The Purification and Partial Characterization of a Soluble Elastin-like Protein from Copper-Deficient Porcine Aorta*

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ABSTRACT: An elastin-like protein is present in the aortas of copper-deficient swine. This protein has been extracted with a pH 3 low-salt buffer and subjected to purification by a method of heat precipitation followed by ion-exchange chromatography. N-Terminal analysis of this protein indicated the sequence to be Gly-Gly-Val-Ile-Gly. The C-terminal amino acid is probably glycine. The molecular weight was evaluated by a sodium dodecycl sulfate disc electrophoretic system. This indicated the molecular size to be about 67,000 or 800 residues. The amino acid analysis of this protein is very much like that of porcine-insoluble aortic elastin except for the absence of methionine and histidine. Also, isodesmosine and dismosine are not seen and there is a lysine content of 48 residues/1000. It is suggested that this protein is the precursor of insoluble elastin and that it be referred to henceforth as tropoelastin.

The search for a soluble precursor of the insoluble elastin fiber has been underway for many years. With its isolation would come a much better understanding of elastin metabolism and fiber formation. Because mature elastin is so insoluble, studies in the past have been focused on methods by which the elastin fiber could be degraded to provide soluble fragments. Several early workers described solubilization of elastin with strong reagents and with enzymes (Richards and Gies, 1902). Recently, Partridge and coworkers applied modern chemical analytical methods to determine the nature of these soluble products. Soluble elastins were produced by boiling the purified insoluble fibers with oxalic acid (Adair *et al.*, 1951). This was done in the hope that these soluble fragments would prove to represent some form of monomeric units which are combined chemically to form the insoluble fiber. However, their polydispersity as to molecular size and their heterogeneity of end groups discouraged any such possibilities (Partridge and Davis, 1955; Partridge *et al.*, 1955). Studies of these fragments did, however, lead to the discovery of the unusual cross-linkages (desmosine, isodesmosine, and lysinonorleucine) which occur in insoluble elastin (Thomas *et al.*, 1963; Franzblau *et al.*, 1965), lending new insight into the metabolism of this unique protein and its method of aggregation. These linkages appear to be derived from condensation products of modified lysine residues in the elastin polypeptide chain (Miller *et al.*, 1964; Partridge *et al.*, 1964; Cleary *et al.*, 1966). Unlike the relatively labile disulfide bonds found in some other proteins, they were found to be remarkably resistant to any form of chemical degradation. It soon become apparent that it would not be possible to isolate a monomeric subunit from insoluble elastin by breaking of cross-linking bonds.

Recently, several reports have appeared suggesting the presence of an elastin-like soluble component in the aortas of copper-deficient swine (Weissman *et al.*, 1963; Smith *et al.*, 1968). Because copper deficiency is known to prevent cross-link formation in both elastin and collagen (Chou *et al.*, 1968), it was speculated that this component may represent the long-sought elastin precursor. With this possibility in mind, studies were undertaken to develop a method for isolation of the soluble component in pure form, and once isolated, to evaluate some of its properties, particularly with respect to its chemical homogeneity. This paper describes the results of these studies and discusses the probability of this component being the soluble polypeptide precursor of insoluble elastin.

Methods

Protein Extraction and Precipitation. Freshly removed thoracic aorta from copper-deficient swine of 90–120-days old (Weissman *et al.*, 1965) was stripped of its adventitia, minced with scissors into small fragments, and frozen with liquid nitrogen in a stainless steel mortar (Graeser *et al.*, 1934). It was then crushed into a finely powdered form. Further maceration was accomplished by using a high-speed Virtis homogenizer, mixing the frozen, weighed, tissue powder with approximately ten volumes of cold pH 2.8, 0.02 M formic acid buffer prior to homogenization. After homogenization, the pH of the buffer–tissue mixture was tested and, if necessary, adjusted to pH 2.8 with 0.1 M formic acid. Extraction was carried out for two 24-hr periods at 4° with continuous gentle agitation. The residue was separated from the supernatant after each extraction by centrifugation in the cold at 10,000*g* for 2 hr. For the second extraction, the residue was again suspended by use of the Virtis homogenizer. The supernatants from each extract were pooled and frozen, then thawed, and recentrifuged at 10,000*g* for 1 hr. The above residues were discarded. After filtration through a fast-flow paper (SS 588), the solutions were placed in an Amicon pressure dialysis apparatus and concentrated in the cold to approximately one-fourth their original volume over an Amicon XM-50 membrane (Amicon Corp., Lexington, Mass.). This membrane selectively prevents the passage of macromolecules greater than 50,000 molecular weight. The

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concentration step gave a protein content to the retentate of approximately 5 mg/ml as determined by the Lowry method using an albumin standard. The filtrate was discarded.

