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Prolyl hydroxylation in elastin is not random

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ABSTRACT

Background: This study aimed to investigate the prolyl and lysine hydroxylation in elastin from different species and tissues.
Methods: Enzymatic digests of elastin samples from human, cattle, pig and chicken were analyzed using mass spectrometry and bioinformatics tools.
Results: It was confirmed at the protein level that elastin does not contain hydroxylated lysine residues regardless of the species. In contrast, prolyl hydroxylation sites were identified in all elastin samples. Moreover, the analysis of the residues adjacent to prolines allowed the determination of the substrate site preferences of prolyl 4-hydroxylase. It was found that elastins from all analyzed species contain hydroxyproline and that at least 20%–24% of all proline residues were partially hydroxylated. Determination of the hydroxylation degrees of specific proline residues revealed that prolyl hydroxylation depends on both the species and the tissue, however, is independent of age. The fact that the highest hydroxylation degrees of proline residues were found for elastin from the intervertebral disc and knowledge of elastin arrangement in this tissue suggest that hydroxylation plays a biomechanical role. Interestingly, a proline-rich domain of tropoelastin (domain 24), which contains several repeats of bioactive motifs, does not show any hydroxyproline residues in the mammals studied.
Conclusions: The results show that prolyl hydroxylation is not a coincidental feature and may contribute to the adaptation of the properties of elastin to meet the functional requirements of different tissues.
General significance: The study for the first time shows that prolyl hydroxylation is highly regulated in elastin.

1. Introduction

Post-translational modifications (PTMs) extend the structural and functional diversity of proteins. Among hundreds of known PTMs [1], hydroxylation is a highly abundant modification [2] as it is present in collagen that quantitatively represents about one third of all animal proteins. Collagen is a fiber protein of the extracellular matrix (ECM) and is structurally organized in a right-handed superhelix composed of three left-handed helical strands. The hydroxylation of collagen is catalyzed by the family of hydroxylases and occurs at the β- or γ-C atoms of P residues, forming 3- or 4-hydroxyproline, respectively, or at the δ-C atom of K, forming 5-hydroxylysine (HyK). HyK is required for the formation of stable intermolecular collagen cross-links, and the hydroxyl groups of HyK residues are involved in the reaction with glucosyl or glucosylgalactosyl residues during enzymatic glycosylation [3,4]. It has been proposed that the low abundant 3-hydroxyproline plays a role in collagen fibril supramolecular assembly [5], and 4-hydroxyproline (HyP) is essential for the stability of the triple helix of collagen at physiological temperature [6]. The stabilization is induced by the electron-withdrawing nature of the hydroxyl substituent, which makes prolyl nitrogen more acidic and influences the backbone orientation of the collagen chains [7]. HyP shows a preference for the C^{γ}-*exo* conformation and for the formation of *trans*-peptide bonds, while P adopts the C^{γ}-*endo* conformation and favors the formation of both *cis* and *trans* peptide bonds [7]. HyP is also found in other substrates including prion protein, argonaute 2, conotoxins, α-fibrinogen, hexokinase 2, pyruvate kinase, hypoxia-inducible factor-α and elastin [7–10].

Elastin is another abundant ECM protein and the major component of elastic fibers, which are present in tissues that undergo continuous deformation including the aorta, lungs, elastic cartilage, fibrocartilage, elastic ligaments or skin. Depending on the mechanical function of the

Abbreviations: ACN, acetonitrile; ECM, extracellular matrix; HD, hydroxylation degree; HPLC, high performance liquid chromatography; HyK, hydroxylysine; HyP, hydroxyproline; IVD, intervertebral disc; LH, lysyl hydroxylase; mRNA, messenger ribonucleic acid; MS, mass spectrometry; P4H, prolyl 4-hydroxylase; PE, pancreatic elastase; PP-II, polyproline-II; PTM, post-translational modification; SD, standard deviation; TE, tropoelastin; TFA, trifluoroacetic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.

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tissues, elastic fiber networks are organized differently and, for instance, occur as concentric fenestrated lamellae in the medial layer of the aorta or as honeycomb-like structures in elastic cartilage [11]. Moreover, the abundance of elastic fibers varies greatly in different tissues. Aortic tissue, for instance, contains between 30% and 57% elastin, whereas skin contains only about 2%–5% elastin based on the dry weight of the tissue [11]. Elastic fibers are composed of a central core of elastin, surrounded by a mantle of microfibrils. The assembly of the fiber network, the so called elastogenesis, is a complex process that requires the involvement of numerous proteins such as fibrillins, fibulins, lysyl oxidase as well as many others [12]. The presence of HyP in mature elastin has already been described in the 1950s [13], however, its existence as shown by amino acid analyses of purified elastin was subject to some controversy as the detection of HyP was frequently related to collagen contamination [14]. Recently, our group has unambiguously shown the hydroxylation of mature elastin by identifying sites of partial modification in human skin elastin at the protein level using mass spectrometry (MS) [15,16]. It was found that about one fifth of the P residues in tropoelastin (TE) undergo partial hydroxylation [17]. 3-Hydroxyproline has not been identified in elastin [18], which may be due to the lack of preferred substrate sequences for prolyl 3-hydroxylase. Prolyl 3-hydroxylase has been described to modify GPHyPG sequences in collagen [19,20] and such sequences are not present in elastin.

