Isolation and Proteolytic Cleavage of the Intact Subunit of Concanavalin A†

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ABSTRACT: Incubation of solutions of concanavalin A in 1% NH_4HCO_3 at 37° results in the formation of a precipitate which contains the intact subunit (mol wt 27,000) and the naturally occurring fragments of the molecule. The supernatant from this precipitation is essentially free of fragments and contains only the intact subunit, representing about 50% (by weight) of the total material. The precipitation thus affords a rapid, bulk method for the purification of the intact subunit of concanavalin A. A similar separation can be achieved by affinity chromatography of concanavalin A on columns of Sephadex G-75. Analysis of the circular dichroic spectra of concanavalin A under conditions similar to those used for precipitation of the naturally occurring fragments shows a very slow change in the far-ultraviolet region. The spectrum of the intact subunit undergoes a similar change but at a slower rate. These findings suggest that molecules containing fragmented subunits have physicochemical properties which differ from molecules composed entirely of intact subunits. The availability of methods for the rapid preparation of the intact subunit of concanavalin A has enabled us to study the action of trypsin and chymotrypsin on the intact polypeptide chain. Under carefully controlled conditions, both tryptic and chymotryptic digestion of the intact subunit produce some high molecular weight fragments as well as smaller peptides. The high molecular weight fragments obtained are not the same as the naturally occurring fragments of concanavalin A.

Concanavalin A (Con A),[1] a lectin isolated from jack beans (Sumner, 1919), binds specifically to mono-, oligo-, and polysaccharides with terminal nonreducing α-D-mannopyranosyl, α-D-glucopyranosyl-, or β-D-fructofuranosyl residues (Goldstein *et al.*, 1965a,b; Goldstein and So, 1965). This plant protein agglutinates a variety of animal cells (Sumner and Howell, 1935, 1936) and induces mitosis and blast transformation in lymphocytes (Beckert and Sharkey, 1970; Powell and Leon, 1970). Variations in the agglutinability of normal, trypsinized and transformed cells have suggested that Con A may serve as a molecular probe of architectural changes in the plasma membrane (Inbar and Sachs, 1969a,b). It has also been observed that, after treatment with trypsin, Con A can restore the growth pattern of transformed fibroblasts to that of normal cells (Burger and Noonan, 1970). Recent experiments have shown that Con A inhibits cap formation by immunoglobulin receptors on lymphocyte surfaces (Yahara and Edelman, 1972) as well as phagocytosis (Berlin, 1972) suggesting that the interaction of Con A with membrane receptors can restrict the mobility of certain membrane components.

Studies of the structure of Con A have shown that the molecule is composed of identical subunits of mol wt 27,000 (Olson and Liener, 1967; Wang *et al.*, 1971). Below pH 6, the protein exists as a dimer of these subunits, but above pH 7, tetramers predominate (Kalb and Lustig, 1968). Each subunit contains one Mn^{2+} ion and one Ca^{2+} ion and also has a single carbohydrate binding site (Kalb and Levitzki, 1968).

We have previously reported that commercially available Con A as well as fresh preparations isolated by three different procedures consist of mixtures of the intact subunit and naturally occurring fragments of the subunit, A_1 and A_2 (Wang *et al.*, 1971). Combined chemical and crystallographic evidence suggests that the tertiary structures of the intact subunit and the subunits constructed from the fragments are similar (Becker, 1971). We now report two independent observations which suggest that, even though the two kinds of subunits have similar tertiary structures, subtle differences in their properties may be detected. First, we have noted that upon standing at 37°, solutions of Con A formed a precipitate which contained the intact subunit as well as the fragments while the supernatant contained the intact subunit relatively free of the fragments. Second, gradient elution with dextrose of Con A adsorbed to Sephadex G-75 separated the intact subunit from the Con A mixture, indicating a difference in the interaction of carbohydrate with the two kinds of subunits.

We have taken advantage of the precipitation to design an improved method for the rapid preparation of large amounts of the intact subunit. The availability of large amounts of the intact subunit have in turn enabled us to examine the action of trypsin and chymotrypsin on the intact polypeptide chain of Con A. We were particularly interested in comparing the fragments obtained after proteolysis to the naturally occurring fragments.

Materials and Methods

Preparation of Con A. Con A was prepared by the method of Agrawal and Goldstein (1967). After elution from the column, the protein was dialyzed against 0.01 M Tris (pH 7.4) followed by extensive dialysis against water and lyophilization.

Preparation of the Intact Subunit by Precipitation. Con A was dissolved in 1% NH_4HCO_3 to a final concentration of 10 mg/ml. The solution was incubated at 37° for 12–16 hr and the precipitate was removed by centrifugation. The clear supernatant was dialyzed extensively against water and then lyophilized. Yields were generally about 50% (by weight).

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[1] Abbreviation used is: Con A, concanavalin A.

