Demonstration of a Precursor-Product Relationship between Soluble and Cross-linked Elastin, and the Biosynthesis of the Desmosines in Vitro*

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Direct evidence showing that a soluble form of elastin is the precursor of cross-linked elastin was obtained from pulse-chase experiments using chick embryo aortas and by demonstrating the conversion of soluble elastin into cross-linked elastin in a cell-free system. Acetic acid extracts of embryonic chick aortas pulse-labeled with [14C]lysine contain two radioactive proteins of molecular weights 74,000 and 138,000 which have been identified previously as soluble elastin and the pro-α chains of collagen, respectively. In pulse-chase experiments, the radioactivity incorporated into the soluble elastin during the pulse with [14C]lysine disappeared during a 24-hour chase with [3H]lysine and 89% of that which disappeared was accounted for in the desmosines of alkali-insoluble elastin. The disappearance of the radioactivity from the soluble fraction and its appearance in the desmosines of elastin were inhibited by β-aminopropionitrile, a specific inhibitor of the cross-linking enzyme lysyl oxidase. In additional in vitro experiments, it was shown that the radioactivity in the desmosines of elastin can arise only from that present in an acid-soluble precursor protein. This precursor protein is soluble elastin, as demonstrated by the formation of desmosines when a homogeneous preparation of soluble elastin was incubated with purified lysyl oxidase.

A soluble protein which resembles cross-linked elastin in amino acid composition accumulates in the aorta of copper-deficient and lathyritic animals (1-4). This protein lacks the desmosines, a family of lysine-derived cross-links unique to elastin (5), but its elevated lysine content (42 to 48 residues/1000) is sufficient to account for the desmosines and other cross-links present in elastin (6). This protein also possesses certain sequence regions in common with elastin (7, 8) and some of its physical properties resemble α-elastin, a soluble degradation product of cross-linked elastin (9). These observations have provided the basis for the widely held idea that it is the soluble precursor of cross-linked elastin and that the maturation of soluble elastin into insoluble elastin occurs with the conversion of lysine residues into cross-links such as the desmosines (5, 10). The initial step in formation of the desmosines is oxidative deamination of lysines to α-aminoacidic-δ-semialdehydes by lysyl oxidase, a copper-dependent enzyme (11); subsequently, the α-aminoacidic-δ-semialdehydes condense into cross-links (5). Therefore, inhibition of lysyl oxidase activity by copper deficiency or by lathyrogens such as β-aminopropionitrile results in the accumulation of soluble elastin.

So far no direct evidence has been presented to show that soluble elastin is in fact the precursor of cross-linked elastin, and not an uncross-linked segment or degradation product of the latter accumulating in copper-deficient and lathyritic tissues. We report pulse-chase experiments demonstrating a precursor-product relationship between soluble elastin and cross-linked elastin, and conversion of soluble elastin into elastin in vitro which show that the former is indeed the soluble precursor to the latter.

EXPERIMENTAL PROCEDURE

Materials

Pig and chick soluble elastin were gifts from Dr. L. B. Sandberg of the University of Utah and Dr. R. B. Rucker of the University of California, respectively. L-[U-14C]Lysine, L-[4,5-3H]lysine, and Aquasol were obtained from New England Nuclear, Boston, Mass. Lathyritic rat skin collagen was prepared by the method of Piez et al. (12). Bovine serum albumin, cyclobeximide, dithiothreitol, and β-aminopropionitrile-HCl were the products of Calbiochem, La Jolla, Cal. Ovalbumin and pepsin were obtained from Worthington Biochemical Corp., Freehold, N. J. Chemicals used for sodium dodecyl sulfate polyacrylamide gel electrophoresis were purchased from Bio-Rad, Richmond, Cal. All other chemicals not listed above were of analytical grade and products of Mallinkrodt, Baker, or Fisher Chemicals.

