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Research paper

Aspartic acid racemization as a dating tool for dentine: A reality

Trinidad Torres [a,*], José E. Ortiz [a], Eva Fernández [b,c], Eduardo Arroyo-Pardo [b], Rainer Grün [d], Alfredo Pérez-González [e]

[a] Laboratory of Biomolecular Stratigraphy, E.T.S.I. Minas, Universidad Politécnica de Madrid, C/Ríos Rosas 21, Madrid 28003, Spain
[b] Forensic Genetics and Population Genetics Laboratory, Department of Toxicology and Health Legislation, Faculty of Medicine, Universidad Complutense, de Madrid, Spain
[c] Instituto de Arqueologia e Paleociencias, das Universidades Nova de Lisboa e do Algarve, Lisbon, Portugal
[d] Research School of Earth Sciences, The Australian National University, Canberra, ACT 0200, Australia
[e] Centro Nacional de Investigación sobre Evolución Humana, Pso Sierra de Atapuerca s/n, 09002 Burgos, Spain

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ABSTRACT

Given the interest in dating sediments from numerous caves, lakes and fluvial terraces containing fossils and lithic components in Europe, here we provide a complete revision of the amino acid racemization (AAR) (aspartic acid in dentine) dating method in vertebrates. To examine the reliability of this method, which is based on a straightforward sample preparation (previous 3.5 kDa dialysis), we used a range of dental material. We examined human dentine collagen from living donors and remains from historical (16th and 19th centuries) and prehistorical (Neolithic) periods. On the assumption that genus does not affect collagen racemization rates, we also studied Neanderthal material and material from carnivores (cave bear) and several other mammals. To validate our age calculation algorithm, we used a wide series of radiometric datings (ESR and ^{14}C), along with thermoluminescence and AAR dating on invertebrate (ostracode) samples. Our results demonstrate that AAR shows satisfactory correlation between age and the extent of aspartic acid racemization for material from modern humans and for ancient (Pleistocene) mammal remains (cave bears, horses and Neanderthals) and highlight a strong correlation between ages derived from dentine collagen aspartic acid and other dating methods. However, in samples from burial sites (19th and 16th century and Neolithic samples from Syria), it was impossible to establish age at death. We assume that taphonomic processes (time and geochemistry) greatly contribute to the denaturation of the collagen triple helix and higher order structures, thereby allowing the racemization of peptide-bound aspartic acid.

1. Introduction

In many ways the use of amino acid racemization (AAR) as a method to date vertebrate remains for forensic and stratigraphic purposes resembles Jason and the Argonauts' quest for the Golden Fleece. In this regard, our personal challenge has been to place hundreds of bone- and tooth-rich continental deposits (lacustrine, caves and fluvial terraces, among others) in the correct geological time-scale. Many of the problems that we have encountered have arisen from not considering what is referred to as the *site effect*, which includes taphonomy, time-averaging, anthropogenic influence and geological evolution.

The first paper on amino acids in fossilized bone appeared in Ezra and Cook (1957). Since then, the use of collagen amino acid (mostly aspartic acid; Asp) racemization has been widely reported (Arany et al., 2007; Bada et al., 1973, 1974, 1984; Bada and Helfman, 1975; Masters and Bada, 1975; Helfman and Bada, 1975, 1976; Hare, 1980; Bada, 1981, 1985a, 1985b, 1990; Belluomini, 1981; McMenamin et al., 1982; Julg et al., 1987; Blackwell et al., 1990; Marshall, 1990; Belluomini et al., 1991; Elster et al., 1991; Kimber and Hare, 1992; Pfeiffer et al., 1995; van Duin and Collins, 1998; Stone and Stoneking, 1999).

AAR has also been used as a technique to estimate ancient DNA preservation (Poinar et al., 1996; Poinar and Stankiewicz, 1996; Krings et al., 1997, 2000, 1999; Hofreiter et al., 2001, 2004a; Pääbo et al., 2004), although some authors express their reservations about its use for this purpose (Collins et al., 2009; Fernández et al., 2009). In addition, this methodology has been applied to determine the age of modern and historical humans (Helfman and

* Corresponding author. Tel.: +34 913366970.
E-mail address: trinidad.torres@upm.es (T. Torres).

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Bada, 1975, 1976; Ohtani and Yamamoto, 1991, 1992; Ohtani, 1995; Ohtani et al., 1998, 2002, 2003, 2004), and a complete revision of the sample preparation method has been described in forensic studies (Waite et al., 1999). However, Hare (1980) considered the use of collagen Asp racemization unreliable for dating and criticized the results of Bada et al. (1974), Bada and Deems (1975) and Bada and Helfman (1975) because of the lack of coincidence found between ^{14}C and AAR ages.

