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Glutamine deamidation: an indicator of antiquity, or preservational quality?

Elena R. Schroeter[1]* and Timothy P. Cleland[2]**

[1]Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA
[2]Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12182, USA

RATIONALE: Much credence has been given in the paleoproteomic community to glutamine deamidation as a proxy for the age of proteins derived from fossil and subfossil material, and this modification has been invoked as a means for determining the endogeneity of molecules recovered from very old fossil specimens.
METHODS: We re-evaluated the relationship between glutamine deamidation and geologic time by examining previously published data from five recent mass spectrometry studies of archeaological fossils. Deamidation values recovered for fossils were graphed against their reported chronologic age using WebPlotDigitizer.
RESULTS: The experimental data that has been produced from fossil material to date show that the extent of glutamine deamidation does not correspond to the absolute age of the specimens being examined, but rather show extreme variation between specimens of similar age and taxonomic affinity.
CONCLUSIONS: Because deamidation rates and levels can be greatly affected by numerous chemical and environmental factors, we propose that glutamine deamidation is better suited as an indicator of preservational quality and/or environmental conditions than a mark of the endogeneity or authenticity of ancient proteins.

The challenge of discerning contaminant molecules from preserved, endogenous molecules is particularly problematic in paleoproteomics studies; if peptides are recovered, questions about their authenticity will likely persist unless a reliable marker for age is found. Deamidation of glutamine is one proposed marker of age that has recently been measured in an extensive set of archaeological and paleontological bone.[1–5] This modification became the focus of age studies because it has been shown to function as a molecular clock for biological processes in modern proteins.[6–8] However, the use of this modification as a definitive marker of antiquity overlooks a number of factors that can complicate the expected progression of deamidation as well as its assessment, and using it as a definitive support of endogeneity in paleoproteomic studies may lead to the incorrect characterization of exceptionally well-preserved, incompletely deamidated ancient sequences as contamination. Here, we propose that extensive deamidation is far from conclusive as a criterion of authenticity for ancient proteins and peptides; rather, if sequence data support the endogeneity of recovered peptides, deamidation should be viewed as an indicator of their preservational quality rather than their exposure to contamination.

* *Correspondence to*: E. R. Schroeter, Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA.
E-mail: easchroe@ncsu.edu
** *Correspondence to*: T. P. Cleland, Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12182, USA.
E-mail: tpcleland@utexas.edu

Deamidation of glutamine and asparagine residues is a common, non-enzymatic modification that results in a +0.98402 mass shift caused by the conversion of a side-chain amide group with a carboxylate via a cyclic intermediate (glutarimide/succinimide) under alkaline and neutral conditions (Fig. 1(A)) or direct hydrolysis under acidic conditions (or in the presence of metal cationic species) (Fig. 1(B)).[9–11] The regularity of deamidation rates for individual residues when held at constant environmental conditions has led researchers to hypothesize that deamidation functions as a molecular clock for *in vivo* biological processes, such as aging and development.[6–8] Because the turnover rate of glutaminyl residues is slower than that of asparaginyl residues,[9] it has been proposed that glutamine deamidation may even be useful as a tool for investigating non-biological processes outside of living tissues, such as the aging of historical artifacts (e.g., artwork using proteinaceous paints).[3,12] The post-mortem deamidation of glutamine residues in peptides of collagen I, the most abundant protein in bone,[13,14] has also been used to study protein breakdown in archeological remains that are tens of thousands of years old.[2,3] Subsequently, extensive glutamine deamidation has been invoked as confirmation that collagen I peptides recovered from fossils are endogenous to the animal being analyzed (and not contamination from recent sources) because they are degraded (e.g., Welker[1]). However, the use of deamidation in this context employs degradation as a proxy for age, even though this relationship is limited based on empirical evidence (Fig. 2) and precludes the possibility that proteins may be both well preserved and ancient.

Many environmental conditions to which proteins are subjected, both *in vivo* and during diagenesis, can affect the rate and total levels of deamidation observed in a sample,

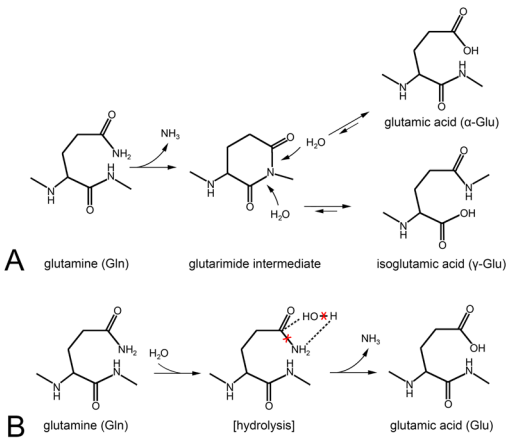

Figure 1. Schematic representation of deamidation of glutamine to glutamic acid via (A) the formation of a glutarimide intermediate and (B) direct hydrolysis. Depiction of cyclical intermediate deamidation (A) is adapted from Li *et al*.[9] Depiction of glutamine hydrolysis is based upon the equivalent reaction hypothesized for asparagine.[10]

