Deep Time Paleoproteomics: Looking Forward

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Abstract

The goal of paleoproteomics is to characterize proteins from specimens that have been subjected to degrading and obscuring effects of time, thus obtaining biological information about tissues or organisms both unobservable in the present and unobtainable through morphological study. Although the description of sequences from *Tyrannosaurus rex* and *Brachylophosaurus canadensis* suggested that proteins may persist over tens of millions of years, the majority of paleoproteomic analyses have focused on historical, archeological, or relatively young paleontological samples that rarely exceed 1 million years in age. However, recent advances in methodology and analyses of diverse tissues types (e.g., fossil eggshell and dental enamel) have begun closing the large, unexplored window of time that remains in the fossil history of the Cenozoic. In this perspective, we discuss the history and current state of deep time paleoproteomics (DTPp), here defined as paleoproteomic study of samples ~1 million years (1 Ma) or more in age. We then discuss the future of DTPp research, including what we see as critical ways the field can expand, advancements in technology that can be utilized, and the types of questions DTPp can address if such a future is realized.

Keywords: Paleoproteomics, Deep Time, Dinosaurs, Enamel, Eggshell

Introduction

The field of paleoproteomics (i.e., the characterization of proteins from archaeological and paleontological tissues using mass spectrometry [MS]) has grown exponentially since the first application of matrix-assisted laser desorption ionization (MALDI) MS to mammal remains 800-450,000 years old in 2000.[1] In the 21 years that followed, a variety of mass spectrometry-based methods have been used to investigate the preserved proteomic content of a diverse array of biological tissues (e.g., bones, teeth, baleen, turtle shell, mummified tissues),[2-14] objects (e.g., paintings, ethnologic objects, potsherds, parchment),[15-24] and species (e.g., mammoth, moa, giant beaver, whales, sea turtles).[2, 3, 11, 12, 17, 25-31] However, the vast majority of these studies have focused on relatively young (< 100 thousand years old) paleontological and archaeological materials and remains. In this perspective, we discuss the development and progress of "deep time paleoproteomics (DTPp)," here defined as MS characterization of material older than ~1 million years (1 Ma). We begin with a short retrospective on our experience in paleoproteomic analyses of Mesozoic dinosaurs, followed by a brief review of the current state of deep time paleoproteomics. We then summarize what we see as possible future directions for the field, and propose questions/hypotheses that may be tested as MS investigations employ new technologies and explore new taxa, tissues, and geochemical environments from deep time. Finally, we discuss the relevance and impact of deep time paleoproteomics to issues affecting our own lineage, now and in the future.

The beginning of deep time paleoproteomics

The examination of specimens older than 1 Ma for preserved proteins began as early as the 1950's[32] with non-MS approaches, including immunology,[33-36] amino acid analyses,[37-41] and non-proteomic MS approaches such as pyrolysis gas chromatography mass spectrometry (PY-GC-MS)[42, 43] and time of flight secondary ion mass spectrometry (TOF-SIMS)[44]. However, although the molecular

techniques used to detect original organic constituents in fossils (e.g., Raman spectroscopy,[45, 46] Fourier transform infrared spectroscopy [FT-IR],[47, 48] TOF-SIMS[49]), have become increasingly sophisticated and have typically remained more accessible and inexpensive than MS, only MS approaches (and MS/MS in particular) have the ability to generate the peptide sequence information.[50] Thus, the development of tandem mass spectrometry and its first application to ancient fossils was a crucial step in the then-incipient field of molecular paleontology.

At the time Asara et al.[51] published the first *Tyrannosaurus rex* (MOR 1125) peptides obtained by LC-MS/MS, paleoproteomic methodology faced a number of limitations. First, sample preparation methodology was generally limited to in-solution digestion, followed by C18 (or similar) cleanup methods.[35, 52, 53] Second, protein search databases were comprised almost entirely of mammal-derived sequences. The scant representation of archosaurian taxa (i.e., birds, crocodiles, and their relatives) was limited to a few select gene products used for molecular phylogenetic reconstructions and the *Gallus gallus* genome.[54] As a result of this limited search space, homology searching of collagen I sequences from a broad set of predominantly mammalian species was necessary to identify relevant sequences. Finally, high mass accuracy instrumentation was limited in availability, and the low-resolution data produced using ion trap mass detection led to questions about the accuracy of identified peptide sequences and the location of post-translational modifications (PTM).[55] In particular, incorrect assignment of hydroxylations to glycine residues required subsequent correction to reflect their much more likely localization on proline residues, a characteristic feature of collagen I.[56] When re-searched using alternative software, the identification of collagen in raw MS data from the *T. rex* was supported by Bern et al.,[57] though the authors raised some concerns about potential contamination of the specimen.

