PUTATIVE FORMS OF SOLUBLE ELASTIN AND THEIR RELATIONSHIP TO THE SYNTHESIS OF FIBROUS ELASTIN*

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SUMMARY. Radiochemical labeling experiments suggest that there may be forms of soluble elastin which behave as proforms to tropoelastin, a known precursor to fibrous elastin. These protein subunits behave as 135,000 and 100,000 dalton subunits in SDS-polyacrylamide gels. However, they are only observed after chemical modification, specific for sulfydryl groups.

Tropoelastin is a soluble protein subunit which can be isolated from the elastin-rich tissue of lathyritic or copper-deficient animals (1). Tropoelastin is approximately 72,000 daltons in size. Recently, it has been suggested that a proform of this protein exists which is approximately 135,000 daltons (2). However, components behaving as proforms of elastin have been difficult to identify using radiochemical techniques. Only tropoelastin, the 72,000 dalton component, has been reported previously to be radiochemically labeled in typical experiments (cf. ref. 1 and references cited).

Reported here are data which suggest at least two proforms of tropoelastin may exist. These forms were observed after chemical modification of protein extracted from cultured chick aorta segments. Furthermore, data are presented which suggest that tropoelastin and not smaller elastin-like peptides is the direct precursor to fibrous elastin.

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MATERIALS AND METHODS

Most of the experiments were performed in the manner of pulse-chase experiments. Thoracic aortas from 3-week-old chicks were used. One gram samples of fresh tissue were first minced ($1mm^3$) in Medium 199 containing N-2-hydryxyethylpiperazine-N'-2-ethane sulfornic acid (HEPES) buffer (pH 7.4). The tissue was then transferred to the same medium containing 20 μ or 50 μCi of ^{3}H-(G)-L-valine per ml. Following the incubations in the medium labeled with ^{3}H-val, aliquots of the tissue were then transferred at varying times to medium containing no isotope for further incubation or incubations were stopped by immersing the incubation flasks in ice. All of the incubations were carried out with agitation in an atmosphere of 95% O_2/5% CO_2 using 10 ml of medium per gram of tissue.

In one series of experiments, the tissue following incubation was homogenized and extracted (4) with 0.05 M phosphate buffer (pH 7.0) containing 0.2% sodium dodecyl sulfate (SDS), 4 M urea, and proteinase inhibitors (N-ethylmaleimide, 0.05 M; EDTA, 0.05 M; and phenylmethylsulfonyl fluroide, 0.01 M). The extraction solution was added at 2 ml per 100 mg of wet tissue. In another series of experiments, the phosphate buffer mixture was used as extractant, but mercaptoethanol was added at 0.1%. Also, a portion of the extracted protein was modified by reaction with iodoacetate (3). The purpose of this step was to carboxymethylate sulfydryl groups in the extracted protein. Furthermore, some of the modified and non-modified samples were subjected to fractionation using n-propanol and n-butanol, using a procedure somewhat specific for the isolation of tropoelastin (4). To prepare the samples for alcohol fractionation, two ml aliquots were used. The protein was first equilibrated in 0.1 M acetic acid by passing the aliquots through 1 x 20 cm columns of Sephadex G-50. This step also removed urea and other components in the initial extracting solution. Following concentration or lyophylization, the distribution of the radioactively labeled products in each of the samples was then assessed using polyacrylamide gel electrophoresis (PAGE) techniques as previously described (7). The PAGE systems employed SDS buffers (5). Following electrophoresis, the gels were sliced into contiguous sections in preparation for counting (4, 7). Highly purified tropoelastin (4), bovine albumin and ovalbumin were used as molecular weight markers.

In addition, tissue extracts obtained from the aortas of copper-deficient chicks (4) were partially purified with respect to tropoelastin. The isolation method was similar to that described by Foster et al. (2) using their buffer-proteinase inhibitor mixture. Briefly, the aortas (2 to 3 grams) were extracted and the resulting supernatant fraction was acidified with acetic acid to precipitate collagen. After this step, the pH of protein extract was adjusted to 7.0 using a solution saturated with Na_3PO_4. NaCl was then added to 15% precipitate elastin peptides. A part of this precipitated material was reacted with iodoacetate (3). Approximately 100 μg of the modified or non-modified protein fractions were electrophoresed using a SDS-PAGE system (5).

