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Research paper

Testing the limitations of artificial protein degradation kinetics using known-age massive *Porites* coral skeletons

P.J. Tomiak [a,*], K.E.H. Penkman [b], E.J. Hendy [a,c], B. Demarchi [b], S. Murrells [b], S.A. Davis [d], P. McCullagh [d], M.J. Collins [b]

[a] School of Earth Sciences, University of Bristol, Wills Memorial Building, Queen's Rd, Bristol BS8 1RJ, UK
[b] BioArCh, Departments of Biology, Archaeology and Chemistry, University of York, York YO10 5DD, United Kingdom
[c] School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK
[d] School of Chemistry, University of Bristol, Bristol BS8 1TS, UK

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ABSTRACT

High-temperature isothermal heating of biominerals has commonly been used to artificially accelerate protein degradation in order to extrapolate kinetic parameters to the lower temperatures experienced *in vivo* and in the burial environment. It is not easy to test the accuracy of these simulations due to the difficulty of finding samples of known age held at a known temperature. We compare protein degradation in the intra-crystalline organic matrix of heated (80 °C, 110 °C, and 140 °C) massive *Porites* sp. coral to that directly measured in the skeleton of colonies growing at ~26 °C and deposited over the last five centuries. This provides the opportunity to critically evaluate the underlying assumption that high-temperature experiments accurately mimic degradation processes and kinetics occurring in a 'naturally aged' biomineral. In all samples the intra-crystalline protein fraction was isolated and the L- and D-concentration of multiple amino acids measured using reverse-phase high-performance liquid chromatography (RP-HPLC). There was no evidence of a failure of the closed system in the high-temperature experiments (assessed by X-ray diffraction, thermogravimetric analyses and determination of leached amino acid concentration). We compared conventional methods for estimation of kinetic parameters with a new 'model-free' approach that makes no assumptions regarding the underlying kinetics of the system and uses numerical optimisation to estimate relative rate differences. The 'model-free' approach generally produced more reliable estimates of the observed rates of racemization in 'naturally aged' coral, although rates of hydrolysis (as estimated from the release of free amino acids) were usually overestimated. In the amino acids for which we were able to examine both racemization and hydrolysis (aspartic acid/asparagine, glutamic acid/glutamine and alanine), it was clear that hydrolysis was less temperature sensitive than racemization, which may account for the differences in degradation patterns observed between the 'naturally aged' coral and high-temperature data. It is clearly important to estimate the individual temperature dependence of each of the parallel reactions.

1. Introduction

1.1. Amino acid racemization (AAR) and high-temperature experiments

The temperature-dependence of reactions is often exploited in controlled high-temperature experiments to explore processes occurring over geological time scales that would otherwise be too slow to observe. This approach has many advantages for the study of amino acid racemization (AAR), the inter-conversion of L-amino acids (which constitute protein in living organisms) into an equilibrium mixture of L- and D-amino acids after tissue death. In addition to being a time-dependent process (and therefore used in geochronology), AAR is also strongly temperature-dependent and so isothermal heating of biominerals has been used as a convenient method of artificially accelerating protein degradation reactions (e.g. Bada, 1972; Mitterer, 1975; Masters and Bada, 1977; Kriausakul and Mitterer, 1978; Kimber and Griffin, 1987; Goodfriend and Meyer, 1991; Miller et al., 1991, 1992, 2000; Roof, 1997; Manley et al., 2000; Kaufman, 2000, 2006; Clarke and Murray-Wallace, 2006). In practice, these experiments typically involve heating samples isothermally at a minimum of three elevated

* Corresponding author. Tel.: +44 117 331 5003.
E-mail address: peter.tomiak@bristol.ac.uk (P.J. Tomiak).

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temperatures, over a range of time-lengths, and determining the extent of racemization (D/L value) and in some studies, hydrolysis.

1.2. Kinetic models

The estimate of a reaction rate constant (k) is typically achieved by transforming D/L values so that they are linear with respect to time, thereby allowing quantification of k from the slope of the resulting regression line (see Clarke and Murray-Wallace, 2006, and references therein). When heated, free amino acid racemization conforms to reversible first-order kinetics (RFOK) (Bada, 1984 and references therein). Racemization of bound amino acids in the proteins of both fossil and (heated) modern biominerals, on the other hand, can deviate significantly from RFOK (see Clarke and Murray-Wallace, 2006, and references therein), a pattern that is particularly apparent at high D/L values, and for the rapidly racemizing amino acid aspartic acid (e.g. Goodfriend, 1991; Goodfriend and Meyer, 1991; Manley et al., 2000). Consequently, empirical-based transformations have become more commonly used to calculate an "effective" or "pseudo" rate constant (reviewed by Clarke and Murray-Wallace, 2006). Irrespective of the transformation used, the calculated rate or "pseudo" rate constant characterizes the "observed" racemization reaction, which in reality is influenced by a network of underlying reactions, including hydrolysis and decomposition reactions (such as decarboxylation, deamination or deamidation; e.g. Wehmiller et al., 1976; Goodfriend and Meyer, 1991; Collins and Riley, 2000).