Following publication of the collagen I peptides and supporting molecular evidence for their persistence in *T. rex* fossils, we sought to apply these methods to a second dinosaur specimen that had

been collected under tightly controlled conditions, using aseptic field techniques for the express purpose of molecular analyses.[58] Thus, the second application of LC-MS/MS to dinosaur remains was to fossil tissue from *Brachylophosaurus canadensis* (MOR 2598), which was excavated encased in its entombing sediment and without the use of any field preservatives or glue. When analyzed with a high resolution mass spectrometer and Orbitrap detection of precursor ions to increase accuracy of peptide identification,[58] this second dinosaur showed that, like the *T. rex*, peptide sequences could be recovered and identified with confidence. Further, it showed that when different types of fossil tissues isolated from the same specimen were subjected to paleoproteomic analyses, different proteomic profiles consistent with those of comparable extant tissues were observed (i.e., collagen I from bulk bone[58] and actin, tubulin, and myosin from isolated vessels[59]). A follow-up study of this same specimen, conducted almost a decade later in different laboratories from the original study and using different MS sample preparation methods and Orbitrap detection of both precursor and fragment ions, reproduced some of the previously reported peptides, as well as several additional collagen I peptides.[4]

Despite these successes, questions still remain about the endogeneity of sequences detected in Mesozoic specimens,[57, 60] because traditional MS bioinformatic approaches bias results toward the identification of highly conserved peptides in extinct taxa.[61] When coupled with the difficulty, expense, and inaccessibility of MS techniques, and the destructive nature of proteomic sampling, the resulting perception that deep time paleoproteomics is "high-risk" has discouraged wider application within the paleontological community. Without concerted efforts, detection of peptide sequences from other species of this age has been elusive.

Current state of deep time paleoproteomics

Since the publication of *T. rex* and *B. canadensis* sequences, interest in applying a broad range of molecular techniques to fossil tissues has grown (e.g., immunogold,[62] Raman spectroscopy,[45, 46] FT-IR,[47,

[48] pigment analysis[63], TOF-SIMS[49]), and the application of paleoproteomics has been no exception. Recent technological and methodological advances have contributed to progress in overcoming some of the inherent challenges of working on deep time samples, improving upon what could be produced by the earliest examples of DTPp. Below we discuss the current state of several aspects of DTPp research.

Anti-contamination protocols

The inherent challenges to obtaining sequence data in deep time has revealed the need for protocols unique to deep-time specimens to support endogeneity of recovered sequence data. Ensuring that proteins from extant sources do not come into contact with ancient samples,[57] and including experimental controls to detect whether contamination has occurred at any stage of analysis are now routinely part of any experimental approach. When applied to DTPp samples, these precautions and controls are typically much more rigorous than those conducted in studies of recently extinct or archaeological samples,[4] and include employing best-practices during excavation and initial sample handling, before and during protein extraction, and during MS sample preparation and analyses. At the most basic level, it is generally agreed that all preparation and analyses of specimens greater than 1 Ma take place in an environment in which extant/modern samples are not analyzed, and that negative controls (e.g., sediment from the excavation site and laboratory reagent blanks) be run simultaneously and under identical parameters to the fossil samples.[64]

Authentication methods

The goal of paleoproteomics is to identify endogenous proteins in long dead tissues; thus, attempts to develop a method or metric to authenticate ancient sequences as endogenous (above and beyond taxonomic specificity and the monitoring of negative controls) remain ongoing. Glutamine deamidation has generally been used for this purpose but is rife with issues that cast serious doubt on

its reliability to support or falsify advanced age (and thus endogeneity)[65-67] of deep time sequences.[68] Efforts to examine specific sites for deamidation may have more utility,[65] but overall, the variation observed in deamidation levels and patterns shows that caution should be used when applying it for authentication of ancient sequences.[68-70] Efforts to find an alternative to deamidation for authenticity that is reliable, consistent, and easily identified has, to date, not been unsuccessful.

