Isolation and Characterization of Concanavalin A Polypeptide Chains*

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ABSTRACT: Two polypeptide chains were isolated from concanavalin A, the lectin from jack beans. The chains were separated on Amberlite CG-50 by stepwise elution with urea at pH 1.9. Chains I and II differed in electrophoretic mobility, molecular weight, and amino acid composition, but were identical in sequence for the first 13 residues of the N termini. A third chain was detected electrophoretically, but this component was difficult to isolate because it was labile in acid. It was concluded that chain II (mol wt 27,000) was the primary gene product, and that chains I (N terminus, mol wt 12,500) and III (C terminus, mol wt 14,000) were derived from it by chemical or enzymatic hydrolysis. The chains associate strongly, and the results suggest that the crystallographic asymmetric unit, the Con A protomer, may consist of either a single polypeptide chain of 27,000 molecular weight or a 1:1 mixture of complementary fragments.

Our principal interest in concanavalin A is in the correlation of chemical results with the crystalline structure, which is simultaneously being determined at Argonne (Hardman *et al.*, 1970, 1971; see also Greer *et al.*, 1970). Sumner (1919) first crystallized Con A.[1] Sumner and Howell (1936a) classified the protein as a phytohemagglutinin and proposed that the biological activity of Con A derived from the binding of carbohydrates. They also recognized the importance of bivalent cations (*e.g.*, Ca^{2+}, Mg^{2+}, Mn^{2+}) in the binding process (Sumner and Howell, 1936b). The saccharides, transition metal ions, and calcium ions are bound to the protein in three interacting sites (Yariv *et al.*, 1968; Kalb and Levitzki, 1968). Calcium ions can be bound only after the transition metal site is occupied, and occupancy of both sites is required before the binding of a saccharide like α-methyl D-glucopyranoside can occur. Each set of binding sites is associated with a protein unit which has a molecular weight of 27,000. The binding unit has the same molecular weight as the crystallographic asymmetric unit, which Hardman *et al.* (1971) refer to as a protomer.

In the electron density map at 4.25 Å, four protomers are clustered around the origin and around the center of the unit cell (Hardman *et al.*, 1971). The protomers are paired, with contacts between protomers of the same pair being more extensive than those in different pairs. Hardman *et al.* proposed that the presence of the four-protomer unit would account for the molecular weight of 96,000 obtained by Sumner *et al.* (1938) for Con A in solution at ~pH 6.7. Values given by Kalb and Lustig (1968) also averaged ~100,000 for the pH range of 6.7–7.8. From pH 3.5 to 5.8 they reported values of 55,000, corresponding to a two-protomer unit. Intermediate values found by Olson and Liener (1967a) and Agrawal and Goldstein (1967) probably reflect mixtures of the two-protomer and four-protomer units.

In the current article we describe efforts to dissociate Con A into single polypeptide chains. For correlation with the crystallographic study, it was of particular interest to determine the number of chains within each protomer, and to ascertain whether the sequences of the chains are identical or different.

Materials and Methods

Like hemoglobin, Con A can be dissociated in acidic solutions of urea. The method chosen for initial fractionation of the components of Con A therefore involved use of urea in a chromatographic system similar to that employed earlier for the separation of the α and β chains of hemoglobin (Chernoff, 1961; Wilson and Smith, 1959). Before the isolation of the polypeptide chains, special care was taken to maximize the purity of the Con A starting material.

Preparation of Concanavalin A. Con A was prepared by a modification of the method of Olson and Liener (1967a). The jackbean meal was obtained from the Sigma Chemical Co., St. Louis, Mo. Con A was extracted for 1 hr at 4° from 300 g of meal with 1.5 l. of 0.01 M Tris-HCl (pH 7.4), 0.001 M in $CaCl_2$ and $MgCl_2$. The suspension was centrifuged for 30 min at 8000 rpm in a Sorvall RC-2B refrigerated centrifuge. The residual meal was mixed with 750 ml of fresh Tris buffer and stirred for 30 min. After centrifugation the supernatant was combined with the first extract and adsorbed to Sephadex G-100 in a batch process. Approximately 800 ml of wet, packed Sephadex, previously equilibrated at pH 7.4 with Tris buffer, was added to the extract, and the mixture was stirred at 4° for 30 min. The gel was collected on a filter and washed with 4 l. of cold Tris buffer. The Sephadex was suspended in an equal volume of Tris buffer and poured into a chromatographic tube, in which the settled gel formed a 7 × 20 cm column. Tris buffer (about 8 l.) was passed through the column until the effluent contained less than 0.02 mg of protein. The Con A was then eluted with 0.2 N KCl–HCl (pH 2.3). The absorbance of the effluent at 280 nm was determined continuously with a recording LKB uv monitor. Fractions of 20 ml were collected.