The concentrated protein solution obtained by pressure dialysis was adjusted to pH 7 and 0.5 M salt by the addition of an equal volume of cold pH 7, 1.0 M potassium phosphate buffer with stirring over a 2–3-hr period. This buffer was 1.0 M with respect to potassium ion concentration.[1] A slight precipitate resulted with the addition of the phosphate buffer. This was removed by centrifugation in the cold at 10,000*g* for 1 hr and discarded.

Reversible heat precipitation (coacervation) was carried out by allowing the protein solution to warm slowly in a water bath with gentle agitation to 25°. The clear protein solution became markedly opalescent with warming. The opalescent material was removed by centrifugation at 5000*g* for 30 min at 25°. This "coacervate" represents the crude soluble elastin fraction. Heat precipitation was carried out again by redissolving the elastin fraction in the original volume of cold pH 7, 0.05 M potassium phosphate buffer and repeating the warming and centrifugation procedure. The supernatants were discarded. The final residue was dissolved in the first buffer used for ion-exchange chromatography.

Ion-exchange chromatography was carried out at pH 7 in solutions of freshly deionized 8 M urea (passed through a Barnstead mixed-bed deionizer) containing the specified molarity of potassium phosphate buffer and 0.1% 2-mercaptoethanol. The resin utilized for ion-exchange chromatography was an Amberlite IRF-97M synthetic cation-exchange resin (Rohm and Haas) in its acidic form. This resin is a fine mesh size, weakly acidic, acrylic-type resin, containing dissociable carboxylic acid groups. It has properties similar to Amberlite IRC-50. After washing and removing fines, the resin was equilibrated with a 1.0 M solution of potassium phosphate buffer. It was stored in this fashion. At the time of use, a 10 × 2.5 cm column of the resin was poured and washed through with 200 ml of a 0.01 M solution of potassium phosphate buffer. The protein sample (approximately 200 mg), which had been dissolved in 35 ml of 0.01 M potassium phosphate buffer in 8 M urea, was dialyzed against this same buffer for 12 hr. This starting buffer (50 ml) was pumped through the column before applying the protein sample. After application of the sample, the column was developed with another 50 ml of this buffer. Two more stepwise elutions of the column were made, one with 0.1 M potassium phosphate buffer in 8 M urea, the second with 1.0 M potassium phosphate buffer in 8 M urea (Figure 1). All ion-exchange chromatography was performed at 8° by the use of a jacketed column. Flow rates were maintained at 100 ml/hr with a peristaltic pump. All buffer solutions described above contained 0.1% 2-mercaptoethanol to protect the proteins from the effect of cyanate ion produced by urea degradation. The protein fractions were freed of urea prior to lyophilization by dialysis against 0.02 M formic acid.

Disc Electrophoresis. The disc electrophoretic method of Shapiro *et al.* (1967) was utilized, which employs a 5% acrylamide gel in the presence of 0.1% sodium dodecyl sulfate. The protein solutions, layered over the tops of the gels, also contained sodium dodecyl sulfate and 0.1% 2-mercaptoethanol. A modification was introduced into this technique by including freshly deionized 8 M urea in the gels and also in the solutions containing the protein samples. Standards for evaluation of molecular weight were myoglobin and ovalbumin (Mann Research Laboratories). Electrophoresis was carried out in 12.5 × 0.5 cm gel tubes at 3 mA/tube at 23° for 4.5 hr. The gels were stained with Coomassie blue.

