

# Posttranslational Processing of Concanavalin A Precursors in Jackbean Cotyledons

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**Abstract.** Metabolic labeling of immature jackbean cotyledons with  $^{14}\text{C}$ -amino acids was used to determine the processing steps involved in the assembly of concanavalin A. Pulse-chase experiments and analyses of immunoprecipitated lectin forms indicated a complex series of events involving seven distinct species. The structural relatedness of all of the intermediate species was confirmed by two-dimensional mapping of  $^{125}\text{I}$ -tryptic peptides. An initial glycosylated precursor was deglycosylated and cleaved into smaller polypeptides, which subsequently reannealed over a period of 10–27 h.  $\text{NH}_2$ -terminal sequencing of the abundant precursors confirmed that the intact subunit of concanavalin A was formed by the reannealing of two fragments, since the alignment of residues 1–118 and 119–237 was reversed in the final form of the lectin identified in the chase and the precursor first labeled. When the tissue was pulse-chased in the presence of monensin, processing of the glycosylated precursor was inhibited. The weak bases  $\text{NH}_4\text{Cl}$  and chloro-

quine were without effect. Immunocytochemical studies showed that monensin treatment caused the accumulation of immunoreactive material at the cell surface and indicated that the ionophore had induced the secretion of a component normally destined for deposition within the protein bodies. Consideration of the tertiary structure of the glycosylated precursor and mature lectin showed that the entire series of processing events could occur without significant refolding of the initial translational product. Proteolytic events included removal of a peptide from the surface of the precursor molecule that connected the  $\text{NH}_2$ - and  $\text{COOH}$ -termini of the mature protein. This processing activated the carbohydrate-binding activity of the lectin. The chase data suggested the occurrence of a simultaneous cleavage and formation of a peptide bond, raising the possibility that annealment of the fragments to give rise to the mature subunit involves a transpeptidation event rather than cleavage and subsequent religation.

**D**URING legume seed development substantial quantities of reserve metabolites are synthesized by the cells of the cotyledons for storage and subsequent use upon germination. The formation of the dessicated mature seed can take up to 3 mo after fertilization and involves a regulated sequence of events in which a period of cell division is followed by cell enlargement, synthesis, and accumulation of reserves and controlled dehydration (5). The principal storage compartment for protein reserves is the protein body. At seed maturity parenchyma cells of the cotyledons are filled with these small ( $1\text{--}10\ \mu\text{M}$ ) membrane-bound organelles. Protein bodies are ultimately derived from the single large vacuole present initially in dividing cells which fragments into progressively smaller units as seed development proceeds (1, 17, 18, 55, 57, 78). Protein loading into the lumen of the storage compartment occurs throughout this fragmentation process. Details of this have already been elucidated for some components that accumulate to high levels in protein bodies, including several storage proteins (2, 15, 19, 20, 28, 37, 62, 63) and lectins (12, 13, 33, 38, 39, 46, 47,

69–71). The proteins are synthesized on membrane-bound ribosomes, co-translationally segregated into the endoplasmic reticulum, and transported to the protein bodies where further processing occurs. Posttranslational processing steps that involve trimming and terminal glycosylation have been demonstrated for the lectins of *Phaseolus vulgaris* and *Ricinus communis*, implying use of an intracellular traffic route through the Golgi apparatus similar if not identical to that established for other eukaryotic systems (reviewed in reference 14). However, nothing is known of the targeting mechanisms involved in sorting products between the secretory pathway to the cell surface and cell wall and the intracellular pathway to the vacuoles and protein bodies (reviewed in reference 7).

In contrast to the information available on the synthesis of storage proteins and lectins in other legumes, the biosynthesis of jackbean proteins is only poorly understood. The most abundant protein in the cotyledons is the storage reserve concanalin, with the endogenous lectin concanavalin A (ConA)<sup>1</sup> next most abundant, representing some 20% of the

<sup>1</sup> Abbreviation used in this paper: ConA, concanavalin A.

total protein (23). An unusual feature of mature jackbeans is the lack of glycoproteins, since neither canavalin nor the lectin is glycosylated, and, other than minor components, there is only one ConA-binding species, a subunit of the principal exoglycosidase of the seed,  $\alpha$ -mannosidase (8, 9). Despite the presence of endoglycosidase H-susceptible oligosaccharide(s) attached to copies of the heavy subunit of the tetrameric  $\alpha$ -mannosidase, no interaction between it and ConA occurs until the native enzyme is dissociated into subunits (10). We have shown that although endogenous "receptors" to ConA are lacking at seed maturity, the abundance of polypeptides able to interact with  $^{125}\text{I}$ -ConA overlays varies during cotyledon development, and in particular a putative precursor form of ConA is itself glycosylated (51). This suggested that processing events during ConA synthesis were atypical of legume lectins and would probably involve some form of posttranslational deglycosylation such as that described for the synthesis of eukaryotic peptide hormones in mammalian and yeast systems (34, 42).

Another unusual feature of jackbeans as compared with other legumes is the proportion of lectin protein composed of naturally occurring fragments. The multiple forms of ConA were first characterized in 1971 when it was noted that the ratio of intact subunit ( $M_r$  27,000) to fragments was 3:2 (26, 73). By contrast, although fragments of legume storage proteins (67), as well as soybean agglutinin (48), have been described, the smaller polypeptides are always very minor components of any preparation. For ConA, it has been assumed that the fragments are degradation products of the  $M_r$  27,000 polypeptide since their sequences align to the  $\text{NH}_2$ - and  $\text{COOH}$ -termini of the intact subunit (76). ConA however, is resistant to proteolysis by endopeptidases found in the mature seed (24), and by using immunoblots of extracts prepared from developing cotyledons we have shown that fragments appear concurrently with the high  $M_r$  precursor forms of the lectin (51).

In the present study we have used metabolic labeling of immature cotyledons to clarify the processing events involved in the assembly of ConA and drug treatment to determine characteristics of the intracellular traffic route to the protein bodies. While this work was in progress, two studies were recently published in which alternative mechanisms for the synthesis of ConA were suggested (11, 35). In one investigation, the sequence of the cDNA thought to code for ConA was determined (11). Comparison of the nucleotide sequence of the proposed lectin mRNA with the established amino acid sequence of the ConA subunit led the authors to suggest that a posttranslational transposition and ligation had to occur to produce the mature lectin. An alternative mechanism, involving a removal of a glycosylated oligopeptide, has been suggested as the sole processing step between the glycosylated precursor and the final lectin form (35). This was inferred from metabolic labeling in which ConA forms were identified after a pulse for 90 min and a single chase time of 24 h. The results presented in this paper demonstrate that the initial glycosylated precursor is indeed deglycosylated but subsequently is cleaved to fragments that constitute the direct precursors of the intact subunit of the lectin, and that reanneal to form mature ConA. The assembly of the final form of the lectin is prevented by pretreatment of the cotyledons with monensin, where immunogold labeling of the tissue indicates

an ionophore-induced secretion of the glycosylated precursor to the cell surface rather than to the protein bodies. Comparison of the  $\text{NH}_2$ -terminal sequences of six of the lectin precursors identified in our study, together with the lectin mRNA sequence (11) and computer modeling of the crystal structure of ConA (60), leads us to suggest that processing steps do not involve a transposition but could occur in the absence of any unfolding or gross disturbance of three-dimensional structure of the initial precursor.

## Materials and Methods

### Seeds, Radiolabeling, Immunoprecipitation, Affinity\* Precipitation, Peptide Mapping, and Electrophoresis

Pods were harvested at intervals during seed maturation of plants grown either in an environmental chamber (12 h light/dark, 25/20°C, 90% relative humidity) or a glass-house. For metabolic labeling, pairs of cotyledons (environmental chamber grown) were each incubated in 2  $\mu\text{Ci}$   $^{14}\text{C}$ -amino acid mixture (Amersham, UK) in a final volume of 500  $\mu\text{l}$  sterile  $\text{H}_2\text{O}$  under fluorescent lighting in a laminar flow cabinet at 22°C. For pulse-chase experiments (Fig. 2, glass-house; Fig. 3, environmental chamber), after the 3-h labeling period cotyledons were washed and transferred to a sterile solution of 1 mM amino acids (as in the  $^{14}\text{C}$ -mixture) and incubated as before for up to 37 h. For monensin treatment, cotyledons (glass-house grown) were incubated in 10  $\mu\text{M}$  monensin (Sigma Chemical Co., Poole, England) for 3 h before incubation with  $^{14}\text{C}$ -amino acids and chased as above. Soluble extracts were made by homogenization in phosphate-buffered saline (PBS), Triton X-100 (0.1% wt/vol), methyl  $\alpha$ -mannoside (200 mM), phenanthroline (10 mM),  $\text{NaN}_3$  (0.1% wt/vol), sonication ( $4 \times 30$  s), and centrifugation (1,000  $g \times 5$  min). Protein was estimated using a modified procedure of Lowry (50) with bovine serum albumin (BSA) as standard. For immunoprecipitation, post-1,000  $g$  supernatants were further centrifuged at 30,000  $g$  for 10 min. A 500- $\mu\text{l}$  extract ( $\sim 2.0$  mg protein) was incubated with 100  $\mu\text{l}$  anti-ConA IgG fraction for 1 h on ice followed by 1 h at 4°C with shaking. Prehydrated protein A-Sepharose (8 mg) was added, and the mixture was further incubated for 1 h on ice before centrifugation at 12,000  $g$ /1 min followed by three wash cycles. For affinity precipitation, ovalbumin was coupled to CNBr-activated Sepharose, 20 mg glycoprotein/g Sepharose. Post-30,000  $g$  supernatants were dialyzed exhaustively against PBS, Triton X-100 (0.1% wt/vol) and recentrifuged as before. A 500- $\mu\text{l}$  extract was incubated with 10  $\mu\text{l}$  ovalbumin-Sepharose, shaken for 1 h at 4°C, incubated on ice for 1 h, and processed as above. Protein was precipitated for electrophoresis by a fivefold excess of  $-20^\circ\text{C}$  acetone, resolubilized in sample buffer, and heated at 95°C for 3 min. For immunoprecipitates, sample buffer was added directly to the washed precipitates. SDS PAGE was carried out on 10–15% (wt/vol) gradient gels, under both reducing and nonreducing conditions, and protein was visualized by Coomassie Blue and/or by fluorography after acetic acid/PPO impregnation.  $^{14}\text{C}$ -Methylated protein mixture (Amersham) was used to calculate the  $M_r$  of the labeled bands using log percent acrylamide  $v$ . log  $M_r$ .  $^{125}\text{I}$ -ConA-overlays were carried out as described (10). Bands were cut out of Coomassie Blue-stained gels and electroeluted into Spectrapor 3 dialysis tubing (Fawell, S. E., D. J. C. Pappin, C. McDonald, and S. J. Higgins, unpublished data) over 18 h at 80 mA using continuously circulating buffer (100 mM Na phosphate pH 7.5, 0.1% [wt/vol] SDS). The gel was removed and the polypeptides further dialyzed against 0.1% (wt/vol) SDS (12 changes/4 d) and then distilled  $\text{H}_2\text{O}$  as before and lyophilized.

