IDENTIFICATION OF A SOLUBLE INTERMEDIATE DURING SYNTHESIS OF ELASTIN BY EMBRYONIC CHICK AORTAE

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1. Introduction

In vertebrates, the protein elastin is a vital component of major blood vessels, imparting to them their elastic, rubber-like quality. Purified elastin appears as an interlacing network of fibers and fibrils when examined in the electron microscope; and its unusual physical properties are reflected in a peculiar, and in some respects unique, amino acid composition. Glycine composes 33% of its residues, and the apolar amino acids proline, alanine and valine constitute approx. another 47% [1]. Elastin is very inert and completely insoluble in all solvents which do not break covalent bonds. Partridge and coworkers demonstrated that the polypeptide chains of elastin are linked together by the desmosines, amino acids unique to elastin [2]. They and Miller et al. [3, 4] showed that these cross-linkages were derived from the oxidation of lysine by a copper requiring enzyme which was inhibited by β-aminopropionitrile. Franzblau et al. [5] have discovered another crosslink in elastin, lysinonorleucine, which is also derived from lysine.

Because of its insolubility, comparatively little was known concerning the chemical and physical structure of the elastin molecule. Recently Sandberg et al. [6] extracted aortae from copper-deficient pigs with 0.02 M formic acid and isolated a protein whose amino acid composition closely resembled that of insoluble elastin except for the absence of the desmosines. They estimated the molecular weight of their material to be 67,000 and called it tropoelastin. Subsequent structural studies by Sandberg et al. [7] have indicated that the linkage regions are rich in alanine. In this last paper the possibility was raised that the 67,000 molecular weight molecule may be a dimer.

In the present communication we report the identification of a soluble protein of a molecular weight of approx. 68,000 which is synthesized by embryonic chick aortae incubated *in vitro* in the absence of inhibitors of crosslinking. From the pattern of labeling with various ^{14}C-amino acids, it appears to be tropoelastin.

2. Materials and methods

A section of the thoracic aorta extending from the root to a point just proximal to the entrance of the ductus arteriosus was dissected with sterile precautions from 9–18 day old chick embryos. The innominate arteries to the point of their bifurcation into the subclavian and carotids were also included. Loose connective tissue was stripped from the aortae which were washed in warm Krebs–Ringer and placed in the same medium. ^{14}C-amino acids were added and the aortae incubated in air at 37°. The aortae were then homogenized and treated as described in the tables and figures.

Disc gel electrophoresis was performed as described by Weber and Osborn [8] using 10% acrylamide gels and half the standard amount of cross-linker. Calibra-

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tion of molecular weight as a function of position in the gel was made by using standard proteins of known molecular weight (fig. 2). The gels were cut into 1.5 mm fractions which were placed into counting vials and 0.25 ml of 30% hydrogen peroxide solution added. The vials were capped and incubated at 55° overnight. Four ml of a Triton X-100 based counting solution and 0.15 ml water were added and the samples counted in a scintillation counter [9]. In some cases in which the samples were labeled with ^{14}C-proline the fractions were placed directly into 6 N HCl and hydrolyzed in sealed tubes for 24 hr at 110°. The polyacrylamide was not appreciably solubilized by this treatment and the proteins contained in the gel were hydrolyzed. The hydrolysates were filtered and the ^{14}C-hydroxyproline content was assayed by a specific chemical procedure [10].

3. Results and discussion

When aortae from 9–18 day old embryos were incubated with ^{14}C-amino acids, they incorporated label at a linear rate for at least 4 hr, thus demonstrating the viability of the system during this period. A representative experiment using aortae from 12 and 18 day old embryos and ^{14}C-valine is illustrated in fig. 1.

Elastin composes roughly 36% and collagen 24% of the dry weight of the thoracic aortae of the vertebrate species which have been analyzed [11–13]. We would therefore expect rapid synthesis of these proteins by chick embryo aortae. In order to characterize the types of proteins being synthesized by the tissues, aortae from 12 day old embryos were incubated with various ^{14}C-amino acids for 90 min, after which they were extracted with sodium dodecyl sulfate (SDS) and the soluble proteins electrophoresed on polyacrylamide gels. Fig. 2 demonstrates that when the proteins were labeled with ^{14}C-glycine, ^{14}C-proline or ^{14}C-alanine, a large fraction of the radioactivity appeared in 2 regions of the gel corresponding to proteins of molecular weight approx. 68,000 and 100,000 Daltons. While these are only estimates of the molecular weights good to roughly ± 10%, the radioactive peak in the 68,000 region appeared to coincide with the serum albumin marker while the peak in the 100,000 region appeared to have a molecular weight somewhat greater than the α-chain

