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Proteome degradation in ancient bone: Diagenesis and phylogenetic potential

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ABSTRACT

The species composition of vertebrate remains found on archaeological and palaeontological sites has proved to be a valuable source of information for reconstructions of past animal husbandry practices as well as for recovering palaeobiological and palaeoecological information. Molecular analyses provide an objective alternative method of species identification to traditional morphological approaches, particularly useful with fragmentary material; the most well-known being the analysis of DNA. However, more recent proteomics techniques are proving to offer powerful new approaches for obtaining molecular species identification and molecular phylogenies from much deeper within the archaeological record. Collagen is the most abundant and stable protein that can survive for millions of years in biomineralised tissues, but it has been well established for several decades that many other, potentially more informative, non-collagenous proteins (NCPs) also survive long into the archaeological and even geological records. This study investigates the potential mechanisms for biomolecule survival in ancient bone, as well as the extent to which the NCPs that do survive over hundreds of thousands of years yield useful phylogenetic information. Some of these NCPs are shown to yield species-specific information making them ideal for palaeoenvironmental inferences.

1. Introduction

1.1. Palaeoecology and past animal husbandry from vertebrate remains

The identification of both ancient animal and plant species is often used to indicate past climate as well as other local environmental conditions (Cruz-Uribe, 1988; Cuenca-Bescós et al., 2009; López-García et al., 2010) or alternatively to investigate biogeographical ranges of extinct species (Álvarez-Lao and García, 2010). The species composition of an assemblage can also be used for biostratigraphy, where the particular set of taxa may be linked to a specific chronological period in limited geographical areas (e.g. Currant and Jacobi, 2001; Stuart and Lister, 2001). In Britain, biostratigraphical evidence from fossil mammal assemblages has also been used as an effective tool for establishing the number and nature of different climatic episodes in the Middle Pleistocene (Schreve, 2001).

Where more detailed information is known about faunal assemblages, such as those pertaining to archaeological rather than palaeontological sites, species identification is not used as much for environmental interpretations, but incorporated into Minimum Number of Individuals (MNI) and/or Number of Individual Specimen (NISP) records. These are often used to investigate dietary strategies and economic practices at individual sites, where the majority of archaeozoological research questions focus on themes that benefit from accurate identifications, such as early animal husbandry practices and investigating the 'secondary products revolution' (Sherratt, 1981).

However, despite appropriate analytical expertise, species discrimination can be irrefutably complicated by unintentional taphonomic processes that cause fragmentation of skeletal remains (i.e., trampling) and, in archaeological assemblages, contemporary intentional fragmentation (i.e., butchery, marrow processing, creation of bone tools), resulting in material for which diagnostic criteria are no longer present. This is apparent in the many archaeological and palaeontological site species lists that have a significant proportion of samples remaining unidentified (e.g., Stuart, 1975, 1982; Thackeray, 1979) as well as those in which the total number of morphologically unidentifiable fragmentary remains are not reported (e.g., Kansa et al., 2009; Murray, 2010). To address these difficulties in identification, the use of biomolecules for making objective identifications has been considered in detail, initially focussing on DNA-based methods (Burger et al., 2001; Bar-Gal et al., 2003; Blow et al., 2008). However, some of the most interesting geographical regions of interest to studies in human evolution and cultural development are located in warm and arid environments, which are not typically conductive to biomolecule survival. For example, research into retrieving amplifiable DNA sequences from Near Eastern archaeological sites often fails (Bar-Gal et al., 2003; Larson et al., 2007). This is one research area where proteinaceous species identification is becoming more commonplace (Buckley et al., 2010; Buckley and Kansa, 2011; Price et al., 2013). As the greatest hindrance to the biomolecular

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archaeologist is the non-uniform preservation of these ancient biomolecules, bone diagenesis and biomolecular degradation needs to be understood in greater detail.

This study aims to: 1) investigate the types of non-collagenous proteins (NCPs) that survive longest in archaeological bone devoid of ancient DNA (aDNA), and 2) explore the phylogenetic information retrievable from proteins that out-survive DNA, and by inference their potential for species identification.

1.2. Bone formation and structure

Bone, one of the most abundant tissues in the archaeological and palaeontological records, is a specialised form of dense connective tissue in vertebrates. It has the mechanical function of giving the skeleton the necessary rigidity to be an attachment and lever for muscles, supports the body against gravity, protects the internal organs and is a ready source of the key regulatory inorganic ions of calcium, magnesium and phosphate. Bone also contains cells and growth factors that in turn control tissue properties (Boskey, 2007). It is a natural composite material, consisting of about 70% inorganic material, predominantly the calcium phosphate hydroxyapatite (HAP), and an organic (mostly protein) component dominated by the structural protein collagen (Currey, 2002). The inorganic phase gives the tissue resistance to compression forces whereas the organic phase gives it resistance to tension forces (Currey, 2002). Bone cells called osteoblasts control the mineralization of the extracellular protein matrix. Once these osteoblasts are engulfed in mineral they become a different type of cell, called osteocytes, which are metabolically barely active, but continue to communicate with each other via interconnecting channels (canaliculae) throughout the tissue and retain function in phosphate and calcium metabolism (Boskey, 2007). Ultimately the bone mineral is removed by osteoclast cells. In this way, bone cells regulate the formation and turnover or resorption of bone, a key step in regulating body calcium, magnesium and phosphate levels.