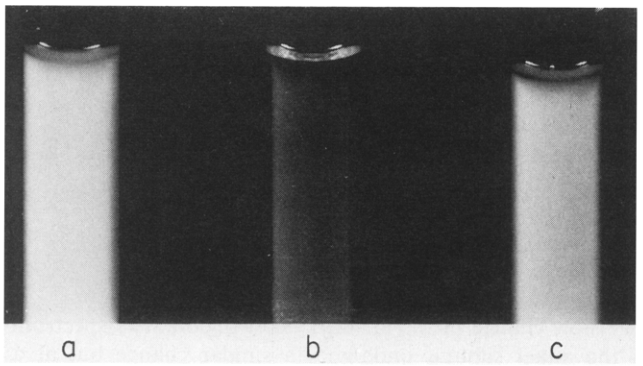

FIGURE 1: Solutions of Con A after 12-hr incubation in 1% NH_4HCO_3 at 37°: (a) 10-mg/ml Con A solution; (b) 10-mg/ml Con A solution, 0.1 M α-methyl D-mannoside; (c) 10-mg/ml Con A solution, 0.1 M D-galactose.

Preparation of the Intact Subunit by Affinity Chromatography. Con A (280 mg) was dissolved in 30 ml of 0.02 M Tris (pH 7.4) and centrifuged. The supernatant was loaded on a column (2.2 × 80 cm) of Sephadex G-75 in the same buffer. After about 150 ml was collected, the column was eluted with a linear gradient of 750 ml of starting buffer and 750 ml of starting buffer which was 0.08 M in dextrose.

Analytical Methods. Procedures used for amino acid analysis according to the technique of Spackman *et al.* (1958) have been described (Edelman *et al.*, 1968). Molecular weight determination by equilibrium sedimentation (Yphantis, 1964) in 0.1 M Tris–6 M guanidine hydrochloride (spectrophotometric grade, Heico Inc., Delaware Water Gap, Pa.) (pH 7.0) were performed at 36,000 rpm as described by Waxdal *et al.* (1971). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborn (1969). To characterize various protein components, 16 identical gels (0.5 × 10 cm) were sliced in 1-mm sections. The corresponding sections from the gels were collected, ground to small pieces, and extracted once with gel electrophoresis buffer and twice with 0.02 M NaCl. The combined extracts were centrifuged and the supernatants were then lyophilized.

Circular Dichroism (CD) and Light-Scattering Measurements. Con A solutions were prepared immediately prior to recording CD spectra. Approximately 6–7 mg of protein was dissolved in 5 ml of fresh 1% NH_4HCO_3. The pH of this buffer was determined to be between 7.9 and 8.1. Solutions were then passed through a Millipore filter (0.45 μ) and their ultraviolet (uv) spectra were recorded on a Cary Model 14 or Model 15 recording spectrophotometer. Concentrations were determined using an extinction coefficient of 1.37 for a 1-mg/ml solution at 280 nm (Yariv *et al.*, 1968). Optical densities varied from 0.7 to 1.6 at 280 nm but were generally between 1.2 and 1.4.

CD spectra were recorded on a Cary Model 60 recording spectropolarimeter with a Cary Model 6001 CD attachment. A 1-mm jacketed cell, thermostatted by a Lauda K-2R circulating methanol bath, was used. The temperature of the circulating liquid was maintained at 37° by means of a Lauda R-20 Electronic Controller. When changes in spectra were followed as a function of time at 37°, the base line was recorded both before and after each experiment to correct for any shifts. The data are expressed in terms of $[\theta]_{MRW}$ using a mean residue weight of 110.

Estimates of the relative rates of precipitation were made by measuring the amount of light scattered perpendicular to the incident beam using a spectrofluorometer described by Rosen and Edelman (1965). Both the excitation and emission monochromators were operated at 300 nm with slit widths of 2 and 6 mm, respectively.

Activity Assays. Carbohydrate binding activity was tested by affinity chromatography on a column of Sephadex G-75 in 0.01 M Tris (pH 7.4). After a column volume of buffer had been collected, a solution of 0.1 M dextrose–0.01 M Tris (pH 7.4) was used to elute the sample fractions which were bound to Sephadex. Hemagglutinating assays were performed by making serial dilutions of the protein fractions in phosphate-buffered saline. A 50-μl suspension of mouse erythrocytes (~3 × 10^7 cells/ml) in phosphate-buffered saline was added to each tube containing 250 μl of the protein fractions. The resulting suspension was mixed by shaking and allowed to settle for 3 hr at room temperature. Hemagglutination titers were determined by eye.