Recently, it was proposed that deep time peptides cannot be considered authentic unless at least two novel peptides are found (i.e., two peptides that are not found in any other taxon).[60] We argue that this is an all but impossible threshold to meet for bioinformatics analyses that rely on matching experimental data to a database of already known sequences—a process that biases results toward conserved peptides and can fail to identify novel sequences even when they are present.[61] Additionally, confident identification of peptides with even a single amino acid variant from the search database remains quite difficult,[61] even in modern tissues,[71] and would likely be more difficult to assess for taxa in deep time. The use of more advanced peptide spectral match (PSM) quality metrics than a basic 1% false discovery rate (FDR) (e.g., posterior error probability [PEP] or more strict FDR values) may be necessary for variant peptides or rare peptides to support their detection when species-specific peptides are not found. Until a reliable metric can be identified, debate about endogeneity of deep time results will remain a lively part of this field, and corroboration of DTPp data with independent molecular techniques (e.g., immunology, Raman, TOF-SIMS) is encouraged.

Range of tissue types analyzed

Although early protein studies relied primarily on fossilized bone tissue, preserved proteins have now been recovered from alternative tissues such as enamel and eggshell [7, 8, 10, 72]. These studies have begun to fill the temporal gap between paleoproteomic analyses of more recent archaeological samples and Mesozoic dinosaur peptides, extending recovery of sequence data deeper into the Cenozoic (Fig. 1).

Enamel paleoproteomics has helped elucidate hominin and primate relationships from a variety of depositional environments[7, 8, 10] and has identified some of the oldest in vivo PTMs that are typically labile (e.g., phosphorylation[7]). Additionally, digestion-free analysis of enamel peptides has directly shown, for the first time, the types of diagenetiforms (i.e., protein forms arising from diagenesis[73]) that may be expected from fossil remains. Because of the obscuring effects of the enzymatic digestion required for bottom-up MS analyses, prior to these studies, patterns of non-tryptic peptides[2] were the only glimpse the community had into how diagenesis altered proteins.