Furthermore, it was also of interest to determine if the disappearance of radioactively labeled protein in the soluble protein extracts corresponded to the appearance of label in insoluble protein fractions during the "chase" periods. For some of the incubations, the insoluble residue after extraction and centrifugation (25,000 x g, 60 min.) was washed and re-extracted two times with a solution containing 4 M urea and 0.1% SDS in 0.05 M phosphate buffer (pH 7.0). In final preparation for counting, the

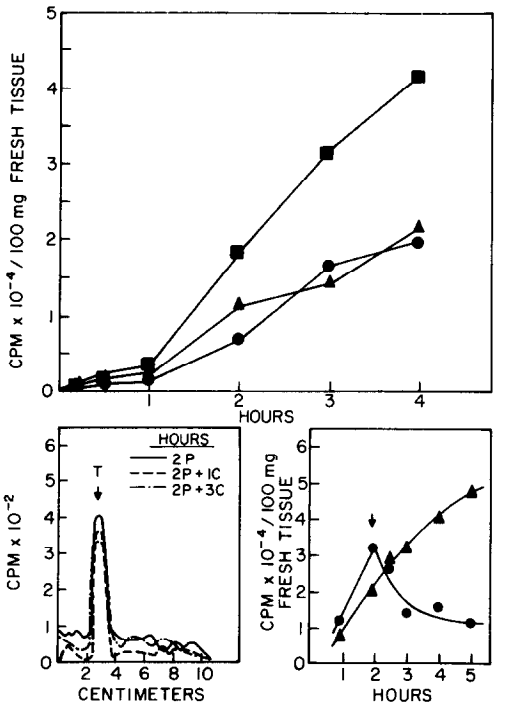

Fig. 1. The incorporation of ^{3}H-val into the total (■), soluble (●) and insoluble (▲) protein from chick aortas segments. Approximately 45 minutes are required before the uptake and incorporation becomes linear. The small figure (bottom-right) indicated that after 2 hours of incubation most of the ^{3}H-val labeled soluble protein appears as insoluble protein after a 3 hour chase. The other small figure (bottom-left) indicates that at selected times (P = pulse, C = chase, number = hours) most of the labeled soluble protein in SDS-PAGE gels behaves as tropoelastin. For this figure, 10% acrylamide was used to make the gels. In addition, during the incubations represented by the data in the large figure, the same pattern of labeling was observed after each extraction at the time periods indicated. For periods, less than one hour, greater than 85% of the total radioactivity in the gels was observed to migrate as tropoelastin. Even after 5 hours, 60-70% of total radioactivity was observed as tropoelastin. The medium contained 20 μCi/ml ^{3}H-val.

residue was washed with distilled water and dissolved using elastase (2 units of Type 1 from Sigma Chem. Co. per mg of residue in 0.1 M TRIS buffer, pH 8.8).

RESULTS AND DISCUSSION

The time course for the uptake and incorporation of ^{3}H-L-valine into soluble and insoluble protein fractions from the minced aortas is shown in Fig. 1. After a 45 to 60 minute lag, the incorporation and secretion

