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Dinosaur Peptide Preservation and Degradation

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Abstract

Soft tissue recovered from purportedly 68 million years old fossils of *Tyrannosaurus rex Brachylophosauraus canadensis* have been analyzed, and preservation mechanisms have been suggested by workers. A "preservation motif" based upon structure-function relationships at the molecular level is thought to explain the selective survival of soft tissue and particular peptide sequences. Preservation motifs include molecularly sheltered environments such as collagen fibril domains that offer tight molecular packing thereby conferring resistance to degradation. Sequences enriched with hydrophobic amino acid residues and depleted in acidic residues suggest another preservation pattern. More recent work by the same group adds that Fenton type reactions, arising from trace iron discovered with the bone tissue, is responsible for "fixing" the collagen for deep time survival. The present paper reviews these preservation motifs and supportive data in light of fast-degrading amino acids such as asparagine and glutamine, and oxidatively sensitive markers tyrosine, methionine, and histidine that survived the long burial age.

Introduction

Discoveries of biomolecular remnants found in ancient organisms are mounting, including the startling discovery of dinosaurian soft tissue, which has opened new lines of investigation thought impossible a few years ago. In 2005 Mary Schweitzer et al. (2005a, 2005b) reported soft tissue found in a *Tyrannosaurus rex* fossilized femur dated at 67 million years old. A detailed documentation of the discovery of soft tissue can be found elsewhere in this issue, and details of Dr. Schweitzer's story, which has been called the greatest discovery of the twenty-first century, is available (e.g., see Fields, 2006), but a brief account will be offered here.

Remarkably the recovered tissue specimen from the femur was found to be "flexible and resilient and when stretched returns to its original shape" (Asociated Press, 2012). Subsequent polypeptide analysis of the tissue showed well-preserved amino acid sequences. In late 2012, Schweitzer reported an even more startling discovery.

> Schweitzer and her team also tested for the presence of DNA within the cellular structures, using an antibody that only binds to the "backbone" of DNA. The antibody reacted to small amounts of material within

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> the "cells" of both the *T. rex* and the *B. canadensis*. To rule out the presence of microbes, they used an antibody that binds histone proteins, which bind tightly to the DNA of everything except microbes, and got another positive result. They then ran two other histochemical stains that fluoresce when they attach to DNA molecules. Those tests were also positive. These data strongly suggest that the DNA is original, but without sequence data, it is impossible to confirm that the DNA is dinosaurian. (Peake, 2012)

While the discovery is surprising, if not shocking, it may turn out to be common. Trace organic matter has been found in Mesozoic fossil bone for decades dating to the 1960s (Schweitzer et al., 2013a). In addition to discernable cells and vessels, chemical matter such as collagen fibrils, lipids and polysaccharides have been reported. Roman Pawlicki has authored or coauthored multiple papers almost 40 years on the subject of Dinosaur soft tissue studies (e.g., Pawlicki et al., 1966; Pawlicki, 1977, Pawlicki and Nowogrodzka-Zagórska, 1998). In the past decade, paleontologists have discovered proteinacious matter in a variety of fossilized remains and are using detailed chemical analysis to elucidate chemical structures.

For example, Scheweitzer et al. (2005b, p. 780) reported that organic residue was recovered from a sauropod dinosaur eggshell, noting, "We have shown that in these well-preserved fossils, the molecular structure of endogenous antigens may be preserved for more than 70 Myr." Schweitzer et al. (2007, p. 188) continued their work and reported finding "thin, flexible and fibrous, and emerged as sheets of material" in demineralized *Triceratops horridus* (MOR 699, 65 Ma) bone. (Ma and Myr mean million years ago and MOR is Museum of the Rockies).

Lindgren et al. (2011) "demonstrate that endogenous proteinaceous molecules are retained in a humerus from a Late Cretaceous mosasaur (an extinct giant marine lizard)." Cody et al. (2011) reported a signature of a "relict chitin-protein complex preserved in a Pennsylvanian (310 Ma) scorpion cuticle and a Silurian (417 Ma) eurypterid cuticle via analysis with carbon, nitrogen, and oxygen X-ray absorption near edge structure (XANES) spectromicroscopy." In addition, an international group of scientists (2012) has managed to identify 126 distinct protein sequences from a 43,000-year-old bone from a woolly mammoth (*Mammuthus primigenius*) (Cappellini, et al., 2012).