including (but not limited to) pH, temperature, ionic strength, and presence of buffer ions.[15–22] It is for this reason that studies assessing the deamidation rates of model peptides *in vivo* have required strict environmental control (e.g., 37°C, pH 7.4 in 1.5 M Tris•HCl buffer).[6–8] In fossils, the environmental factors experienced by preserved peptides cannot be fully known, and have likely fluctuated over time – certainly at least three times, as they transition from life through death and decay and ultimately into entombed and altered tissue. Furthermore, these conditions may vary on local- to nano-scales (as they do in other processes dependent on environmental factors, such as elemental uptake, recrystallization, and isotopic composition)[23,24] causing differing states of deamidation between individual bones in the same locality, or even separate regions of the same bone. This variation will be even greater between fossils from localities with different sedimentary environments, which can be deposited under highly disparate physical, biological, chemical, and hydrodynamic conditions.[25] Previous studies have attempted to correct for the effects of the environment on expected deamidation rates using 'thermal age', a metric that uses environmental data (e.g., current/prehistoric temperatures and fluctuations, burial depth over time, sediment type, hydration of sediment) to normalize the thermal history of fossils between different localities.[1,26] However, while some deamidation rate studies have shown a broad correlation with thermal age,[1–3] the majority of critical environmental factors experienced by fossils over time, including how long each of those conditions dominated local environments, are unknown and unknowable for most fossil localities, obscuring the calculation of an accurate thermal age as well as an accurate prediction for deamidation rates.

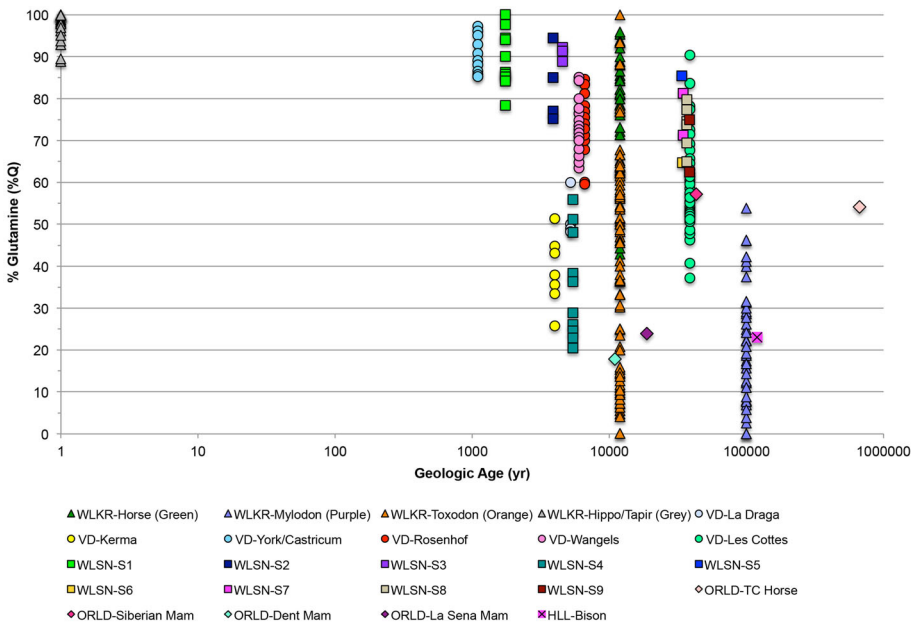

Figure 2. Graph of previously published data for deamidation values of archaeological fossils against their reported chronologic age. Shape of data points correspond to studies of origin (Δ: Welker *et al*.[1]; ○: van Doorn *et al*.;[2] ■: Wilson *et al*.;[3] ◆: Orlando *et al*.;[5] X: Hill *et al*.[4]). Individual species and/or localities are represented by different colors. Data is given as %Glutamine, or %Q (i.e., percentage of remaining glutamine that has not been deamidated). Note the high level of variation of %Q in specimens of similar age clusters. Interestingly, the oldest data point on this graph (Thistle Creek Horse, 560–780 Ka[5]) is less than 50% deamidated. Data was acquired from published graphs using WebPlotDigitizer.[37] Where data was published as %Deamidation, it was converted here into %Q for consistency across studies. Age axis is in logarithmic scale.