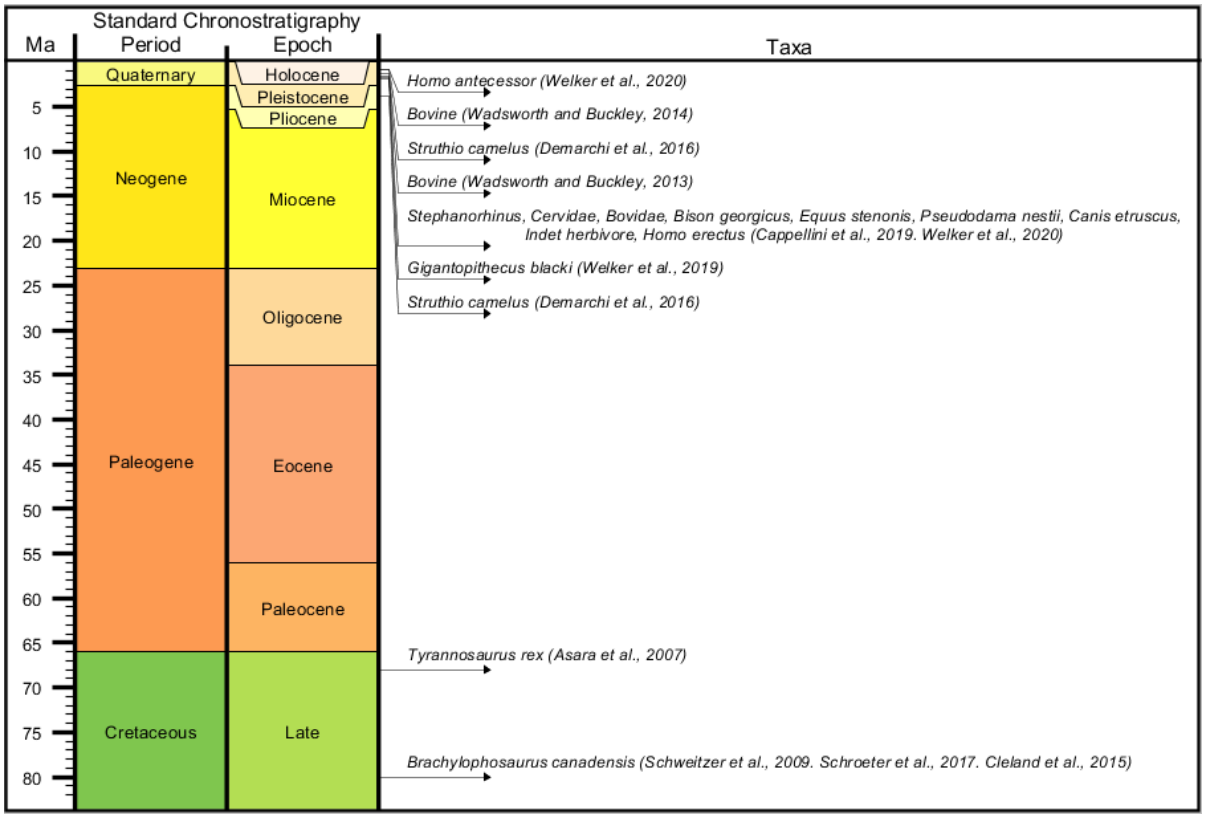

Figure 1. Geologic time scale[74] showing taxa ~1Ma and older from paleoproteomic studies.

Peptides derived from fossil ostrich eggshells represent the oldest Cenozoic peptides to date (3.8 Ma).[72] Peptides from these samples were found to be highly acidic and tightly bound to the calcium carbonate of the eggshells, providing testable hypotheses for diagenetic pathways leading to

preservation. Demarchi et al.[72] hypothesize that mineral interaction is critical for long-term survival of proteins into deep time, consistent with earlier hypotheses for bone.[75] This hypothesis is further supported by the relatively high quality of protein preservation within the various enamel samples.[7, 8, 10]

Advancements in bioinformatic analyses and search databases

Bioinformatic innovations within the greater proteomic community have allowed DTPp to improve peptide identifications from fossil taxa. These have included the advent of new search software (e.g., MaxQuant, Metamorpheus[76, 77]), as well as algorithms to detect unspecified sequence alterations (e.g., PeaksPTM, Byonic, MaxQuant, MSFragger, MetaMorpheus[78-82]). In younger specimens, these advances have allowed the detection and localization of a number of *in vivo* and diagenetic post-translational modifications (PTMS) not previously known, including advanced glycation end products (AGEs),[2, 3] alkylations,[2] phosphorylation,[7] and others. As our knowledge of diagenetic change in archeological samples increases, it can increasingly be applied in bioinformatics approaches towards DTPp.

Additionally, the expansion of known genomes for extant, non-model taxa (for a full perspective see Heck and Neely[83]) has directly benefited DTPp research, as exemplified by recent DTPp studies on enamel[7, 8, 10] and eggshell[72] as well as re-evaluation of *B. canadensis*[4]. Much like proteomic studies of extant taxa that do not have known proteomes in search databases, paleoproteomics benefits from the increasing addition of well-annotated genomes of taxa related to the target species in molecular databases. Such additions provide predictive protein sequences that allow for the construction of a search space phylogenetically closer to the extinct target species, and therefore less likely to miss identifying peptides that are present but too evolutionarily distinct from the in-silico examples used for comparison. However, because most extinct species will never have a sequenced genome (to date the oldest sequenced genome is a 1.0-1.2 Ma mammoth[84]), reliance on extant phylogenetic bracketing[85] in

database construction, as well as continued genomic investigation of extant groups whose genomes are still poorly represented, will remain critical for DTPp studies into the future—particularly for groups with no living descendants.

Future directions in DTPp

Although paleoproteomics of both recent and deep time samples has progressed rapidly in the last two decades, there are still many gaps in our knowledge and technological limitations to be overcome. However, the future of paleoproteomics is bright. Below, we discuss emerging innovations in MS methodology and technology that may be applied to DTPp to further advance our understanding of extinct species.