At the Biomolecular Stratigraphy Laboratory, we opted to work with dentine and not with bone samples, as the latter material is porous and more susceptible to degradation and contamination by recent amino acids than the more compact dentine. We have extensive experience in the application of Asp racemization as a dating tool (Torres et al., 1998, 1999, 2000a, 2000b). Our laboratory has also studied the racemization kinetics of amino acids in dentine collagen in the presence of water vapor (Llamas et al., 1999; Canoira et al., 2003) (Fig. 1 Supplementary Data). Those results are consistent with the ones reported by Hare (1980), who indicated that the presence of water is a key factor to explain AAR in collagen. However, it was demonstrated that Asp racemization occurs below the temperature of collagen degradation. Canoira et al. (2003) calculated the Arrhenius parameters in thermally-induced racemization of Asp in solid dentine samples from an extant mammal (black bear) and fossil bear remains from three radiometrically dated palaeoentological sites. These authors reported different behavior of Asp racemization in both cases. This observation reinforces the relevance of taphonomy (not controlled experimental conditions), which influences AAR in collagen at low temperatures. The geochemical homogeneity of carbonate-rich cave loams ensures homogeneous "site effects" (Hoyos et al., 1998a; Torres et al., 2003), which is consistent with Hare (1980), as in the cave infills there is little temperature oscillation and a constant moisture content in most cases. In fact, according to Smith et al. (2003), and Sasowsky and Mylroie (2004), humidity in caves is high and not prone to large fluctuations. In fact, deep in caves, temperature may vary only tenths of a degree, and relative humidity changes in fractions of a percent (Toomey, 2009).

In our first collagen analysis performed in bear teeth, we applied the protocol described by Meyer and Goodfriend (1991) for mollusk samples; our results were disappointing because other organic compounds, apart from amino acids, occurred in chromatograms, sometimes interfering in the separation of Asp enantiomers (see section 4.1). Later, using the sample preparation procedure reported by Lafont et al. (1984) and Marzin (1990), we achieved satisfactory results, as organic compounds that interfered in the separation of amino acids were eliminated through a previous dialysis step. We then published the first age calculation algorithm calibrating the Asp D/L values in dentine collagen with Th/U and ^{14}C datings (Torres et al., 2001, 2002).

The present study has two objectives:

1) to attest the all-purpose (forensic science and geological dating) utility of the dentine sample pre-preparation methodology using a dialysis at 3.5 kDa. For this purpose, we discuss the Asp racemization rate in dentine over time using samples from modern humans, from individuals from the 19th and 16th centuries, and from Neolithic and Paleolithic periods of distinct Asian localities, in addition to Upper and Middle Pleistocene mammals (Fig. 1).
2) to report on the reliability of AAR as a dating tool for Pleistocene remains through the improvement of the age calculation algorithm for Asp D/L values in dentine collagen reported by Torres et al. (2002), by using a series of new electron spin resonance (ESR) dates, together with previously used radiocarbon and U/Th ages. We validate the reliability of this equation using samples from archaeological and palaeontological sites (El Sidrón-northern Spain, Ambrona-central Spain, Pinilla del Valle-central Spain, and cave bear sites in Austria and Italy) previously dated by other methods.

2. Material and methods

Fig. 1 and Table 1 show the geographical location of the sites studied, together with some information about the localities, which is completed in Table 2. Most of the sites correspond to inner sediments of the caves (far from the cave entrance). According to our measurements, the sediments show very low annual temperature oscillation (less than 1 °C annual variation, Torres et al., 2003).

2.1. Electron spin resonance (ESR) dating

ESR was carried out on cave bear teeth from several localities in northern Spain (Table 3). Due to the value of the material, we followed semi non-destructive analytical procedures (see Grün, 1995, 2006; Grün et al., 2003). A small piece of tooth enamel was removed in each case. During this process, the enamel was separated from the dentine with a needle and was not cleaned further.

Enamel fragments were mounted in a Bruker ER 218PG1 programmable goniometer and measured at each dose step at 10° angle intervals for 420° (spectra past 360° were used to check for short-term fading effects). ESR measurements were carried out on a Bruker 106 spectrometer with a 15 kG magnet and a rectangular 4102 ST cavity. The samples were recorded with the following measurement parameters routinely applied in the laboratory: accumulation of between 1200 (natural sample) and 200 scans (for the higher dosed samples) with 1.015 Gpp modulation amplitude, 10.24 ms conversion factor, 20.48 ms time constant, 2048 bit spectrum resolution (resulting in a total sweep time of 20.972 s), 120 G sweep width and 2 mW microwave power. The enamel pieces were successively irradiated with the following cumulative doses: 0, 11.9, 23.5, 45.2, 68.4, 113, 152, and 197 Gy. The total measuring time of the machine was 37 days. ESR intensity values were obtained by natural spectrum fitting (see Grün, 2002), dose values by applying a single saturating function with linear conversion, and errors by Monte Carlo simulation (for more details see Grün and Brumby, 1994).

The beta dose rate was determined from large sediment samples from the sites in which the teeth were found. In Amutxate cave, the gamma dose rate was measured *in situ* with a Digidart portable CsI gamma spectrometer.

Uranium (U) in the enamel and dentine was determined with laser ablation (for more details, see Eggins et al., 2003, 2005). The enamel was scanned twice, both scans resulting in similar U distributions. Two models of U uptake (early and linear uptake) were considered for age calculation of the samples.

2.2. AAR

AAR analyses were undertaken on a wide range of samples that can be classified into the following four groups:

- *Modern humans*: samples from age-controlled individuals that were obtained from the Odontological Faculty of the *Universidad Complutense* of Madrid.
- *Historical humans*: samples obtained from the anatomical collection of the Forensic School of the *Universidad Complutense* of Madrid. All these corpses came from dismantled cemeteries from the 16th century (Fortress of La Mota) and 19th century (Monastery of San Francisco). The approximate age of each