Methods

Preparation and Extraction of Labeled Aortas—Eighteen aortas obtained from 17-day-old embryonic chicks were pulse-labeled with 20
Acetic acid extracts of aortas from 17-day-old embryonic chicks pulse-labeled with \(^{14}C\)lysine for 30 min have two major radioactive components of molecular weights 74,000 and 100,000, and 100,000, 100,000, and 95,000, respectively. When the gels were cut in 2-mm slices, broad peaks of pro-\(\alpha_1\), pro-\(\alpha_2\), and \(\alpha_1\) chains with poor resolution resulted. However, when cut in 1-mm slices, peaks of molecular weight 100,000, 100,000, and 95,000 were present, corresponding to pro-\(\alpha_1\), pro-\(\alpha_2\), and \(\alpha_1\) chains. We routinely sliced the gels in 2-mm slices and for simplicity we have pooled the radioactivity under pro-\(\alpha_1\) and pro-\(\alpha_2\) chains or \(\alpha_1\) and \(\alpha_2\) chains and referred to them as pro-\(\alpha\) or \(\alpha\) chains. In a typical experiment the acid-soluble fraction contained S.1 x 10^6 cpm, whereas the residue had 7.2 x 10^4 cpm.
For the corresponding periods, the collagen (procollagen) peaks had respectively 787 cpm (4%), and 2, 4, and 6 hours of chase, elastin peaks was 1666 cpm (100%), 220 cpm (13%), 70 cpm (4%), and 62 cpm (6%) after the pulse and 2, 4, and 6 hours of chase, respectively. The radioactivity under the soluble elastin (S) is indicated. A, 30-min pulse; B, 2-hour chase; C, 4-hour chase; and D, 6-hour chase. The migration of fl and (Y chains of collagen and authentic chick soluble elastin is indicated. The radioactivity under the collagen (or procollagen) peaks for the corresponding time periods was respectively 678 cpm, 1029 cpm, 941 cpm, and 747 cpm.

Fig. 2 (center). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of acid extracts of aortas incubated in the absence of ß-aminopropionitrile. The extracts were treated and equal amounts separated as described in Fig. 1. A, 30-min pulse; B, 2-hour chase; C, 4-hour chase; and D, 6-hour chase. The migration of soluble elastin (S) and α and ß chains of collagen is indicated. The radioactivity under soluble elastin peaks was 1722 cpm (100%), 1111 cpm (65%), 510 cpm (30%), and 501 cpm (29%) after the pulse and 2, 4, and 6 hours of chase, respectively. The radioactivity under the collagen or procollagen peaks for the corresponding time periods was respectively 787 cpm, 706 cpm, 484 cpm, and 379 cpm, respectively.

and if the radioactivity is equally distributed between desmosine and isodesmosine. Therefore, the following transfer experiments were done.

Aortas were pulse-labeled for 15 min followed by cold chase for varying periods of time in the presence or absence of ß-aminopropionitrile. The aortas were defatted and acid-extracted as described previously and the soluble elastin separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The residue was extracted with urea, and alkali-insoluble elastin prepared. The insoluble elastin was hydrolyzed and the radioactivity in the desmosines measured using an amino acid analyzer and liquid scintillation counting. The results of experiments of this type are shown in Table I. After a pulse of 15 min, the soluble elastin contained 6104 counts and no radioactivity was present in the desmosines of the cross-linked elastin. When the chase was done in the absence of ß-aminopropionitrile, there was a linear increase with time in the radioactivity of the desmosines and a decrease in that of soluble elastin. After 24 hours of chase, 89% of the radioactivity lost from the soluble elastin fraction could be accounted for as cross-linked elastin (Table I, Experiment 1). The presence of ß-aminopropionitrile during the chase period markedly inhibited the formation of radioactive desmosine. After 24 hours, only 31% of the expected radioactive cross-linked elastin was observed, and 40% of the label was still present as soluble elastin (Table I, Experiment 2). When ß-aminopropionitrile was present during the pulse as well as the chase period, no radioactivity appeared in the desmosines (Table I, Experiment 3).

While the above observations indicate rather strongly that a precursor-product relationship exists between soluble elastin and cross-linked elastin, it remains possible that the radioactivity in the desmosines arises from labeled substances present within the acid-insoluble aorta fraction, since large amounts of unidentified radioactive material were always observed in that fraction. In order to determine whether or not this is the case, the following experiments were done. Acid-soluble and insoluble residue fractions were prepared from aortas pulse-labeled for 15 min without chase and from unlabeled control aortas. Various combinations of the labeled and unlabeled extracts and residue fractions were incubated in the presence and absence of ß-aminopropionitrile, and the amount of radioactivity appearing in desmosine and isodesmosine measured. The results of these experiments are presented in Table II. Incubation of the labeled residue fraction with unlabeled extract gave rise to only insignificant amounts of radioactivity in desmosines. However, the addition of labeled extract to this mixture resulted in the appearance of 658 counts distributed between desmosine and isodesmosine, and this reaction was inhibited by the presence of ß-aminopropionitrile. In addition, the incubation of labeled extract and unlabeled residue resulted in the appearance of 479 counts in the desmosines and this reaction too was ß-aminopropionitrile-inhibitable. Thus, the radioactivity appearing in the