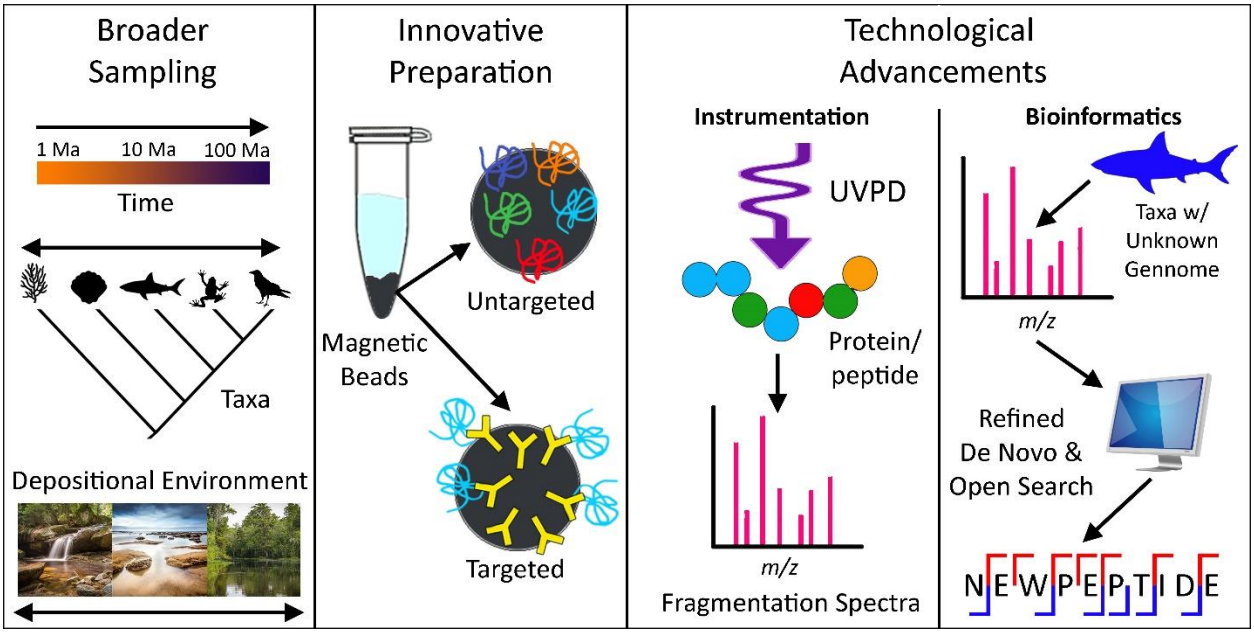

Figure 2. Schematic of possible future directions in DTPp.

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DTPp can be applied to an increasingly broad range of taxa, depositional environments, and age ranges

Taxa: To date, peptide sequences have been detected from few vertebrate taxa and no invertebrates ~1 Ma or more in age. In total, peptide sequences have been recovered from 17 species of tetrapods (3 archosaurs, 13 mammals) (Fig. 1).[4, 7, 8, 10, 51, 58, 59, 72, 86] This asymmetry of data is undoubtedly an artifact of sampling bias rather than preservation potential; indeed, recent paleoproteomic studies on archaeological mollusk shells and Pleistocene coral[87, 88] have shown that biomineralized invertebrates also provide an excellent avenue for future deep time research, one that has been to date left unexplored. Additionally, paleoproteomics of invertebrates might enjoy an abundance of samples because invertebrate fossils of some taxa (e.g., shelled mollusks) are much more numerous than vertebrate remains, allowing greater access for the destructive sampling usually required for proteomics. If pursued, proteomic analyses of taxa such as corals and other marine and freshwater invertebrates may provide valuable insights into biochemical and physiological response to paleoenvironments in deep time.[88]

Depositional Environments: To fully evaluate the protein preservation landscape in deep time, an intentional and methodical effort must be made to unravel the relationship between the geochemical environment and the preservation of molecules that persist through diagenesis. This should include both analyzing specimens from varied depositional environments and a more comprehensive characterization of the geochemical factors to which the fossil was subjected than current paleoproteomics studies typically perform. These steps are critical, because a more complete understanding of the geochemical controls on molecular preservation will enable us to identify reliable metrics that can be used as proxies to select specimens with a high potential for protein preservation. Given that paleoproteomics—and especially DTPp—can be costly in both money and permanently destroyed fossil tissue, a reliable way to choose which samples are most likely to yield results is of great

interest. For example, recent research has suggested that evaluation of characteristics such as the rare earth element (REE) uptake profile[89] or FT-IR peak ratios[90-92] of a fossil may reflect its preservational state/level of inorganic diagenetic alteration, and therefore its suitability for proteomic analyses.

