ELASTIN COACERVATE AS A MATRIX FOR CALCIFICATION

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SUMMARY: A calcifying system has been developed which, for the first time, demonstrates the capacity of α-elastin, as the coacervate, to initiate calcification in an in vitro system. This is achieved with blocking the amino and carboxyl charged groups by N-formylation and O-methylation. The calcification system is of particular interest as it demonstrates the initiation of calcification after blockage of the charged amino and carboxyl groups. This leaves either neutral sites or the charged pyridinium moieties as the sites of initiation.

INTRODUCTION

A well recognized process that occurs during aging, as well as in various pathological conditions, is the accumulation of insoluble calcium salts within the walls of arteries. In humans, calcium levels can range from .13% in young arteries to greater than 5% in arteries from older patients (1). These calcium deposits are intimately associated with the elastin component of the arterial wall (2,3,4). Although several proposals have been put forward (5), a fundamental mechanism of initiation, which would explain why elastin is such an effective initiator of calcification, has yet to be verified.

Previously, it has been shown that elastin fibers can provide a nucleating matrix for calcification in vitro, initiating the precipitation of calcium phosphate from otherwise stable solutions (2,6-8). Previous proposals have been that ionized groups on the organic matrix are involved in the nucleation process. A new theory on binding of calcium ion to elastin has recently been proposed (9), which implicates neutral binding sites on the protein polypeptide backbone in the initiation process. In the present report, a solubilized elastin, studied in

a conformation shown to interact with calcium ion (10) and chemically treated such that all the carboxyl and amino groups have been blocked, was investigated for it's ability to provide a matrix for the initiation of calcification.

METHODS

Elastin was prepared from porcine aorta as described previously (10). Soluble elastin was prepared from the purified elastin by oxalic acid extraction as described by Partridge et al. (11). Separation of the α and β fractions was achieved by coacervation as follows. After oxalic acid extraction, the extracts (with the exception of the first one) were combined, cooled in an ice bath, and the pH adjusted to 5.2 with NaOH. Upon warming to 30°C for 1 hour, a yellow, viscous coacervate (α-1) formed in the bottom of the flask. The supernatant which will coacervate at 37°C was removed, dialyzed for 72 hours against distilled water, and freeze dried (α-2).

To form the blocked elastin 2 gm of the α-2 elastin was dissolved in 100 ml formic acid in a 500 ml round bottom flask. The temperature was lowered to 4°C and 30 ml acetic anhydride was slowly added with stirring over a 4 hour period. Five ml H_2O was then added and the mixture evaporated under vacuum to dryness and dried over P_2O_5 over night. The dry N-formyl α-2 elastin was dissolved in redistilled trifloroethanol (100 ml) at 4°C. Diazomethane (12) in ether was slowly added with stirring until the solution maintained a pale yellow color and ceased to evolve N_2. If the elastin precipitates before the completion of the reaction, more TFE can be added. After sitting at room temperature for 1 hour following completion of the reaction, the solution was evaporated to dryness and washed three times with ether and air dried. The N-formyl-O-methyl ester of α-2 elastin was dissolved

in a minimum of ice water, NaCl added until 0.05 molar and the temperature raised to 37°C. The supernatant was discarded and the coacervate dissolved in ice water, dialyzed, and freeze dried.

The mineralization solution consisted of 20 mM barbital buffer pH 7.41 containing 55 mM KCl, 1.25 mM KH_2PO_4, and 1.50 mM $CaCl_2$. Radioactive calcium was added to follow calcification. All water used to make the solutions was glass distilled and boiled prior to use. All glassware was coated with siliclad to provide an inert surface.

The calcification matrix was made by dissolving 3 mg of blocked elastin in 2 ml cold mineralizing solution in small flat bottom shell vials. The vials were capped and allowed to sit at room temperature for 1 hour. They were then incubated with shaking at 37°C for the desired length of time. Calcification was determined by measuring the disappearance of ^{45}Ca from the media or measured directly by removing the solution from the coacervate, washing the coacervate with warm buffer and dissolving it in 0.5 ml trifloroacetic acid. Aliquots of 0.1 ml were mixed with Bray's solution and the radioactivity measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

The first coacervate (α-1) did not redissolve readily, either before or after blocking. For this reason the second coacervate (α-2) was used in these studies. Formation of the N-formyl-O-methyl derivative of α-2 elastin gives rise to a protein that is quite soluble in cold buffered solutions, yet will coacervate at any pH upon warming to 37°C. This is an essential feature in utilizing the coacervate for calcification studies

since α-elastin will not normally coacervate at the physiological pH required to study calcification.

Complete blocking of carboxyl and amino groups was confirmed by acid-base titration and nuclear magnetic resonance. By ratioing the formyl and ester methyl peaks to the Val, Leu, and Ileu methyl protons, it was also shown that there are approximately 16 chains present in the 70,000 mw protein.