*The acid extracts did not contain radioactive desmosines as revealed by amino acid analysis.
Elastin Biosynthesis

TABLE I

**Synthesis of elastin by embryonic chick aortas**

| Treatment                        | Radioactivity × 10⁻¹² in | Elastin × 10⁻² |
|----------------------------------|--------------------------|----------------|
|                                  | cpm                      | cpm            |
|                                  | Expected*                | Obtained*      |
| Pulse, 10 min                    | 61.0                     | 0              |
| Pulse + 3-hr chase               | 4.6                      | 3.1            |
| Pulse + 6-hr chase               | 2.4                      | 4.3            |
| Pulse + 24-hr chase              | 1.3                      | 4.9            |
| **Experiment 2**                 |                          |                |
| Pulse, 15 min                    | 30.0                     | 0              |
| Pulse + 3-hr chase               | 21.5                     | 0.2            |
| Pulse + 6-hr chase               | 18.2                     | 0.4            |
| Pulse + 24-hr chase              | 12.3                     | 0.7            |
| **Experiment 3**                 |                          |                |
| Pulse, 1 hr                      | 40.4                     | 0.3            |
| Pulse + 24 hr chase              | 19.1                     | 0              |

*The values were corrected for 57% recovery obtainable from the gel.

(Counts per min of soluble elastin after pulse) − (counts per min of soluble elastin after chase).

Counts per min (desmosine + isodesmosine) × 5.75.

**TABLE II**

**Synthesis of desmosines by acid extract and residue fractions of pulse-labeled aortas**

| Incubation of aorta fractions | Radioactivity in cpm | Isodesmosine Desmosine Isodesmosine + desmosine |
|-------------------------------|----------------------|-----------------------------------------------|
| Labeled residue + unlabeled extract | 34 | 25 | 59 |
| Labeled residue + unlabeled extract + β-aminopropionitrile (400 µg) | 0 | 0 | 0 |
| Labeled residue + unlabeled extract + labeled extract | 390 | 268 | 658 |
| Labeled residue + unlabeled extract + labeled extract + β-aminopropionitrile (400 µg) | 17 | 0 | 17 |
| Labeled aorta extract + unlabeled residue | 193 | 286 | 479 |
| Labeled aorta extract + unlabeled residue + β-aminopropionitrile (400 µg) | 34 | 20 | 59 |

**DISCUSSION**

During recent years, evidence has been obtained supporting the idea that soluble elastin is the precursor of cross-linked elastin. For example, Miller et al. demonstrated that lathyrogens and copper deficiency interfere with the incorporation of lysine into cross links of mature elastin (19). Subsequently, Smith et al. (1) and Sandberg et al. (21) showed that an elastin-like soluble protein accumulates in the aorta of copper-deficient pigs. This protein was isolated and partially characterized and, because of its unique amino acid composition and several other elastin-like properties, it was assumed to be the precursor of cross-linked elastin. Observations by others have provided additional support for this assumption. For example, a non-collagenous substance with physical and chemical properties identical with authentic soluble elastin but which, in addition, serves as substrate for lysyl oxidase, has been extracted from embryonic chick aortas pulse-labeled in vitro (13). This substance disappears from the soluble fraction with increasing incubation times (13). Although all of these observations are consistent with a precursor-product relationship between soluble elastin and cross-linked elastin, no compelling experiments have been done previously definitely documenting such a relationship. The embryonic chick aorta appears to...
Soluble elastin was prepared from aortas pulse-labeled with [4,5-\(^{3}H\)lysine by acetic acid extraction and purified as described under "Methods" (Fig. 3). The preparation was dialyzed versus 0.1 M NaH\(_2\)PO\(_4\), pH 7.7, containing 0.15 M NaCl. Lysyl oxidase was purified from embryonic chick cartilage by affinity chromatography, followed by diethylaminoethy-cellulose chromatography (29). In order to supplement or replace enzymes or other substances which may be required for desmosine synthesis but lost during purification, the residue fraction from unlabeled aortas was added to the incubation mixtures. The soluble elastin preparation, containing 8436 cpm in 1.0 ml of buffer, was preincubated at 37°C for 1 hour and the other ingredients in 0.1 M NaH\(_2\)PO\(_4\), pH 7.7, buffer containing 0.15 M NaCl in a volume of 1 ml each were added. The total volume was brought to 5.0 ml. The mixtures were incubated for 24 hours, after which they were dialyzed and lyophilized. The desmosines were measured as described under "Methods" after hydrolysis.