### Sequencing

Lyophilized protein was dissolved in 0.07 ml 0.2 M  $\text{NaHCO}_3$  containing 0.25% wt/vol SDS and added to 20 mg of 170 Å pore-size *p*-phenylene diisothiocyanate glass (72). The glass was incubated for 60 min at 56°C under  $\text{N}_2$ , then washed with water and methanol to remove noncovalently bound material. The glass-coupled peptide was then sequenced by automated solid-phase Edman degradation (44) using the Solid Phase facility of the AFRC sequence unit (Department of Biochemistry, University of Leeds). Anilinothiazolinone amino acids were converted in 0.05 ml 20% aqueous trifluoroacetic acid for 12 min at 70°C (under  $\text{N}_2$ ), and the corresponding phenylthiohydantoin amino acids were identified by reverse-phase high-performance liquid chromatography using a method adapted from (80). The phenylthiohydantoin amino acids were quantified at 265 nm by a Spectra-Physics SP-4100 computing integrator (Spectra-Physics Inc., Mountainview, CA), and Ser and Thr residues were confirmed by detection of their dehydro-derivatives at 313 nm.

## Electron Microscopy

Immature cotyledons (seed fresh weight 600 mg) were placed adaxial surface down on 20  $\mu$ l of 10  $\mu$ M monensin for up to 21 h at 22°C under fluorescent lighting. Controls consisted of freshly dissected cotyledons without incubation, cotyledons incubated in sterile H<sub>2</sub>O, and cotyledons incubated on 10  $\mu$ M monensin containing 1 mM cycloheximide after 1 h of preincubation on 1 mM cycloheximide. After incubation, tissue layers closest to the droplet were removed and cut into 1-mm cubes under 5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.0. After fixation (1 h at 0°C), washing (16 h in 50 mM cacodylate buffer at 4°C), and postfixation (veronal buffered 1% osmic acid at 0°C for 1 h), the tissue was further washed in distilled H<sub>2</sub>O, dehydrated through a graded ethanol series, and embedded in Araldite. Thin sections were cut from the embedded tissue, mounted on 200 mesh gold grids, and incubated (1 h, room temperature), submerged in 20  $\mu$ l anti-ConA in 500 mM Tris-HCl, pH 7.4, containing 0.1% gelatine 0.1% Triton X-100 and 2% BSA. Grids were washed for 30 s in tap H<sub>2</sub>O, incubated for 1 h at room temperature in 20  $\mu$ l goat anti-rabbit IgG adsorbed onto 20 nM colloidal gold particles in Tris buffer as before. The grid was then washed for 30 s in tap H<sub>2</sub>O, stained in saturated aqueous uranyl acetate solution (20 min), lead citrate (2 min), and viewed in an JEOL electron microscope 100B operating at 60 kV. Thick sections (0.5  $\mu$ m) were mounted directly on glass slides and stained with toluidine blue (0.1% wt/vol in 1% Borax, 30 s) for examination under the light microscope.

## Results

### Metabolic Labeling of Developing Cotyledons

The period of cell division in jackbeans is completed by 30–40 d after anthesis when the seed fresh weight is ~100–200 mg. Rapid synthesis and accumulation of proteins destined for storage within the protein bodies then commence. As shown in the SDS PAGE analysis of total extracts prepared from developing cotyledons (Fig. 1*A*) canavalin (subunit  $M_r$  49,300) became abundant by 700 mg fresh wt, and the polypeptide of  $M_r$  33,500, previously identified as a ConA precursor by immunoblotting (51), also appeared at early stages and was present for some time before the appearance of any other forms of the lectin (compare Fig. 1*A*, lanes *c–f* with lanes *g* and *h*). Fragments were only detected at stages when ConA polypeptides of  $M_r$  similar to that of the mature lectin subunit were also present (Fig. 1 lanes *g* and *h*).

Analysis of polypeptides in the dry mature seed (Fig. 1*A*, lane *i*) depicts the typical ConA intact subunit and the two principal naturally occurring fragments. The molecular weights of these species are established by amino acid composition at 26,000, 13,000, and 11,000 (73). In our gel system with the  $M_r$  markers cited, the final form of the intact subunit was  $M_r$  30,000 and of the fragments was 16,200 and 14,200. Fig. 1*B* is the fluorograph of the Coomassie Blue-stained gel shown in Fig. 1*A* and depicts the range and amount of polypeptides that have been synthesized during a 3-h pulse-label of the immature cotyledons with <sup>14</sup>C-amino acids. When intact cotyledons were incubated in <sup>14</sup>C-amino acids, the precursors were rapidly taken up into the tissue, and incorporation of radioactivity into trichloroacetic acid-precipitable material proceeded linearly with time (Marcus, S. E., and D. J. Bowles, unpublished data). From a maximum incorporation of >2,500 cpm/100  $\mu$ g protein over a 3-h incubation in precursor at seed fresh weight 500 mg (Fig. 1, lane *d*), levels decreased as development proceeded, until by 1,500 mg fresh weight (Fig. 1, lane *h*), total incorporation was reduced to ~300 cpm/100  $\mu$ g protein, reflecting a change in the rate of protein synthesis and/or in relative pool sizes of amino acids. Although the ConA precursor of  $M_r$  33,500 was a major component of the cotyledons synthesized during the 3-h incubation, no other forms of the lectin were radioactively

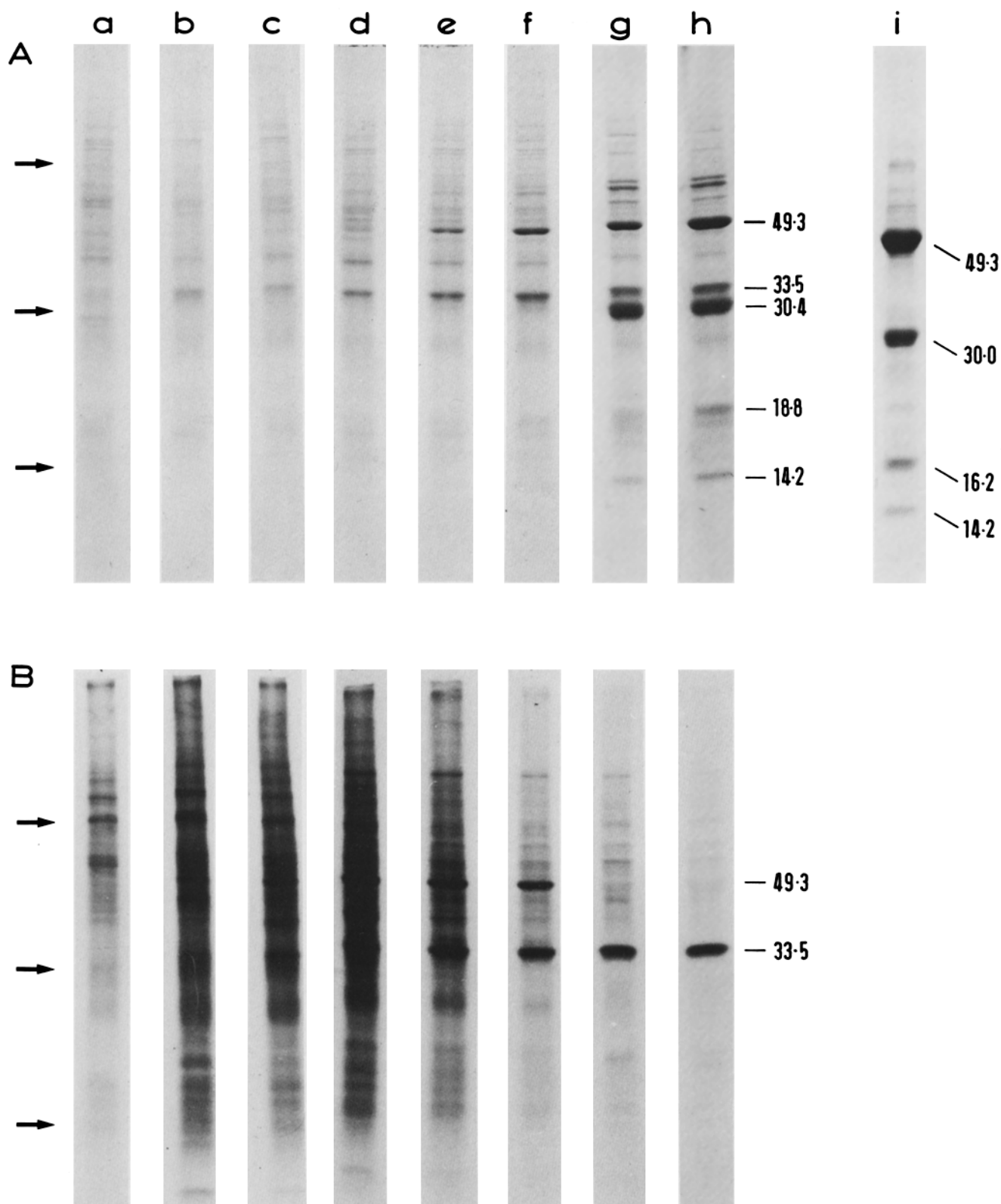
labeled within that period. Since the other forms were present in the Coomassie Blue-stained gels (compare lanes *g* and *h* of Fig. 1, *A* and *B*) the result indicated that processing of the  $M_r$  33,500 polypeptide did not commence for at least 3 h.