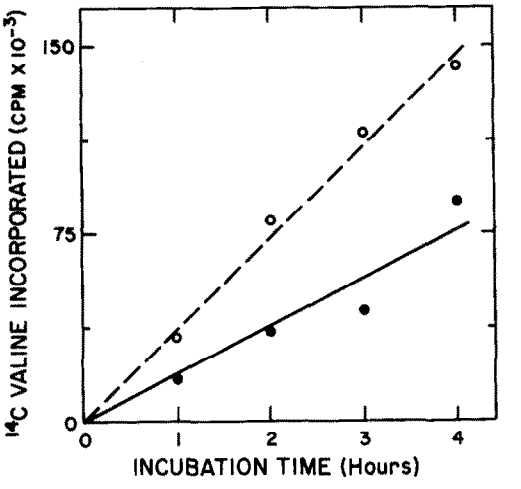

Fig. 1. Incorporation of ^{14}C-valine by aortae from 12 and 18 day old embryos. Eight aortae from either 12 or 18 day embryos were incubated in 2.5 ml of Krebs–Ringer containing 1 μCi ^{14}C-L-valine. At 1 hr intervals 2 aortae were removed and homogenized in 0.02 M formic acid. After dialysis against 0.02 M formic acid at 4°, the homogenates were hydrolyzed and the samples then evaporated and total incorporated radioactivity determined in a scintillation counter. (o–o–o): 18 day; (•–•–•): 12 day.

collagen standard. When ^{14}C-valine was used as the label, only one predominant peak in the 68,000 region was observed. The higher molecular weight peak clearly contained most of the collagen as proven by ^{14}C-hydroxyproline analyses of hydrolyzed proteins from various regions of the gel (table 1). The 68,000 molecular weight peak also contained a small but significant amount of ^{14}C-hydroxyproline. This result is consistent with this peak being a soluble elastin, since it is unlikely that more than 10% of the amino acid residues in elastin are hydroxyproline [1, 6]. However, this criterion must be used with caution until it is demonstrated that the ^{14}C-hydroxyproline did not arise from a small amount of degradation of labeled collagen.

Since the intracellular specific activities of each ^{14}C-amino acid could not be measured, it was not possible to quantitate the amounts of each incorporated amino acid in the peaks. However, since both collagen and elastin contain glycine as approx. one-third of their residues, the quantities of the other amino acids in each of the 2 peaks can be compared by making them relative to ^{14}C-glycine (table 2). When this is