The Con A was dialyzed successively against deionized water and 0.01 M Tris-HCl (pH 7.4), 0.001 M in $CaCl_2$ and $MgCl_2$. For additional purification, the solution was recycled

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[1] Abbreviations used are: Con A, concanavalin A; Temed, *N,N,N′,N′*-tetramethylethylenediamine; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin.

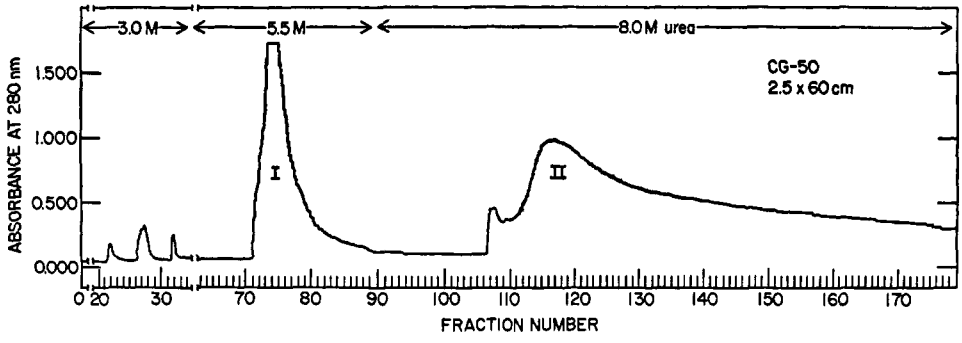

FIGURE 1: Separation of polypeptide chains of Con A on a 2.5 × 60 cm column of Amberlite CG-50. Chains I and II were eluted at pH 1.9 by the stepwise addition of urea, the concentrations of which are listed over the effluent curve. The first series of small peaks correspond mainly to nonprotein material from the CG-50 resin. Fractions of 12 ml were collected.

through a second 7 × 20 cm column of Sephadex G-100.

To remove both carbohydrate and metal ions from the protein, the column effluent was dialyzed at 4° against 0.1 M acetic acid, 10% saturated with EDTA. The volume of solution was decreased by pressure dialysis against deionized water until the concentration of protein was about 35 mg/ml. A small amount of precipitate was removed by centrifugation. The pH of the supernatant was 5.5.

To crystallize the protein, the solution was made 0.01 M in $MnCl_2$ and $CaCl_2$, allowed to stand at 22° for 30 min, and brought to 2 M in phosphate by the addition of an equal volume of 4 M sodium phosphate, pH 5.6 (Hardman *et al.*, 1971). After 2 weeks, the crystals were harvested by filtration and washed with 2 M phosphate on the filter. Samples containing about 30 mg of Con A/ml were recrystallized in 2 M phosphate.

Commercial samples of Con A (three-times crystallized) were obtained from Miles Laboratories, Elkhart, Ind.

Separation of Polypeptide Chains of Con A. To ensure removal of metal ions before chromatography, a more extreme procedure than that preceding crystallization was used. Lyophilized, salt-free Con A (2 g) was dissolved in 200 ml of 0.02 M HCl saturated with EDTA, and the pH was adjusted to 1.7. The protein was dialyzed exhaustively at 22° against HCl–EDTA of the same concentrations (*i.e.*, 2 days, ten changes of 4 l. each). The solution was dialyzed against distilled, deionized water at 4° and lyophilized.