### Synthesis of ConA

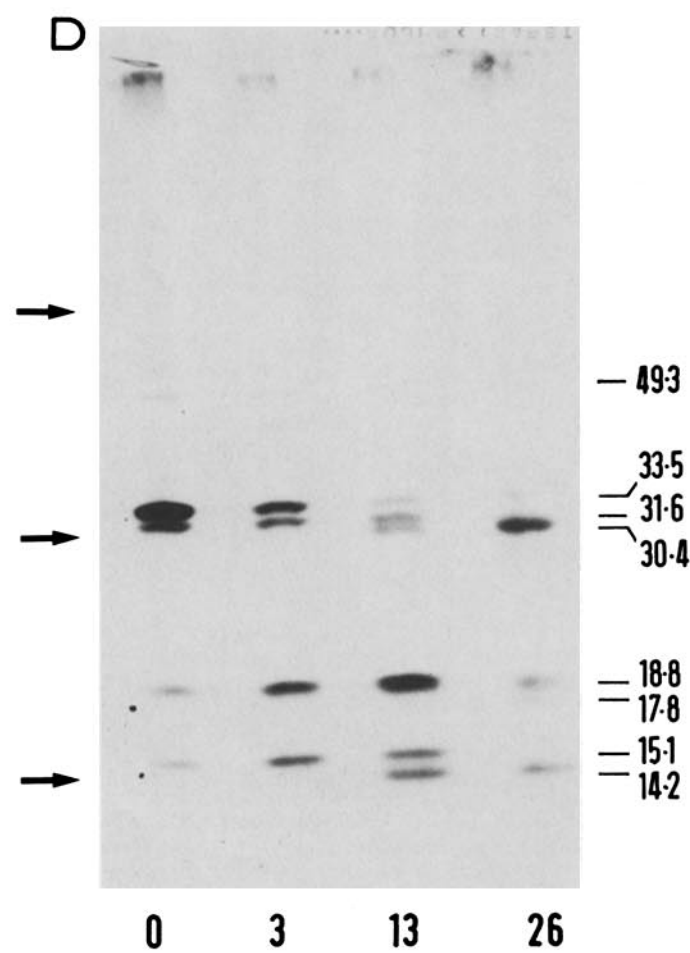
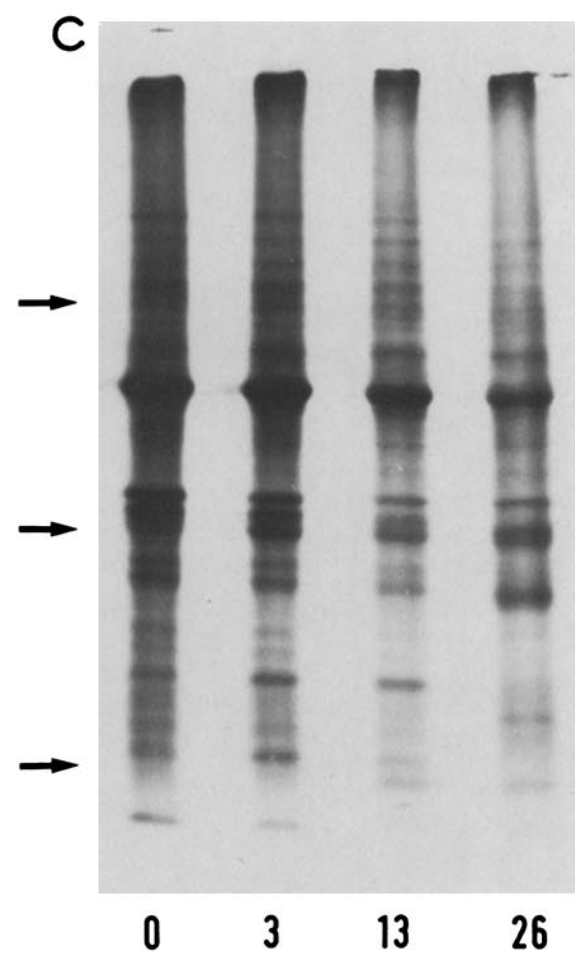
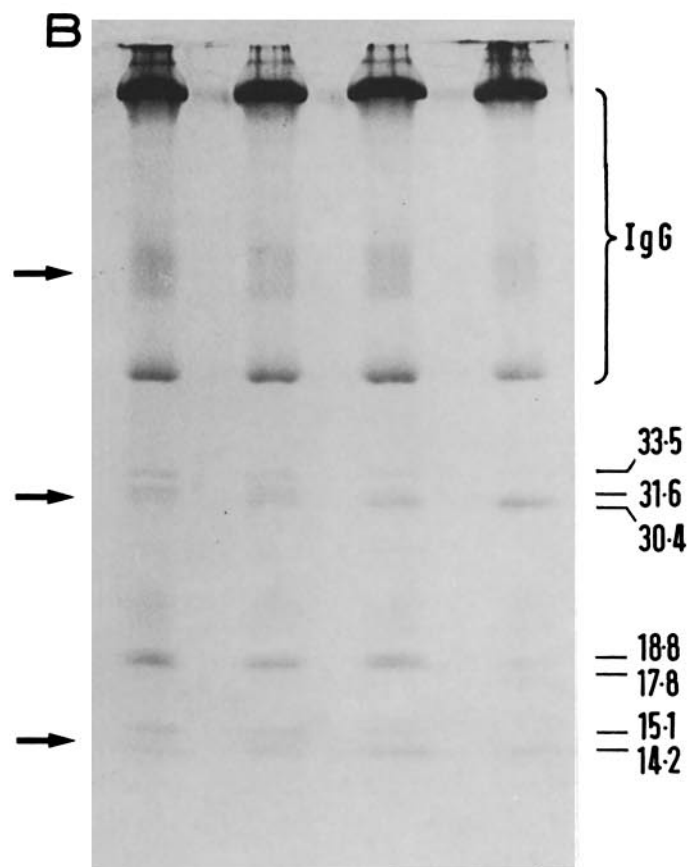
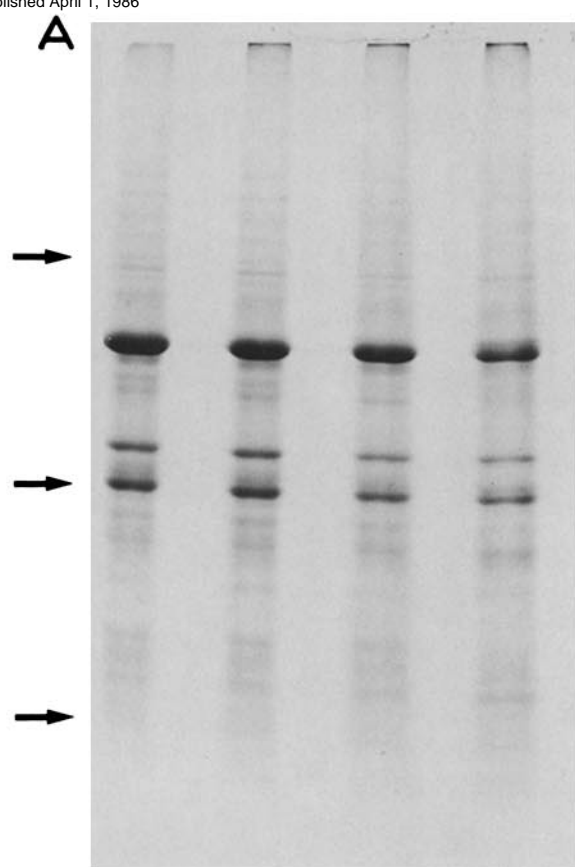
To study the assembly of ConA we used metabolic labeling of immature cotyledons (seed fresh weight 0.66 g) in a pulse-chase and analyzed the interrelationship of lectin precursor forms by SDS PAGE/fluorography of protein A-Sepharose immunoprecipitates. Sonication of the tissue led to a partial release of luminal contents from membrane compartments, including the protein bodies, but Triton X-100 was also used to solubilize membrane-bound proteins. We included methyl  $\alpha$ -mannoside in the analyses to prevent lectin-receptor complexes from forming between active species of ConA and endogenous glycoconjugates and to inhibit any carbohydrate-binding between the lectin and antisera. Phenanthroline, an inhibitor of the neutral metalloproteinase activity prominent during cotyledon development (Marcus, S. E., and D. J. Bowles, unpublished data), was also included to prevent artifactual proteolysis before analysis. After 3 h of labeling the cotyledons with <sup>14</sup>C-amino acids (time 0 of the chase), the principal lectin form in the fluorograph of the immunoprecipitate shown in Fig. 2 was a polypeptide of  $M_r$  33,500. Small amounts of radioactivity were also present in a polypeptide of  $M_r$  31,600, and trace levels were incorporated into bands at  $M_r$  18,800 and 15,100. After 3 h of chase, the <sup>14</sup>C label in  $M_r$  33,500 band had substantially decreased, whereas that into the 18,800 and 15,100 species had increased. By 13 h, the radioactivity associated with the polypeptides of  $M_r$  33,500 and 31,600 had fallen to trace levels and two additional labeled bands at  $M_r$  30,400 and 14,200 had appeared. After 26 h total chase time, no label remained in the  $M_r$  33,500 and 31,600 forms, and only trace levels occurred in the polypeptides of lower  $M_r$ . At this point, most of the radioactivity was now to be seen in the  $M_r$  30,400 form. This pulse-chase experiment was repeated seven times over the course of 12 mo. In all cases the same pattern of processing events was seen with radioactivity shifting sequentially during the chase from polypeptides of high  $M_r$  to those of low  $M_r$ , and then after 20–36 h back to a polypeptide of  $M_r$  30,400. In some instances, for example Fig. 3, no radioactivity was found in the polypeptides of 18,800 and 15,100 after the 3-h in vivo labeling period, and at 27 h chase some label remained in the smaller polypeptides. For all pulse-chase experiments, as exemplified in Fig. 2*D*, the pattern of processing of ConA species was visible both in the fluorograph and in the equivalent Coomassie Blue-stained gel (Fig. 2*B*) of the immunoprecipitates. In particular, the polypeptide of  $M_r$  33,500 declined rapidly in amount over the incubation period, despite the observation that the component is maintained for many weeks in seeds maturing on the parent plant. This indicated that the incubation conditions, involving the culture of isolated cotyledons, may have shifted the tissue from a steady-state, e.g., by deprivation of nutrient inflow from the parent plant.