The mineral phase has been demonstrated to consist of small tabular or plate-shaped crystals (Lees, 1979; Weiner and Price, 1986; Landis, 1996) with dimensions typically ranging from 2–5 × 20–50 × 12–20 nm, although this varies with age and species (Millard, 2001; Glimcher, 2006). The small size of these crystallites gives bone mineral a large surface area of 85–170 m^2g^{-1} (Lowenstam and Weiner, 1989), and makes it chemically very reactive. In bone, these apatite crystals develop with their long *c*-axes parallel to the collagen fibril (Posner, 1969). The collagen, along with associated proteins, plays an important part in determining nucleation, growth and proliferation of these crystals. Initially, the mineral crystals are formed in an environment rich in the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) proteins, and as bone crystals grow with age, association with proteins that regulate remodelling, such as osteocalcin (OC), increases (Boskey, 2007).

The organic protein component secreted by osteoblasts provides flexibility and forms the extracellular matrix (ECM) upon and within which mineral crystals are grown. In bone the protein phase accounts for ~30% (by dry weight), of which collagen predominates accounting for ~90% (by weight) of the constituents in the organic matrix (Millard, 2001). Collagens are the most abundant structural protein in the animal kingdom and of the more than 27 types of collagen, the fibrous collagen type I (hereafter written as 'collagen (I)') is prevalent, particularly so in bone. To distinguish one collagen type from another, vertebrate collagens were assigned Roman numerals in order of discovery (I, II, III, etc.) and to distinguish between chains that constitute the individual collagen molecules, they were called α1, α2, α3, etc., followed by the collagen type in which they occur (Kadler, 1994). For example, collagen (I), which consists of two identical chains and one dissimilar chain, all of which are unique to collagen (I), can be written [$\alpha1(I)_2$ α2(I)]. Each alpha chain coils in a left-handed alpha helix, whereas the combined three chains supercoil in a right-handed manner to form tropocollagen triple helix molecules (Smith and Wood, 1991). These tropocollagen molecules combine and line up head-to-tail to form fibrils, which also combine to form repeating arrays of fibres that give flexibility to the non-mineralised tissues (Currey, 2002). When reinforced with mineral particles, the resulting composite increases in strength and becomes capable of bearing weight. Spaces between the molecules, and between the fibrils, can accommodate the mineral particles (Weiner and Traub, 1986; Hodge, 1989) which appear to deposit first within the holes and then spread throughout the matrix (Lees, 1979).

Associated amongst the complicated networks of collagen molecules are many other proteins loosely termed 'non-collagenous proteins' (NCPs). Several families of proteins associated with the collagen matrix are involved in the regulation of the mineralisation process, although recent research into the characterisation of bone matrix has emphasised its complexity, with Jiang et al. (2007) showing as many as 2479 unique proteins associated with bone; some of these proteins have multiple functions beyond their role in mineralisation (Zhu et al., 2007). The proteins include phosphorylated proteins, proteoglycans, glycoproteins, and gamma-carboxy-glutamic acid(Gla)-containing proteins. Amongst the phosphorylated proteins, the SIBLINGs are the most widely studied (Qin et al., 2004); all have cell-binding domains and all interact with fibrillar collagen. Some of these proteins act as both inhibitors and promoters of mineralisation, depending on the extent of post-translational modification and/or concentration (Boskey, 2007). Similarly, small leucine-rich proteoglycans (SLRPs) interact with fibrillar collagen. The Gla-protein family has fewer members, but one, the small mineral-binding protein OC, is the most abundant of all NCPs found in bone, making up approximately 20% by weight of the total NCPs.

1.3. Bone in the burial environment

Bone survives better than most other tissues due to the mutual stability induced by association of water-insoluble protein to thermostable mineral, constrained further by packaging arrangements. As this is a mutual relationship, once the degradation of one phase occurs, the other will likely soon follow (Hedges, 2002). Bone degradation is considered to occur mainly by two processes; one mediated by microorganisms and fungi (Hackett, 1981; Bell et al., 1996), and the other much slower process of chemical degradation (Collins et al., 2002; Berna et al., 2004). Regarding microbial attack, within a very short time of death decay processes take over a body, with gut organisms invading tissues following the circulatory system (Jans et al., 2004). Even with the inhibition of microbial degradation under favourable burial conditions, both the organic biomolecules and the inorganic bioapatite mineral remain subject to chemical degradation. All living bones contain very small thermodynamically unstable HAP crystals, hence changes in crystallinity after burial are likely; crystallinity is a concept that includes crystal size, crystal order, and the number of defects in the crystal (Grynpas, 1976). The mineral alterations that occur in the burial environment result in re-crystalisation into larger crystals via a process of Ostwald ripening (Trueman et al., 2004). Collagen decay and loss are the primary diagenetic changes to the organic phase of bone which is likely to be demineralised before it can be altered, and then undergoes a variety of hydrolysis reactions (Collins et al., 1995). Although a number of micro-organisms can produce collagenases that are capable of hydrolysing collagen and, under laboratory conditions, degrade bone in a matter of days (Child and Pollard, 1991), in general, mineralised bone collagen is considered not susceptible to enzymatic degradation (Collins et al., 2002). Therefore non-enzymatic hydrolysis, followed by loss of hydrolysed peptide fragments, has been considered a major pathway for collagen degradation and this has been modelled by Collins et al. (1995).

In studying protein loss from bone, Hare (1980) simulated the effects of environmental leaching and showed that with liquid water present, dramatic effects due to leaching were apparent, with about 85% protein leached out when the water:bone ratio was 10:1. Some