NEUTRAL SITE BINDING OF CALCIUM ION TO ELASTIN COACERVATE

IR SPECTROSCOPY

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SUMMARY: Infrared spectroscopy of the N-formyl-O-methyl ester blocked α-elastin coacervate demonstrated that calcium specifically affected the amide I band, shifting it to shorter wavenumbers. Calcium chloride, calcium bromide, and calcium phosphate introduced a second peak in the C=O stretch region, sodium chloride and sodium phosphate were ineffectual. Correlation with IR spectra of synthetic elastin peptides, known to bind calcium with peptide acyl oxygens, indicated the calcium ion coordinated with neutral carbonyl sites on the peptide backbone. This is the first report of calcium ion binding to peptide carbonyls in the aqueous milieu of an α-elastin coacervate.

INTRODUCTION

A molecular mechanism for calcium-elastin binding was proposed over two years ago (1). The significance of the mechanism lay in its ability to explain the dual nature of elastin pathogenesis, that is the ability of this highly hydrophobic protein both to initiate arterial wall calcification (2) and to serve as a substrate for lipid deposition (3). Combined, these two processes constitute fundamental aspects of atherosclerosis. The model proposed that calcium ion bound elastin at formally neutral sites, the peptide carbonyl oxygens, which could favorably orient with respect to the peptide backbone due to specific glycine containing amino acid sequences. It was indicated that elastin sequences could form β-turns (4,5) orienting the peptide carbonyl oxygens perpendicular to the peptide backbone and presenting polarizable, but not ionizable, sites for calcium ion binding (6). The proposal was that once coordinated to the neutral matrix

of the elastic fiber, the calcium ions attracted counterions, particularly phosphate, to maintain charge neutrality. The phosphate having relaxed charge repulsion between calcium ions on the neutral matrix, in turn enhanced further calcium ion deposition. It was proposed that this cascading series of events initiated calcification. In addition the calcium ions effectively shielded the polar peptide oxygens, forced exposure of nonpolar side chains and allowed the elastic fiber to retain a hydrophobicity for lipid binding (7). Thus the neutral site model provided for elastin's high affinity for both calcium ion and lipid.

Evidence supporting the neutral site binding of calcium ion to elastin is accumulating. Alpha elastin, a 70,000 molecular weight water soluble protein preparation from aortic elastic fibers, coacervated at 37°C and pH 7.4 when all the ionizable carboxyl and amino groups were blocked and, in this state, initiated calcification (8). The most probable explanation was that calcium ion was binding to the acyl oxygens of the peptide moieties.

It had previously been shown that α-elastin interacted with calcium ions in solution (9,10). The circular dichroism solution spectrum of blocked α-elastin in TFE changed with calcium ion addition, a change indicative of a conformational transition from an ordered to a less ordered state. This interaction was specific for calcium ions; magnesium ions had some effect while univalent cations had none.

The amino acid sequence of elastin dictates its conformation which in turn determines its affinity for calcium ions (1). Knowing part of the primary structure of tropoelastin (11), this laboratory synthesized tetra-, penta-, and hexapeptides which

were reported to repeat in the native protein. As anticipated (1), these peptides bound calcium with specific avidity at neutral sites (6,12,13). For example, the cyclododecapeptide, $(Gly\text{-}Gly\text{-}L\text{-}Val\text{-}L\text{-}Pro)_3$ containing only the peptide functional group, specifically coordinated calcium ions in TFE (12). Interestingly, it had the same affinity toward magnesium and strontium as blocked α-elastin (9). The hexapeptide N-Formyl-VAPGVG-OMe (13) and its high polymer, N-Formyl-V$(APGVGV)_n$OMe (6) exhibited high affinities for Ca^{++}, Sr^{++}, with little or no affinity for Mg^{++}, Na^{+}, and K^{+} in TFE and in methanol.

Thus experimental support for the neutral site theory accrues, both from α-elastin and synthetic peptide studies. To date, however, there is no direct evidence for calcium-carbonyl coordination in the α-elastin coacervate, i.e. in an aqueous milieu. The elastin-calcium ion titrations were done in organic solvents, not H_2O. Such data would serve as a link between the in vitro studies on elastin coacervate calcification and the spectroscopic solution experiments on blocked α-elastin and its synthetic analogs. Such a link is necessary to prove that, in an aqueous environment, the elastic fiber not only chelates calcium ion but also chelates it through acyl oxygens. This paper reports the first IR spectroscopic data demonstrating calcium ion binding to neutral sites, i.e. to the peptide carbonyls, in the α-elastin coacervate.

MATERIAL AND METHODS

The α-elastin coacervate was formed from the N-Formyl-O-methyl ester of α-2 elastin (8). For each spectrum, 20 ml. of blocked α-elastin in D_2O (1.5 mg protein/ml.) were allowed to incubate over a Beckman KRS-5 IR window for 12-14 hours at 43°C, in order to coacervate the protein on the window. The column of