### Structural Relatedness of the Different ConA Forms

Two-dimensional mapping of iodinated tryptic peptides can be used to establish structural relatedness between trace levels of potential precursor and final proteins forms such as those



**Figure 1.** Metabolic labeling of immature cotyledons with  $^{14}\text{C}$ -amino acids for 3 h analyzed by SDS PAGE. 200  $\mu\text{g}$  protein was loaded per lane. (A) Coomassie Blue-stained gel. (B) Fluorograph of gel shown in A. Seed fresh weights (milligrams): lane a, 10; lane b, 140; lane c, 300; lane d, 500; lane e, 600; lane f, 700; lane g, 1,200; lane h, 1,500. Lane i was prepared from mature cotyledons in a separate electrophoretic run. 10–15% gradient gels were developed under reducing conditions. Arrows correspond to 69,000, 30,000, and 14,300.  $M_r$  49,300 corresponds to the subunit of the storage reserve, canavalin.



detected in fluorographs of immunoprecipitates (27). We used this method to analyze each of the polypeptides visualized in the fluorograph shown in Fig. 2D. Since maps of polypeptides of identical  $M_r$  were consistently the same, only one representative example is shown in Fig. 4. Maps of five of the polypeptides of  $M_r$  33,500, 31,600, 30,400, 18,800, 17,800

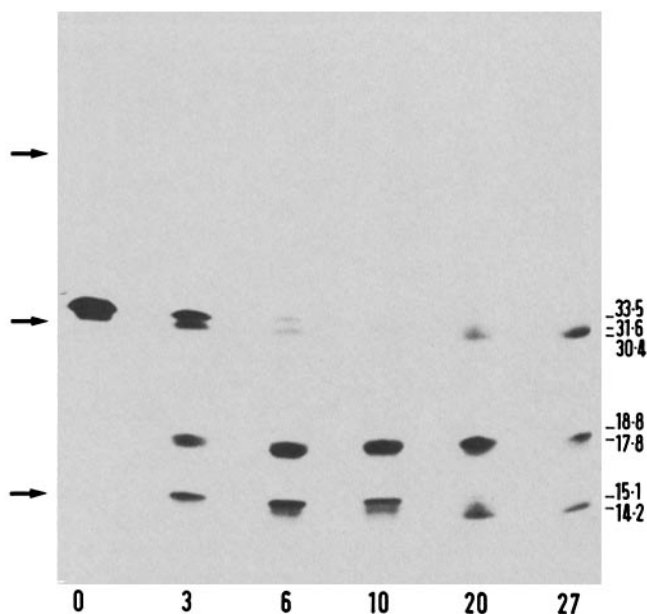


Figure 3. Metabolic pulse-chase labeling of immature cotyledons (seed fresh weight 660 mg) with  $^{14}\text{C}$ -amino acids for 3 h with 0–27 h chase, analyzed by SDS PAGE using 10–15% gradient gels under reducing conditions. A fluorograph of immunoprecipitated protein is shown. Arrows correspond to 69,000, 30,000, and 14,300.

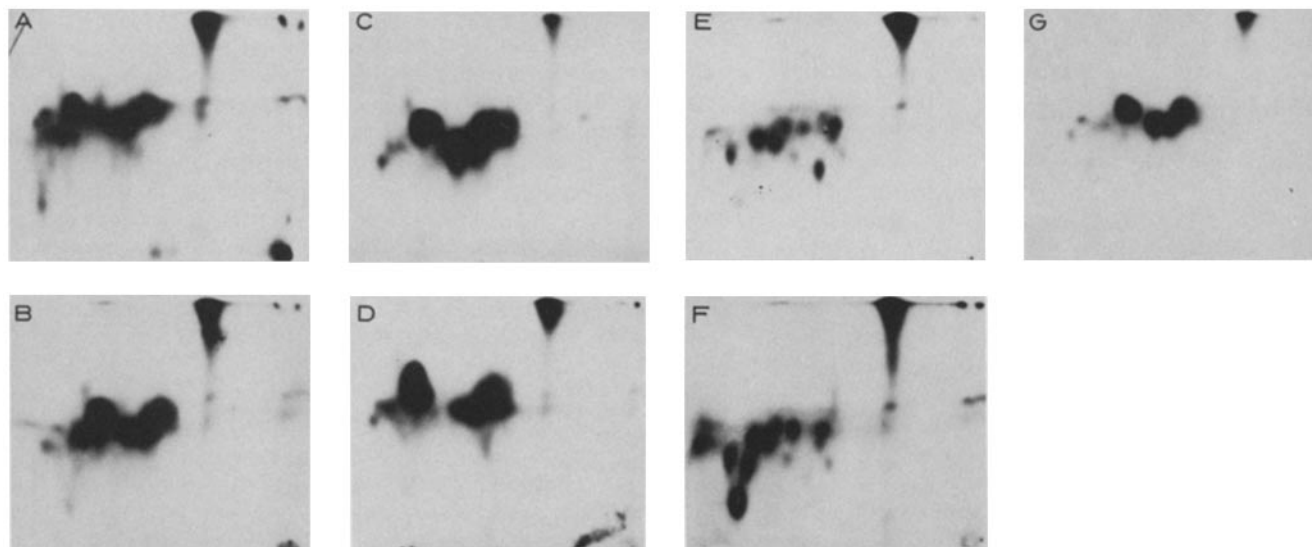


Figure 4. Analysis of  $^{125}\text{I}$ -peptide maps after in situ iodination of bands depicted in Fig. 2D and trypsin digestion. Autoradiographs of maps shown corresponding to: (A) 33,500, (B) 31,600, (C) 18,800, (D) 17,800, (E) 15,100, (F) 14,200, (G) 30,400.

Figure 2. Metabolic pulse-chase labeling of immature cotyledons (seed fresh weight 660 mg) with  $^{14}\text{C}$ -amino acids for 3 h with 0–26 h chase. Total protein (A and C) and immunoprecipitates (B and D) were analyzed by SDS PAGE and fluorography. (A and B) Coomassie Blue-stained gels; (C and D) corresponding fluorographs. 10–15% gradient gels were developed under nonreducing conditions. Arrows correspond to 69,000, 30,000, and 14,300.  $M_r$  49,300 corresponds to the subunit of the storage reserve, canavalin. Under nonreducing conditions, IgG is visualized as three bands, as depicted.

closely resembled one another and our previously published map of the ConA intact subunit (23). Although it was immediately clear that the polypeptides of  $M_r$  15,100 and 14,200 were related to one another, their relationship to the other five was less obvious. This dissimilarity could have arisen if the principal  $^{125}\text{I}$ -labeled peptides of  $M_r$  15,100 and 14,200 were only minor components in the other maps.

Our pulse-chase data had suggested that the production of the intact subunit of ConA involved the formation of a covalent bond between two smaller polypeptides, but the relative position of the two fragments in the polypeptide of  $M_r$  30,400 remained unknown. The only method of establishing unequivocally their relationship was the use of N-terminal sequencing. First, sequencing studies (Table I) on ConA polypeptides of  $M_r$  30,000, 16,200, and 14,200, immunoprecipitated and electroeluted from fully mature jackbeans such as those depicted in Fig. 1, lane i, confirmed that the species of lectin occurring in the jackbeans used in our study were identical to those studied by Edelman and co-workers (21, 73, 74, 76). Species of polypeptides coincident with the radio-labeled bands in the immunoprecipitates from pulse-chase experiments were then electroeluted and sequenced. In each case, only a single sequence was observed. As shown in Table I, the  $\text{NH}_2$ -termini of the 33,500, 15,100, and 14,200 precursors were all the same and corresponded to residues from 119 onwards in the mature intact subunit of ConA (21). Polypeptides at 18,800, 17,800, and 30,400 shared a different  $\text{NH}_2$  terminal sequence, which was that of the mature ConA subunit preceded by a four-residue extension. Therefore, the precursor that was first labeled in the pulse-chase began with an amino acid sequence that was subsequently found in the middle of the mature protein.

Table 1. Sequencing Studies on ConA Polypeptides

	Polypeptide	NH <sub>2</sub> -terminal sequence	Initial sequence yield*
	<i>M<sub>r</sub></i>		<i>pm</i>
Mature	30.0	ADTIVAVELD	250
	16.2	ADTIVA	185
	14.2	STHETN	115
Immature	33.5	STHETNALHFMF	25
	18.8	AAYNAD	90
	17.8	AAYNAD	30
	15.1	STHETN	25
	14.2	STHETN	35
	30.4	AAYNADTIVA	45

\* For each sample a plot of Log PTH yield versus residue number was constructed, and a line was fitted by regression analysis (phenylthiohydantoin-Ser, phenylthiohydantoin-Thr excluded). The initial sequence yields were estimated from the intercept. Ave. repetitive sequence yields (derived from the slope) were typically >96%.

### Effect of Drugs on the Intracellular Transport and Processing of ConA Precursors

Several agents including ionophores and lysosomotropic agents have been particularly useful for the delineation of intracellular traffic routes in mammalian cells. Monensin, a carboxylic ionophore, is known to intercalate into lipid bilayers, permit transmembrane exchange of protons for monovalent cations, and thereby affect any intracellular traffic routes dependent on the maintenance of proton gradients (reviewed in reference 65). Although the ionophore affects a variety of cellular functions, one of its primary sites of action is the Golgi complex, where it has been shown to interfere with terminal glycosylation and transport in and out of the dictyosomes. Incubation of jackbean cotyledons in the ionophore before and during metabolic pulse-chase labeling inhibited conversion of the polypeptide of *M<sub>r</sub>* 33,500 to any other molecular species (Fig. 5).