The chains were separated on Amberlite CG-50. The processing of the resin to obtain particles 25 to 50 μ in "diameter" was described previously (Edmundson and Hirs, 1962; Edmundson, 1968). After successive treatment with 2 N NaOH for 2 hr and 4 N HCl for 1 hr, the resin was washed with water on a filter and equilibrated with 12% (v/v) formic acid. A 2.5 × 60 cm column was prepared at 22°.

An aliquot containing 1.0 g of demetalized Con A in 100 ml of 12% formic acid was driven into the column under nitrogen pressure (5 psi). Fractionation of the polypeptides was initially carried out with a linear gradient of urea from 3 to 8 M. This method was superseded by a stepwise procedure, in which the first eluate was 3 M urea, freshly prepared and adjusted to pH 1.9 with concentrated HCl. After 360 ml was forced through the column with a metering pump at a rate of 5 cm/hr, the 3 M urea was removed from the top of the column and replaced with 5.5 M urea (pH 1.9). When the tracing indicated that the first polypeptide chain had emerged, the concentration of urea was increased to 8 M to elute the second component.

The components from the CG-50 column were tested for purity by gel electrophoresis (see next section). The components were purified by recycling chromatography (ascending system) on a 3.3 × 92 cm column of Bio-Gel P-10. The eluent was 8 M urea (pH 1.9). Samples contained 60 mg of protein in 20 ml of 8 M urea (pH 1.9). The effluent was recycled two to four times, and the stream was diverted to a fraction collector when the absorbance tracing indicated that the appropriate resolution had been achieved.

Metal-free Con A was also fractionated on the Bio-Gel column.

At the suggestion of K. G. Mann, the dissociation of Con A in 6 M guanidine hydrochloride at pH 6.5 was examined by gel filtration on a 1.9 × 88 cm column of Sepharose 6B (Fish *et al.*, 1969). The components were tested for electrophoretic homogeneity before and after rechromatography on CG-50.

Removal of Urea and Salts. Urea and salts were removed from the components either by exhaustive dialysis against deionized water over a period of 4–5 days or by a column procedure with Amberlite CG-50 (Hirs, 1967).

Electrophoresis of Con A and Its Components. The purity of each sample of Con A was assessed by disc electrophoresis at pH 4.5 according to the method of Reisfeld *et al.* (1962). The gels were stained with Amido Schwarz and scanned with a Canalco Model E microdensitometer. The isolated polypeptide chains, as well as Con A, were subjected to polyacrylamide gel electrophoresis in freshly deionized 8 M urea (pH 3.3). To prepare the 5% acrylamide gels, the following solutions were combined in the order listed and in the ratio (v/v) of 1A:8B:7C:16D; solution A: 1.0 ml of *N,N,N',N'*-tetramethylethylenediamine diluted to 100 ml with 8 M urea, pH 7.0; solution B: 20 g of acrylamide, 0.8 g of *N,N'*-methylenebis(acrylamide) in 100 ml of 8 M urea, pH 7.0; solution C: 0.375 g of KCl and 1.25 ml of concentrated HCl in 1 l. of 8 M urea; final pH 3.3; solution D: 0.56 g of ammonium persulfate in 100 ml of 8 M urea, pH 7.0. Aliquots of 1.0 ml of the mixed solutions were polymerized in 0.5 × 8 cm tubes for 1 hr under fluorescent light.

Before application of the samples, the gels were run under a current of 5 mA/gel at room temperature for 2.5 hr or until the tracking dye (4-phenylazo-1-naphthylamine) had moved completely through the gel. The resistance of the gels stabilized during this period, and the pH dropped to that of the tray solution (solution C above) as the Temed was eluted from the gels (for a discussion of the effects of "prerunning" gels in the presence of urea, see Panyim and Chalkley, 1969). Samples were dissolved in solution C and stabilized with glucose or glycerol. They were allowed to enter the gels under a current of 2.5 mA/gel for 15 min, after which the electrophoresis was carried out at 5 mA/gel for 2 hr.

