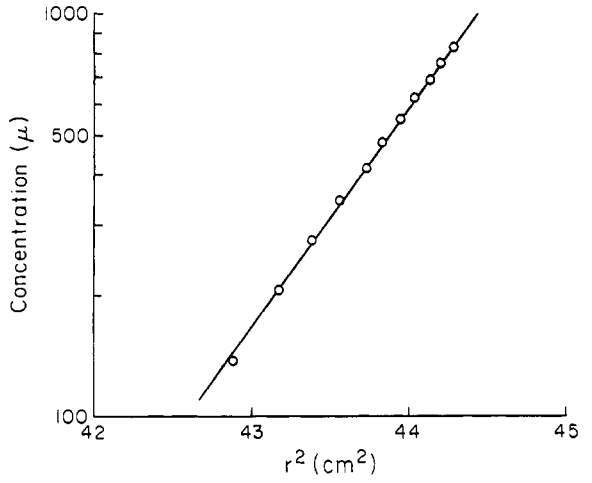

FIGURE 1: Plot of the logarithm of the concentration *vs.* (radius)^2 used for the determination of the molecular weight of the Con A subunit. Protein concentration: 0.4 mg/ml in 0.1 M Tris–6 M guanidine hydrochloride, pH 7.0. Concentrations are expressed in microns of fringe deviation.

of Con A in 0.1 M Tris–6 M guanidine hydrochloride (spectrophotometric grade, Heico Inc., Delaware Water Gap, Pa.), pH 7.0. The high-speed sedimentation equilibrium experiments (Yphantis, 1964) were performed at 36,000 rpm for the intact subunit and 40,000 rpm for the CNBr fragments. In those experiments in which meniscus depletion was not achieved, the data were analyzed by the method of Nazarian (1968). Partial specific volumes were calculated (McMeekin *et al.*, 1949) from amino acid compositions of Con A and its CNBr fragments.

Uncertainty in the molecular weight due to errors in the determination of the concentration distribution in the cell was estimated to be about 5%. Uncertainty in the calculated partial specific volume would introduce further errors, estimated to be about 5%.

Estimation of molecular weights by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the procedures of Weber and Osborn (1969).

Results

The intact polypeptide chain of Con A was separated from the naturally occurring fragments A_1 and A_2 by ion-exchange chromatography on DEAE-cellulose (Wang *et al.*, 1971). The purified material showed a single band on gel electrophoresis in sodium dodecyl sulfate, with a mobility corresponding to mol wt 27,000. More accurate estimates of the molecular weight were made by high-speed equilibrium sedimentation (Yphantis, 1964) in 6 M guanidine-HCl–0.1 M Tris (pH 7.0). As shown in Figure 1, the plot of the logarithm of the protein concentration *vs.* the radius squared had no apparent curvature, suggesting that the preparation is homogeneous with respect to molecular weight. The weight-average molecular weight of the polypeptide chain of Con A was 25,800 (Table I).

TABLE I: Molecular Weight and Terminal Amino Acid Residues of Con A and Its CNBr Fragments.

Sample	Mol Wt	NH_2 Terminus	COOH Terminus
Con A	25,800	Ala	Asn
F_1	4,700	Ala	Hsr
F_2	9,100	Glx	Hsr
F_3	10,700	Phe	Asn

TABLE II: Amino Acid Compositions[a] of Con A and Its CNBr Fragments.

	F_1	F_2	F_3	Sum ($F_1 + F_2 + F_3$)	Subunit
Lys	3.8	6.2	3.3	13.3	12.7
His	1.1	3.3	2.0	6.4	5.9
Arg	0.9	2.5	2.8	6.2	5.9
Trp	1	2	2	5	5
Asp	7.5	12.2	13.5	33.2	33.1
Thr[b]	4.0	7.3	7.1	18.4	17.8
Ser[b]	2.9	10.3	13.5	26.7	29.1
Glu	1.0	4.1	6.1	11.2	12.1
Pro	2.5	2.0	5.1	9.6	11.2
Gly	2.2	4.0	8.5	14.7	14.9
Ala	3.4	6.1	9.0	18.5	18.1
Cys	0	0	0	0	0
Val[c]	3.3	9.2	6.4	18.9	18.2
Hsr[d]	0.7	0.7	0	1.4	0
Met[e]	0	0	0	0	1.6
Ile[c]	4.9	2.9	6.2	14.0	13.6
Leu[c]	0.9	7.5	8.9	17.3	18.5
Tyr	2.0	4.2	2.1	8.3	6.5
Phe	0	2.4	7.2	9.6	11.5
Yield	96%	92%	98%		