Results

Precipitation. Incubation of solutions of Con A at 37° leads to the formation of a precipitate (see Figure 1). We have observed this type of precipitation over a range of Con A concentrations from 1 to 10 mg per ml. In 1% NH_4HCO_3 (pH 7.9), a precipitate begins to appear within 30–60 min and visibly increases in amount for at least 4 hr. In contrast, comparable solutions maintained at room temperature or at 4° give very little precipitate; the absorbance of the supernatant has been observed to decrease by ~3% after 8 hr at room temperature. At 37° and Con A concentrations of 10 mg/ml, we have also observed precipitation at pH 5.0 (0.124 M NaOAc), pH 6.0 (0.124 M NaOAc), pH 7.0 (0.124 M Tris-HCl), pH 8.0 (0.124 M Tris-HCl), but not at pH 9.0 (0.124 M Tris-HCl). Less precipitate forms at pH 5.0 than at pH 6.0, 7.0, or 8.0 and less forms in 0.124 M Tris-HCl (pH 8.0) than in 1% NH_4HCO_3 (pH 7.9). Increasing salt concentrations (KCl and NH_4HCO_3) increased the rate of visible precipitation as did increasing concentrations of Con A. While this process is apparently enhanced by a number of salts, HCO_3^- and SO_4^{2-} salts are particularly effective. Precipitation also occurs under the conditions described by Burger and Noonan (1970) for treatment of Con A with trypsin (1 mg of Con A dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) for 5 hr at 37°).

Much less precipitate formed in the presence of 0.1 M α-methyl D-mannoside (Figure 1) than in the absence of this sugar or in the presence of 0.1 M galactose. Estimates of the increase in light scattering of solutions of Con A (5 mg/ml) at 37° also indicated that the relative rate was decreased by the presence of α-methyl D-mannoside but not by galactose. Increasing the concentration of α-methyl D-mannoside up to 1 M decreases the rate and extent of precipitation even further.

CD Measurements. In order to gain some insight into the processes responsible for precipitation at 37°, circular dichroic spectra were recorded on solutions of Con A under a variety of conditions. At 37°, the CD spectrum of Con A in 1% NH_4HCO_3 between 250 and 200 nm undergoes several distinct changes as a function of time (Figure 2a). The trough at 222–224 nm is blue shifted to ~215 nm. There is a concomitant increase in negative intensity and bandwidth. The change is gradual over a period of 8–9 hr, and is undergone by both Con A and the isolated intact subunit. In the latter case, however, there is an apparent lag, with relatively little change occurring in the first hour (Figure 2b).

The presence of α-methyl D-mannoside in the solutions slows

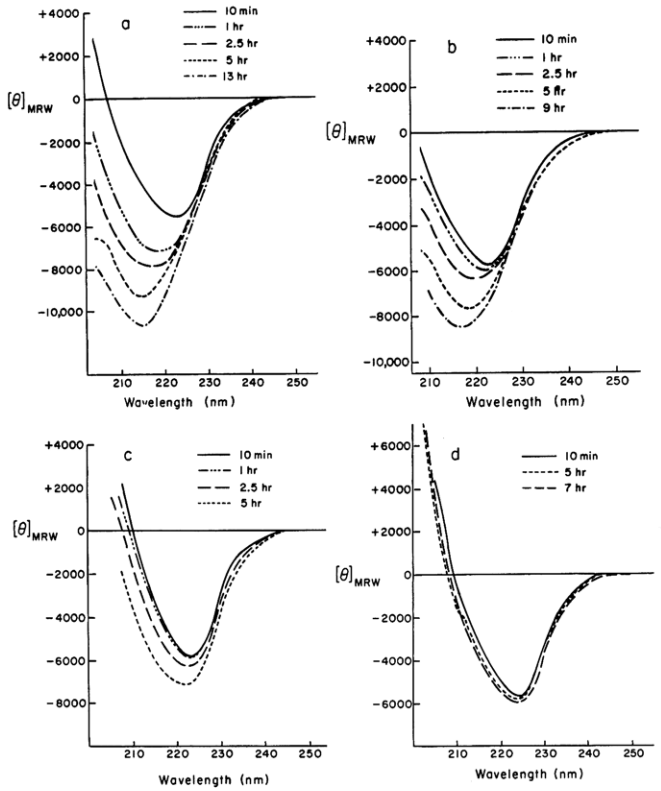

FIGURE 2: Changes in the far-ultraviolet circular dichroic spectra of solutions of Con A after incubation at 37°: (a) Con A in 1% NH_4HCO_3; (b) intact subunit in 1% NH_4HCO_3; (c) Con A in 1% NH_4HCO_3, 0.1 M α-methyl D-mannoside; (d) Con A in distilled water, pH 8.0.