Beyond environmental factors, deamidation is also greatly affected by the primary sequence and resultant three-dimensional (3D) structure of proteins.[6–8,21] For a given residue, charge distributions, steric factors, and other considerations of the peptide chemistry of the surrounding sequence govern its initial deamidation rate, which is then further modified by the 3D folding to which it is subject.[8] While the expected rates for synthetic peptides of known length and sequence can be predicted under controlled conditions,[7] these factors cause deamidation rates to vary greatly even between peptides of the same protein, beyond what is driven by environmental conditions. Indeed, in a study of collagen I peptides from multiple archeological samples, Wilson *et al*.[3] found that deamidation patterns of specific preserved peptides varied greatly: some peptides were found to always be deamidated in all samples regardless of age or preservational quality of the specimen, some showed no deamidation in any samples, and some showed deamidation levels correlated with the estimated thermal age of the sample, though the study was not able to pinpoint a structural reason for these disparate patterns.[3] This indicates that specific peptides may have more utility for comparing deamidation rates between samples than others, but also illustrates the potential difficulty of applying this framework to fossil specimens of vastly different ages and taxonomic affinities – especially ones without closely related modern descendants. For example, the subset of peptides that deamidate at a useful rate may not be known for a given species. Additionally, it may not be feasible to extrapolate them from other species because sequence variation between taxa, even for conserved proteins like collagen I, could potentially lead to changes in steric interactions that may affect rate.[27] Without being able to assess this subset in advance, researchers are left to calculate deamidation levels for any peptides recovered, some of which may be useful and some not, but which may give conflicted or skewed results when considered together *a priori*. Even if the best peptides to use for degradation assessment can be reliably predicted in advance from a closely related species, they may not always be preserved in a given fossil, or may not be recovered in analysis even when they are preserved.[28] Furthermore, it is unknown how changes to the sequence and 3D structure of proteins by diagenetic modifications, such as formation of advanced glycation end products (AGEs)[29] or inclusion of diagenetic iron on the proteins,[30] may further alter deamidation rates, potentially arresting this process while preserving peptides into geologic time and resulting in extreme deviations from 'expected' glutamine deamidation levels.

Additional factors may further complicate efforts to establish a reliable relationship between age and deamidation. Notably, while deamidation may occur non-enzymatically, enzymatic activity can also cause deamidation. Transglutaminases (TGases or TGs), a widespread group of enzymes that are found in vertebrates, plants, and microorganisms, are known to catalyze the deamidation of glutamine into glutamic acid.[31–33] The possible presence of these enzymes in burial environments and decaying tissues, and their effect on the deamidation rate of bones during diagenesis, has not been explored. The presence of metal ions has also been implicated in the hydrolysis of amides,[11,34] which may differentially affect fossils with even slight chemical differences in burial environments.

Ultimately, the best indication that glutamine deamidation is not suitable as a strict marker for age is that the published empirical evidence for deamidation in fossil specimens shows that deamidation is *not* correlated with chronologic time. We have reanalyzed the data presented in five studies[1–5] that use deamidation as a proxy for age, which examined bones up to 560–780 Ka (Fig. 2), by plotting the reported level of non-deamidated glutamine (%Q) for each specimen against its reported chronologic age (i.e., the geologic age of its excavation locality). From these combined data, the more recent bones (0–2000 years) generally fluctuate between 80–100% non-deamidated glutamine (%Q). However, older fossils of similar ages display extreme variance in their total levels of deamidation, even between specimens of the same species – as much as the full range between 0–100% (e.g., *Toxodon*[1]). In cases like these, using deamidation as a marker for antiquity and authenticity would not only require that a subset of samples from the same species and age be disregarded, but also that researchers designate an *a priori* threshold along a sliding scale as a cut-off for what is authentic and what is not. When conducting paleoproteomics on an individual specimen, recovered peptides may be disregarded on the basis of having an arbitrary 'low' level of deamidation in its peptides, when they actually represent one end of a range of values that is not apparent when considered in isolation. In either case, valuable proteomic data may be lost by the arbitrary assignment of a threshold based upon a single metric that has been shown not to correlate with specimen age.

The susceptibility of glutamine deamidation to environmental conditions we cannot trace accurately through geological history, the inherent variability of the rate of this reaction between different proteins as a result of their unique sequences and 3D structures, and the lack of empirical data supporting any reliable relationship between deamidation level and chronologic age preclude glutamine deamidation as a meaningful marker of antiquity or authenticity. The *a priori* assumptions that must be made as to the levels of deamidation that are 'expected' for a fossil of a given age, and what threshold of variability might be acceptable, means that it is not a criterion that can be applied robustly or consistently to individual specimens, particularly not on progressively more ancient ones. Thus, invoking it as a 'proof' of endogeneity, or requiring it as a criterion for acceptance of ancient proteomic data, is inappropriate. Other markers of protein breakdown (e.g., backbone cleavage, glutamic semialdehyde)[35,36] may be better markers of protein age than deamidation, but this remains to be fully tested. Alternatively, we propose that glutamine deamidation levels should be considered as a preservational marker on an individual basis, informing on the preservational quality of specific peptides and specimens rather than determining their chronological placement. Indeed, it has been proposed that the retention of amides might be an indicator that structural aspects of a protein are still intact,[7] which might be expected for proteins that persist beyond mathematical models for their predicted degradation. Further, as deamidation rates of fossil samples were found not to correlate with traditional metrics of bone quality that rely on mineral properties (e.g., crystallinity, CO_3/PO_4 and amide/PO_4 ratios),[2] using glutamine deamidation as a preservational indicator might provide a better, more direct assessment of protein degradation in ancient samples.

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