Amino Acid Analysis. Samples of proteins with the exception of insoluble elastin were hydrolyzed for 20 hr at 110° with redistilled 6.2 N HCl in sealed evacuated tubes. Insoluble elastin was hydrolyzed in the same manner for 72 hr. The HCl was removed in a rotary flash evaporator at 40°. The Technicon TSM amino acid analyzer or the Beckman Model 120B automatic analyzer were utilized for analysis using accelerated physiologic columns. The values for serine and threonine were not corrected for destruction losses during hydrolysis. Hydroxyproline contents were determined by the method of Woessner (1961).

Evaluation of N-Terminal Amino Acids. N-Terminal amino acids were evaluated by the three-cycle phenyl isothiocyanate reaction of Edman (Doolittle, 1965), using some modifications to improve yields (L. Hood, 1967, personal communication). Chromatographic identification of the phenylthiohydantoin amino acids was achieved by utilizing systems D and E of Edman and Sjöquist (1956).

Evaluation of C-Terminal Amino Acids. The hydrazinolytic procedure for myoglobin described by Braun and Schroeder (1967) was utilized without modification. The hydrogen form of Amberlite CG-50 was used as a catalyst. Hydrazinolysis was carried out at 80° for 88 hr in sealed evacuated tubes. A sample of ribonuclease (Worthington) was also subjected to hydrazinolysis as a control. Amberlite IR-120 was used in a 1 × 10 cm column to remove the amino acid hydrazides, eluting the free amino acids with a pH 3.1 pyridine–acetate buffer. Final identification and quantitation of the free amino acids was accomplished on the Beckman Model 120B amino acid analyzer.

Fingerprint Analysis. Proteins for fingerprint analysis were dissolved at a concentration of 10 mg/ml in a pH 8, 2.2 M ammonium bicarbonate solution. Digestion was accomplished with electrophoretically purified elastase (Worthington) in a 1 to 100 enzyme:substrate ratio. Digestions were carried out for 4 hr at 37°. Samples were spotted in duplicate (2 mg of digested protein per spot) on a 46 × 90 cm sheet of Whatman No. 3MM paper, allowing 6 cm between each spot. Electrophoresis was carried out in a 10% acetic acid and a 1% pyridine pH 4 buffer at 30° and 40 V/cm for 2.5 hr using a Savant high-voltage tank electrophoretor. After drying, the paper was cut into 6-cm strips, each strip representing an individual sample. One strip was stained by dipping in a cadmium–ninhydrin solution (Dreyer and Bynum, 1967) and developed to determine the peptide movement in the first dimension and to make a comparison with amino acid standards which had been subjected to electrophoresis concomitantly. The duplicate strip was stitched to a second 46 × 57 cm sheet of Whatman No. 3MM paper and chromatography was performed in the second dimension using 1-butanol–glacial acetic acid–water (3.4:1:5, v/v) for 18 hr. During this time, the chromatographic front had progressed approximately 45 cm. The use of a stained duplicate electrophoretic strip permitted a more precise location of peptides to be made for the chromatographic development in the second dimension. This gave more reproducible results than two-dimensional analysis performed on a single

[1] All references to potassium phosphate buffer throughout this paper will signify the molar content of potassium ion.

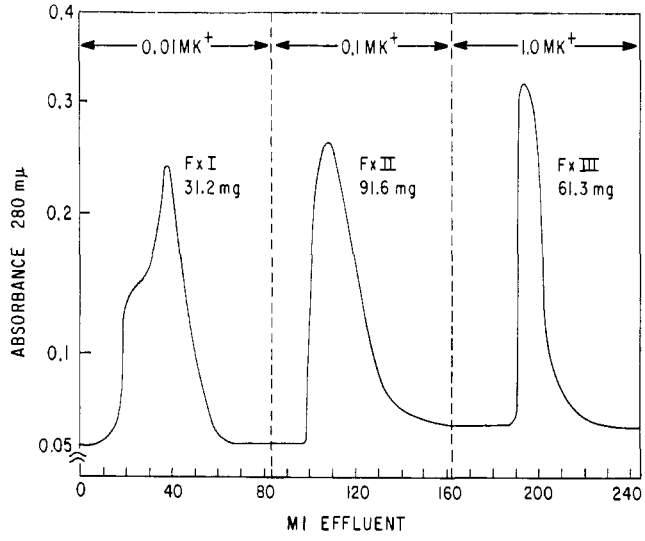

FIGURE 1: IRF-97M elution pattern of proteins obtained from acid extraction of copper-deficient porcine aorta. Chromatography was performed at pH 7.0 and 8° on a 2.5 × 10 cm column at 100 ml/hr. Sample size was approximately 35 ml, containing 200 mg of protein. The column was developed with three concentrations of potassium phosphate buffer, all in 8 M urea and 0.1% 2-mercaptoethanol. The buffers were applied in a stepwise fashion, the steps being indicated by the vertical dotted lines. Yields from each step are shown on the figure.