Age range: Along with the broader sampling across more fossil taxa and depositional geochemistries, the third axis of study that requires a concerted expansion of sampling is time. In the 14 years since peptides from *T. rex* peptides were first published, the next-oldest data thus far produced by the paleoproteomics community is 3.8 Ma.[72] Thus, fossils across 62.2 Ma of time—nearly the whole of the Cenozoic—await paleoproteomic investigation (Figure 1). This is, in part, because of the reining paradigm that proteins are incapable of persisting into deep time. This assumption, produced prior to the development and application of paleoproteomic techniques, is based upon in-solution kinetic models that did not consider the potentially stabilizing effects of tissue structure, microbial activity, or geochemistry,[93] and discounted the possibility of protein preservation greater than 1 Ma.[94, 95] However, as even conservative estimates of "the oldest verified" peptides are well beyond the 1 Ma mark, it has become clear our sampling strategies can extend deeper into the Cenozoic. The analyses of additional, older timepoints will allow direct evaluations of protein preservation, leading to a more complete understanding of how and why proteins can persist to the Mesozoic. Additionally, deeper examination of the Cenozoic fossil record will provide access to more diverse extinct evolutionary lineages, as well as ancestral lineages for extant taxa.

DTPp can explore innovative approaches in sample preparation

Future discoveries in paleoproteomics will depend not just on expanded sampling efforts, but also on innovative approaches to sample preparation, and the development of bioinformatic tools to address challenges inherent to the proteomic study of fossils (and absent from the analysis of more typical proteomic samples).

Bead-based methods: Recent developments in bead-based sample preparation for paleoproteomics[87, 96-100] have allowed rapid separation of co-extracting molecules (e.g., humic substances) that prevent detection and characterization of ancient proteomes through ion suppression, as well as host of other issues (e.g., column clogging).[101] Although bead-based methods have, to-date, only been paired with bottom-up MS approaches, the wide range of protein/peptide sizes that is possible to capture with this methodology[102, 103] make it suitable for future use with digestion-free and top-down approaches as well. Bead-based methods have also been shown to be effective in automated sample preparation of archaeological material.[98] Automation has great potential for deep time paleoproteomics because sample preparation can be completed within a controlled environment. This has the potential to reduce laboratory contamination (e.g., keratins) that may overwhelm the low levels of preserved endogenous proteins expected from deep time samples, and may improve repeatability. However, to effectively utilize the bead-based methods, optimization of protein extraction for each tissue/sample type will be necessary. Recently optimized applications to bone[98] and enamel[7, 8, 10] show that fossil protein extractomics[104] is an area that has much room for development and optimization, especially with an eye towards increasing yield for samples from deep time.

Targeted paleoproteomics/Immunoprecipitation: Targeted paleoproteomics is another avenue that has received little or no exploration in DTPp, despite its common usage in proteomics experiments of extant tissues.[105, 106] In particular, using antibodies or aptamers[107] to enrich specific proteins from a complex fossil extract mixture may allow greater sequence coverage of these targets, thus increasing confidence of species identifications and the accuracy of phylogenetic placements. This selective enrichment may present an alternative method for removal of humics and other co-extracting suppressants by isolating only proteins of interest, even low abundance ones, while leaving the remainder of the extract behind. Immunoprecipitation methodology could also increase confidence in the use of antibodies for direct analysis of fossil soft tissues because it can remove the concerns of non-

specific binding by validating the antibody target.[108] Because mass spectrometry provides direct identification of isolated/enriched proteins, it is possible to directly evaluate the specificity of the antibodies/aptamers and rule out cross-reactivity with humics or other diagenetic molecules as the source of immunological response.[109] Further, we have found that some low-abundance bone proteins (e.g., hemoglobin) solubilize better in extraction buffers that are non-optimal for general proteome,[104] suggesting that extraction reagents may themselves be optimized for more targeted investigations.