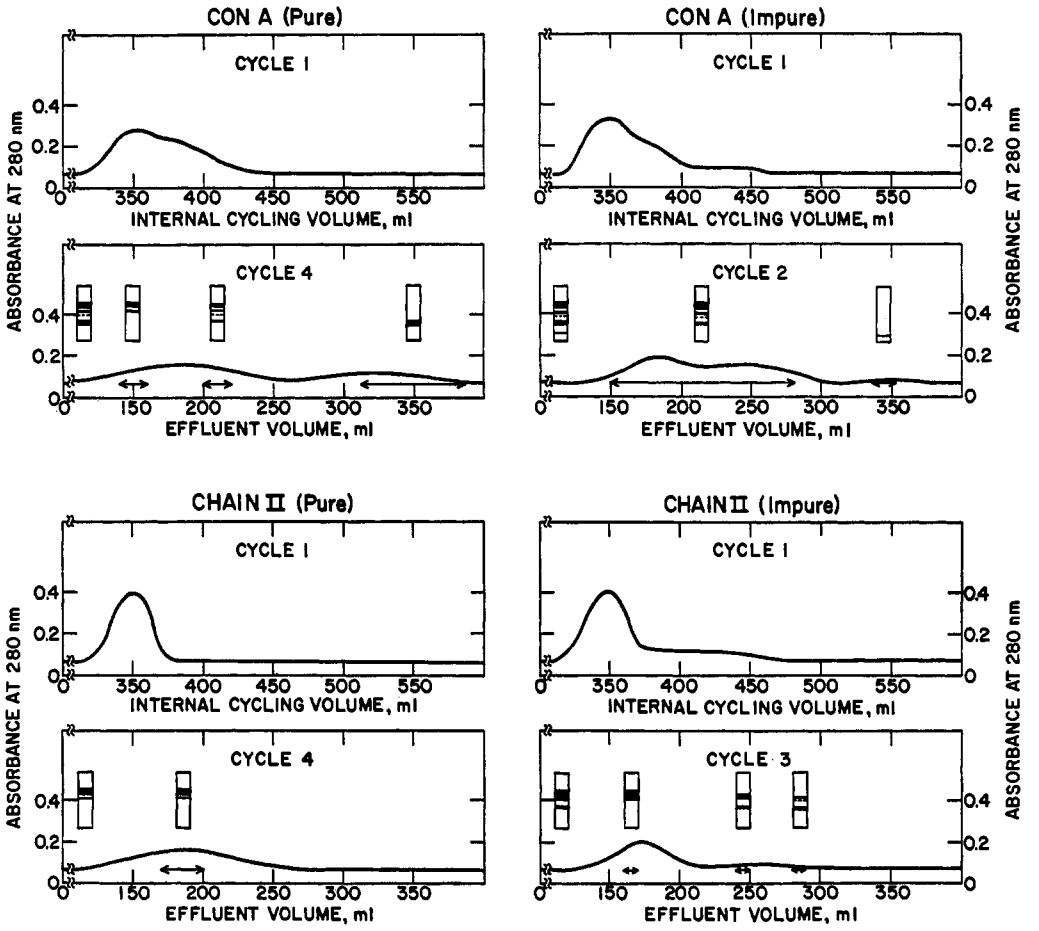

FIGURE 2: Recycling chromatography of Con A and chain II on a 3.3 × 92 cm column of Bio-Gel P-10, with pH 1.9, 8 M urea as eluent. The absorbance tracings for the first and last cycles are given for each sample. Electrophoretic patterns for components in 8 M urea, pH 3.3, are shown above the appropriate sections of the effluent curves. The patterns at the far left of the panels represent the gels for the starting material used in each fractionation.

Attempts to establish the molecular weights of the polypeptide chains by ultracentrifugal analysis have thus far been unsuccessful, principally because the chains tend to aggregate in dilute aqueous salt solutions. However, estimates were obtained from measurements of the electrophoretic mobilities in 10% acrylamide gels containing 1% (w/v) sodium dodecyl sulfate (Shapiro *et al.*, 1967; Weber and Osborn, 1969). All samples of the Con A components dissolved in the sodium dodecyl sulfate buffer, but the time required varied from 1 hr to 2 days.