| Incubation                      | Isodesmosine | Desmosine | Isodesmosine + desmosine |
|--------------------------------|--------------|-----------|-------------------------|
| Soluble elastin + lysyl oxidase | 262          | 143       | 605                     |
| + unlabeled pellet             |              |           |                         |
| Soluble elastin + lysyl oxidase | 0            | 0         | 0                       |
| + unlabeled pellet + \(\beta\)  |              |           |                         |
| aminopropionitrile (500 \(\mu\)g) |              |           |                         |

provide a system with which this documentation can be obtained.

Acetic acid extracts of pulse-labeled aortas contain two radioactive proteins of molecular weights 74,000 and 138,000 which have been identified as soluble elastin and the pro-\(\alpha\) chains of collagen, respectively (13, 14). The soluble elastin fraction rapidly disappears from the extracts during chase with \(^{14}C\)lysine, and there is a concurrent loss of lysyl oxidase substrate. Since this loss is prevented in major part by \(\beta\)-aminopropionitrile, it is likely that cross-linking into an insoluble form, presumably elastin, rather than degradation, is the cause for the disappearance of soluble elastin. If this were true, the lysines of soluble elastin would be converted to desmosines and other elastin cross-links resulting in the transfer of radioactivity from the soluble elastin into the alkali-insoluble elastin; this transfer would be prevented by \(\beta\)-aminopropionitrile. As seen in Experiment 1 (Table I) cross-linked elastin was not present during the pulse period, but it appeared during the chase; after 24 hours, 89% of the radioactivity lost from the soluble elastin was recovered in the alkali-insoluble fraction. The formation of cross-linked elastin was inhibited by the presence of \(\beta\)-aminopropionitrile during the chase period\(^a\) (Experiment 2, Table I) and essentially prevented when the inhibitor was present during both the pulse and chase periods (Experiment 3, Table I).

Evidence obtained from other experiments demonstrates conclusively that cross-linked elastin derives from soluble elastin. As shown by the mixing experiments described in Table II, the cross-linked radioactive elastin could not arise from the insoluble aorta residue fraction. Nor could it arise during the chase periods (Experiment 2, Table I) and essentially during the chase periods (Experiment 3, Table I).

\(^a\)In this experiment the apparent inability of \(\beta\)-aminopropionitrile to completely prevent desmosine formation is probably a result of lysyl oxidase activity during the pulse period when the inhibitor was not present, because when \(\beta\)-aminopropionitrile was present during the chase as well as the pulse, no desmosines were formed (Experiment 3, Table I).

Two other aspects of the conversion of soluble elastin to cross-linked elastin merit comment. While \(\beta\)-aminopropionitrile completely inhibits the cross-linking when it is present during both the pulse and chase periods (Table I), it does not prevent the insolubilization of the soluble elastin (13) (Fig. 2). Thus, reactions other than cross-linking which are not susceptible to \(\beta\)-aminopropionitrile inhibition appear to be involved in the insolubilization process. The nature of these reactions is not clear, although two possibilities are apparent. Insolubilization may result from the binding of soluble elastin to non-elastin components of the extracellular matrix such as the microfibrillar protein or, the newly synthesized soluble elastin may bind through the lysyl side chains to aldehyde groups produced on the surface of cross-linked elastin prior to the exposure to \(\beta\)-aminopropionitrile. Another interesting aspect of the conversion of soluble elastin to insoluble elastin is the time course of the reaction. While over 90% of the soluble elastin is lost from the soluble fraction after 3 hours of chase, only 48% of the expected radioactivity appears in the elastin desmosines during this period. The time lag in the formation of desmosines may be a consequence of additional reactions, some of which may involve currently unidentified enzymes, required for the conversion of \(\alpha\)-aminoacidic-\(\delta\)-semialdehyde to the desmosines and other cross-links.

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