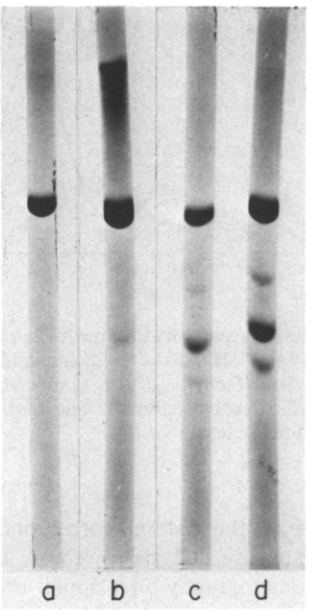

FIGURE 3: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the supernatant and precipitate obtained when solutions of Con A (10 mg/ml) are incubated in 1% NH_4HCO_3 at 37°: (a) 25 μg of supernatant, (b) 50 μg of supernatant, (c) 25 μg of precipitate, and (d) 50 μg of precipitate.

down the rate of increase in intensity at 215 nm (Figure 2c). The change is also influenced by the composition of the solvent. Solutions of Con A in 0.01 M Tris–0.1 M NaCl (pH 8.0) show an increase of negative intensity with time, but the spectral shift is much less pronounced. If Con A is dissolved in distilled water and the pH brought to 8.0 with a minimal amount of NaOH, only very slight spectral changes are observed within 7-hr incubation at 37° (Figure 2d).

To test whether the spectral change is simply a result of light scattering as precipitate is formed, a solution of Con A (1 mg/ml) in 1% NH_4HCO_3 was incubated at 37°. At various time intervals, aliquots of this solution were removed and passed through a Millipore filter and immediately the CD spectrum was recorded at 37°. The CD change for these Millipore-filtered solutions as a function of time was the same as that shown in Figure 2a where the spectra were recorded on a solution of Con A (1 mg/ml in 1% NH_4HCO_3) maintained at 37° in the spectropolarimeter cell.

Intact Subunit Prepared by Precipitation. When solutions of Con A (10 mg/ml) in 1% NH_4HCO_3 were maintained at 37° for 12 hr and centrifuged, the clear supernatant was found to be essentially free of the naturally occurring fragments while the precipitate was enriched in these fragments. Evidence for this conclusion was provided by gel electrophoresis in sodium dodecyl sulfate (Figure 3) and by estimates of the weight-average molecular weight by equilibrium sedimentation in 0.1 M Tris–6 M guanidine hydrochloride (pH 7.0) (Table I). Precipitation under these conditions thus serves as a rapid method for the large-scale preparation of the intact subunit of Con A. Like the preparations that contain the naturally occurring fragments, after dialysis and lyophilization the intact subunit will precipitate from solutions (10 mg/ml) in 1% NH_4HCO_3. However, estimates of the rate by light scattering measurements suggest that precipitation of the intact subunit proceeds more slowly than the mixture from which it was isolated.

Affinity Chromatography. The observation that the fragments A_1 and A_2 were apparently precipitated preferentially in 1% NH_4HCO_3 suggested that molecules containing the fragments might have slightly different properties than molecules made up entirely of intact subunits. To test for any difference in their ability to bind carbohydrates, Con A was chromatographed on columns of Sephadex G-75 (Figure 4) by elution with a shallow dextrose gradient. Two major fractions were obtained. Fraction A (Figure 4) contained the intact subunit plus fragments, while fraction B (Figure 4) was essentially free of fragments. Under certain conditions the two fractions were not adequately resolved, but the intact subunit

TABLE I: Molecular Weights[a] of the Supernatant and Precipitate Obtained by Incubation of Con A in 1% NH_4HCO_3 at 37° for 12 hr.

Sample	Mol Wt
Con A[b]	22,000
Intact Subunit[c]	25,800
Supernatant	26,900
Precipitate	21,000

[a] Weight-average molecular weights were obtained by sedimentation equilibrium in 6 M guanidine hydrochloride. [b] Con A preparation containing the naturally occurring fragments was isolated by the method of Agrawal and Goldstein (1967). [c] The value for the molecular weight of the intact subunit is taken from Wang *et al.* (1971).

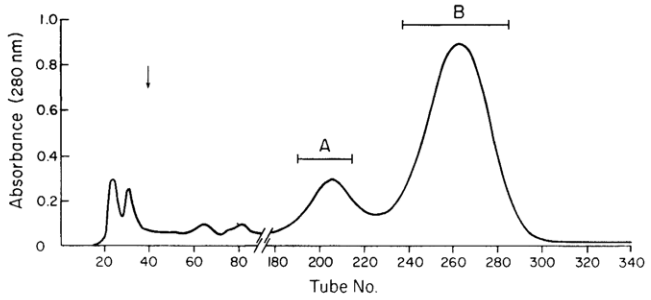

FIGURE 4: Affinity chromatography of Con A (280 mg) on Sephadex G-75 in 0.02 M Tris (pH 7.4). At the point indicated by the vertical arrow, a linear gradient from 0 to 0.08 M dextrose was initiated. Each tube contained 3.7 ml of effluent. The material in fractions A and B is described in the text.

could be purified from the material corresponding to fraction B (Figure 4) by extensive dialysis to remove bound dextrose followed by rechromatography on a similar column. Preparation of the intact subunit by this procedure was generally more time consuming and gave lower yields (~30%) than by the precipitation technique. Total recovery of material was generally low (~40%) possibly because of precipitation of the fragments on the Sephadex column.