Amino Acid and End-Group Analyses. Each polypeptide chain was subjected to quantitative amino acid analysis on a Spinco 120B instrument (Spackman *et al.*, 1958; Spackman, 1963). The tryptophan content was determined by the methods of Beaven and Holiday (1952) and of Bencze and Schmid (1957).

Amino end-group analyses (Edman and Begg, 1967) of 10-mg samples of Con A and its polypeptide chains were performed with an Illinois Tool Works (Illitron) sequenator, Sequenator programs were those of Edman and Begg (1967). and O. Smithies (1970, personal communication), as well as those developed in our laboratory (available upon request.).

The proteins were dissolved in trifluoroacetic acid, dried to a thin film in the cup of the sequenator, and coupled with phenyl isothiocyanate. The cleavage step was performed with a 3:2 mixture of heptafluorobutyric acid–trifluoroacetic acid. PTC-amino acids were converted to the PTH derivatives with 1 N HCl for 10 min at 80° (W. M. Fitch and C. Nolan, 1970, personal communication). Before identification on a Beckman GC-45 gas chromatograph, the PTH derivatives of lysine, serine, threonine, aspartic acid, and glutamic acid were converted to trimethylsilyl derivatives with *N,O*-bis(trimethylsilyl)acetamide by the procedure of Pisano and Bronzert (1969). In some cases, results of gas chromatography were verified by thin-layer chromatography on silica gel sheets by the methods of Jeppsson and Sjöquist (1967), and of T. Baldwin (1970, personal communication).

Results

Separation of Polypeptide Chains. The elution profile for the separation of Con A components is presented in Figure 1. As in the case of hemoglobin (Chernoff, 1961), the first series of peaks on the chromatogram correspond mainly to nonprotein material from the column of CG-50 resin. The principal peaks in Figure 1 represent two polypeptide chains, I and II, which are widely separated on the column. The recoveries of chains I and II from the column were 75–100% on a weight basis.

The effluent curves for the fractionation of Con A and chain II on Bio-Gel P-10 are shown in Figure 2. The polypeptide chain designated as II in the CG-50 system emerged first from the P-10 column. Recent commercial samples contained small amounts (~5%) of a low molecular weight (~9000)

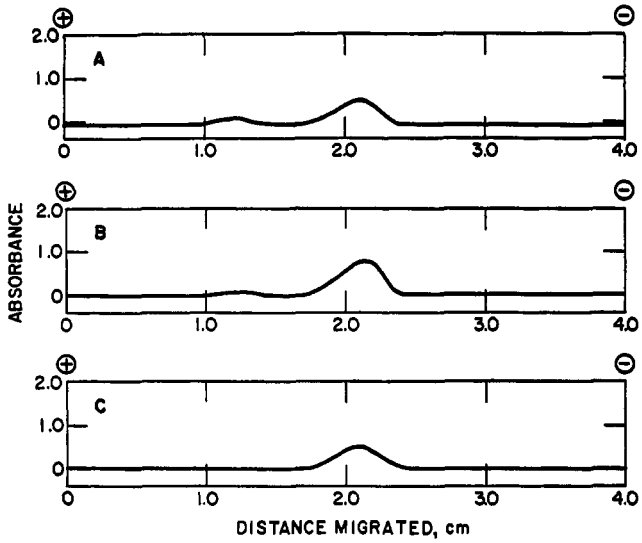

FIGURE 3: Microdensitometer scans of disc electrophoresis gels for Con A at pH 4.5. Panel A: Con A prepared by adsorption and elution from Sephadex; panel B: Con A prepared with Sephadex and crystallized once; and panel C: Con A crystallized three times. Absorbance of white light is plotted against length of gel in centimeters (4 cm on the scan is equal to 1 cm of the gel). The stacking gels were omitted from the scans.

