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ScienceDirect
Methods 45 (2008) 65–74
METHODS
www.elsevier.com/locate/ymeth

Advances in collagen cross-link analysis

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Accepted 30 January 2008

Abstract

The combined application of ion-trap mass spectrometry and peptide-specific antibodies for the isolation and structural analysis of collagen cross-linking domains is illustrated with examples of results from various types of collagen with the emphasis on bone and cartilage. We highlight the potential of such methods to advance knowledge on the importance of post-translational modifications (e.g., degrees of lysine hydroxylation and glycosylation) and preferred intermolecular binding partners for telopeptide and helical cross-linking domains in regulating cross-link type and placement.

Keywords: Collagen; Cross-links; Hydroxylysine; Pyridinoline; Lysyl oxidase; Mass spectrometry

1. Introduction

The basic mechanism and principal pathways of collagen cross-linking were defined in the late 1960s, 1970s, and early 1980s [1–9]. Techniques included labeling reducible cross-links with tritiated borohydride, isolating them for structural analysis after proteolysis or acid hydrolysis as tritiated peptides or amino acids and similarly pursuing 3-hydroxypyridinium cross-linking residues by their inherent fluorescence [9,10]. Different tissue types showed distinctive patterns of cross-linking chemistry, though all were essentially based on the reactions of peptide-bound aldehydes created from specific lysine and hydroxylysine side chains by the action of lysyl oxidase(s) during the assembly of collagen subunits into fibrils. All the fibril-forming collagens (types I, II, III, V, and XI) and the fibril-associated type IX collagen rely on this mechanism of cross-linking to provide tissue structural integrity and material function [11]. Using relatively standard methods of peptide isolation by HPLC and other separation techniques followed by sequence analysis, many of the cross-linking interaction sites in the major tissue collagens have been worked out [9–11]. There are still many questions and gaps in knowledge, however, including whether for any tissue the full complement of cross-linking maturation products has been identified and the manner and extent to which evolved tissue differences in cross-linking confer functional advantages. For example, bone collagen has a unique and characteristic cross-linking phenotype [10–12]. Is this relevant to its ability to mineralize as we suspect [13], and, if so, what is the molecular benefit bestowed by the distinctive cross-linking?

In order to explore these and related questions in collagen biology, we have refined protein mass spectrometric methods to give a new approach. This promises greatly increased sensitivity, selectivity, and speed for profiling collagen cross-linking domains from small samples of polymeric collagen. We focus here on collagens of the skeletal tissues, bone, and cartilage, but the methods exemplified are applicable to all forms of cross-linked collagen. In addition, antibodies raised against peptide sequences that adjoin cross-linking sites have proven to be very useful tools for probing the extent to which heterotypic cross-linking can occur between different chains and molecular types of collagen in different tissues [14–16]. This added

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1046-2023/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2008.01.002

level of tissue and fibril complexity is emerging as a common evolutionary theme in matrix biology. Finally, we provide examples of applying related methods to identify the structure of cross-linked collagen peptides recovered from urine and potentially other body fluids. Such peptides have seen extensive use as biomarkers of the normal turnover and pathological breakdown of bone, cartilage, and other tissues.

These evolving techniques are summarized as a series of examples of results from bone and cartilage collagens.

2. Methods

2.1. Electrospray mass spectrometry

Ion-trap instruments seem particularly well suited to giving informative fragmentation patterns with the type of cross-linked peptides derived from collagen. For the results presented here we used an LCQ Deca XP ion-trap mass spectrometer with in-line liquid chromatography (LC) (ThermoFinnigan) [15,17]. A C8 capillary column 300 μm i.d. × 150 mm long (Grace Vydac 208MXS5.315) flowing at 4.5 μl/min feeds directly into the mass spectrometer. We use a C8 column rather than the standard C18 because we have found that cross-linked structures and some of the larger collagenous peptides elute more readily from this less hydrophobic column. The mobile phase consists of buffer A: 0.1% formic acid in MilliQ water with 2% buffer B, and buffer B: 0.1% formic acid in 3:1 acetonitrile:*N*-propanol (v/v). Samples are loaded in 0.1% formic acid containing 5% buffer B and run with a complex gradient of 2–52% buffer B over 40 min. The sample stream is introduced into the mass spectrometer with an atmospheric pressure electrospray ionization source (ESI). The spray voltage is set at 3 kV and the inlet capillary temperature is 160 °C. We routinely set the Deca XP to run in an automatic triple-play mode, which cycles through a full scan, zoom scan and MS/MS every few milliseconds.