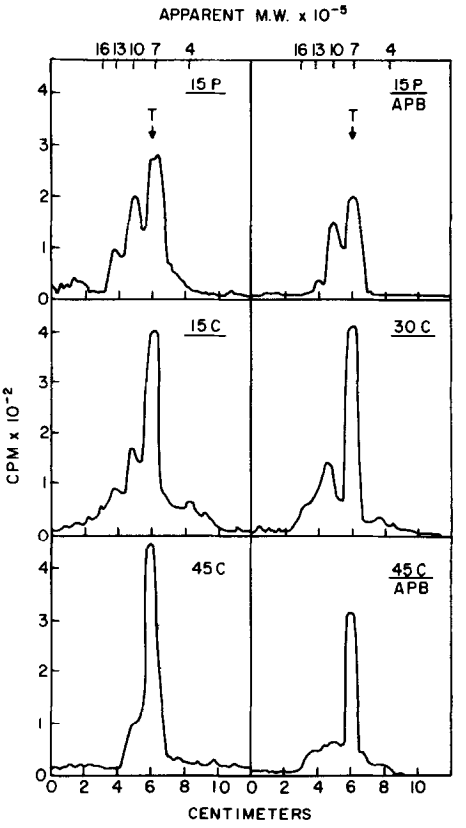

Fig. 2. The distribution of ^{3}H-val in soluble protein after incubation aorta segments, extraction and carboxymethylation. The symbols to define the figures indicate whether the protein was extracted after a "pulse" (P) or a "chase" (C) period. The pulse was 15 minutes followed by chases of 15, 30, or 45 minutes. Those figures in which the symbol APB appears represent the distribution of labeled protein soluble in 0.1 M acetic acid-N-propanol-n-butanol (2:3:5). After a 15 pulse, peaks of radioactivity appears as 135,000, 100,000 and 72,000 dalton subunits. Most of this radioactivity could be recovered in the aqueous alcohol mixtures. After the pulse, the 135,000 dalton subunit was not well defined. The 100,000 dalton subunit during the chase, however, appeared converted to the subunit corresponding to tropoelastin. The incubation medium for these experiments contained 50 μCi/ml ^{3}H-val.

of labeled protein appeared linear. The majority of the extracted protein comigrated with tropoelastin in gel systems suitable for the visualization of soluble elastin subunits at each of the time intervals (Fig. 1).

When the tissue was pulsed for two hours with ^{3}H-val and chased for 3

hours in non-labeled medium, radioactively labeled protein appeared to disappear from the extract into the insoluble residue (Fig. 1). After a 2 hour pulse followed by a 3 hour chase, only the 72,000 dalton was observed in soluble extracts (Fig. 1). This point was considered important with respect to the overall process of elastin fibrogenesis. Although elastin-like peptides smaller than 72,000 daltons have been observed in tropoealstin isolates (4), we have never observed these peptides in short term labeling studies using young chicks aortas (5, 6). One would expect that in this type of experiment, if elastin peptides smaller than 72,000 daltons are directly incorporated into insoluble elastin they would have been observed in the SDS-gels. Since smaller peptides were not observed at any of the time periods indicated in Fig. 1, one interpretation is that the most immediate precursor to insoluble elastin is tropoelastin as the 72,000 dalton species.

The question of a possible proform or forms of tropoelastin, however, was more difficult to resolve. In experiments on the metabolism of elastin reported earlier (5, 6) we were unable to detect putative elastin subunits greater than 72,000 daltons, even when short pulse periods (15 to 30 min) were used. It is now felt that reason for this is perhaps related to the ability of elastin proforms to form high molecular weight aggregates even under denaturing conditions. Although tropoelastin contains no cysteine, we have recently observed that highly purified samples of tropoelastin, if carboxymethylated, appear to contain components greater than 72,000 daltons when examined using SDS-PAGE gels. As shown in Fig. 2, ^{3}H-val labeled components estimated to be 135,000 and approximately 100,000 daltons appear to be chased into tropoelastin, if the protein from radioactively labeled extracts is reacted with iodoacetate before application and electrophorsis. At all of the time periods examined, protein samples which were not modified contained only a major component migrating as tropoelastin (Fig. 1).