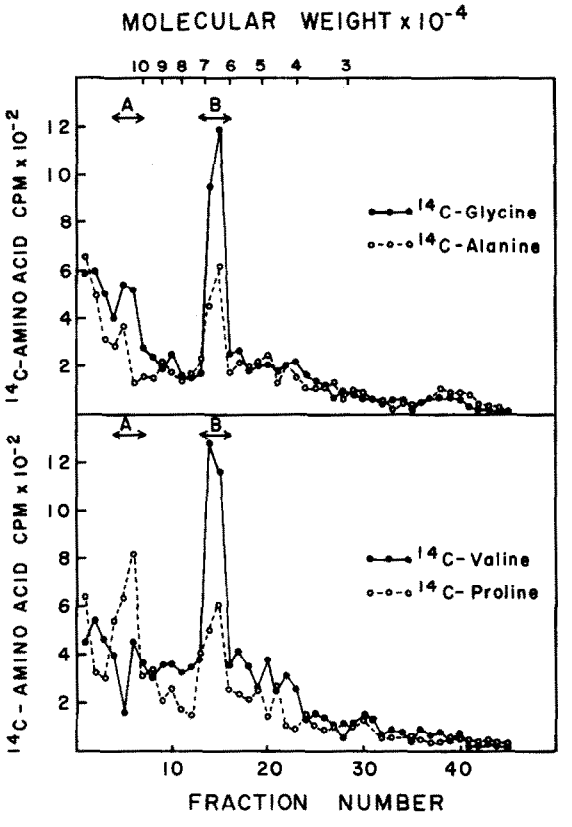

Fig. 2. Gel electrophoresis of SDS soluble proteins from aortae labeled with ^{14}C-amino acids. Four sets of 4 aortae from 12 day old embryos were incubated for 1.5 hr in 1.0 ml of Krebs–Ringer to which 2 μCi of either ^{14}C-glycine, ^{14}C-proline, ^{14}C-alanine, or ^{14}C-valine had been added. They were then homogenized in 0.45 ml of 0.01 M $NaPO_4$ pH 7.0 in a Dounce homogenizer. The homogenate was then transferred to another tube and 50 μl of 10% SDS solution and 5 μl of mercaptoethanol were added and the homogenate incubated at 37° for 2 hr. The homogenate was dialyzed overnight at room temp against 0.01 M $NaPO_4$, pH 7.0, 0.1% SDS and 0.1% mercaptoethanol. After dialysis the homogenate was centrifuged at room temp for 10 min at 3,000 rpm. Fifty μl aliquots of the supernatant were then electrophoresed on polyacrylamide gels as described by Weber and Osborn [8]. The gels were fractionated and counted as described in Materials and methods. Calibration of molecular weight as a function of position in the gel was made by electrophoresing the following standard proteins and staining with Coomassie blue: Lactic dehydrogenase (36,000), creatine kinase (40,000), pyruvate kinase (57,000), serum albumin (68,000), rat tail α-collagen (95,000).

Table 1
Distribution of ^{14}C-hydroxyproline after electrophoresis of soluble proteins labeled with ^{14}C-proline.

Fractions pooled	Total ^{14}C (dpm)	^{14}C-hypro (dpm)	Degree of hydroxylation* (%)
1–7	7196	1339	18.6
8–11	2791	50	1.8
12–16	5592	223	4.0
17–22	2063	0	0

Samples were obtained from gels containing proteins which had been prepared as described in fig. 2. Hydrolysis conditions and assay for ^{14}C-hydroxyproline are as described in Materials and methods.
* Values are 100 times ^{14}C-hydroxyproline per total ^{14}C.

done, it is seen that the 68,000 molecular weight peak contains relatively less ^{14}C-proline, more ^{14}C-alanine, and much more ^{14}C-valine than the 100,000 molecular weight peak. These relative ratios are in qualitative agreement with the proportions of these amino acids in elastin and collagen. Although it is clear that the 68,000 molecular weight peak contains large amounts of ^{14}C-alanine the difference in the relative ratios of ^{14}C-alanine to ^{14}C-glycine between the 2 peaks is probably within the error in the method. More precise agreement with the actual chemical ratios is precluded by the fact that other labeled proteins appear throughout the gel.

If the 68,000 molecular weight peak is a soluble elastin, then we might expect it to become insoluble and disappear in an experiment in which the aortae were first labeled and then incubated in medium containing cold amino acids. Such proved to be the case as illustrated in fig. 3 for experiments with ^{14}C-proline and ^{14}C-valine. The distribution of radioactivity in the gel after a 30 min incubation with either isotope was similar to that after a 90 min incubation except that the 68,000 molecular weight peak now contained a greater proportion of the total soluble incorporated radioactivity. In the case of ^{14}C-proline 33% of the total was in this peak after a 30 min incubation as opposed to 22% after a 90 min incubation. In the case of ^{14}C-valine the percentages were 40% after 30 min and 24% after 90 min (compare figs. 2 and 3). However, when the 30 min incubation was followed by a 3 hr chase period

Table 2
Distribution of ^{14}C-amino acids in soluble proteins from aortae after gel electrophoresis.

Isotope	Region A		Region B	
	(cpm)	Relative to ^{14}C-glycine	(cpm)	Relative to ^{14}C-glycine
^{14}C-Proline + ^{14}C-Hydroxyproline	2291	1.31	1759	0.68
^{14}C-Alanine	928	0.53	1440	0.56
^{14}C-Valine	1385	0.79	3154	1.22
^{14}C-Glycine	1744	1.00	2582	1.00

Values represent total radioactivity found in regions A and B of fig. 2. Ratios in columns 3 and 5 were obtained by dividing the cpm in each amino acid by the cpm in ^{14}C-glycine in that region.

there was a 73% decrease in ^{14}C-proline and a 82% decrease in ^{14}C-valine in this putative elastin peak. There was only a 14% decline of ^{14}C-proline and no decline of ^{14}C-valine in the collagen peak. Concomitant with the decrease in counts solubilized by SDS, there was a marked increase in radioactivity recovered in the insoluble residue (table 3). After a 30 min incubation, 9.0% of the incorporated valine and 4.9% of the ^{14}C-proline was insoluble, while after the 3 hr chase, 45.6% of the ^{14}C-valine and 36% of the ^{14}C-proline was insoluble. These observations suggest that the soluble protein is being incorporated into the insoluble elastin fibrils.