We used immunogold labeling with anti-ConA of thin sections of cotyledons incubated in the presence or absence of monensin to determine whether any morphological changes were induced by the ionophore. As shown in Fig. 6, *A* and *B*, at a seed fresh weight of 600 mg the cells of the immature cotyledons were highly vacuolated and contained a few large amyloplasts. In control tissue (i.e., that incubated in H<sub>2</sub>O or incubated in cycloheximide before monensin treatment) immunoreactive material was found associated with the endoplasmic reticulum and electron-dense deposits within protein bodies (Fig. 6 *C*). Only background levels of staining were located in the cell walls or cytoplasm. After monensin treatment, pockets of electron dense and immunoreactive material were found also outside the cell between the plasma membrane and cell wall (Fig. 6 *D*). Since the changes induced by monensin were prevented by prior incubation in cycloheximide, the immunoreactive material directed to the cell surface in monensin-treated cells was probably synthesized during incubation in the ionophore. Lysosomotropic agents such as the weak bases chloroquine and NH<sub>4</sub>Cl raise the pH of acidic intracellular compartments in mammalian cells, thereby affecting the function of such organelles as endosomes and lysosomes and processes such as receptor recycling (reviewed in reference 25). The pH of the protein body in the developing seed is unknown, but since the organelle is derived from the vacuole, and many of the hydrolases that accumulate

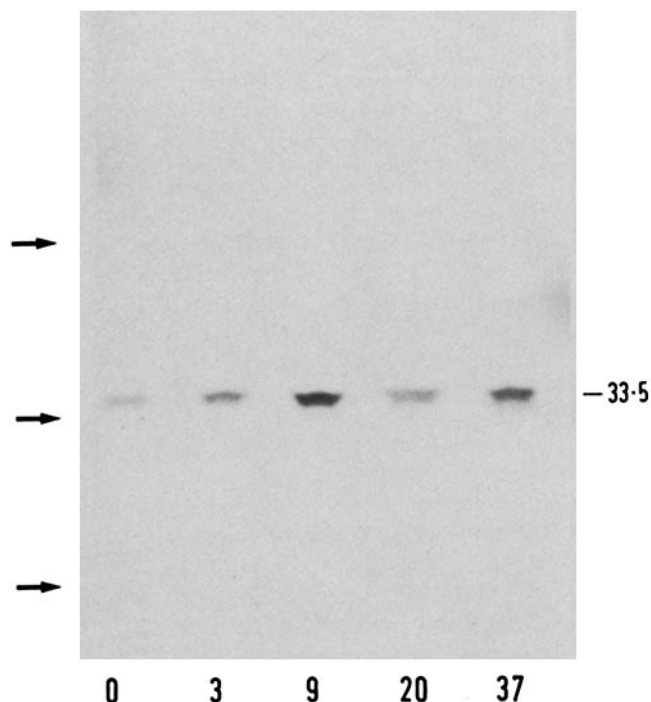


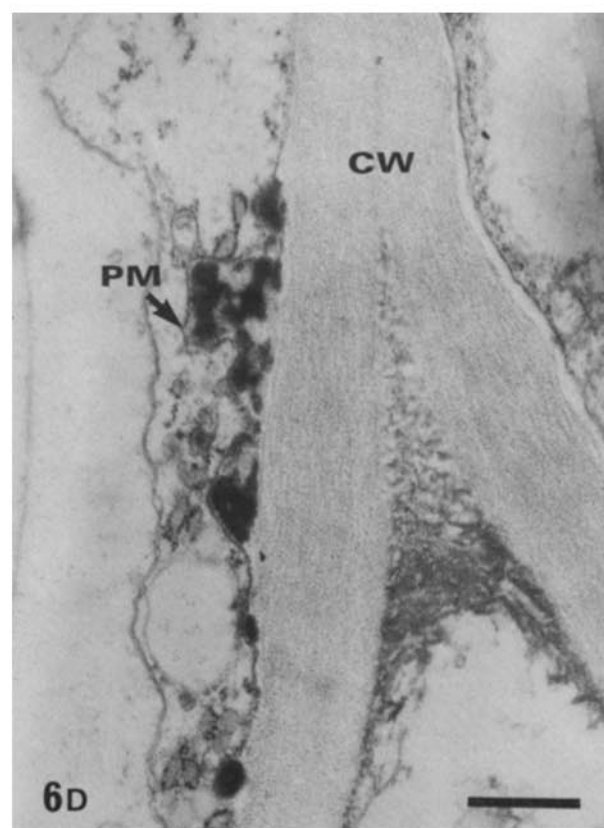
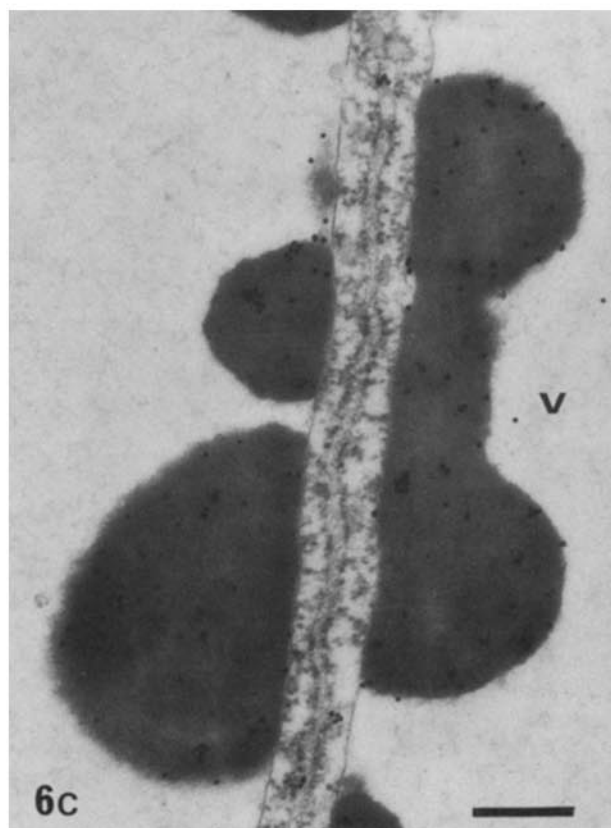
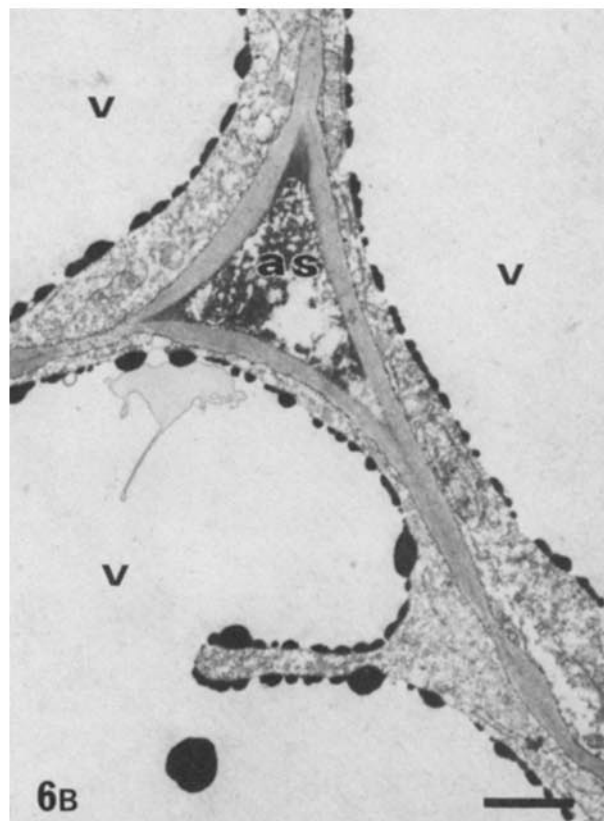
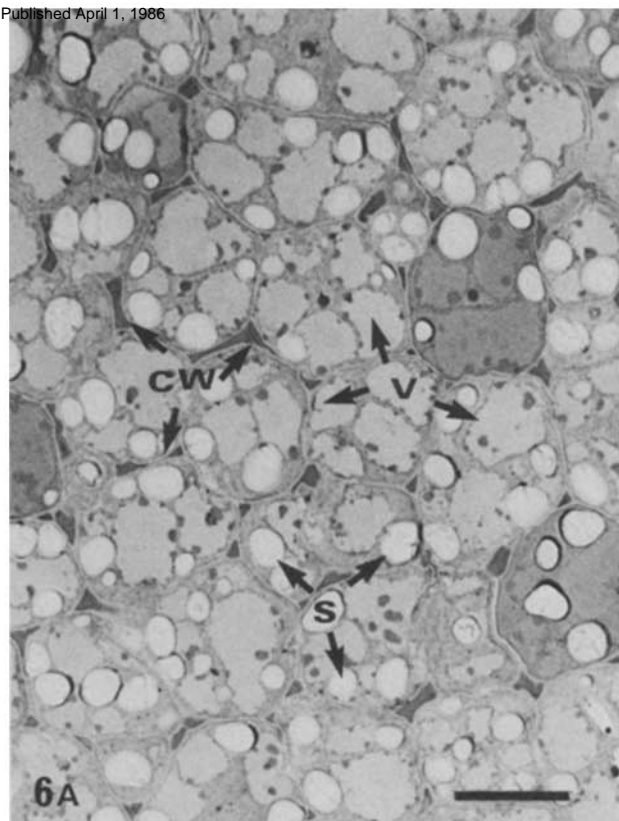
Figure 5. Metabolic pulse-chase labeling of immature cotyledons (seed fresh weight 600 mg) with <sup>14</sup>C-amino acids for 3 h with 0–37 h chase after preincubation in 10  $\mu$ M monensin and co-incubation of ionophore throughout. Protein was immunoprecipitated with anti-ConA/protein A-Sepharose and analyzed by SDS PAGE using 10–15% gradient gel under nonreducing conditions. The gel was stained with Coomassie Blue, destained, and subjected to fluorography. The fluorograph is shown, and the single radiolabeled band co-migrated with the *M<sub>r</sub>* 33.5 K polypeptide as visualized with the dye. Arrows correspond to 69,000, 30,000, and 14,300.

in that compartment as well as in the protein bodies exhibit an acidic pH optimum (6, 56, 68), the luminal pH of the protein body may be maintained by a proton pump analogous to that of the vacuolar tonoplast (reviewed in reference 52) and acidic compartments of mammalian cells (31, 79). When jackbean cotyledons were incubated before and during the pulse-chase in either chloroquine or NH<sub>4</sub>Cl, the processing of the ConA precursor of *M<sub>r</sub>* 33,500 was unaffected in that the pattern and dynamics of its conversion to other species were as shown in Fig. 2. Two other drugs, vinblastine and colchicine, were also without effect.