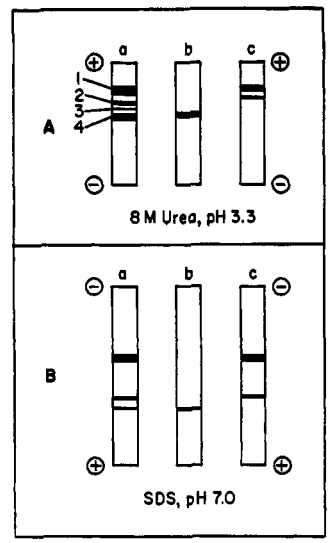

FIGURE 4: Gel electrophoresis of Con A and isolated chains. Panel A: gels containing 8 M urea, pH 3.3, prepared as described in the text. Samples: a, Con A; b, chain I; c, chains II and III. Panel B: gels containing 1% sodium dodecyl sulfate, pH 7.0 (Weber and Osborn, 1969). Samples: a, Con A; b, chain I; c, chains II and III. Proteins used to calibrate the gels included: ovalbumin (43,000), chymotrypsinogen (25,700), ribonuclease A (13,700), and cytochrome *c* (11,700). Samples were dissolved in 0.01 M sodium phosphate, pH 7.0, 1% (w/v) in sodium dodecyl sulfate and 0.1% (v/v) in 2-mercaptoethanol. The mercaptoethanol was omitted from Con A and its constituent chains, since these molecules are devoid of half-cystine (Olson and Liener, 1967a).

polypeptide which was eluted with both chains I and II in the CG-50 system. The removal of this contaminant on the Bio-Gel column is illustrated in the upper right panels of Figure 2. The separation of residual quantities of chain I from a sample of chain II is shown in the lower right panels. The chromatogram obtained with a pure sample of chain II is presented for comparison. Absorbance measurements before and after fractionation on Bio-Gel indicate recoveries of 90 to 100% of the polypeptide chains.

An additional component (III) remained associated with chain II in both the CG-50 and Bio-Gel procedures (see next section). When placed on a Sepharose column in 6 M guanidine·HCl, however, Con A was separated into chain II and a mixture of components I and III. The mixture of I and III could be resolved on CG-50. Chain III proved unstable in the acidic urea solutions and could not be isolated in sufficiently pure form for reproducible amino acid analysis (see following section).

Electrophoresis of Con A and Its Components. Microdensitometer scans of the stained gels after electrophoresis of various Con A samples at pH 4.5 are shown in Figure 3. Con A prepared by adsorption and elution from Sephadex alone was contaminated to the extent of about 10% (see panel A in Figure 3). The impurities were largely removed by crystallizing the protein once (panel B in Figure 3). The major component in samples crystallized more than once (panel C in Figure 3) comprised $\geq 95\%$ of the total protein.

Representative electrophoretic patterns obtained with Con A and its components in urea and sodium dodecyl sulfate are shown in Figure 4.

In 8 M urea at pH 3.3, Con A was separated into three major bands and one minor band (sample a in Figure 4A). The protein was distributed among the bands as follows: band 1 = 47%, bands 2 and 3 together = 20%, and band 4 = 33%. When the chains isolated from CG-50 columns were run under the same conditions, chain I (sample b in Figure 4A) migrated as one band, corresponding to band 4 of Con A. Chain II (sample c in Figure 4A) appeared as two bands, the principal one (77%) in approximately the same position as band 1 of Con A, and the second (23%) corresponding to band 2 (chain III).

In sodium dodecyl sulfate gels, Con A was separated into three components, with molecular weights calculated to be 27,000, 14,000, and 12,500 (sample a in Figure 4B). Chain I usually appeared as a single band with a molecular weight of 12,500 (sample b in Figure 4B). Chain II migrated as two bands with molecular weights of 14,000 and 28,000 (sample c in Figure 4B).

When dissociated from component III in guanidine·HCl and separated on Sepharose, chain II was electrophoretically homogeneous in SDS gels. After it was allowed to stand for 24 hr at 22° in 8 M urea, pH 1.9, and again subjected to electrophoresis in SDS gels, chain II gave a multibanded pattern. Components I and III were present in approximately equal amounts (together representing ~15% of the total). Chain II was still the predominant component (80%), but minor bands (<5%) with molecular weights of 24,000, 18,000, and 9000 were also observed. Crystals freshly prepared by the method used by Hardman *et al.* (1971) for X-ray diffraction studies gave patterns indistinguishable from that just described, with the exception that the relative quantity of chain II varied from 60 to 75% of the total.