Edwards et al. (2011, p. 3217) studied a 50-million-year-old reptile concluding, "taken together, all the analyses performed in this study strongly suggest that the fossilized reptile skin… is not a simple impression, mineralized replacement or an amorphous organic carbon film, but contains a partial remnant of the living organism's original chemistry, in this case derived from proteinaceous skin."

Paleontologist Robert Reisz with the University of Toronto, Mississauga, led an international team of scientists from Canada, Taiwan, the People's Republic of China, Australia, and Germany to excavate a site in South Africa yielding a 190-million-year-old dinosaur nesting site of the prosauropod dinosaur Massospondylus. The clutch of eggs produced over 200 bones from individuals at different stages of embryonic development (Reisz, et al., 2012; 2013). Robert Reisz, the principle investigating of the work observed that "the bones of ancient animals are transformed to rock during the fossilization process … to find remnants of proteins in the embryos is really remarkable, particularly since these specimens are over 100 million years older than other fossils containing similar organic material" (Crucefix, 2013). In fact, we are not aware of any significant technical challenges to the protein identification that has been given. Also, Glass et al. (2012) identified eumelanin, an organic biopolymer that makes up the black ink found in modern squids, still present in an ink sac from a 160-million-year-old squidlike giant.

Recently, Armitage and Anderson (2013) were the first to report detection of flexible tissue and observed osteocytes in the supraorbital horn of *Triceratops horridus*. This significantly broadens the scope of specimens found to contain nonfossilized material. In fact, it would seem that numerous additional findings are forthcoming since investigators are now aware of the probable existence of organic matter in alleged ancient specimens.

Before the "Brex" Findings

Schweitzer's work prior to the discovery of a *T. rex* [museum designation of MOR 1125] was helpful and possibly profoundly preparatory for future discoveries (Schweitzer et al., 1997). In 1997, she reported that a "near-complete specimen of the Late Cretaceous dinosaur T-Rex "Big Mike" [MOR 555] was collected by the Museum of the Rockies (MOR) from the Hell Creek formation (67–65 million years ago) of eastern Montana in 1990" adding that "burial was rapid enough to forestall damage by scavenging and weathering" (Schweitzer et al., 1997, pp. 6291–6292). They eventually removed "trabecular tissues" from MOR 555 after removal of surrounding sediment from the bones. The team studied isolated protein fractions using HPLC, NMR, ESR, Raman Spectroscopy and several biochemical techniques. HPLC revealed the presence of heme-containing compounds in the bone (absorption spectrum at 405–410 nm) but not the surrounding sediments or extract solvents showing no contamination. NMR showed the "presence of a paramagnetic atom, such as those seen in various metallo-proteins. The spectrum was consistent with degraded heme proteins in the met (Fe^{3+}) state." A Raman spectra of the extracts

showed marker bands consistent with the "heme prosthetic group." Using ELISA (Enzyme-Linked ImmunoSorbent Assay; see http://www.immunochemistry.com/what-immunoassay), the group reports that immunological studies reveal "striking evidence for the presence of hemoglobin derived peptides in the (*T-rex*) bone extract" (Schweitzer et al., 1997, p. 6295). "Rats immunized with *T. rex* tissue extracts produced antibodies that recognized hemoglobin" (Schweitzer et al., 1997, p. 6294), indicating the presence of hemoglobin in the *T. rex* tissue. The same response was not observed for the surrounding sandstone matrix and plant extracts. The researchers concluded that "six independent lines of evidence point to the existence of heme-containing compounds and/or hemoglobin breakdown products in extracts of trabecular tissues of the large theropod dinosaur *Tyrannosaurus rex*" (Schweitzer et al., 1997, p. 6291).