A typical experiment showing calcification of the coacervate with time is illustrated in Figure 1. In the presence of the coa-

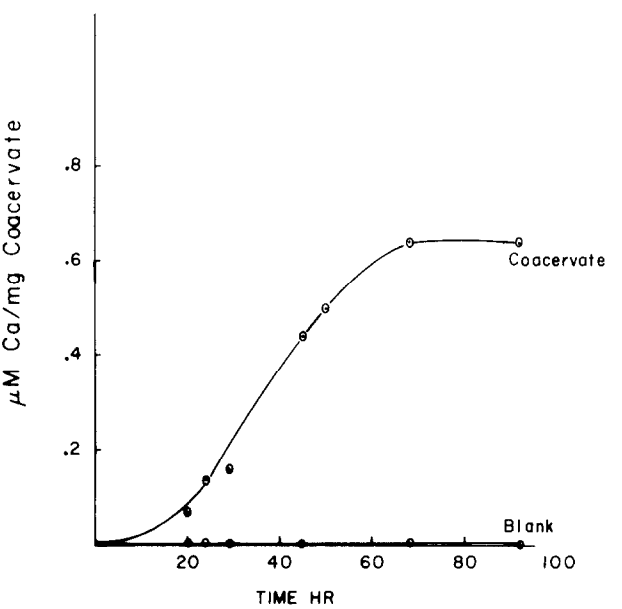

Fig. 1 Calcification of Blocked α-Elastin Coacervate. Preparation of the calcifying system was as described in the text. Calcification was measured by uptake of ^{45}Ca by the coacervate and is expressed as μM Ca^{++}/mg coacervate.

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cervate matrix there is an initial lag period of about 16 hours, after which calcification proceeds rapidly, reaching a maximum at around sixty hours. At this point about 0.6 moles of calcium had been bound per mg coacervate. The Ca/PO_4 ratio of the calcified

elastin was around 1.6/1 which is typical of hydroxyapatite structure (13). The coacervate itself was visably different after calcification. Initially, the coacervate was a clear, faintly yellow film with a very glue-like consistency. After calcification, the coacervate became opaque and lost much of it's adhesive properties. In the absence of the coacervate there was no precipitation of $CaPO_4$ from the mineralizing solution.

Ligamentum nuchae (LN) elastin fibers, purified by NaOH extraction, also act as initiators of calcification in this system (Figure 2a and 2b). When using LN, the onset of calcifica-

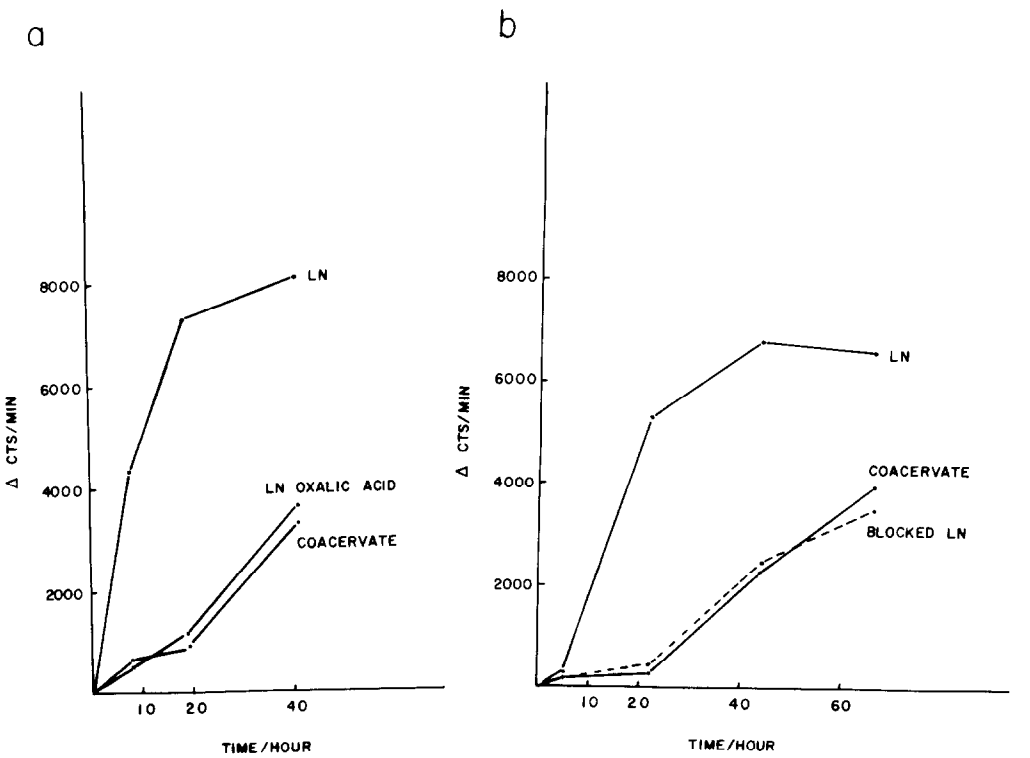

Fig. 2 Calcification of Ligamentum Nuchae Elastin (LN). Preparation of the calcifying system was as described in the text. (a) Comparison of the calcifying ability of LN with the coacervate before and after oxalic acid wash. (b) Comparison of calcifying ability of the demineralized and blocked LN elastin with the coacervate.

tion is very rapid with no observable lag period. If, however, the LN elastin is demineralized by washing with oxalic acid prior to being added to the mineralizing solution, calcification proceeds in a manner very similar to the coacervate. Formation of the N-formyl-O-methyl ester derivative of the oxalic acid washed LN elastin does not alter it's calcifying properties.

The initial lag period observed in these studies has been reported previously with aortic slices in serum (2,14), and has been shown to be the time required for the formation of amorphous calcium phosphate (14). The length of this lag period is quite dependent on the pH, ionic strength and [Ca] x [P] product of the system being used.

Elastin is known to have a greater calcification tendency than most other proteins, yet in it's native state it is one of the most non-polar proteins known, with fewer than 10% of it's amino acid side chains being chemically reactive. It would seem unlikely that the few charged groups that are present would play the major roll in the interaction of calcium with this protein. What we have shown in this report is that in vitro elastin will initiate calcification just as well after all the charge has been removed from the protein by blocking the free amino and carboxyl groups. This strongly supports the theory of neutral site binding. A possibility, however, that has not been eliminated is anion interaction with the positively charged pyridinium ring of the crosslinks.

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