sheet without duplicates. The two-dimensional fingerprints were stained with the cadmium–ninhydrin reagent. Insoluble porcine aortic elastin, prepared by standard methods (Jackson and Cleary, 1967), was digested with the soluble elastin samples.

Results

Purification Procedure. The extraction of the pulverized aorta with pH 3 low ionic strength buffer is based on a recent finding which indicates that the soluble elastin-like protein is maximally solubilized under these conditions (Sandberg *et al.*, 1969). Heat precipitation or "coacervation" was recognized by early workers to be a property of solubilized, degraded elastin (Richards and Gies, 1902). The method was utilized by Smith *et al.* (1968) for isolation of the elastin-like-soluble component from crude porcine aortic extracts. Further study has revealed that heat precipitation of this protein occurs most readily around neutral pH and at salt concentrations between 0.5 and 1.0 M (D. W. Smith, 1969, unpublished data). This observation provided the rationale for the addition of the pH 7, 1.0 M phosphate buffer to the crude pH 3 extract prior to heat precipitation.

Stepwise elution of the proteins from the IRF-97M resin at 0.01, 0.1, and 1.0 M pH 7 phosphate buffer in 8 M urea gives three distinct protein fractions. The elution patterns of these fractions are shown in Figure 1. Yields from a 200-mg loading are given. This amount of material resulted from the extraction of three 20-g aortas. If a linear elution from 0.1 to 0.5 M phosphate buffer is utilized to replace the elution step with 1.0 M potassium phosphate buffer to remove the third fraction from the resin, the results are identical. With this latter procedure, the third fraction is eluted as a symmetrical peak at 0.2–0.3 M potassium buffer. The stepwise elution is a much simpler procedure; and, therefore, it was used in this study. Urea (8 M) was utilized throughout the ion-exchange procedure to ensure complete separation of the individual protein fractions. Exclusion of the urea from the buffers gives inconsistent results. Also, it seemed desirable to operate at low temperatures to prevent protein aggregation due to hydrophobic interaction (Smith *et al.*, 1968). Therefore, 8° was selected because this is the lowest temperature at which 8 M urea will not crystallize.

TABLE I: Amino Acid Values for Porcine Aortic Protein Fractions Obtained from Ion-Exchange Chromatography on IRF 97M Resin. [a]

Amino Acid	From Ion-Exchange Chromatography			Insoluble Porcine Control Aortic Elastin
	Fraction I	Fraction II	Fraction III	
Asp	96.9	50.8	2.9	8.8
OH-Pro	1.9	91.7	11.2	14.5
Thr	49.2	23.4	13.8	7.4
Ser	54.9	38.0	9.4	8.1
Glu	126.5	75.4	18.3	20.9
Pro	69.3	107.4	108.7	93.8
Gly	84.7	296.6	333.7	328.9
Ala	71.7	114.2	218.1	233.3
Cys (1/2)	24.7	Trace	0.0	0.0
Val	70.4	38.0	120.6	124.9
Met	24.6	7.6	0.0	1.7
Ile	43.9	14.3	18.8	19.6
Leu	85.5	33.6	46.2	57.4
Tyr	29.7	3.4	15.6	16.9
Phe	29.0	14.5	27.9	32.3
Isodes (1/4)	0.0	0.0	0.0	8.1
Des (1/4)	0.0	0.0	0.0	8.3
Lys	53.7	28.8	47.5	7.6
His	18.4	6.3	0.0	1.1
Arg	65.3	56.1	7.1	6.6

[a] In residues/1000 residues. The analysis of insoluble porcine aortic elastin is included for comparison with fraction III. It was carried out on a 72-hr hydrolysate of autoclaved, hot sodium hydroxide treated tissue.