The Arrhenius relationship (Equation (1), Box 1) describes the relationship between temperature and the rate of a reaction. Using k values acquired experimentally at elevated temperatures, and providing certain assumptions are met as discussed below, the Arrhenius relationship can be applied to quantify the temperature-sensitivity of k. This subsequently enables an estimate of the rate of racemization at the lower environmental temperatures that fossils are naturally exposed to (Box 1). In some cases this method has been used to estimate absolute ages for fossil biominerals (see Equation (3), Box 1, e.g. Mitterer, 1975; Mitterer and Kriausakul, 1989; Brooks et al., 1990; Miller et al., 1991). By providing estimates of the temperature sensitivity (i.e. activation energy, E_a) of racemization, isothermal heating experiments also enable palaeotemperature estimations based on the D/L values of fossils of known age (e.g. Kaufman, 2003). It is worth noting however, that in all cases (including this study), when the value of k for the observed degradation reaction does not represent a "true" rate constant, the activation energy (as well as other Arrhenius parameters) derived using k, is considered an "apparent" or "effective" activation energy.

There are several theoretical issues associated with extrapolating from high to low temperatures that may affect the validity of this approach (Kimber and Griffin, 1987; Goodfriend and Meyer, 1991; Walton, 1998; Collins et al., 1999; Collins and Riley, 2000; Demarchi et al., 2013a). Firstly, the use of kinetic models that do not adequately represent the observed reaction will result in inaccuracies in the derived rate constant and Arrhenius parameters (Goodfriend and Meyer, 1991). Secondly, fundamental to these experiments is the assumption that when heated, the protein fraction follows the same degradation pathways occurring under "normal" burial temperatures. This in turn relies on the assumption that either (i) the temperature sensitivities (the activation energies) of the parallel reactions contributing to k do not differ significantly, or, (ii) that these underlying reactions are inconsequential in determining the rate of racemization. If these assumptions are true, the line connecting the k values estimated at different temperatures on an Arrhenius plot (Box 1) should be linear. However, if the temperature-sensitivity of each reaction contributing to k varies, then these underlying reactions will influence k differently at

Box 1. Kinetic experiments and the Arrhenius relationship.

The Arrhenius relationship can be used to determine how the rate of an ideal gas phase reaction is affected by temperature. The relationship is widely used to explore the temperature-dependence of reactions, many of which do not conform to this ideal behaviour.

The Arrhenius equation can be given as:

$$k = A\, e^{(-E_a/RT)} \quad (1)$$

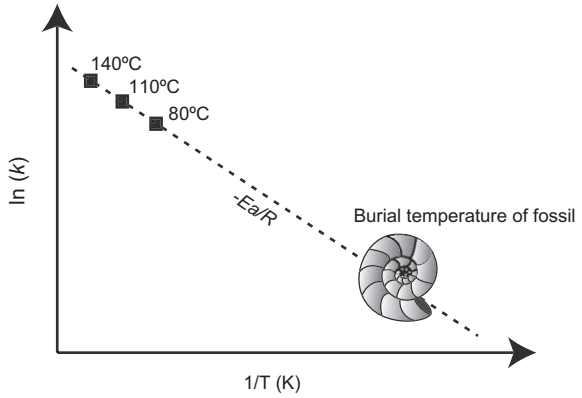
k = the rate constant; A = the Pre-exponential factor (yr^{-1}); E_a = the activation energy (J mol^{-1}); R = the universal gas constant (8.314 J mol^{-1}); T = the absolute temperature (K).

However, in order to calculate the temperature dependence of the rate constant, the two unknown parameters of the Arrhenius equation must be solved.

Equation (1) can be rearranged into "$y = mx + c$" form, to give:

$$\ln k = -E_a/RT + \ln(A) \quad (2)$$

In an "Arrhenius plot", the natural logarithm of the rate constant (ln k) is plotted against the reciprocal of the absolute temperature in K ($1/T$).



Schematic Arrhenius plot.

If values of ln k acquired at 3 or more temperatures are plotted, according to equation (2), a straight line connecting these points will yield a slope (m) equal to $-E_a/R$, with a y-intercept of ln(A). The activation energy for the reaction is therefore equal to the gradient (m) × R.

It is therefore theoretically possible to determine k at any temperature, by substituting the values for E_a and A, and the desired temperature, into equation (1) (shown graphically in the schematic Arrhenius plot). Therefore, the rate constant for racemization can be calculated for a fossil, if its effective burial temperature (the integrated effect of all temperatures to which the fossil sample has been exposed (Wehmiller, 1977)) is known. The rate constant can subsequently be substituted into an appropriate 'age equation' to estimates the fossil's age; for example, if the rate constants are derived assuming reversible first order kinetics (RFOK) the following equation can be applied:

$$t = \frac{\ln\{(1 + \mathrm{D/L})/(1 - \mathrm{D/L})\} - \mathrm{constant}}{2k} \quad (3)$$

constant = racemization induced during sample preparation; t = age of the sample (yrs); D/L = ratio of D to L enantiomers