These observations also explain the differences in distribution of radioactivity in the gel between samples from 30 and 90 min incubations. During a 30 min incubation very little of the labeled elastin monomer has become crosslinked, but after 90 min a significant fraction is crosslinked. The result then is a relative decrease in the elastin peak with incubation time and a relative increase in the collagen peak. However, the relative ratios of ^{14}C-amino acids (e.g. ^{14}C-valine: ^{14}C-glycine) within a peak is fairly independent of the incubation time.

There was little incorporation of any of the isotopes

Table 3
Recovery of ^{14}C-amino acids in proteins soluble and insoluble in SDS after pulse and pulse chase.

Incubation condition	Soluble ^{14}C (cpm × 10^{-3})	Insoluble ^{14}C (cpm × 10^{-3})	Total ^{14}C (cpm × 10^{-3})	$\frac{\text{Insoluble } ^{14}\text{C}}{\text{Total } ^{14}\text{C}}$ (%)
Expt. 1				
0.5 hr ^{14}C-Val	42.7	4.2	46.9	9.0
0.5 hr ^{14}C-Val + 3 hr ^{12}C-Val	21.6	18.1	39.8	45.6
Expt. 2				
0.5 hr ^{14}C-Pro	39.3	2.0	41.3	4.9
0.5 hr ^{14}C-Pro + 3 hr ^{12}C-Pro	27.6	15.5	43.1	36.0

In experiment 1, 2 sets of 4 aortae from 12 day old embryos were incubated in 1.0 ml Krebs–Ringer containing 2 μCi ^{14}C-valine for 0.5 hr after which one set was homogenized and prepared as described in fig. 2. The second set was rinsed with medium containing 50 μg/ml of ^{12}C-valine and then incubated for another 3 hr in the same medium after which it was treated identically to the first set. The second experiment was identical to the first except ^{14}C-proline and ^{12}C-proline were used. The values represent the average of 3 separate experiments.

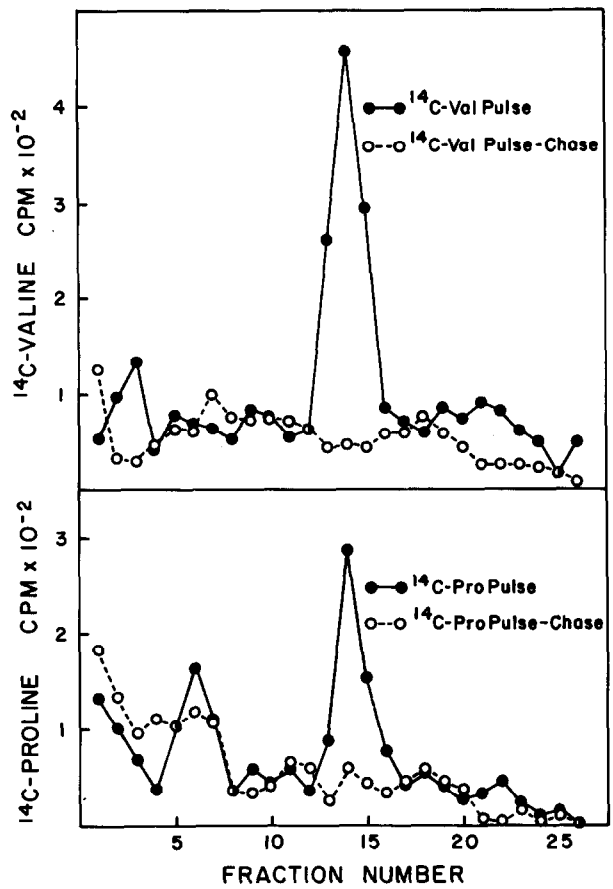

Fig. 3. Gel electrophoresis of SDS soluble proteins after pulse and pulse-chase with ^{14}C-proline or ^{14}C-valine. In one experiment two sets of 4 aortae from 12 day old embryos were incubated in 1.0 ml Krebs–Ringer containing 2 μCi ^{14}C-valine for 0.5 hr after which one set was homogenized and the soluble proteins prepared and electrophoresed as described in fig. 2. The second set was rinsed with medium containing 50 μg/ml of ^{12}C-valine and then incubated for another 3 hr after which it was treated identically to the first set. The second experiment was identical to the first except ^{14}C-proline and ^{12}C-proline were used. (●—●—●): 0.5 hr pulse; (o----o----o): 0.5 hr pulse + 3 hr chase.

tested at any time into a fraction containing proteins of approx. 34,000 molecular weight. It appears therefore from these results that the tropoelastin monomer polypeptide chain is of a molecular weight of approx. 68,000.

Acknowledgements

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