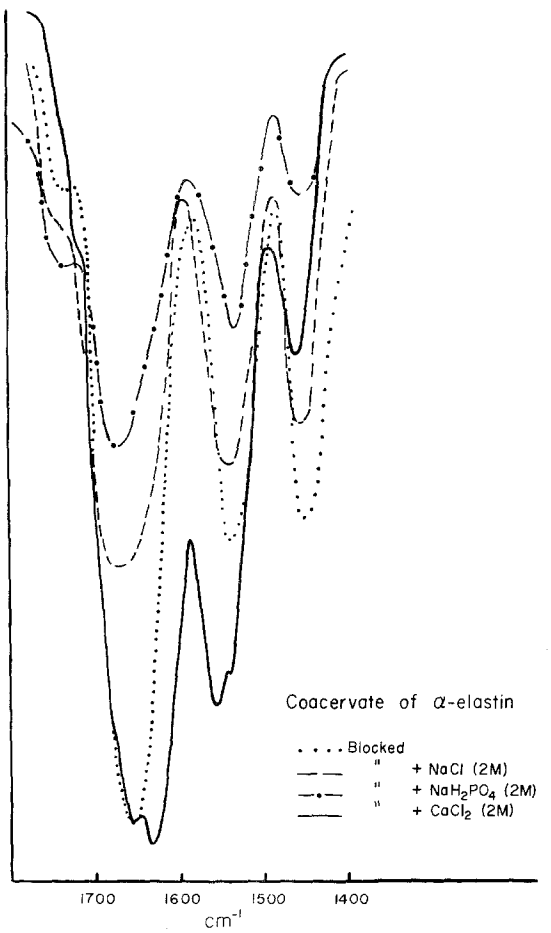

Fig. 1 IR spectra of blocked α-elastin, percent transmission versus wavenumber: The coacervate medium was incubated in the presence and absence of 2M $CaCl_2$ (—), 2M $CaBr_2$ (not shown), 2M $CaCl_2$+KH_2PO_4 (not shown), 2M NaCl (– –), and 2M NaCl+KH_2PO_4 (– . –). The resultant film was dried and examined with a Beckman IR 12 spectrophotometer.

fluid resting on the window was 15 mm in height. During the incubation period α-elastin settled out as the coacervate film on the window. Excess water was removed and the film examined in the IR. Where indicated coacervation was carried out with 2M $CaCl_2$, 2M $CaBr_2$ or 2M NaCl. The NaCl solution also contained 66mM KCl, 1.25mM KH_2PO_4, 20mM sodium barbital, pH 7.4 which constituted the calcification medium, minus $CaCl_2$, of Starcher and Urry (8).

The spectra, recorded as percent transmission versus wavenumber, were obtained with a Beckman IR 12 spectrophotometer at a scanning speed of $8cm^{-1}$/min, and a period of 2. Slower scanning did not change the spectra. The relative intensities of the spectra were arbitrary.

RESULTS AND DISCUSSION

In the region between 1400 and $1700cm^{-1}$, the α-elastin coacervate had three minima (Figure 1): one at $1662cm^{-1}$ (the amide I, C=O stretch), another at $1530cm^{-1}$ (the amide II, N-H deformation) and a third at $1450cm^{-1}$ which is a non-amide vibration (14). 2M $CaCl_2$ had a marked effect on the carbonyl stretch, moving it to shorter wavenumbers with a biphasic minimum at $1655cm^{-1}$ and $1630cm^{-1}$. This shift was specific for calcium ion; calcium chloride was almost as effective as calcium bromide. With 2M $CaCl_2$, KH_2PO_4 (not shown), and the other calcification reagents (8), the wavenumber shift was quantitatively identical although the $1655cm^{-1}$ minimum was further dampened in magnitude. The large shift to lower energies of the amide I band was unique to calcium ion addition; with 2M NaCl or 2M NaCl,KH_2PO_4 and the calcification reagents, there was a slight change toward higher energy.

The amide II band moved to longer wave numbers with both calcium ion and sodium ion additions, although the shift was largest about $15cm^{-1}$ for $CaCl_2$, $CaBr_2$, and $CaCl_2$+KH_2PO_4 and about $5cm^{-1}$ for sodium ions. For $CaCl_2$ and $CaBr_2$ the minimum moved from $1530cm^{-1}$ to $1555cm^{-1}$, while in the case of $CaCl_2$ and the calcifying system the $1530cm^{-1}$ band was almost obliterated. The amide II band in the coacervate with 2M NaCl was centered at $1540cm^{-1}$ and at $1535cm^{-1}$ for 2M NaCl+KH_2PO_4. The band at $1450cm^{-1}$ showed little or no variation, a finding consistent with the fact

that this band is not in the peptide moiety (14). This negative piece of data underscores the relevancy of the calcium ion specific shift to the amide moiety.

In addition to the changes in the amide frequencies, the ester carbonyl absorption, seen as a small shoulder at 1735cm^{-1} shifted to lower frequencies only with Ca^{++}, indicating that the ester carbonyls also participated in calcium ion binding.

The question arises whether the calcium ion specific effect observed in the amide I region is due either to a conformational change in the macromolecule or to calcium ion binding of the peptide carbonyls. There is also the possibility that both occur; i.e. a calcium ion-acyl oxygen coordination dependent structural change. Studies with the synthetic elastin peptide, N-Formyl-VAPGVG-OMe indicate that these IR changes also occur on peptide carbonyl coordination of Ca^{++}.

When N-Formyl-VAPGVG-OMe was titrated with calcium chloride, carbon-13 and proton magnetic resonance data (D.W. Urry and T. Ohnishi, in preparation) and CD data (13) indicated that the elastin hexapeptide bound the cation by its acyl oxygens. IR spectra of the hexapeptide with and without $CaCl_2$ or $CaBr_2$ were qualitatively similar to those of blocked α-elastin reported here. Therefore, we conclude that the calcium ion specific binding site in blocked α-elastin coacervate is the peptide carbonyl oxygen. It is significant that this study was done with coacervated elastin because in this state the protein units have reassociated to form fibers (15), that is, the coacervate approximates an in vivo configuration. Consequently we have demonstrated that calcium ion binds elastin at neutral sites when in a biologically relevant state.

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