[a] Values are expressed as moles/mole, based on molecular weight values shown in Table I. [b] Extrapolated to zero time. [c] 72-hr hydrolysate. [d] Sum of homoserine and homoserine lactone. [e] Determined as methionine sulfone.

The amino acid composition (Table II) of Con A indicated two methionyl residues per subunit of 25,800. Only Ala was found as the NH_2-terminal residue of the intact subunit. After CNBr cleavage, two new NH_2-terminal residues, Glx and Phe appeared, suggesting cleavage into the expected three fragments. The CNBr fragments were separated by gel filtration on Bio-Gel P-60 in 20% formic acid (Figure 2). Amino-terminal analysis of the material in fraction A yielded only Phe (Table I). No homoserine was found in this fraction and it was tentatively concluded that fraction A contained the COOH-terminal fragment. The material in fraction B (Figure 2) arose from incomplete cleavage of one of the methionyl residues and will be discussed below. Fraction C (Figure 2) contained a fragment having Glx at the NH_2 terminus (Table I) and homoserine at the COOH terminus (Tables I, II). These data suggest that this fragment is derived from the middle of the polypeptide chain. The material in fraction D (Figure 2) contained the remaining CNBr fragment. The NH_2 terminus of this fragment was Ala (Table I) and the COOH terminus was homoserine (Tables I, II), indicating that the material in fraction D was the NH_2-terminal CNBr fragment. The material from fractions D, C, and A were named F_1, F_2, and F_3, respec-

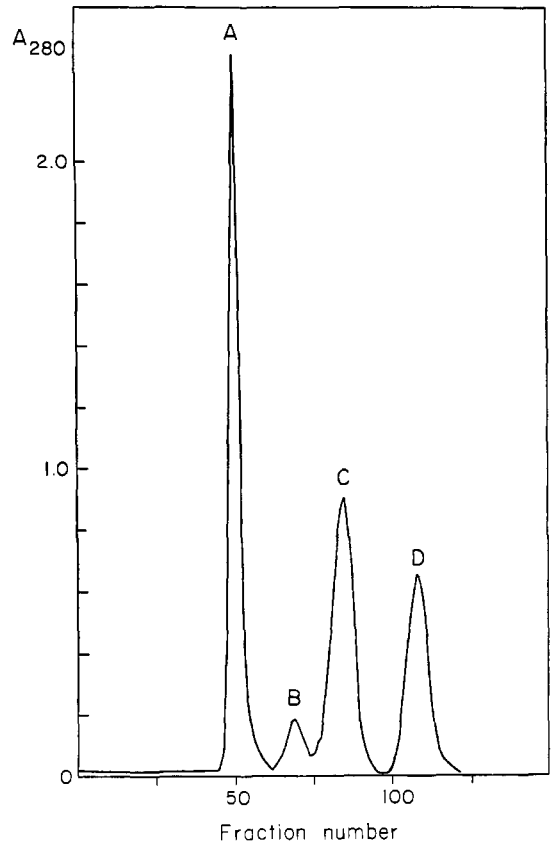

FIGURE 2: Separation of the CNBr fragments of Con A (75 mg) by gel filtration on Bio-Gel P-60 in 20% formic acid. Column dimensions, 2.5 × 80 cm; volume per tube, 2.6 ml.

tively, in the order of their tentative arrangement in the polypeptide chain.

In Table I are shown the molecular weights of F_1, F_2, and F_3, obtained by equilibrium sedimentation experiments in 6 M guanidine-HCl–0.1 M Tris (pH 7.0). The sum of the molecular weights of the fragments was equal to that of the intact polypeptide chain within experimental error. Amino acid compositions (Table II) obtained from 24-, 48-, and 72-hr hydrolysates of each of the CNBr fragments were calculated on the basis of the molecular weights given in Table I. The amino acid compositions are compared (Table II) to that of the intact subunit, which had a slightly higher molecular weight than the sum of the molecular weight of the fragments (Table I).