Component III became progressively more heterogeneous during the fractionation procedures. Three additional bands (totaling ~20% of the protein) were detected in an SDS gel after an attempt to purify this component on CG-50.

Amino Acid and End-Group Analyses. The amino acid compositions of chains I and II are given in Table I. The results are expressed as molar ratios based on molecular weights of 12,500 and 27,000, respectively.

The sequence of the N-terminal segment of chain I is: Ala-

TABLE I: Amino Acid Composition of Polypeptide Chains I and II.

Amino Acid	Chain I	Chain II
Tryptophan	3.02	5.53
Lysine	8.04	13.1
Histidine	2.09	6.27
Arginine	2.67	6.44
Aspartic acid	16.8	34.6
Threonine	10.1	20.5
Serine	14.0	30.8
Glutamic acid	4.98	13.1
Proline	4.62	11.7
Glycine	6.14	17.1
Alanine	7.71	19.1
Half-cystine		
Valine	10.1	18.2
Methionine	0.97	1.98
Isoleucine	6.61	15.8
Leucine	8.12	19.7
Tyrosine	5.07	7.35
Phenylalanine	2.04	11.3
Integral no. of residues	114	253
Calculated molecular weight	12,504	27,458

Asp-Thr-Ile-Val-Ala-Val-Glu$\begin{matrix}\text{-Ile-}\\\text{-Leu-}\end{matrix}$Asp-Thr-Tyr-Pro. Chain I was degraded for as many as 36 steps, but after stage 13, some hydrophilic residues could not be accepted with certainty because of the rapid decrease in yields of the PTH derivatives. N-Terminal analysis of samples of chain I containing the low molecular weight impurity (see Figure 2) revealed a proline end group in addition to PTH-alanine. The yields of the proline derivative were 5–10% of the theoretical value.

The sequence of the first 13 residues in the N-terminal segment of chain II is identical with that of chain I.

In a sample of chain III in which the degradation products were at a minimum, the N terminus was blocked in at least 90% of the molecules.

Discussion

Highly purified Con A, suitable for both X-ray diffraction and chemical studies, can be prepared by the combination of adsorption and elution from Sephadex and subsequent crystallization. The protein can be separated into three principal chains which differ in molecular weight, electrophoretic properties, and amino acid composition. To achieve maximum purity and yields of the isolated chains, it was necessary to remove bound carbohydrate and metal ions from Con A before transfer to an Amberlite CG-50 column.

Chains I and II could be separated on Bio-Gel P-10, but in smaller quantities than those conveniently handled with Amberlite CG-50. The P-10 columns proved most useful in freeing component II of small amounts of component I or in removing a low molecular weight contaminant from Con A. Complete separation of components II and III by column chromatography alone required the presence of guanidine · HCl.

After dissociation, both chains II and III became more susceptible to cleavage in acidic solutions of urea. It is unlikely that this cleavage is mediated by enzymes, which would have to be sufficiently stable to survive the fractionation procedures and treatment with guanidine and urea. Peptide bonds that are labile in acid tend to involve hydroxy amino acids (serine and threonine) and dicarboxylic acids (aspartic and glutamic acid) and their amides (asparagine and glutamine). The end groups liberated by cleavage of chain II are consistent with the expected products. Recent evidence indicates that asparagine is the C-terminal residue of chain I. The blocked end group of component III suggests the presence of N-terminal glutamine, which has cyclized to pyrrolidonecarboxylic acid. The present results do not rule out enzymatic hydrolysis for the heterogeneity in SDS gels of samples of Con A not previously exposed to acids or denaturants.

The tendency of polypeptide chains II and III to associate is reflected in the electrophoretic patterns. From a comparison of the gels we concluded that the three major bands for Con A in 8 M urea probably represented the primary gene product, chain II, and two fragments, I and III, derived from it. The sequence results indicate that chain I is the amino-terminal segment of the intact subunit. The combined molecular weights of components I and III are identical with that of component II, and the release of equal quantities of the two components from chain II further suggests that component III represents the carboxyl part of the parent chain.