Proteolysis of the Intact Subunit. Under carefully controlled conditions, limited digestion of the intact subunit with trypsin yielded high molecular weight fragments of the intact polypeptide chain as evidenced by the appearance of new protein bands on polyacrylamide electrophoresis in sodium dodecyl sulfate. Figure 5 shows the results of one of our representative experiments. The intact subunit (100 mg) isolated by NH_4HCO_3 precipitation described above was dissolved in 0.2 M phosphate buffer (pH 7.0). The turbid solution was passed through a Millipore filter and appropriately diluted to give solutions of approximately 10 mg/ml and 1 mg/ml in protein. Aliquots

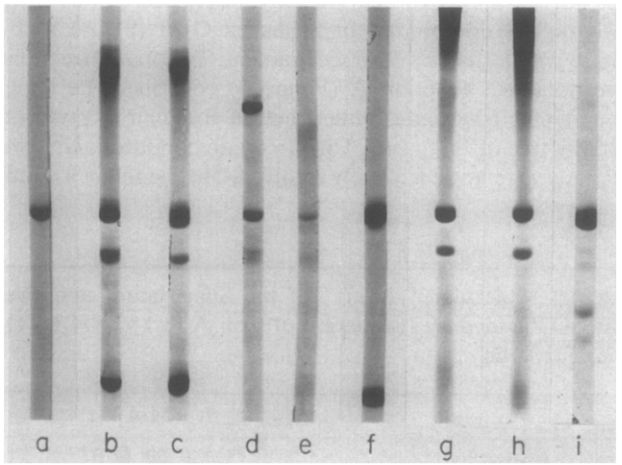

FIGURE 5: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of tryptic digest of the intact subunit of Con A: (a) intact subunit before addition of enzyme; (b) 10-mg/ml Con A, 0.1-mg/ml trypsin; (c) 1-mg/ml Con A, 0.01-mg/ml trypsin; (d) 10-mg/ml Con A, 1-mg/ml trypsin; (e) 1-mg/ml Con A, 0.1-mg/ml trypsin; (f) components of the tryptic digest which did not bind Sephadex (fraction A of Figure 6); (g–h) components of of the tryptic digest which were bound to Sephadex and eluted with dextrose (fractions B and C of Figure 6); (i) Con A preparation containing the naturally occurring fragments. The band at the top of gels b, c, and d probably represents formation of high molecular weight aggregates which resulted in precipitation.

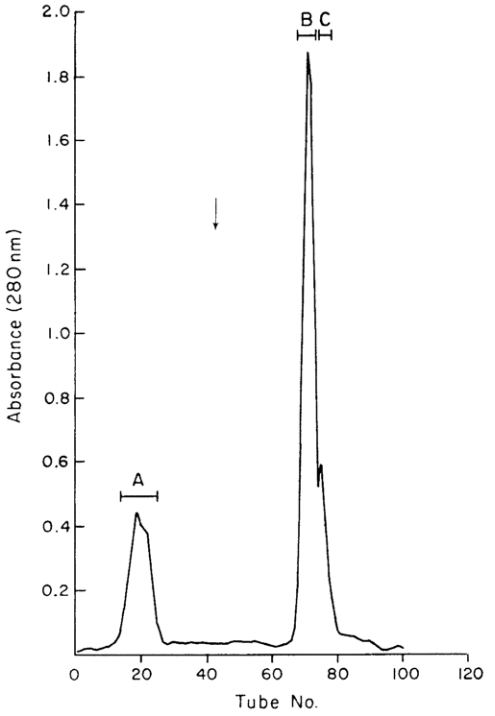

FIGURE 6: Fractionation of tryptic digest of Con A (Figure 5b) (30 mg) on Sephadex G-75 in 0.01 M Tris (pH 7.4). At the point indicated by the vertical arrow, a linear gradient from 0 to 0.3 M dextrose was initiated. Column dimensions, 1.4 × 20 cm; volume per tube, 1.5 ml. The material in fractions A, B, and C is described in the text.

of these solutions were taken for zero-time controls (Figure 5a). Trypsin (10 mg, PTCK-trypsin, Calbiochem lot no. 73325) was dissolved in 1 ml of water and aliquots of this enzyme solution were added to the Con A solutions to give (b) 10-mg/ml Con A, 0.1-mg/ml trypsin; (c) 1-mg/ml Con A, 0.01-mg/ml trypsin; (d) 10-mg/ml Con A, 1-mg/ml trypsin; (e) 1-mg/ml Con A, 0.1-mg/ml trypsin. The solutions were incubated in a 37° water bath for 10 hr. Aliquots were dialyzed against water, lyophilized, and subjected to gel electrophoresis, the results of which are shown in Figure 5b–e. Similar gel patterns were obtained from samples taken after 5-hr incubation. When the ratio of enzyme to Con A was 1% by weight, tryptic digestion yielded new bands with molecular weights of about 20,000 (Tn I) and 8000 (Tn II) (Figure 5b,c) in addition to the band corresponding to a molecular weight of about 27,000. In digests with a higher ratio of enzyme to Con A (10%), bands of molecular weights 27,000 and 20,000 remained while the lower molecular weight band disappeared (Figure 5d,e). The band on top of each gel was assumed to contain high molecular weight aggregates.