Ion-exchange chromatography as described here, when utilized in conjunction with the heat precipitation technique, appears to yield one major protein constituent with each of the elution steps. The three protein fractions have been analyzed further by disc electrophoresis (Figure 2) and amino acid analysis (Table I).

The first fraction, by amino acid analysis, appears to be similar to ground substance proteins (Barnes and Partridge, 1968). This can be deduced from the high content of polar amino acids as well as appreciable amounts of cystine and methionine. On disc electrophoresis, this fraction appears as one major protein band with a molecular weight greater than 100,000. The second fraction consisted largely of collagen, as judged by its amino acid analysis. Its disc electrophoretic pat-

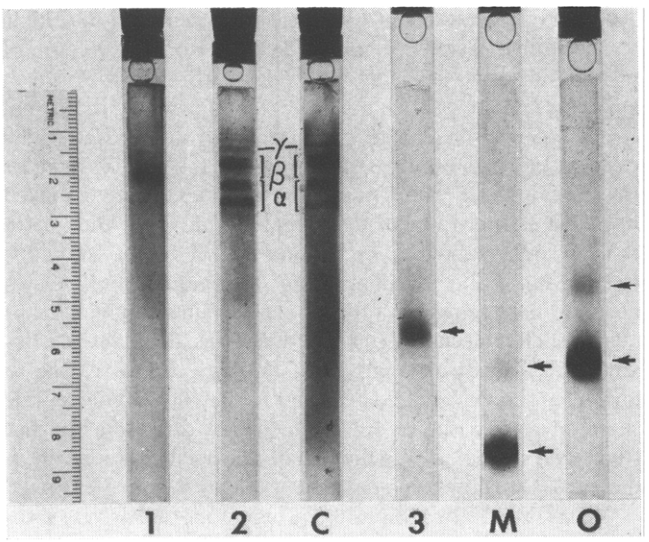

FIGURE 2: Disc electrophoresis using the sodium dodecyl sulfate system of Shapiro *et al.* (1967) in the presence of 8 M urea. This representative run contains the three ion-exchange fractions (1, 2, and 3), guinea pig collagen (C), myoglobin (M), and ovalbumin (O). Arrows indicate the dense center of migration used to measure mobility of each fraction. Slight dimerization of myoglobin and ovalbumin occurred because of the absence of 2-mercaptoethanol in the medium.

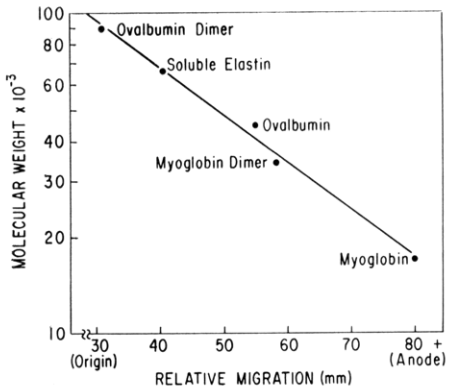

FIGURE 3: Semilog plot of molecular weight against distance of migration of proteins by disc electrophoresis. The line of best fit is drawn through the data points for the known proteins (myoglobin and ovalbumin). The mean data point for the soluble elastin is fitted to this plot. Standard error of the mean for five determinations was ±2600.

tern was very similar to a collagen standard containing α, β, and γ components. The third fraction appears to be an elastin on the basis of the small proportion of polar amino acid residues and the high contents of glycine, alanine, valine, and proline. The amino acid recovery, on analysis of this sample, was 97%, based on the weight of the sample hydrolyzed. This result indicates that fraction III is pure protein with little or no other components; *i.e.*, carbohydrates of lipids. Also, the hydrolysate was remarkably free from humins, a further indication of low carbohydrate and tryptophan content. Disc electrophoresis showed only a single band for this fraction.

Properties of the Elastin-like Protein. Some of the physical and chemical properties of the purified, elastin-like protein were studied. Table I shows its amino acid analysis as compared with the other fractions obtained by ion-exchange chromatography and as compared with insoluble porcine aortic elastin prepared by autoclaving and sodium hydroxide treatment (Jackson and Cleary, 1967). It can be seen that the fraction III protein obtained by ion-exchange chromatography is considerably different than the insoluble aortic elastin with respect to several amino acids; *i.e.*, it has a lower content of aspartate and hydroxyproline and lacks methionine, histidine, desmosine, and isodesmosine. Lysine is markedly increased.