### Carbohydrate-binding Activity and Glycosylation of ConA Precursors

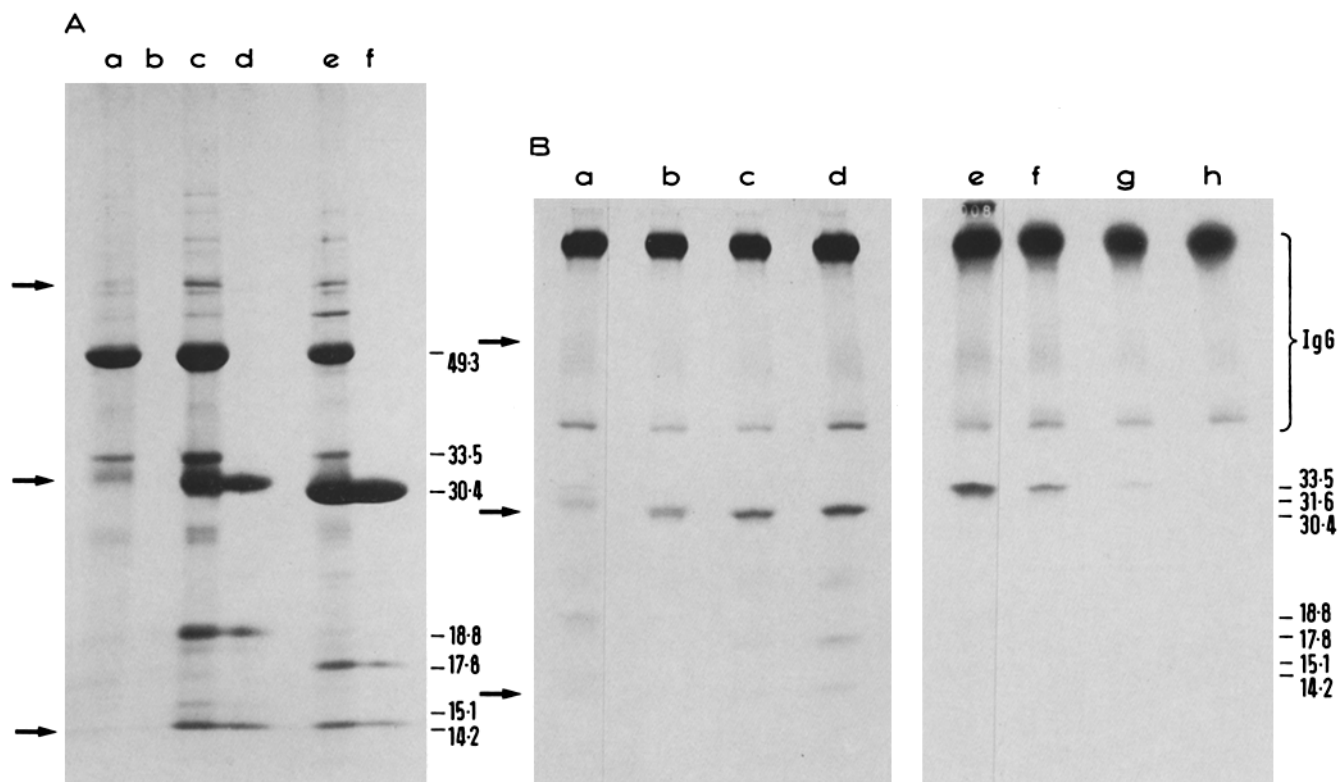
We used affinity precipitation with ovalbumin-Sepharose to determine which abundant precursor forms of ConA exhibited carbohydrate-binding activity. As shown in Fig. 7 *A*, the polypeptide of *M<sub>r</sub>* 33,500 was not recovered in the affinity precipitates (Fig. 7, lanes *a–f*), whereas those corresponding to *M<sub>r</sub>* 30,400, 18,800, 17,800, 15,100, 14,200 (Fig. 7, lanes *c* and *d*) and *M<sub>r</sub>* 30,000, 16,200, and 14,200 (Fig. 7, lanes *e* and *f*) could interact with the immobilized glycoconjugate. <sup>125</sup>I-ConA overlays of SDS PAGE analyses were used to determine which of the lectin precursors were ConA-binding polypeptides (Fig. 7 *B*). Lectin species were immunoprecipitated from immature cotyledons (660 mg fresh wt), pulsed with <sup>14</sup>C-amino acids for 3 h (Fig. 7, lane *a*), cotyledons of





**Figure 6.** (A) Light micrograph of a section through an immature cotyledon showing thin-walled (cw) parenchyma cells containing vacuoles (v) and amyloplasts (s). Bar, 100  $\mu$ m. (B) Electron micrograph of a thin section of the same cotyledon, showing part of three cells with dark staining protein deposits within the vacuoles (v) and airspaces between the cells (as). Bar, 2.5  $\mu$ m. (C) Electron micrograph of similar tissue to that in B. This section has been stained with anti-ConA goat anti-rabbit-adsorbed 20-nm gold (antiConA Gar-G20). The gold-labeled antibody has bound to electron-dense material within the vacuoles and also to material within a strand of rough endoplasmic reticulum, which in this section lies in the cytoplasm between the vacuoles. Bar, 0.5  $\mu$ m. (D) Electron micrograph of a thin section of immature cotyledon tissue fixed after 6 h of incubation in the presence of monensin. Electron-dense material is visible in a space between the cell wall (cw) and the plasma membrane (pm) of the left-hand cell. Anti-ConA GAR-G20 binds to this material. Export of immunoreactive material in this way is never observed in any of the three control experiments to this series. Bar, 1.5  $\mu$ m.





**Figure 7.** SDS PAGE analysis of ConA polypeptides using affinity precipitations and  $^{125}\text{I}$ -ConA overlays. 10–15% gradient gels were developed under reducing conditions. (A) Extracts prepared from seed fresh weights (milligrams): lanes *a* and *b*, 550; lanes *c* and *d*, 1,200; lanes *e* and *f*, 2,300, and affinity-precipitated with ovalbumin-Sepharose. (*a*, *c*, and *e*) Total extracts. (*b*, *d*, and *f*) Corresponding affinity precipitates. A Coomassie Blue-stained gel is shown. (B) Extracts prepared from immature and dry cotyledons, immunoprecipitated with anti-ConA/protein A-Sepharose and gels stained with Coomassie Blue (lanes *a*–*d*) and overlaid with  $^{125}\text{I}$ -ConA (autoradiographs in lanes *e*–*h*). Fresh weights (milligrams) used were lanes *a* and *e*, 560; lanes *b* and *f*, 2,100; lanes *c* and *g*, 2,300; and lanes *d* and *h*, dry mature seed.

2,100 mg (Fig. 7, lane *b*), those of 2,300 mg (Fig. 7, lane *c*), and dry mature cotyledons (Fig. 7, lane *d*). These samples contained all of the abundant precursor forms as well as the lectin species of the mature seed. Fig. 7, lanes *e*–*h* contain autoradiographs of the tracks shown in lanes *a*–*d* after  $^{125}\text{I}$ -ConA overlays. Only the species of  $M_r$  33,500 carried an oligosaccharide(s) recognized by ConA and by late stages of development quantities of this precursor had substantially decreased (Fig. 7, lane *g*) until by full maturity none remained (Fig. 7, lane *h*).

### ***The Lack of Necessity of Precursor Unfolding During Assembly of ConA***

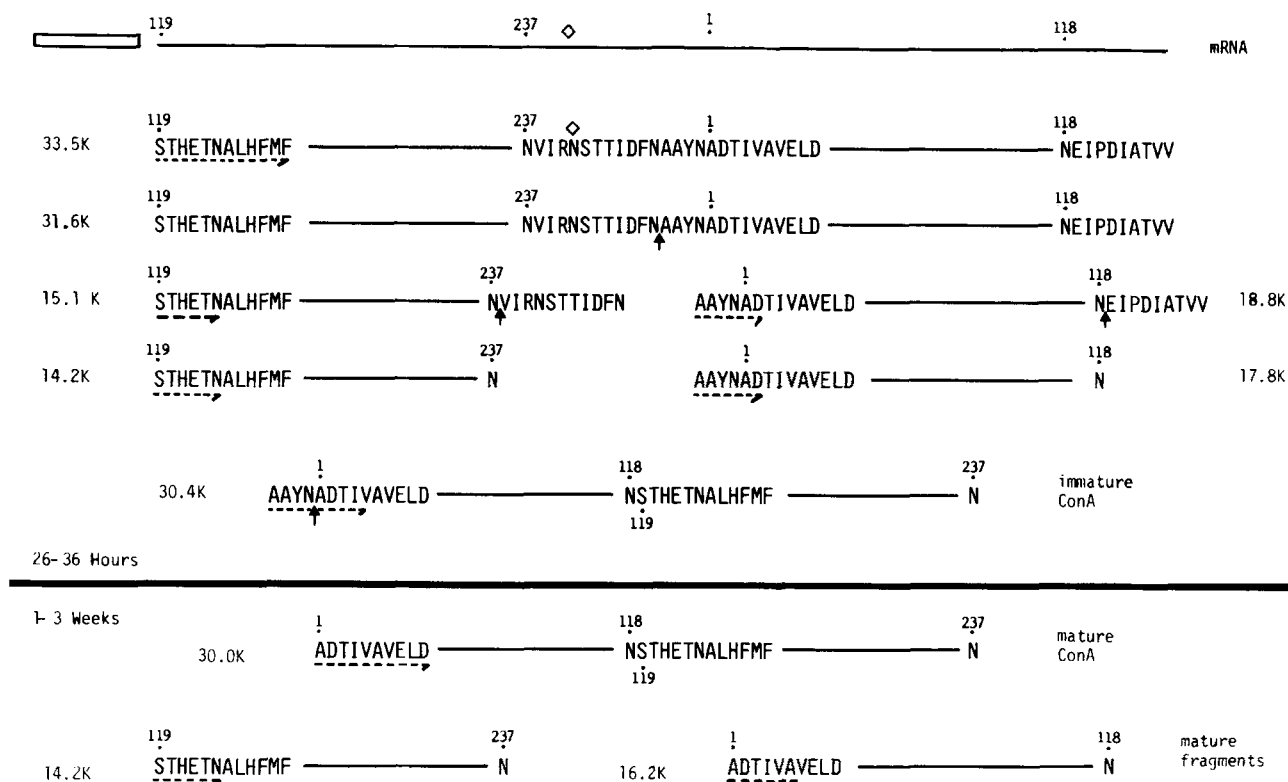
The  $\text{NH}_2$ -terminus of the ConA precursor of  $M_r$  33,500 first labeled in our pulse-chase analysis is identical to that recently predicted from the mRNA sequence for the primary translational product after removal of the putative signal peptide (11). This indicates that processing events for which we have evidence can be considered both within the context of the entire predicted sequence of the first precursor form of the lectin as well as the relevant features of the final protein. The proposed identities of the precursors involved in the formation of ConA are shown in Fig. 8, with amino acid sequences that we have positively identified underlined (see Table 1).