Figure 6 shows the chromatographic profile when one-third of the digestion mixture from Figure 5b was subjected to affinity chromatography on a column of Sephadex G-75 in 0.01 M Tris (pH 7.4). After a column volume of buffer had been eluted, a 0–0.3 M dextrose gradient in 0.01 M Tris (pH 7.4) was used to elute the digestion products which were bound to Sephadex. Each of the fractions from this column was dialyzed against water, lyophilized, and subjected to gel electrophoresis in sodium dodecyl sulfate. The gel patterns yielded by each of the fractions in Figure 6 are compared to the entire digestion mixture in Figure 5f–h. Fraction A (Figure 6) contained two bands (mol wt ~27,000 and ~8000) (Figure 5f). Fractions B and C (Figure 6) yielded essentially identical gel

TABLE II: Amino Acid Compositions[a] of Fractions[b] Obtained from a Tryptic Digest of the Intact Subunit of Con A.

	Intact Subunit (27,000)	Tn I (20,000)	Tn II (8000)
Lys	12.0	7.0	3.7
His	6.2	4.0	2.0
Arg	6.2	4.5	1.7
Asp	33.0	25.8	7.3
Thr	21.8	18.6	5.4
Ser	24.4	15.4	7.8
Glu	20.5	13.8	5.0
Pro	13.5	9.8	3.5
Gly	18.6	14.8	3.8
Ala	19.0	15.9	9.1
Val	14.5	9.8	6.5
Met[c]	—	—	—
Ile	14.5	13.9	4.3
Leu	19.0	17.2	6.2
Tyr	3.8	3.7	1.8
Phe	10.5	5.4	5.9
Total residues	239.5	181.6	74.0

[a] Values are expressed as residues per mole, based on molecular weight values shown in parentheses under each fraction. [b] The fractions were obtained by polyacrylamide electrophoresis of the tryptic digest. The material in each fraction was eluted from sliced gel sections whose mobility corresponded to the molecular weights shown in parentheses. [c] Trace amounts of methionine were present in the fractions; no attempt was made to determine this value accurately.

patterns: two bands with molecular weights of about 27,000 and 20,000 (Figure 5g,h).

To characterize the various components, polyacrylamide gels identical with that of Figure 5b were sliced and eluted. Table II shows the results of amino acid analysis of the various fractions. From the amino acid compositions, material in two of the fractions can be tentatively identified. The band which has a mobility corresponding to a molecular weight of about 27,000 yielded a composition similar to the Con A polypeptide chain. Both the amino acid composition and mobility of Tn II suggest that it is a polypeptide containing about 76 residues. From the results of our sequence studies on Con A (G. M. Edelman *et al.*, 1972, unpublished data) we have tentatively placed this 76 residue peptide at the NH_2-terminal portion of the cyanogen bromide fragment F_3 (Waxdal *et al.*, 1971). The results of analysis of Tn I (mol wt ~20,000) did not indicate any outstanding features and therefore we cannot as yet assign its position in the Con A polypeptide chain.

Digestion of the intact subunit with chymotrypsin (Worthington CDI 8LK) following the same procedure as that given for tryptic digestion also yielded high molecular weight fragments of the intact polypeptide chain (Figure 7). With 1% (weight ratio of enzyme to Con A) chymotrypsin, a high molecular weight product (~25,000) is obtained (Figure 7b,c) while with 10% chymotrypsin, other intermediate sized fragments (mol wt 15,000 and 20,000) are formed in addition to the major product (mol wt 25,000) (Figure 7d,e). In both tryptic and chymotryptic digests, appreciable amounts of protein with carbohydrate binding and hemagglutinating activity remained.

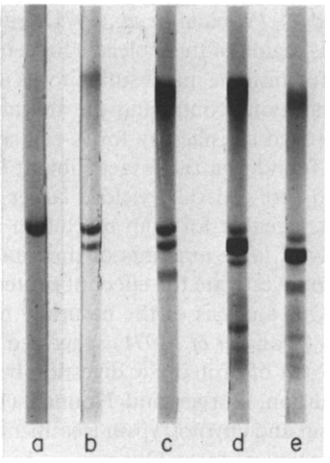

FIGURE 7: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of chymotryptic digest of the intact subunit of Con A: (a) Con A before addition of enzyme; (b) 10-mg/ml Con A, 0.1-mg/ml chymotrypsin; (c) 1-mg/ml Con A, 0.01-mg/ml chymotrypsin; (d) 10-mg/ml Con A, 1-mg/ml chymotrypsin; (e) 1-mg/ml Con A, 0.1-mg/ml chymotrypsin.