Estimation of molecular weight of fraction III by disc electrophoresis, using the method of Shapiro *et al.* (1967), gave reproducible results. These are shown in Figures 2 and 3. Figure 2 is a representative run of the three ion-exchange fractions, myoglobin, ovalbumin, and collagen. A continuous electrophoresis system such as this gives somewhat diffuse protein bands when stained with Coomassie blue. However, there is always a sharp dense center line from which accurate measurements can be made. A good linear distribution was obtained from proteins of known molecular size (myoglobin and ovalbumin) when plotting the log of molecular weights *vs.* relative mobilities (Figure 3). This has been confirmed by other investigators (Peel, 1968). When fitted to this plot, the molecular weight of soluble elastin (fraction III) appears to be 67,000. The standard error of the mean for five determinations on different preparations of fraction III was calculated at ±4%. The accuracy with respect to determination of the molecular size of soluble elastin is dependent upon the assumption that the protein is in a state of random configuration, similar to the denatured standards.

As a further criterion of purity and homogeneity, the N-terminal amino acid sequence was determined by a modified three-cycle Edman procedure. The sequence thus far is Gly-Gly-Val-Ile-Gly-. The amino acid in position 4 is either isoleucine or leucine, probably the former. Differentiation of these two phenylthiohydantoin amino acids is difficult with the chromatographic systems utilized. Sequence determinations were performed on three samples of 32, 40, and 40 mg with yields at each step averaging 74%. The phenylthiohydantoin derivatives identified by thin-layer chromatography were homogeneous. Therefore, we feel we have a protein of reasonable purity whose N-terminal sequence appears to be unique.

Evaluation by hydrazinolysis indicated that glycine was the probable C-terminal amino acid. Four determinations on different preparations all yielded glycine as the major amino acid with minor amounts of alanine and serine. Quantitation of two of these determinations on the amino acid analyzer gave molecular weights of 56,000 and 64,000 for the soluble elastin. This is in reasonable agreement with the molecular weight determination by disc electrophoresis. Braun and Schroeder (1967) demonstrated 100% recoveries of the C-terminal glycine of myoglobin by their method. However, we have reservations about this procedure as applied to elastin because of the large amounts of internal glycine present. Some glycine hydrazide may contribute to the amount of free glycine measured.

Fingerprints of the soluble elastin, as well as those of a normal insoluble porcine aortic elastin sample prepared by standard methods (Jackson and Cleary, 1967), were compared for similarity. The results of this experiment are shown in Figure 4. It will be noted that there is a large number of peptides which move relatively slowly by electrophoresis. These

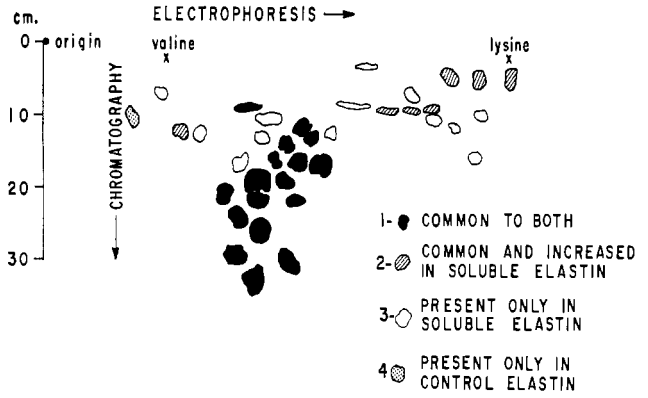

FIGURE 4: A composite of fingerprints of elastase-digested control insoluble aortic porcine elastin and the soluble elastin obtained from copper-deficient porcine aorta. The relative electrophoretic migrations of valine and lysine are indicated.

are common to both proteins (group I). Because of their rapid chromatographic mobility in a nonpolar solvent, it can be assumed these are hydrophobic in nature, consisting largely of such amino acids as glycine, alanine, proline, valine, leucine, and isoleucine. A number of these spots were peach colored, indicating an N-terminal glycine. It is also noted that there are some fast-moving peptides common to both proteins, but they are in a much stronger concentration in the soluble material (group II). Some of these are lysine-containing peptides of small molecular size (L. B. Sandberg, 1969, unpublished data). Some peptides present in both areas of the map are unique to the soluble material (group III).