Material in fraction B (Figure 2) was rechromatographed twice under the same conditions. Amino acid analysis of the purified fraction indicated that it contained equimolar amounts of fragments F_1 and F_2. All of the methionine had been destroyed and 2 moles of homoserine was present. Only Ala was detected as the NH_2-terminal residue. These data are consistent with the conclusion that this fragment results from incomplete cleavage of the peptide bond linking F_1 and F_2 in the intact polypeptide chain and it is therefore designated $F_{1,2}$.

The tentative ordering of the fragments was confirmed by the isolation and sequence analysis of methionine-containing peptides obtained from proteolytic digests of the intact polypeptide chain. The first overlap peptide, 0–1 (Figure 3), was isolated from a 2-hr tryptic digest of Con A. The digest was chromatographed on a column of Sephadex G-50 and the fraction containing small peptides was subjected to high-voltage paper electrophoresis at pH 1.9. Peptide 0–1 was recovered in 60% yield and amino acid analysis showed 0.9 residue of Met (Table III). Using the dansyl-Edman method, the amino acid sequence of peptide 0–1 was shown to overlap the COOH-terminal peptide of F_1 and the NH_2-terminal peptide of F_2 (Figure 3). These data place fragments F_1 and F_2 in the proper order in the polypeptide chain. The remaining overlap peptide, 0–2 (Figure 3), was isolated from an 18-hr tryptic digest of denatured Con A. The digest was subjected to gel filtration on Sephadex G-50 and the methionine-containing fraction was further fractionated by ion-exchange chromatography on DEAE-cellulose and by high-voltage electrophoresis at pH 4.7. Peptide 0–2 was isolated in 11% yield; its amino acid composition is given in Table III. Sequence analysis confirmed the Met–Phe linkage in the polypeptide chain (Figure 3). Although peptide 0–2 was isolated from a tryptic digest, other data (M. J. Waxdal, B. A. Cunningham, G. M. Edelman, 1971, unpublished data) suggest that it was probably the result of a chymotryptic cleavage.

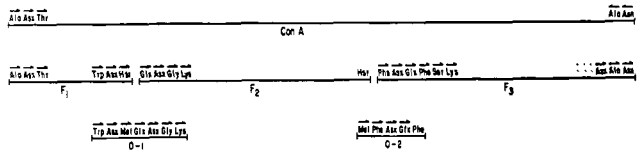

FIGURE 3: Order of the CNBr fragments (F_1, F_2, and F_3) from Con A deduced by comparison of their NH_2- and COOH-terminal amino acid residues with those of Con A and with the amino acid sequence of the methionine-containing overlap peptides, 0–1 and 0–2.

The occurrence of natural fragments of Con A (Wang *et al.*, 1971) provides another opportunity to confirm the ordering of the CNBr fragments. Fragment A_1 has been assigned to the NH_2 terminus and A_2 has been assigned to the COOH terminus of the intact subunit (Wang *et al.*, 1971). Each of these fragments contains a single methionyl residue. Thus, on treatment with CNBr, A_1 should yield F_1 and a large fragment corresponding to the NH_2-terminal portion of F_2. Similarly, A_2 should yield F_3 and a short segment at the COOH terminus of F_2. Fragment A_1 was treated with CNBr and the resulting fragments were separated by gel filtration on Sephadex G-75 in 1 M propionic acid (Figure 4). Amino acid analysis and NH_2-terminal analysis of material in fraction B (Figure 4) indicated

TABLE III: Amino Acid Composition of Peptides Used to Establish the Order of the CNBr Fragments.