Since the  $\text{NH}_2$ -terminus of the polypeptide of  $M_r$  33,500 contained a sequence found at residue 119 onwards in the mature protein, and since the pulse-chase showed that the fragments were direct precursors of that protein, it initially

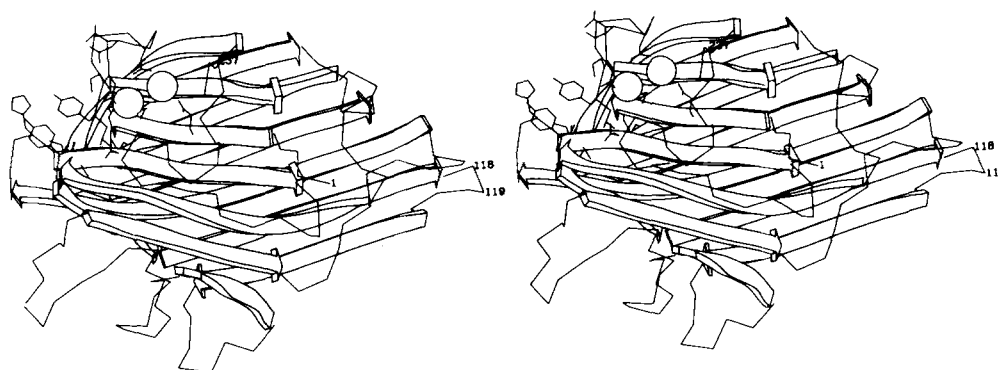
seemed probable that a posttranslational transposition of the fragments had occurred. A consideration of the known structure of the mature ConA monomer, however, suggests that this apparent “reverse” rearrangement of the two polypeptide chains may not require extensive refolding.

A stereo view of the tertiary folding of the mature ConA monomer is shown in Fig. 9. The monomer is a dome-shaped molecule  $\sim 42 \times 40 \times 39 \text{ \AA}$  whose structure is dominated by two large antiparallel  $\beta$ -sheet structures comprising slightly over 50% of the residues of the whole protein. The  $\text{NH}_2$ - and  $\text{COOH}$ -termini of the mature protein (residues 1 and 237) lie on the forward surface of the molecule ( $\sim 18 \text{ \AA}$  apart), and residues 118 and 119 form part of an exposed  $\beta$ -bend structure towards the bottom right. The two metal ions ( $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$ ) lie in a depression in the top left-hand quadrant of the protein which forms part of the sugar binding site (32). Included in the view are the side chains of those residues thought to be involved in the metal and carbohydrate-binding activity (32).

An important constraint on the folding process (noted in reference 60) was that the molecule must be considered as a single folded domain. In addition, the frequent occurrence of adjacent strands of  $\beta$ -structure from widely separated portions of the linear amino acid sequence tends to indicate that the structure has folded in a continuous process. This interpretation severely constrains the degree of three-dimensional rearrangement that the molecule may undergo as a result of posttranslational processing and in effect implies that any



**Figure 8.** Proposed identities of the precursor forms of concanavalin A: residue numbers correspond to residue positions in the mature ConA-monomer. Arrows indicate cleavage sites for the asparagine endopeptidase.  $\diamond$ , proposed site of glycosylation. Residues positively identified by  $\text{NH}_2$ -terminal sequence analysis are underlined (---). The initial mRNA translation product (mRNA) derived from reference 11 is shown schematically at the top. Residue 119 is preceded by a 29 residue signal peptide ( $\square$ ) that is cleaved immediately after co-translational segregation.



**Figure 9.** Stereo view of mature ConA monomer. The view was drawn from the crystal coordinates supplied in reference 60 using the program of reference 45. The twisted arrows denote strands of  $\beta$ -sheet and the two metal ions ( $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$ ) are drawn as closed circles. Also included in the view are those side chains thought to be involved in metal and carbohydrate binding activity (32).

"precursor" form of the protein probably has already assumed a conformation close to that of the mature molecule.

In terms of three-dimensional folding, the ConA precursor of  $M_r$  33,500 may be represented schematically as in Fig. 10. Here, the free  $\text{NH}_2$ -terminus of the precursor molecule starts at position 119 of the mature protein. The chain continues through the molecule to residue 237 (the COOH-terminus of the mature protein) which is linked to residue 1 by a 15-residue section of peptide which may form an extended surface loop and which contains a coding sequence for N-linked glycosylation (Fig. 8). The chain continues through the structure to residue 118 where there is an additional extension of nine residues to the final COOH-terminus of this precursor molecule. The validity of this proposed structural arrangement is strongly supported by the observation that the structure of the two-chain pea lectin, which shows direct linear

sequence homology at this stage, very closely matches the overall tertiary folding of ConA (53).

A consideration of the data in Figs. 2, 3, and 7-10 suggests the occurrence of the following events: (a) The  $M_r$  33,500 precursor is processed within hours to a polypeptide of  $M_r$  31,600. This species appears to represent a very transient processing step and insufficient material could be obtained for direct  $\text{NH}_2$ -terminal sequence analysis. Processing may involve only the removal of glycosyl residues, possibly from the surface loop connecting the terminal residues of the mature protein, since overlay experiments indicated that only the  $M_r$  33,500 form could be recognized by ConA. (b) The molecule is proteolytically cleaved to yield two polypeptide chains of  $M_r$  18,800 and 15,100. This process would require only a single cleavage within the surface loop connecting residues 237 and 1 of the mature protein and appears to

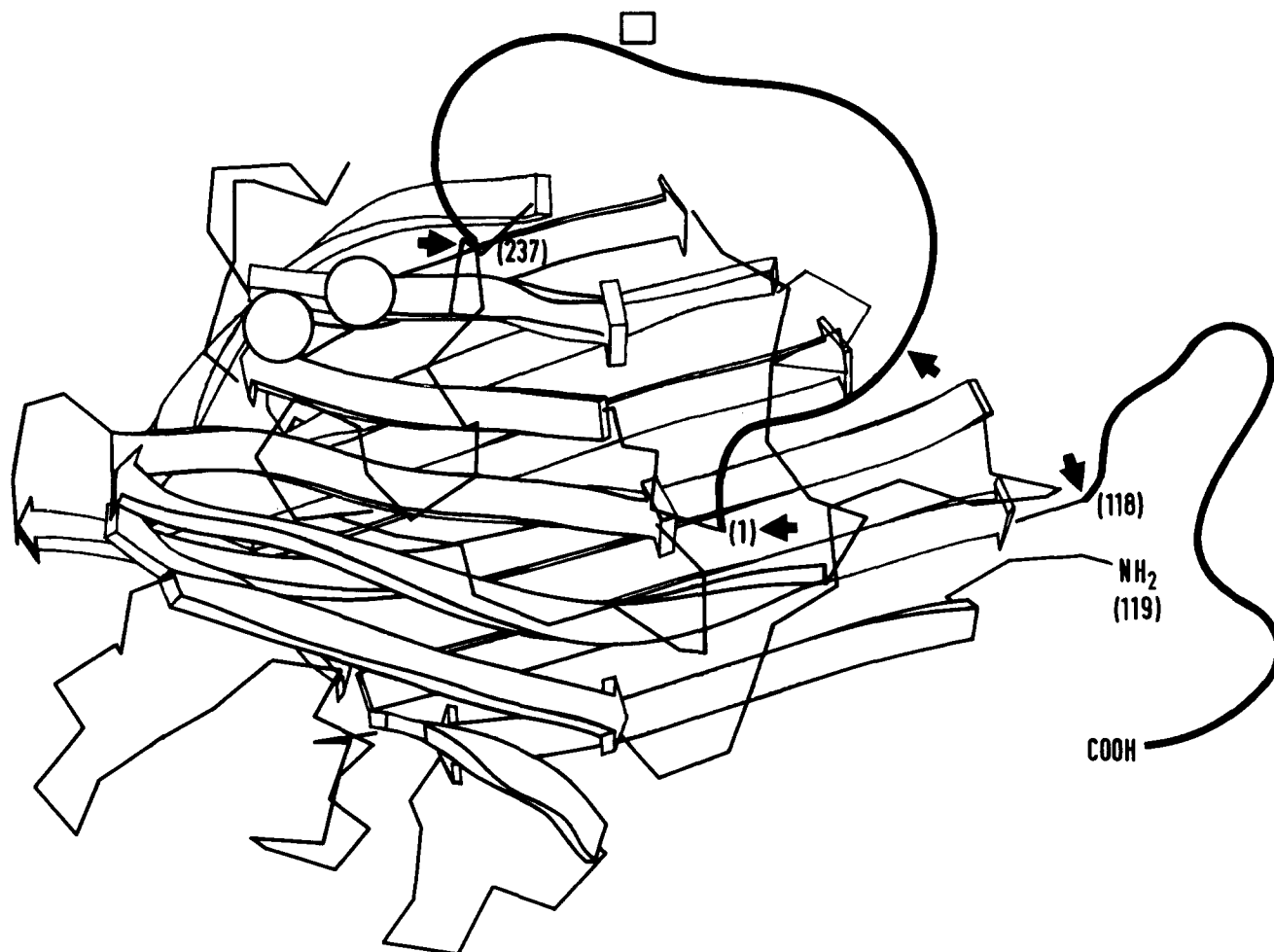


Figure 10. Hypothetical structure for the  $M_r$  33,500 precursor of ConA. Numbers correspond to residue positions in the mature ConA monomer. Arrows indicate the approximate positions of cleavage by the asparagine endopeptidase (refer to Fig. 8 for more details). The proposed location of the oligosaccharide side chains is also marked (◊).