With the exception of crystals of the type used for diffraction, all commercial and Argonne samples of Con A contained a significantly larger quantity of component I than III. The ease with which chain III is degraded into smaller fragments, particularly in acidic solutions, probably accounts for this discrepancy.

The molecular weight of 27,000 for chain II or the combination of chains I and III closely agrees with the estimated mass of the crystallographic asymmetric unit (Hardman *et al.*, 1970, 1971) and is consistent with the molecular weight of 55,000 for Con A at pH values below 5.8 (Kalb and Lustig, 1968). We concluded that, within any one crystal, the crystallographic asymmetric unit may contain either an intact polypeptide chain (II) or a 1:1 mixture of two fragments (I and III). These two forms of the Con A protomers are isomorphous. In view of the marked tendency of chains II and III to associate, it is not surprising that two protomers interact strongly to form the 55,000 molecular weight complex.

Acknowledgments

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Tritiation of Tryptophyl Residues in Proteins*

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ABSTRACT: *S*-Carboxymethyl-lysozyme, myoglobin, and wool keratin were treated with tritiated trifluoroacetic acid with the aim of selectively labeling their tryptophyl residues. The distribution of the tritium labels was determined by amino acid analysis of complete enzyme hydrolysates of the treated proteins. Most of the radioactivity introduced into the first two proteins was present as tryptophyl residues and a large part of the remainder as degradation products of these residues. This degradation could be minimized by curtailing the time of labeling or by adding 2-mercaptoethanol to inhibit aerial oxidation. The former modification gave best results with *S*-carboxymethyl-lysozyme; enzyme hydrolysis of the tritiated protein afforded a 97% yield of tryptophan which bore 95% of the total radioactivity. Tritiation of wool led to the incorporation of about 15 times as much radioactivity as expected if only tryptophyl residues were labeled. This extra radioactivity could be removed only by disruption of the wool's structure, *e.g.*, by reduction and carboxymethylation; the residual activity was present mainly in the tryptophyl residues.

Tryptophyl residues are frequently cited as major sites of photodegradation during irradiation of proteins (*e.g.*, Weil *et al.*, 1953; Luse and McLaren, 1963; Leaver and Ramsay, 1969) and there have been many photochemical studies of tryptophan and tryptophyl peptides (*e.g.*, Yoshida and Kato, 1954; Weil, 1965; Benassi *et al.*, 1967; Savige, 1971). However, the fate of tryptophyl residues during irradiation of proteins is less well known owing to the complexity of these systems. Studies of this nature (*e.g.*, Lapuk *et al.*, 1968; Gomyo and Fujimaki, 1970) would be simplified if it were possible to radioactively label the tryptophyl residues initially as this would facilitate subsequent isolation of photodegradation products.

The boron trifluoride complex of tritiated acetic acid has been used previously to label tryptophyl residues in β-lipoprotein (Gosztonyi *et al.*, 1965) but model experiments with tryptophan, tyrosine, phenylalanine, and serine indicate that the labeling is not specific for tryptophyl residues. A later procedure used by Bak *et al.* (1969) appears to show more promise. These authors showed by nuclear magnetic resonance that all five hydrogen atoms on the aromatic system of the single tryptophyl residue of glucagon were labeled when the protein was dissolved in deuterated trifluoroacetic acid. Seryl and threonyl residues were concomitantly trifluoroacetylated, but these groups were subsequently hydrolyzed during dialysis against water. Bak *et al.* (1969) also labeled glucagon by treatment with tritiated trifluoroacetic acid. Radioassay of solvent distilled from the mixture showed that, for each glucagon molecule, 66 hydrogen atoms had exchanged with tritium, 5 more than the number of hydrogen atoms present at O and N sites. Not all of these extra labels, which presumably reside on the tryptophyl residue, were stable to back-exchange; only two remained after prolonged dialysis.

The methods used by Bak *et al.* (1969) provide mainly indirect evidence for the labeling of the tryptophyl residue in glucagon and might not detect partial labeling at other sites. Therefore we have labeled myoglobin, *S*-carboxymethyl-lysozyme, and wool with tritiated trifluoroacetic acid and then

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