	COOH-Terminal Peptide of F_1	NH_2-Terminal Peptide of F_2	0–1	NH_2-Terminal Peptide of F_3	0–2
Lys		0.9	1.0	1.0	
Trp	1		1		
Asp	1.0	1.0	2.1	1.2	1.2
Ser				1.0	
Glu		1.0	1.0	1.0	1.1
Gly		1.1	0.9		
Hsr	0.7				
Met			0.9		0.6
Phe				1.9	1.9
% yield	35	24	60	52	11

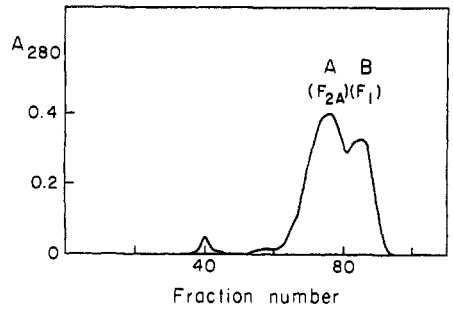

FIGURE 4: Separation of the CNBr fragments of A_1 (27 mg) by gel filtration on Sephadex G-75 in 1 M propionic acid. Column dimensions, 2.5 × 80 cm; volume per tube, 2.0 ml.

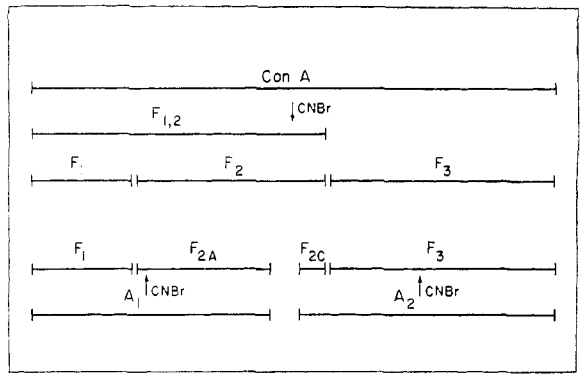

FIGURE 6: Linear model summarizing the relationships of the Con A, CNBr fragments F_1, F_2, $F_{1,2}$, F_{2A}, F_{2C}, and F_3 and the naturally occurring fragments A_1 and A_2.

that it contained only fragment F_1. The material in fraction A (Figure 4) was a new fragment which we have designated F_{2A}. This fragment had a Glx residue at the NH_2 terminus which was the same as that of CNBr fragment F_2. Comparison of the amino acid compositions and peptide maps of tryptic digests of F_2 and F_{2A} indicated that F_{2A} coincides with NH_2-terminal portion of F_2. As in the production of $F_{1,2}$, a small amount of incomplete CNBr cleavage of this methionyl residue may have produced $F_{1,2A}$. No attempt was made to isolate this material.

Treatment of A_2 with CNBr followed by gel filtration on Sephadex G-75 in 1 M propionic acid yielded two fractions. The amino acid composition and NH_2-terminal residues of the material in fraction A (Figure 5) were identical with those of F_3. Fraction B (Figure 5) contained an 11-residue peptide, F_{2C}. This peptide was identical with the COOH-terminal portion of F_2. All of these results are consistent with the previous placement (Wang *et al.*, 1971) of A_1 and A_2 in the intact subunit. Figure 6 summarizes the relationships of the CNBr fragments, F_1, F_2, $F_{1,2}$, F_{2A}, F_{2C}, and F_3 ,and the naturally occurring fragments A_1 and A_2.

Discussion

CNBr cleavage of the subunit of Con A yielded the expected three fragments, and comparison of their NH_2- and COOH-terminal residues to those of the intact subunit suggested that the order of the fragments was F_1–F_2–F_3. This assignment was confirmed by sequence analysis of the two methionine-containing overlap peptides isolated from tryptic digests of the subunit. The amino acid sequence of 0–1 (Figure 3) agrees with the sequences of the COOH-terminal peptide of F_1 and the NH_2-terminal peptide of F_2 (Figure 3) and firmly establishes the order of the first two CNBr fragments. The amino acid sequence of the remaining overlap peptide, 0–2, indicates that the NH_2-terminal residue of F_3 (Phe) is preceded in Con A by a methionyl residue (Figure 3), proving that F_3 arises by the CNBr cleavage of a Met–Phe bond.