Discussion

On the basis of the results obtained from N- and C-terminal analyses, it appears that we have isolated a unique protein. The finding of only one band on disc electrophoresis substantiates its homogeneity as to molecular size. Based on the results of amino acid analysis, it is evident that this soluble fraction has the expected composition of the precursor of insoluble elastin (Petruska and Sandberg, 1968). Its high lysine content is necessary for the production of the desmosine cross-links associated with mature elastin. Its elution as a single, sharp peak from the ion-exchange resin, even with a linear gradient, indicates that one molecular species with a similar charge distribution is present. The elution behavior of fraction III on the cationic exchange resin shows that the ϵ-amino groups of lysine, which provide most of the positive charges on the molecule, are intact. Thus, in addition to partial characterization, we have tested and proven purity and homogeneity by at least four criteria.

The findings of different fingerprint patterns for the soluble and insoluble elastins might suggest there is a contaminant present in the soluble material. However, the majority of the peptides seen are common to both preparations (Figure 3, group I). In addition, there is a mixed group of electrophoretically fast-moving peptides (groups II and III) which are present in much higher concentration or unique to the soluble elastin. This electrophoretic behavior, we believe, is due to the differing lysine contents of the soluble and insoluble material.

High concentrations of lysine residues are found in soluble and insoluble elastins of copper-deficient animals because of the absence of an active amine oxidase, which oxidizes the ϵ-amino group of peptide-bound lysine (Pinnell and Martin, 1968). These lysine residues confer extra positive charge on the peptides in which they are found, causing them to move faster electrophoretically than lysine-free peptides. In cross-linked insoluble elastin, most of these peptides have lost their extra charges and, therefore, are diminished or absent from this fast-moving group. Their position is not certain, but we suspect they have remained at or near the origin of application as a fairly high molecular weight residue. Thus, the difference observed by fingerprint analyses of the two elastins is due to local changes which have occurred in the elastin polypeptide chain, at areas associated with cross-link formation. Further studies on the primary structure of the polypeptide chain in these areas are now being pursued.

We think that the elastin-like protein reported here is actually the soluble precursor of insoluble elastin. Its chemical homogeneity lends support to this hypothesis. Also, it is readily isolated only in an experimental disease state such as copper deficiency in which formation of elastin cross-linkages has been prevented. In normal tissues, we feel that cross-linking occurs much too rapidly to allow detection of this substance. Presumably, this protein, in its noncross-linked form, should also be present in the aortas and other elastin-rich structures of lathyritic animals.

Finally, we feel some consideration should be given to an appropriate name for this material. In keeping with the name for noncross-linked, soluble collagen, we suggest that henceforth it be referred to as *tropoelastin*. This name has already been suggested by Jackson and Cleary (1967) in a discussion of elastin nomenclature. If our estimations of molecular size are correct, porcine tropoelastin is a single polypeptide chain, approximately 800 residues in length, with the following amino acid content: aspartate, 2; hydroxyproline, 9; threonine, 11; serine, 8; glutamate, 15; proline, 87; glycine, 267; alanine, 174; valine, 97; isoleucine, 15; leucine, 37; tyrosine, 13; phenylalanine, 22; lysine, 38; and arginine, 6. Of the 38 lysine residues, approximately one-third are involved in production of the known elastin cross-links. We would like to theorize from the amino acid analyses of insoluble elastin (Table I) and from a model recently proposed by Partridge (1966), that six or seven half-desmosine cross-links per molecule of tropoelastin are formed from twice as many lysine residues. Six lysines remain unaltered and are as such in insoluble elastin. The fate of the remaining 18–20 lysine residues is uncertain, although some of them undoubtedly are converted into intermediates such as lysinonorleucine (Franzblau *et al.*, 1965), merodesmosine (Starcher *et al.*, 1967), or α-aminoadipic δ-semialdehyde (Miller *et al.*, 1967; Pinnell and Martin, 1968).