We have performed proteolytic digestions of both Con A and the intact subunit under a wide variety of other conditions ranging over enzyme concentrations of 0.005–1 mg/ml, temperature 4–37° and times from 1 to 16 hr. These digests often yielded some of the tryptic or chymotryptic peptides expected from a knowledge of the amino acid sequence (G. M. Edelman *et al.*, 1972, unpublished data) and, in cases where mild conditions were used, large fragments of the polypeptide chain. However, in none of our experiments on the digestion of Con A or the intact subunit was the intact polypeptide chain of Con A completely removed and only under the conditions reported in detail above was the conversion to a large fragment observed to occur in good yield (~50%) as estimated by band intensities on polyacrylamide gels.

Discussion

We have previously reported a method for isolating the intact subunit of Con A by ion-exchange chromatography on DEAE-cellulose (Wang *et al.*, 1971). While this method provided a satisfactory product, it required working with relatively small amounts of material and yields were generally low. In addition, some preparations were not fractionated by this procedure. The method described in the present report provides a simple, rapid technique for preparing gram quantities of the intact subunit in about 50% yield. As the starting material is obtained by an equally large-scale process (Agrawal and Goldstein, 1967), the entire preparation can be utilized for rapid production of bulk quantities.

The intact subunit isolated by this technique has proved suitable for biological studies, for the elucidation of the amino acid sequence of the protein and for the determination of the three-dimensional structure by X-ray crystallography. The use of the intact subunit was critical to the analysis of results obtained from cyanogen bromide cleavage of Con A (Waxdal *et al.*, 1971). Furthermore, it has proved even more important in establishing the primary structure. As the cyanogen bromide fragment F_2 contains the region where the polypeptide chain of Con A is cleaved to produce the naturally occurring

fragments A_1 and A_2 (Waxdal *et al.*, 1971), efforts to obtain peptides from this region of the molecule for sequence analysis were not successful until the intact subunit was used. Although the Con A preparations containing the fragments have, for the most part, proved satisfactory for X-ray crystallographic studies, we have found that the intact subunit isolated by the method described here generally yielded larger crystals which were particularly valuable for high resolution studies.

The availability of large amounts of the intact subunit has also enabled us to investigate the effect of proteolytic enzymes on the protein. Our analysis of the naturally occurring fragments A_1 and A_2 (Wang *et al.*, 1971) suggested that they may be formed as a result of proteolytic digestion before or during isolation. In addition, Burger and Noonan (1970) have reported that trypsin and chymotrypsin treatment of Con A converted it to a monovalent form. Our present results show that, under our digestion conditions, trypsin and chymotrypsin do not convert the intact subunit to fragments A_1 and A_2 but produce other high molecular weight fragments (Figures 5 and 7). Trypsin reproducibly gave two new major components—one of molecular weight of about 20,000 (Tn I) and another of molecular weight of about 8000 (Tn II). When the digestion mixture was passed through a column of Sephadex G-75 (Figure 6), Tn I as well as the species of mol wt 27,000 adhered to the column. Since these components were adsorbed to Sephadex and could be eluted with the competitive inhibitor, dextrose, we conclude that they possess carbohydrate binding activity. The component with molecular weight of about 27,000 is probably the undigested intact subunit and we assume it retains carbohydrate binding activity. The large fragment (Tn I) also binds to Sephadex but without further fractionation we cannot establish whether this activity is a property of the fragment or the result of interaction with the active, intact subunit. Our present results suggest that fragment Tn I may be responsible for the observed decrease in molecular weight as reported by Burger and Noonan (1970). Whether it can account for their observed change in the activity of Con A requires further investigation.

From the above results, we also infer that the component of mol wt 27,000 in fraction A (Figure 6) is an inactivated form of the corresponding component in fractions B and C (Figure 6). Fragment Tn II then represents a medium molecular weight component which interacts neither with carbohydrate nor the active fractions (B and C in Figure 6) of the original digestion mixture. Comparison of the amino acid composition of Tn II to the results of our sequence studies of Con A (G. M. Edelman *et al.*, 1972, unpublished data) has allowed us to place it tentatively at the COOH-terminal portion of the molecule.

The precipitate obtained by incubating the protein in bicarbonate solutions at 37° is enriched in the naturally occurring fragments (Figure 3). It would therefore appear to provide excellent starting material for isolating A_1 and A_2 for structure–activity correlations. As fragments A_1 and A_2 have been assigned, respectively, to the NH_2 and COOH terminus of the intact polypeptide chain of Con A (Wang *et al.*, 1971), correlating either carbohydrate binding or mitogenic activity with one of the fragments would be a significant step toward elucidating the mechanism of action of Con A. The major difficulty in using the precipitate for isolating the natural fragments is its limited solubility. Fragment A_1 has been obtained from the precipitate by ion-exchange chromatography in 8 M urea. Preliminary experiments suggest that the material soluble after removal of urea was predominantly A_1 and had hemagglutinating activity. However, yields were very low and the fraction was contaminated with small amounts (less than 5%) of the intact subunit.