Schweitzer began to suspect that remnants of original or endogenous protein could survive in well-preserved fossils. Yet many in the community remained unconvinced. Commenting on the state of the art understanding of protein/DNA stability, she writes, "test tube studies showed organic molecules should not survive more than a million years … (and DNA less than that)" (Schweitzer, 2010, p. 67). One reviewer, she explains, rejected out of hand the possibility of long ages of protein survival whatever data she presented (Schweitzer, 2010). She followed the work with additional studies to confirm the principle of long survival ages. In 2002, she sequenced protein from mammoth fossils using mass spectrometry and "unambiguously identified collagen, a key component of bone, tendon, skin and other tissues" (Schweitzer, 2010, p. 67).

"Brex"

In 2003 the Museum of the Rockies completed excavation of a *T.rex* specimen designated MOR 1125, nicknamed "Brex" after discoverer Bob Harmon. Helicopters were needed to retrieve the remains. One of the plaster jacketed leg bones was split to enable easier removal but many fragments resulted. Schweitzer and assistant Jennifer Wittmeyer examined the fragments using the analytical and biochemical techniques developed with her earlier work. The internal structure of the bone was examined, and to their surprise, lining the inner surface of one of the bone fragments was a fibrous and channeled bone appearing to be medullary bone, which suggested that Brex was pregnant. Medullary bone appears for a very short time when a female bird is "in lay." The rich bone deposit offers a source of calcium for eggshells and is found in birds, and by extension, egg-laying dinosaurs. Demineralization was next attempted with mild acid. A fibrous-stretchy-flexible clump of tissue was left, just as observed when the procedure is applied to fresh samples of bird bones. The experiment was repeated many times. Treatment of the denser cortical (outside or sheath) bone resulted in yet greater quantities of soft tissue but this time they observed "hollow, transparent, flexible, branching tubes … [that] looked like blood vessels" (Schweitzer, 2010, p. 69). Osteocytes that secrete collagen and other organic substances found in bone were also observed.

According to Schweitzer, the results published in 2005 (Schweitzer et al., 2005a, 2005b) but did not immediately attract controversy. The next step concerned analysis of the recovered tissue. Analytical work on the "degraded" protein was attempted. The team was eventually able to sequence the material. These results published in 2007 and 2008 were met with great controversy. Schweitzer reports that they grouped most closely with birds, followed by crocodiles—the two groups that are the closest living relatives.

The dissenting claims charged that the organic matter was "slime" or a biofilm produced by microorganisms. The biofilm theory holds that bacterial polysaccharide slime displaces exogenous soft tissue inside bone cavities. The resulting product resembles soft tissue but is a product of bacteria activity. Examining numerous ancient specimens, Kaye et al. (2008) found biofilm contamination in most specimens. Carbon dating of the film points to its relatively modern origin. In addition to similarities between the infrared spectra of modern biofilms and the subject bone tissue where both had a polysaccharide signature, the group also reported the presence of blood cell size iron-oxygen spheres. The chemical identity was an oxidized form of formerly (FeS) pyritic spherical iron particles. Taken together, they concluded that the biofilm or slime is the identity of the organic residue, not dinosaurian soft tissue. This topic is covered in more detail by Anderson (2015).

Still others believed that not enough sequences were produced for an accurate analysis of the protein (Buckley, et al, 2008; Pevzner, et al., 2008). A subsequent discovery, according to Schweitzer seemed to "calm the storm." A duckbill dinosaur (*Brachylophosaurus canadensis* [Museum of the Rockies (MOR) 2598], "Brachy") was uncovered the following summer, and great precautions were taken to shield the fragile bones and contents from air pollutants, humidity changes and other possible contaminates. The finding was a treasure house of osteocytes, intact cell nuclei, and collagen fibers. The decisive evidence showed that the reportedly 80 million year old duckbill bone "reacted with antibodies that target collagen and other proteins that bacteria does not manufacture" according to Schweitzer (2010, p. 69). After receiving confirmation from 3 different labs, further criticism regarding the biofilm hypothesis ended. A subsequent paper disclosed the peptide analysis of the proteinacious material recovered from Brex (Schweitzer et al., 2011).