Samples can be generated for mass spectrometry by standard protein purification methods such as chromatography, or samples can come directly from SDS–PAGE. A band is cut from the gel lane, washed, and digested with an enzyme (trypsin, endo Asp-N, etc.) to extract peptides from the gel slice [18,19]. We find that a crucial step in sample preparation is adequate peptide solubilization prior to loading on the mass spectrometer. Cross-linked peptides require 5% organic solvent in the LC loading buffer for efficient recovery.

2.2. Antibody production

Antisera were raised in rabbits against synthetic peptides (DGSKGPTISA and GGDKGPVVAA) that were the human homologues of the N-telopeptide sequences found to contain the aldehyde-forming cross-linking lysines in bovine α1(XI) and α2(XI) chains, respectively [20]. The peptides were conjugated to keyhole limpet hemocyanin with glutaraldehyde, which tends to result in the production of antibodies that recognize an epitope that features the free carboxyterminal sequence of amino acids. Thus, the mouse monoclonal antibody, 2B4, recognizes the C-terminus of EKGPDP where K is involved in cross-linking [20], and which can be used to immunopurify cross-linked collagen type II C-telopeptides from urine (see later). A mouse monoclonal antibody (1H11) that recognizes cross-linked N-telopeptides (NTx) from human bone collagen bearing the α2(I) sequence, JYDGKGVG [21], was used similarly to purify such peptides from urine.

3. Tissue-specific patterns of cross-linking

Collagen fibrils in all tissues are heteropolymers, in that the bulk collagen (type I or II) is copolymerized on a template of either type V collagen for type I collagen-based connective tissues [22] or type XI collagen for type II collagen-based cartilages and related tissues [23]. Fig. 1 illustrates this concept for cartilage type II collagen where collagen XI is polymerized in the interior (not necessarily as a single central core as depicted, but, for example, as proposed in the recent model of Holmes and Kadler [24]) and collagen IX is covalently bonded to surface type II molecules and to other type IX molecules [15]. Tissues such as cartilage, bone, high-load-bearing tendon, ligaments, fibrocartilage, etc., make collagen that is extensively cross-linked by trifunctional pyridinolines or pyrroles, the formation of which requires lateral molecular packing in the 3D structure that includes an underlying element of nearest neighbor molecular relationships portrayed in the lower two schemes.

The essential chemistry of cross-link formation for bone, cartilage, and many other tough connective tissue collagens is summarized in Fig. 2. Pyrroles and pyridinolines in about equal amounts are characteristic maturation products of bone collagen, whereas cartilages go on to form predominantly hydroxylysyl pyridinoline. The ultimate chemistry is determined by the degree of hydroxylation of lysine residues at the two telopeptide and two helical cross-linking sites found in the major fibrillar collagen molecules (types I, II, and III), where the cross-links are located as shown in Fig. 1.

3.1. In-gel trypsin digested collagen

Ion-trap mass spectrometry can detect cross-linked peptides in in-gel trypsin digests of collagen chains and peptide fragments. In the two examples shown in Fig. 3, the main cross-linked peptides from the C-telopeptide-to-helix site are recovered and identified from CNBr-digests of pepsin-solubilized type II collagen from fetal epiphyseal and adult articular human cartilages. A band containing the appropriate CB12X cross-linked fragment was digested in-gel with trypsin [18,19] and run on LCMS. In each example, panel a is the ion-current of the LC eluent, panel b is the mass spectrum for the eluent region indicated and