A detailed mechanism for the precipitation of the fragments cannot be offered at this time but the data suggest that the process is more complex than a simple "salting out" of the various components (von Hippel and Schleich, 1969). Moreover, it should be emphasized that the precipitate contains both the intact subunit and the fragments. If the isolated intact subunit is subjected to the same conditions, it also precipitates. The rate appears, however, to be decreased. Precipitation is greatly retarded by the presence of α-methyl D-mannoside (Figure 1), a saccharide which is strongly bound by Con A, but not by galactose which does not bind to the protein (Goldstein *et al.*, 1965b). It has been reported (So and Goldstein, 1967; McKenzie *et al.*, 1972) that glucose inhibits the precipitation of Con A from solutions in phosphate buffer at pH 7. Analysis by circular dichroism of structural changes in Con A under conditions similar to those used for precipitation of the naturally occurring fragments indicates a very slow change in the far-uv region of the spectrum (Figure 2). Like precipitation, the spectral change requires the presence of salts, is temperature dependent, and is not specific for NH_4^+ or HCO_3^- ions. It appears that the spectral shift is not simply a result of light scattering as precipitate is formed. Scattering by particulate matter usually results in a decrease in band intensity with a concomitant shift toward the longer wavelengths (Gordon, 1972; Schneider *et al.*, 1970; Urry and Krivacic, 1970). The change seen here, however, is toward the shorter wavelengths and the band intensity is increased. Furthermore, when the precipitate is removed by passage through a Millipore filter, the spectrum is essentially unchanged.

The increase in intensity at 215 nm (Figure 2) resembles that seen for solutions of Con A at pH 9.0 (Pflumm *et al.*, 1971; Zand *et al.*, 1971) where the molecule undergoes irreversible denaturation. The spectral changes and subsequent precipitation are consistent with a shift from the as yet uncharacterized poly(*S*-carboxymethyl-L-cysteine) β structure (Stevens *et al.*, 1965; Ikeda and Fasman, 1967) to an intermolecular pleated sheet β structure such as that found in poly(L-lysine) solutions (Sarkar and Doty, 1966). The transformed spectrum closely resembles one containing between 60 and 80% β structure and no α helix (Greenfield and Fasman, 1969). A similar spectrum has been observed for solutions of Con A in 0.5 M bicarbonate (pH 8.1) which were 10% in dioxane (McCubbin *et al.*, 1972).

It has been shown previously that the circular dichroic spectrum of Con A undergoes changes in the near-uv region upon binding of α-methyl D-mannoside (Pflumm *et al.*, 1971) or glucose (McCubbin *et al.*, 1971). When glucose was added to Con A, a uv difference spectrum was also obtained (Doyle *et al.*, 1968; Hassing and Goldstein, 1970). These results suggest that carbohydrate binding may be accompanied by a conformational change in the protein. Our data show that the change in the far-uv region of the CD spectrum and precipitation are retarded by α-methyl D-mannoside, suggesting that sugar binding converts Con A to a conformation which is more resistant to the effects of incubation at 37° in 1% NH_4HCO_3.

The combined CD and precipitation data indicate that incubation at 37° in bicarbonate buffer selectively denatures molecules containing fragments. This suggests subtle differences in the properties of Con A molecules containing fragments and molecules composed entirely of the intact subunit. Another independent observation lends further support to

this hypothesis. When Con A is adsorbed to Sephadex and eluted with a linear gradient of dextrose, two fractions are obtained—one containing the intact subunit plus fragments and one essentially free of fragments (Figure 4). Thus, while recent low-resolution X-ray crystallographic studies indicate the tertiary structure of the fragmented subunit does not differ greatly from that of the intact subunit (Becker, 1971), our data show differences in their properties. A detailed comparison of their three-dimensional structures at atomic resolution is currently being undertaken.

The observation that a large percentage of Con A can precipitate from solutions at 37° has a number of implications for biological studies. It remains to be established whether the biological action of Con A is the result of (a) simple binding of Con A to cells; (b) binding followed by a conformational change similar to that proposed in the presence of α-methyl D-mannoside (Pflumm *et al.*, 1971); (c) binding followed by a conformational change or intermolecular interaction similar to that seen in this study. All three possibilities and combinations seem tenable at this time. In any case, under the conditions used in many biological experiments, it should be noted that Con A may precipitate or its concentration may vary with time. This effect may be minimized if the glycoprotein receptors of cells behave in the same way as α-methyl D-mannoside.

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