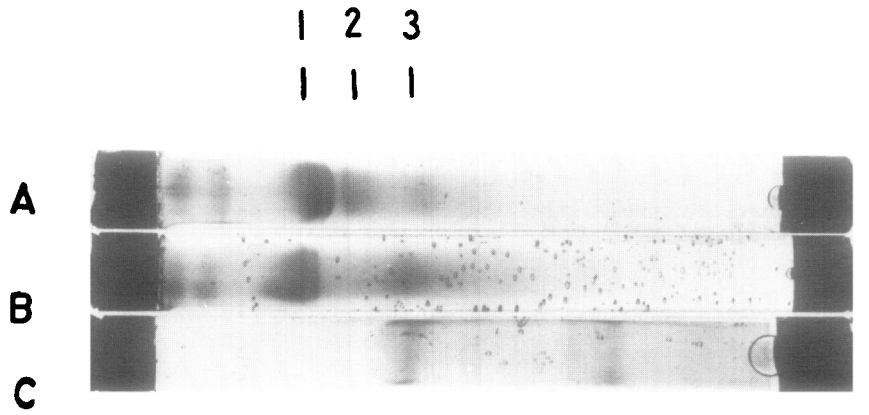

Fig. 3. SDS-PAGE gels of protein partially purified with respect to tropoelastin from the aortas of copper-deficient chicks. One, two and 3 indicate proteins with the mobility of collagen α_1-chains or globular proteins of approximately 135,000 daltons, protein with a mobility of 100,000 daltons, or tropoelastin, respectively. The 100,000 dalton component is only observed in gel A containing protein reacted with iodoacetate. This band is not present in gels B and C, corresponding to the non-modified protein fraction (see text) and tropoelastin (8), respectively. The other material in the tropoelastin gel (C) is partially degraded tropoelastin (4). The gels were 5% acrylamide.

It should also be noted that the migration of the ^{3}H-valine labeled components correspond to the mobility of protein subunits obtained from aorta protein isolates rich in soluble elastin (Fig. 3). Foster et al. (2) have suggested that their putative proelastin migrates as a collagen α_1-chain. Collagen α_1-chains migrate in SDS gels similarly to globular protein subunits of 135,000 daltons. Also, the 100,000 dalton subunit was only observed in samples of protein which had been carboxymethylated.

Discussion

Foster et al. (2) have recently reported that a 135,000 dalton subunit with the characteristics of elastin may be isolated from the aortas of chicks, if crosslinking inhibitors are present. They suggest that this subunit behaves simiarly to collagen α-chains in most elastin isolations, so that its presence is difficult to demonstrate. The fact that its detection in well-designed radiochemical labeling studies has not been

successful, however, is even more difficult to explain. Apparently experimental conditions that would easily demonstrate the presence of procollagens are not ideal for the demonstration of putative proelastins.

Given that the "proelastin" isolated by Foster et al. (2) contained cysteine, we assumed if proelastins exist they may be as high molecular weights aggregates or in combination with other matrix proteins, e.g., microfibrillar protein. This kind of phenomenon could preclude their extraction or penetration into polyacrylamide gels. In fact, previously we have observed very high molecular weight components in pulse-chase type labeling studies which do not penetrate 5, 7.5 or 10% SDS-PAGE gels (6). Furthermore, we have observed that purified tropoelastins even when stored under denaturing conditions undergoes proteolysis and other changes (4). When reacted with iodoacetate, however, the proteolysis is decreased and subunits greater than 72,000 daltons are observed in SDS-PAGE gels (data will be summarized elsewhere). It was for these reasons that the radioactively labeled protein extracts were carboxymethylated. Upon carboxymethylation, radioactively labeled subunits of 135,000, 100,000 and 72,000 daltons (tropoelastin) were observed.

That the 135,000 and 100,000 dalton subunits are collagens is not entirely ruled out. However, it is difficult to detect collagen synthesis with the labeling system that was used (cf. ref. 5). Furthermore, it was possible to recover most of the radioactivity aqueous alcohol solutions (cf. ref. 1). Subsequently, we propose that the 135,000 dalton subunit is probably the same protein described by Foster et al. (2). The 100,000 dalton component is felt most probably to be a truncated and intermediate form of tropoelastin. It is envisioned that analogous to the conversion of procollagens to collagen α-chains, several steps are also involved in the synthesis of tropoelastin. Currently work is in progress to chemically characterize the 130,000 and 100,000 daltons subunits.

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