Acknowledgments

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References

Adair, G. S., Davis, H. F., and Partridge, S. M. (1951), *Nature 167*, 605.
Barnes, M. J., and Partridge, S. M. (1968), *Biochem. J. 109*, 883.
Braun, V., and Schroeder, W. A. (1967), *Arch. Biochem. Biophys. 118*, 241.
Chou, W. S., Savage, J. E., and O'Dell, B. L. (1968), *Proc. Soc. Exptl. Biol. Med. 128*, 948.
Cleary, E. G., Sandberg, L. B., Jackson, D. S. (1966), *Biochem. Biophys. Res. Commun. 23*, 139.
Doolittle, R. F. (1965), *Biochem. J. 94*, 742.
Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol. 2*, 32.
Edman, P., and Sjoquist, J. (1956), *Acta Chem. Scand. 10*, 10.
Franzblau, C., Sinex, F. M., and Faris, B. (1965), *Nature 205*, 802.
Graeser, J. B., Ginsberg, J. E., and Friedman, E. (1934), *J. Biol. Chem. 109*, 149.
Jackson, D. S., and Cleary, E. G. (1967), *Methods Biochem. Anal. 15*, 25.
Miller, E. J., Martin, G. R., and Piez, K. A. (1964), *Biochem. Biophys. Res. Commun. 17*, 248.
Miller, E. J., Pinnell, S. R., Martin, G. R., and Schiffman, E. (1967), *Biochem. Biophys. Res. Commun. 26*, 132.
Partridge, S. M. (1966), *Fed. Proc. 25*, 1023.
Partridge, S. M., and Davis, H. F. (1955), *Biochem. J. 61*, 21.
Partridge, S. M., Davis, H. F., and Adair, G. S. (1955), *Biochem. J. 61*, 11.
Partridge, S. M., Elsden, D. F., and Thomas, J. (1964), *Biochem. J. 93*, 30c.
Peel, D. (1968), *Biochem. J. 108*, 51p.
Petruska, J. A., and Sandberg, L. B. (1968), *Biochem. Biophys. Res. Commun. 33*, 222.
Pinnell, S. R., and Martin, G. R. (1968), *Proc. Natl. Acad. Sci. 61*, 708.
Richards, A. N., and Gies, W. J. (1902), *Am. J. Physiol. 7*, 93.
Sandberg, L. B., Hackett, Jr., T. N., and Carnes, W. H. (1969), *Biochim. Biophys. Acta 101*, 201.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Smith, D. W., Weissman, N., and Carnes, W. H. (1968), *Biochem. Biophys. Res. Commun. 31*, 309.
Starcher, B. C., Partridge, S. M., and Elsden, D. F. (1967), *Biochemistry 6*, 2425.
Thomas, J., Elsden, D. F., and Partridge, S. M. (1963), *Nature 200*, 631.
Weissman, N., Reay, D. T., Coulson, W. F., and Carnes, W. H. (1965), *Lab. Invest. 14*, 372.
Weissman, N., Shields, G. S., Carnes, W. H. (1963), *J. Biol. Chem. 238*, 3115.
Woessner, Jr., J. F. (1961), *Arch. Biochem. Biophys. 93*, 440.

Polarized Fluorescence Decay Curves for β-Lactoglobulin A in Various States of Association*

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With the Technical Assistance of J. C. Auchet

ABSTRACT: The decay curves of the parallel and perpendicular components of fluorescence have been determined on β-lactoglobulin A solutions, at conditions in which this protein exists mainly in the form of a monomer, dimer, or octamer. The measured relaxation times are in fair agreement with molecular parameters determined by small angle X-ray scattering.

The recent development of a technique for measuring separately the decay curves of the parallelly and perpendicularly polarized components of fluorescence has permitted the determination of the relaxation times of macromolecules under various conditions (Wahl, 1965, 1966, 1969). This approach has given the possibility of examining directly the shapes of macromolecules by fluorescence polarization without varying the environment. It seemed of interest, therefore, to apply this technique to a protein which can exist in various well-characterized states of aggregation. For this purpose, we have selected β-lactoglobulin A and have determined its fluorescence depolarization curves under conditions where this protein exists predominantly as a monomer, dimer, or octamer. The results of this study are reported in the present paper.

β-Lactoglobulin is a globular protein, the basic subunit of which consists of a single polypeptide chain with a molecular

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