Acta Histochemica 116 (2014) 1359–1366

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Fluorescence, aggregation properties and FT-IR microspectroscopy of elastin and collagen fibers

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ARTICLE INFO

Article history:
Received 15 July 2014
Received in revised form 18 August 2014
Accepted 19 August 2014

Keywords:
Elastin
Collagen type I
Collagen bundles
Hydrophobicity
Birefringence
FT-IR

ABSTRACT

Histological and histochemical observations support the hypothesis that collagen fibers can link to elastic fibers. However, the resulting organization of elastin and collagen type complexes and differences between these materials in terms of macromolecular orientation and frequencies of their chemical vibrational groups have not yet been solved. This study aimed to investigate the macromolecular organization of pure elastin, collagen type I and elastin–collagen complexes using polarized light DIC-microscopy. Additionally, differences and similarities between pure elastin and collagen bundles (CB) were investigated by Fourier transform-infrared (FT-IR) microspectroscopy. Although elastin exhibited a faint birefringence, the elastin–collagen complex aggregates formed in solution exhibited a deep birefringence and formation of an ordered-supramolecular complex typical of collagen chiral structure. The FT-IR study revealed elastin and CB peptide N—H groups involved in different types of H-bonding. More energy is absorbed in the vibrational transitions corresponding to —CH, —CH_2 and CH_3 groups (probably associated with the hydrophobicity demonstrated by 8-anilino-1-naphtalene sulfonic acid sodium salt [ANS] fluorescence), and to νCN, δNH and ωCH_2 groups of elastin compared to CB. It is assumed that the α-helix contribution to the pure elastin amide I profile is 46.8%, whereas that of the B-sheet is 20% and that unordered structures contribute to the remaining percentage. An FT-IR profile library reveals that the elastin signature within the 1360–1189 cm^{-1} spectral range resembles that of Conex–Toray aramid fibers.

Introduction

Interactions between collagen and elastic fibers have been proposed in reports using histological, histochemical and histophysical methods (Braga-Vilela and Vidal, 2006; Aldrovani et al., 2007) and electron microscopy (Gibson et al., 1997; Kielty et al., 2002). Based on the work of Gibson et al. (1997), Kielty et al. (2002) have suggested that a protein might link elastic fibers to collagen. Hard extraction methods and histochemical methods, accompanied by simultaneous analysis with polarized light microscopy, have suggested a tight collagen–elastic fiber relationship in porcine pericardium and aorta (Braga-Vilela and Vidal, 2006; Aldrovani et al., 2007).

The amount of elastin isolated from some tissues has been surveyed with the following results: 30–43% in aorta, 2–5% in skin, 5–8% in *ligamentum nuchae* (Ayer, 1964), and ~2 ± 1% in the dermis of healthy human skin, as measured using morphometric techniques (Uitto et al., 1983). Regarding the anatomic structure of elastic fibers in the skin, three forms have been described: filaments, a natural network and 20 nm-beaded fibrils (Ayer, 1964). Images of morphological aspects along with biomechanical properties have been reported for elastic fibers and collagen in arteries in a study that combined second-harmonic generation (SHG) signals, two-photon excited fluorescence and multiphoton microscopy which showed the stress–strain distribution of these fibers in the arterial wall (Zoumi et al., 2004).

One of the methods proposed for extraction of elastic fibers is based on boiling in diluted alkali. However, "by extending the extraction time it is found that considerable destruction of elastica occurs" (Ayer, 1964 – review). Another recommended method involves boiling the sample in dilute acetic acid; in this case, no changes in the fibrillar-meshed architecture of the elastica have been reported (Ayer, 1964; Eyre et al., 1984; Mecham, 2008).

Some optical methods have indicated that elastic fibers can exhibit birefringence; this anisotropic optical property has been attributed to the oriented distribution of the filamentous elements

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http://dx.doi.org/10.1016/j.acthis.2014.08.007
0065-1281/

of elastin (Romhányi et al., 1975; Romhányi, 1986). A compositional complexity of molecules interacting with elastin includes type VIII collagen and emilins, as well as the microfibril-associated glycoprotein 1 (MAGP-1), which is an integral microfibril molecule that is important for the structural integrity of the elastic fibers (Kielty et al., 2002). These reports indicate that the composition and structure of the elastic fibers is complex, and that the biological role of many of their molecular components is far from being clear.

A contribution to a comparative study of the macromolecular organization of elastin and collagen fibers and their complexes could be done by evaluating the optical anisotropic properties of these structures in separate and in the aggregates these materials could form in solution, under dialysis conditions. In addition, comparison of Fourier transform-infrared (FT-IR) microspectroscopic signatures between elastin and collagen bundles could probably provide information on differential frequencies of their most important vibrational groups.

In the present study, pure elastin, collagen type I and elastin–collagen complexes, formed in solution, were studied using polarized light microscopy. Additionally, differences between elastin and collagen bundles were investigated at the level of their vibrational groups, as studied by FT-IR. Hydrophobicity by ANS fluorescence, which could add information for the interpretation of FT-IR results, was also searched in pure elastin.

Material and methods

Elastin and collagen type I preparation

Pure elastin obtained from bovine nuchal ligament (Sigma-Aldrich, St. Louis, MO, USA) was used. Small fragments of this material were suspended in distilled water and smears were prepared from that suspension. The smears were dried following three stages: first, they were left in a refrigerator for 24 h, then they were left at 37 °C for 3 h, and finally they were dried at 60 °C for 2 h.