Comparison of the results of CNBr cleavage of the naturally occurring fragments of Con A, A_1, and A_2, with earlier data confirmed our previous assignments (Wang *et al.*, 1971). A_1 is composed of F_1 and the NH_2-terminal portion of F_2 (F_{2A}) (Figures 4 and 6). Fragment A_2 is composed of the COOH-terminal 11 residues of F_2 (F_{2C}) and all of F_3 (Figures 5 and 6). It should be noted, however, that the sums of the amino acid compositions of F_{2A} and F_{2C} showed fewer residues than F_2. This was expected because the sum of the amino acid compositions of A_1 and A_2 had previously been found to be less than the total number of residues in the intact subunit of Con A (Wang *et al.*, 1971).

Of the three CNBr fragments, F_3 showed a strong tendency to aggregate, both with itself and with the other CNBr fragments, F_1 and F_2. Gel filtration on Sephadex G-75 in propionic acid of Con A treated with CNBr resulted in incomplete separation of the CNBr fragments. Rechromatography of the fraction emerging in the void volume on the same column showed that this fraction contained aggregates of F_3 as well as appreciable amounts of F_1 and F_2. Consequently, it was necessary to prepare pure F_3 by gel filtration in 20% formic acid. In this solvent, the F_3 aggregate is essentially free of the other CNBr fragments (Figure 2).

The presence of a small amount of $F_{1,2}$, which probably resulted from incomplete cleavage of a methionyl residue, is in agreement with the order of the fragments. Incomplete cleavage of Met–Thr and Met–Ser bonds by CNBr has been previously reported (Waxdal *et al.*, 1968; Cunningham *et al.*, 1968; Schroeder *et al.*, 1969; Rutishauser *et al.*, 1970). In the present case, CNBr failed to cleave a Met–Glx peptide bond quantitatively although amino acid analysis after acid hydrolysis indicated that all of the methionine had been converted to homoserine and homoserine lactone. The yield of $F_{1,2}$ was only about 2%, however, and the incomplete cleavage did not cause any major difficulties in preparing CNBr fragments for further studies of the amino acid sequence.

Fragment F_3 contains 7 of the 11 phenylalanyl residues present in Con A. The predominance of such a hydrophobic residue may account for the strong aggregation of fragment

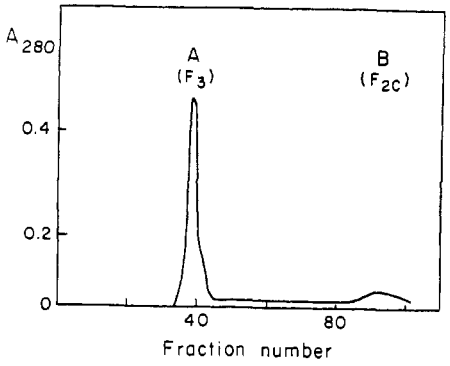

FIGURE 5: Separation of the CNBr fragments of A_2 (20 mg) by gel filtration on Sephadex G-75 in 1 M propionic acid. Column dimensions, 2.5 × 80 cm; volume per tube, 2.0 ml.

F_3 discussed above. Our data also suggest that these residues may play a role in the biological activity of Con A. Circular dichroism studies (Pflumm *et al.*, 1971) indicate changes in the near-uv circular dichroic spectra on binding of α-methyl D-mannoside. This raises the possibility that a conformational change may occur on binding to glycoproteins on the surface of cell membranes. Hydrophobic structures on membranes may be triggered by interaction with this region of the Con A molecule to produce effects such as mitogenesis.

The presence of a nonpolar region near the carbohydrate-binding site of Con A has been implicated by the studies of Poretz and Goldstein (1968) who found that phenyl derivatives of D-glucopyranosides were bound more strongly than the nonderivatized sugar. At present, however, we do not know whether the hydrophobic regions implicated by our structural studies correspond to the nonpolar region near the carbohydrate-binding site. Such an identification must await detailed analysis of the results from amino acid sequence analysis (B. A. Cunningham, M. J. Waxdal, J. L. Wang, and G. M. Edelman, 1971, unpublished data) and X-ray crystallographic studies (J. W. Becker, G. N. Reeke, and G. M. Edelman, 1971, unpublished data) at atomic resolution.

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