Direct Digestion: Another promising sample preparation method is a recently proposed direct digestion of bone after treatment with isopropanol.[110] This direct digestion approach is slightly different from that used by Cleland[111] and Terajima et al.[112] , as it does not rely on removal of bone mineral prior to digestion. Even without demineralizing first, an extensive bone proteome was recovered by this method, including proteins from cellular and vascular compartments. Future developments in direct digestion of fossil bone may overcome issues that result in poor protein extraction yield.[104] These include protein insolubility, which may increase through diagenesis (e.g., addition of AGEs).[113] Direct digestion may also reduce the potential for laboratory contamination by limiting preparation steps, and avoid issues of heterogenous protein preservation across bone[50, 114] by providing a more convenient way to extract multiple bone areas.

DTPp can embrace technological advancements

Instrumentation: Refinement of mass spectrometry methodology over the last 20 years has shaped the current state of paleoproteomics, and inclusion of new developments in instrumentation and fragmentation will be critical to future innovations. The ever-increasing sensitivity of modern instruments[115, 116] will allow paleoproteomics to detect more low abundance proteins or protein fragments than are typically found in DTPp samples, and the use of gas phase separations (e.g., high Field Asymmetric waveform Ion Mobility Spectrometry [FAIMS]) may overcome issues with coextracting

molecules without requiring separation during sample preparation. Alternative data acquisition methods (e.g., data-independent acquisition [DIA] or similar) may potentially increase sensitivity without increasing the amount of sample required. Multiple reaction monitoring (MRM) and/or parallel reaction monitoring (PRM) data acquisition workflows,[117] which can identify target peptides of interest at the sequence level in complex samples,[118] may be employed to interrogate ancient tissues for the presence of phylogenetically or physiologically relevant peptides.[119] These workflows can potentially offer a potential path for species identification with the accuracy of MS2 fragmentation, but in a fraction of the time typically required for discovery mode LC-MS/MS analyses.[120] Newer fragmentation methods (e.g., ultraviolet photodissociation [UVPD],[121] electron transfer dissociation coupled to higher-energy collision dissociation [EThcD][122]) may enable us to more accurately localize in vivo and diagenetic PTMs, including those that may be lost during traditional collision-based fragmentation. Accurate localization of diagenetic PTMs permitted by these technologies will, in turn, elucidate patterns of protein diagenesis, and facilitate the development of predictive models of protein modification and breakdown. Further, the use of digestion-free and top-down paleoproteomics, combined with these alternative fragmentation methods, will help maximize our understanding of diagenetiform formation.

Bioinformatics: Limitations of available bioinformatic technology have always been the biggest challenge faced by paleoproteomic studies. DTPp is even more impacted by bioinformatic challenges because, to date, the limited retrieval of ancient DNA[84] prevents generating genomes from the majority of extinct species. As a result, new bioinformatic tools or add-ons to current tools will be necessary to maximize detection and confidence in identification of sequences that vary from search databases.[61] The difficulties in detecting and controlling false discovery of these variants[71] makes finding changes specific to extinct species very difficult, but the ability to do so is a crucial goal of paleoproteomics as a discipline. Thus, although such variations affect all proteomic disciplines that rely on database searching, the field of paleoproteomics must take a central role in overcoming these challenges. This is especially

true if paleoproteomics is to be applied to extinct taxa that are phylogenetically distant from all living species, and thus will never have known proteomes of close relatives for database comparison. For example, ichthyosaurs are a group of swimming reptiles that are very evolutionarily distant from all living taxa—geckos are more closely related to hummingbirds than an ichthyosaur is to any extant reptile.[123] Therefore, MS studies of such species will remain confined to only the most conserved peptide sequences until there are further advancements in de novo sequencing[124-127] and similar methodologies that maximize confidence in sequence identification.

Along with species-specific changes, unknown diagenetic PTMs continue to pose challenges to peptide identification. Only a limited set of diagenetic PTMs is currently characterized (e.g. AGEs, hydrolytic cleavage, oxidation[2, 3, 7, 8, 10, 15]), and the full scope of how diagenesis may alter protein sequences is unknown. To reduce bioinformatic search space without sacrificing identification of diagenetically altered sequences, it is necessary to more fully explore diagenetic changes so that they can be incorporated into search parameters. In addition to directly examining fossil proteins for alteration, this will require the development of tools that can predict what kind of changes might occur or patterns of change that might develop, and testing for their presence in fossil tissues and actualistic experiments.