Collagen type I was extracted from the rat tail tendons of the experimental animals previously used to study collagen bundle supraorganization in the skin (Ribeiro et al., 2013). The tails were washed extensively, and their skin was removed. The collagen bundles, which were visible in the external surface of the tail, were removed and washed in Milli-Q water from a Millipore Direct-Q3-System (Molheim, France). After three washes, well-separated and clean collagen bundles were immersed in a 3% acetic acid solution. This solution remained in the refrigerator for 2–3 days for complete dissolution of the collagen fibers. Next, a careful filtration was performed. Subsequently, the collagen fibers were reconstituted by adding 20% NaCl solution to the collagen solution at final concentration of 10%. Immediately afterwards, visibly precipitated fibers, showing a transparent aspect, were left in the refrigerator; white floating reconstituted fibers were dissolved again in a 3% acetic acid solution and kept in the refrigerator until they were completely dissolved. Additional acetic acid solution was added when necessary. After complete fiber dissolution, a careful filtration was performed.

In an attempt to form a complex between elastin and collagen, 150 mg of elastin powder was suspended in 3 mL of the collagen acetic acid solution and dialyzed in Milli-Q water in the refrigerator, until reconstituted collagen formed. In this case, reconstitution was performed without using NaCl. The dialysis was interrupted as soon as reconstituted collagen fibers appeared. At this step, a collagen–elastin complex was evident.

All of the protocols involving animal care and use for collagen attainment were approved by the Committee for Ethics in Animal Experimentation of the University of Campinas (CEAE/IB/Unicamp) (protocol 2700-1) and were in accordance with the Guidelines of the Canadian Council on Animal Care.

Elastin fluorescence analysis

Some elastin smears were treated with 0.1% 8-anilino-1-naphtalene sulfonic acid sodium salt (ANS) (T484 Kodak, Rochester, NY, USA) in a non-polar solvent, butanol, for 30 min in the dark; next, unbound ANS was removed from the smears using pure butanol (Vidal, 1978, 1980). Then, the preparations were cleared in xylene and mounted on slides in Nujol mineral oil (M3516; Sigma-Aldrich).

The ANS-stained preparations were examined under a Carl Zeiss Axiophot 2 photomicroscope (Oberkochen/Munich, Germany) equipped for epifluorescence and using an HBO-mercury short-arc 103 W/2 as UV source and a 365 nm filter for the excitation light; FT-395 and LP-420 nm filters were also employed. For image analysis and photomicrographs, a Zeiss Axiocam HRc camera and Kontron KS400-3 software were used.

Optical anisotropy

The identification of birefringence characteristics in unstained elastin fragments and elastin–collagen type I complexes was performed using an Olympus BMX 51 polarization microscope (Olympus Corp., Tokyo, Japan) connected to a computer and equipped with optical devices for differential interference contrast microscopy (DIC). Optical anisotropy documentation was carried out with Image-Pro-Plus 6.3 software (Media Cybernetics, Bethesda, MD, USA).

FT-IR microspectroscopy

FT-IR spectral profiles were obtained for sections of bovine collagen bundles (CB) and elastin smears. CB sections were prepared from bovine flexor tendons acquired from a slaughterhouse, as previously reported (Vidal, 2013). Briefly, tendon fragments cut parallel to the tendon axis and fixed in 4% paraformaldehyde at pH 7.4 under vacuum were embedded in Hystosec medium (Merck, Darmstadt, Germany) to obtain 10 μm thick sections which were dewaxed in xylene, mounted on slides and dried at 37 °C. Because an all-reflecting objective (ARO) was used for the microspectroscopic examination, the materials were mounted on the surface of thick gold-covered reflective glass slides (Mello and Vidal, 2014).

The Illuminat IR II^{TM} microspectrometer (Smiths Detection, Danbury, CT 06810, USA) equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride detector, ARO (16×) and Grams/AI 8.0 software (Thermo Electric, Waltham, MA, USA), and coupled to an Olympus BM51 microscope was used for FT-IR microspectroscopy. The performance of the equipment was validated using a low signal-to-noise ratio (7929:1) (Vidal and Mello, 2011). The absorbances of the samples and background were obtained using 64 scans for each preparation with a spectral resolution of 4 cm^{-1}. The spectral absorption profiles were determined at frequencies ranging from 4000 cm^{-1} to 650 cm^{-1}. A plus-zero baseline was acquired, and the FT-IR spectrum was normalized using the highest absorbance peak as a reference. After 10 spectral profiles were obtained, an average profile was produced using the Grams software (Vidal and Mello, 2011; Vidal, 2013; Mello and Vidal, 2014). The area of the band peaks was determined using the numerical integral statistics of the Grams software. Peak fitting and peak fitting estimate procedures using a Gaussian function and low sensitivity level were also applied to specific spectral regions of absorption band peaks, using the Grams software, enabling the area of selected bands to be calculated (Mello and Vidal, 2014).

An absorbance ratio, previously proposed to determine the integrity of the collagen triple helices or the relative amount of native collagen in a sample (Gordon et al., 1974; Goissis et al., 2001), was also estimated.