With 86 residues, P is the fourth most frequent amino acid in human elastin after G, A and V (based on human TE isoform 2 without signal peptide). 60 of these residues are followed by a G residue and further 5 by an A residue, which makes them potential hydroxylation sites of prolyl 4-hydroxylase (P4H, EC 1.14.11.2) that has been described to show a preference for XPG and XPA motifs [21,22]. To date, the function of the prolyl hydroxylation in elastin has not been identified, and it has even been speculated that the modification could be a coincidental feature, as the precursors of elastin and collagens are synthesized close together in the rough endoplasmic reticulum [23]. However, in more recent studies the role of P residues in elastin has been extensively examined, and it was found that P residues together with G residues present in a ratio of around 1:2 are crucial for preventing amyloid fibril formation in elastin and allowing elastomeric properties of the protein. It has been shown that P is important for maintaining structural heterogeneity of elastin and the flexibility of its backbone. Due to their conformationally restricted main chains, P residues show a propensity for polyproline II helix formation, however also a reduced ability to form hydrogen-bonded turns and β sheets, which increases backbone hydration [24,25]. Overall, it seems likely that these properties may be altered upon partial oxidation of P residues to HyP. Indeed, several studies suggest that the presence of HyP has consequences for mature elastin, *e.g.* through modification of coacervation behavior of TE and fiber assembly during elastogenesis [26–28], while is not essential for biosynthesis or secretion of tropoelastin [23].

In this study, the prolyl hydroxylation in elastin from different species including bird and several mammals was investigated at the protein level. Elastin was first isolated from the tissue samples and then digested using the unspecific protease pancreatic elastase (PE). The resultant peptide mixtures were analyzed using LC-MS and the P hydroxylation sites present in the various elastins were comprehensively studied and compared. The study provides detailed insight into the P hydroxylation sites and the hydroxylation degree (HD) of specific P residues in elastin of different tissues and species. Furthermore, it allows conclusions on a potential biological role of this PTM in elastin.

2. Materials and methods

2.1. Materials

Porcine PE was purchased from Elastin Products Company (Owensville, MO, USA). HPLC-grade acetonitrile (ACN) (VWR Prolabo, Leuven, Belgium) and doubly distilled water were used. Analytical grade 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), formic acid and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade or higher purity.

2.2. Tissue sampling and elastin isolation

9 human skin biopsies (5 mm in diameter) were obtained postoperatively from the tumor-free border of excised skin cancer tissue from different skin areas of individuals aged 15 to 90, and 7 foreskin samples (8 mm × 8 mm) were derived from children aged 4 to 13 years. Moreover, 5 human aortic biopsies (5 mm in diameter) were taken from patients during coronary artery bypass grafting, and 5 ear cartilage samples were taken from patients of chondrodermatitis nodularis chronica helicis from the disease-free area around the affected regions during surgery. 18 intervertebral discs (IVD) from lumbar and thoracic spines were obtained *post mortem* or during routine surgical procedures for treatment of herniation or disc degeneration. The study was approved by the ethics committees of the medical faculties of the Martin Luther University Halle-Wittenberg, Germany and the Technion - Israel Institute of Technology, Haifa, Israel and was carried out in compliance with the Helsinki Declaration. All experiments were undertaken with the understanding and written consent of each subject or each subject's legally authorized representative. Bovine and porcine tissue samples from 3 animals, respectively, were collected at local slaughterhouses. 3 freshly killed chickens were obtained from a local farm, and samples were taken in the lab. An overview on all tissue samples that were analyzed in the scope of this study can be found in Table 1.

Isolation of elastin from all samples was carried out using a gentle method, which removes contaminants by other ECM proteins and prevents damage to elastin [29]. In brief, tissue samples were subjected to treatment with a variety of reagents including organic solvents, cyanogen bromide, formic acid, mercaptoethanol, urea and trypsin from porcine pancreas (all purchased from Sigma-Aldrich, Steinheim, Germany) to remove all components present in the tissue except for elastin which is resistant to this procedure. After the final step, the samples were dried under laminar air flow, subdivided into aliquots and stored at −26 °C until further analysis. It was found that this isolation method does not completely purify elastin fibers from IVD, which still contained residual collagen after isolation. This may most likely be due to the different composition of IVD, which mainly contains collagen type II [30,31], while most of the analyzed tissues contain mainly collagen types I and III, which is easier to remove. Therefore, for IVD samples another isolation protocol was used [32] and completed through addition of a collagenase treatment step. The protocol has been described in detail previously [33].

2.3. Proteolysis of elastin

Isolated elastin samples were dispersed in 50 mM Tris buffer, pH 7.5, at a concentration of 1 mg mL^{-1}. All samples were digested with PE for 24 h at 37 °C using enzyme-to-substrate ratios of 1:100 (*w*/w), respectively. A trypsin digest of TE was prepared for method validation purposes by dissolving human TE isoform 2 in 50 mM Tris buffer, pH 7.5,

Table 1
Overview on species and different tissue samples that were investigated in the scope of this study.

Tissue	*Homo sapiens*	*Sus scrofa*	*Bos taurus*	*Gallus gallus*
Skin	x	x	x	x
Aorta	x	x	x	x
Intervertebral disc	x	x	x	–
Cartilage	x	x	x	–
Ligament	–	x	x	–
Comb	–	–	–	x