Relevance of Paleoproteomics

The recovery and analyses of deep time proteins has the potential to shed light on ancient life independent of morphology, as well as to address issues impacting our own lineage and future. The fossil record represents hundreds of millions of years of evolutionary "experimentation" for which data already exist; by utilizing the technological advances outlined herein, gathering and interpreting these ancient data will be increasingly within our grasp.

Phylogeny, Evolutionary Transitions, and Molecular Clocks

The most obvious outcome of the study of ancient molecules is that sequence data from deep time fossils provides independent tests of phylogenetic hypothesis that are typically based exclusively on morphological features (and are thus more readily influenced by evolutionary convergence than molecular data).[7, 8, 10, 128-130] However, DTPp could also potentially be used to calibrate and fine-tune molecular clock estimates for the origination of crucial evolutionary adaptations. The molecular clock hypothesis proposes that DNA and protein sequences change at a relatively constant rate in different lineages/molecules, and when calibrated to the geologic age of evolutionary events (as estimated from the fossil record), that constant rate can be determined and subsequently used to estimate evolutionary time scales.[131] Sequences from DTPp samples could be used to more precisely determine (and test) the rate of change than can be achieved using extant sequences alone. For example, the evolution of feathers had enormous impact on the evolutionary trajectory of the dinosaur (bird) lineage. Scales, beaks, and feathers are comprised of keratin-like proteins known as corneous β-proteins (CβP),[132-135] which are unique to sauropsids[132] and harder and less flexible than the mammalian α-keratins that comprise skin, hair, and nail or claw sheaths. However, a subset of CβPs (called feather CβPs) have a molecular modification that confer greater flexibility to these CβPs than other sauropsid keratins.[136, 137] Although this modification was hypothesized to originate at ~124 Ma using molecular clock theory,[136] morphological evidence shows the presence of feathers in organisms that predate this estimate by millions of years.[138] Immunological techniques have demonstrated preserved feather CβPs in isolated *Anchiornis* feathers (~160 Ma),[138] making them an excellent target for testing the utility of DTPp for recalibrating molecular clocks.

Physiology

Independent adoption of true homeothermic endothermy in mammals and birds is another evolutionary mystery that could be resolved using DTPp exploration. Although hemoglobin in both birds and mammals is modified to increase efficiency of oxygen downloading to tissues (which is required to sustain elevated metabolic rates),[139] this is accomplished differently in each group; birds incorporate more positively charged residues than mammals in the region of hemoglobin that binds the allosteric effector molecule needed to download oxygen.[140] Given the evolutionary distance between these groups, it is hypothesized that mammals achieved endothermy through a different pathway than birds and their dinosaurian ancestors.[141] Hemoglobin sequence data from fossils may narrow the timepoint at which these changes occurred, and could help point to atmospheric and other factors that may have favored the incorporation of these different effector molecules in each lineage, which ultimately led to the success and diversification of endothermic birds and mammals from different ectothermic ancestors.

Diagenetic alteration on endogenous molecules

Diagenetic alterations of preserved molecules hold potential for the study of human disease. Diagenetic modifications in fossil remains from deep time reflect end-member states of modification and can potentially be used to predict what may be possible in vivo. For example, AGEs, which have been detected in fossil bony remains,[2, 3] lead to reduced bone quality in both aging [142, 143] and/or diabetic living humans.[144] Diagenetic fragmentation of proteins[2, 7, 8, 10] may also lead to evidence for weak or fragmentation-prone areas of proteins that may be targets for therapeutics.

Conclusion

Recent innovations in preparation and analytical techniques, expansion of databases and novel search algorithms, and better understanding of diagenetic alterations to molecules leaves

paleoproteomics well poised to advance our understanding of extinct species. A concerted, community-wide effort to fully evaluate and characterize fossils from deep time for molecular content will yield new approaches to age-old problems, from evolution to human health, making the study of deep time fossils for molecular content an endeavor well worth the expense and effort needed to grow this field.

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