activate the carbohydrate-binding property of the protein since all forms other than those of  $M_r$  33,500 and 31,600 can be found in affinity-precipitates with ovalbumin-Sepharose. It should be noted that after this and subsequent cleavages, the two separate chains probably remain in a stable, noncovalent folding which closely resembles the overall tertiary structure of the mature protein and which can bind carbohydrate. (c) Further proteolytic processing events then occur, all of which involve a single endopeptidase specific for the peptide bond COOH-terminal to an asparagine residue. The occurrence of processing at such a cleavage site has been shown for several legumes (reviewed in reference 36), and the removal of the short (9–11 residue) peptides from the COOH-termini of both fragments is entirely consistent with the observed reduction in  $M_r$ . Major rearrangements in the tertiary folding of the polypeptide chain would not be necessary, and the two polypeptide fragments remain in a folded but noncovalent association. (d) Some 13 h after synthesis of the initial precursor, a covalent linkage is formed between the two polypeptide fragments at positions 118 and 119 of the mature protein. As shown in the proposed precursor structure, these residues may already be positioned reasonably near on one surface of the protein. The ligation produces a form of ConA that is very close to the mature protein but that retains

a four-residue extension  $\text{NH}_2$ -terminal to residue 1 (a form referred to in reference 11 as immature ConA). (e) Removal of the four-residue extension, once again by cleavage at the COOH-terminal side of an asparagine residue, seems to occur much more slowly over a period of weeks (51) and eventually yields the mature form of the protein described by Edelman and co-workers (4, 21, 60, 74).

## Discussion

ConA is a member of the family of legume lectins and shares particularly close sequence homology with the two-chain lectins of *Lens culinaris* and *Vicia faba* (22, 29, 30). A puzzling feature has been that maximum alignment can only be achieved by circular permutation of the amino acid sequence of the jackbean lectin, although the secondary structure of both fava and lentil lectin is analogous to that of ConA (22, 29). The typical assembly process for the two-chain ( $\alpha$ ,  $\beta$ ) lectins such as fava (33) and pea (38) is translation of an mRNA encoding both subunits to give rise to a polyprotein that is subsequently cleaved posttranslationally in the protein bodies to produce the  $\alpha$ - and  $\beta$ -chains. Polyproteins are also intermediates in the synthesis of oligomeric storage proteins where the translational product contains multiple copies of

the subunit sequence (reviewed in reference 36). Our data suggest that the assembly of ConA initially follows an identical processing route as the two chain lectins but that the two "fragment" polypeptides, rather than remaining as a noncovalently associated dimer, become annealed to one another through the formation of a peptide bond.

It had been speculated that genomic reorganization was responsible for the circular permutation of amino acid sequence found in ConA (22, 30), but the sequence of jackbean lectin cDNA, published while this work was being carried out, led the authors to propose that a posttranslational ligation was responsible (11). The initial  $M_r$  33,500 precursor we have identified contains an identical  $\text{NH}_2$ -terminal sequence to that predicted from the lectin cDNA and therefore confirms that the cDNA was derived from a translatable mRNA species. No evidence was given in reference 11 of ligation of fragments, nor was the possibility of multiple genes encoding ConA factually excluded. Evidence that the initial translational product was the direct precursor of both the mature lectin subunit and fragments was recently presented (35), but products formed after 24-h chase were the only species analyzed and no  $\text{NH}_2$ -terminal sequencing was carried out.

The synthesis of a polypeptide through the posttranslational ligation of precursors is an entirely novel means of assembling a protein, and led us to consider alternative explanations for our data. The most obvious is that some other message is present that gives rise to the  $M_r$  30,400 form of ConA. This could occur via a polypeptide intermediate that is rapidly processed and therefore does not accumulate.

The results obtained with monensin clearly argue against this suggestion. Ionophore-induced entrapment of the biosynthetic precursor at a specific point in the temporal sequence of processing, an effect identical to that shown for the insulin receptor (41), does not lead to recovery of any species other than the polypeptide of  $M_r$  33,500. This result, as well as those from the pulse-chase experiments, render it improbable that a lectin message coding directly for the  $M_r$  30,400 ConA polypeptide is only expressed late in the chase incubation.

Our evidence indicates that the point between residues 118 (Asn) and 119 (Ser) is the site in the intact subunit of ConA for the formation of a posttranslational peptide bond. This bond was previously identified as the site in the lectin at which cleavage would result in the formation of the two principal naturally occurring fragments (21). Since that section of the intact subunit sequenced normally when cyanogen bromide fragment F2 was analyzed by Edman degradation (74), it seems probable that a typical peptide bond is formed between residues 118 and 119.

The molecular mechanism used for the production of gramicidin S and tyrocidin offers one precedent for nonribosomal polypeptide synthesis (reviewed in reference 3). However, the complexity of this cytoplasmic process, involving an alternation of transthiolation and transpeptidation, and a requirement for ATP, suggests that it is an unlikely method for joining the two precursors of ConA within the lumen of the protein bodies.

We suggest the likelihood of an alternative energy-independent mechanism that is common in extracytoplasmic peptide formation in prokaryotes. From close inspection of the chase data it would appear that the  $M_r$  30,400 species is formed from the  $M_r$  18,800 and 14,200 polypeptides. This

implies that the cleavage of the nine residue peptide from the COOH-terminus of the  $M_r$  18,800 fragment and the formation of the peptide bond between residues 118 and 119 might occur simultaneously. This strongly suggests that the process is a transpeptidation event, rather than a temporal separation of cleavage and ligation. Although a transpeptidation reaction of this type has been described in detail for the synthesis of bacterial cell walls (40), it has not been reported to occur in higher organisms. If our supposition is valid, the subsequent occurrence of the  $M_r$  17,800 fragment, and the persistence of fragmented forms of the lectin through to seed maturity, may represent the continuing activity of the asparagine endopeptidase.

When the naturally occurring fragments of ConA were first described, it was suggested that oligomers expressing carbohydrate-binding activity could be formed from the intact subunits, from mixtures containing intact subunits and pairs of complementary fragments, or from fragmented species alone (26, 73). Lentil lectin and favin, the two lectins most closely related to ConA, are the molecular equivalent of the non-covalently associated ConA "fragments." This raises the possibility that the joining of the smaller polypeptides to give the single covalent chain of mature ConA is not relevant to the endogenous function of the lectin and may only reflect some feature of the environment of jackbean protein bodies (a compartment within which the lectin is stored for periods of up to 8–10 wk).

Of greater functional significance may be the activation of carbohydrate-binding activity on cleavage of the precursor molecule into two separate chains. Our results imply that elements (protein or carbohydrate) of the surface loop connecting the terminal residues of the mature protein are responsible for inhibiting sugar-binding activity. It is possible that elements of structure formed by this surface loop might physically obstruct access to the binding site, analogous to the protective function proposed for the folding of the polypeptide chain in proinsulin (61). Alternatively, removal of the strand could give rise to local refolding of the chain in this area to form the final "active" architecture of the metal and sugar binding domains. This latter process is directly analogous to the local refolding seen in the activation of chymotrypsin from its inactive precursor (77). Either way, the removal of this structure from the surface of ConA seems to be highly reminiscent of some form of zymogen activation.

Our experiments with monensin demonstrate that the activation process occurs when the precursor has reached the protein bodies. In the presence of ionophore, immunoreactive material, identified as the glycosylated  $M_r$  33,500 precursor, was secreted and accumulated between the plasma membrane and cell wall. The lack of processing of the ConA precursor in the presence of monensin is probably a direct consequence of its delivery into a compartment that does not contain the required proteinases. The molecular basis for this re-routing of a component destined for the protein bodies onto the secretory pathway is unknown. Immunogold labeling of pea cotyledons indicates that vicilin is also re-routed to the cell surface by monensin, but there is no evidence about which forms of the storage protein are secreted (16). Since one defined site of action of the ionophore is in the region of the *cis*-cisternae of the Golgi apparatus (66), acquisition of retention signals may be prevented, which could lead to passive

loss of components from the cell rather than an induced transfer to the protein body. In mammalian cells, monensin has multiple effects on the processing and transport of lysosomal enzymes, leading to the secretion of both precursor and mature forms at different rates for different proteins (59). In a variety of cell systems monensin-stimulated secretion is known to be  $\text{Na}^+$ -dependent and  $\text{Ca}^{2+}$ -independent (54, 58, 75), although there is also evidence to suggest that intracellular  $\text{Na}^+$  levels affect the mobilization of bound intracellular  $\text{Ca}^{2+}$  (49) and thereby influence indirectly secretory events exemplified by catecholamine release from chromaffin cells (64).

Since the endoplasmic reticulum membrane contains *N*-glycosylated components, not least the ribophorins (43), synthesis of an inactive ConA precursor might be necessary for successful transport of the protein from its site of co-translational segregation. It is also possible that activation of carbohydrate-binding activity is significant for the endogenous role of the lectin in the protein bodies. Jackbean maturation is characterized by a progressive decline in *N*-glycosylated polypeptides, until by maturity only one remains that is masked from any carbohydrate-specific interaction with the lectin (10, 51). This suggests that if the function of the sugar-binding sites of ConA in the protein bodies is to complex with endogenous polymeric glycoconjugates this is only important at a specific stage of cotyledon development.

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