Peptide	Species	Chain	Amino Acid Sequence (NH_2 ….COOH)	Position	Observed	Predicted
1	T-Rex	a-1	GA***T***GAPGIAGAPGFPGAR	220–237	0	2.1
2	T-Rex	a-1	GAAGPPGA***T***GFPGAAGR	687–704	0	2.0
3	T-Rex	a-1	GV***Q***GPPGP***Q***GPR	508–519	0	1.4
4	T-Rex	a-1	GVVGLPG***Q***R	781–789	0	1.0
5	B-Can	a-1	GL***T***GPIGPPGPAGAPG***DK***G***E***AGP***S***GPPGP***T***GAR	586–618	2	3.8
6	B-Can	a-1	G***S***AGPPGA***T***GFPGAAGR	687–704	0	2.0
7	B-Can	a-1	GA***T***GAPGIAGAPGFPGAR	220–237	0	2.1
8	B-Can	a-1	GV***Q***GPPGP***Q***GPR	508–519	0	1.4
9	B-Can	a-1	GP***S***GP***Q***GP***S***GAPGPK	238–252	0	1.7
10	B-Can	b-2	G***SN***G***E***PG***S***AGPPGPAGLR	292–309	1	2.0
11	B-Can	b-2	GLPG***ES***GAVGPAGPPG***S***R	502–519	1	2.0

Table 1. List of collagen I peptides found in *Tyrannosaurus rex* and *Brachylophosauraus canadensis* trabecular bone specimens. Reproduced from San Antonio et al. (2011). *Amino acids in bold font* are potentially reactive (authors' insert).

Soft Tissue Analysis

Bone is an organic-mineral composite comprising of mainly collagen I, hydroxyapatite—$[Ca_5(PO_4)_3OH]$—a mineral substance, and populations of cells. The cell types include osteoblasts responsible for producing bones and osteocytes that maintain and remodel bone (Schweitzer et al., 2013b). Schweitzer was well aware of conventional studies that showed fast decomposition rates for the organic phase and especially DNA. Model studies showed an upper theoretical limit of ca.100,000 years for DNA and ca.3,000,000 years for proteins at low temperatures (10°C, 50°F) (San Antonio et al., 2011). Some evidence suggested that DNA can survive thousands of years past the predicted limit (e.g. recovery of a complete, "high-quality mitochondrial [mt] genome from a stratigraphically validated 130,000- to 110,000-year-old polar bear jawbone" [Lindqvist, et al., 2010]). Schweitzer concluded that the degradation models were inadequate to explain deep time survival given the accepted view that the specimens were 68 million years old. Yet, their analysis successfully showed the dinosaurian peptide sequences drawn from *Tyrannosaurus rex* and *Brachylophosauraus canadensis*. In two studies, they reported the recovery of collagen I and osteocytes and sequencing of these protein materials (Table 1 and 2). The first report appeared in 2011 concerning the collagen I mass spectral (MS) data (Table 1) and the second in 2013 concerning MS data for protein segments found in osteocytes (Table 2).

In the second report, Schweitzer et al. (2013b) more closely examined the "soft, transparent microstructures" appeared to be osteocytes. They reported immunological and mass spectral evidence consistent with extant proteins including actin, tubulin, PHEX and histone H4. They "reanalyzed mass spectrometry data collected on tryptic digests of extracts of powdered dinosaur bone, containing osteocytes as well as other cell and tissue types, from *T. rex* and *B. canadensis* to support the presence of actin, tublin and *PHEX*… Osteocytes are characterized by expression of several proteins including…actin, tubulin…and proteins that play a role in bone mineralization, such as *PHEX*" (Scheitzer et al., 2013b, p. 418). The table presented here (Table 2) summarizes data from Schweitzer's report. We will highlight some chemically active amino acids discussed below.

It is also noteworthy that Cleland et al. (2012) published an excellent work concerning the evaluation of protocols used for their specimens. Well aware of the potential to alter the soft tissue remains, they attempted to review known analytical procedures that were considered "clean, efficient extraction (methods) to obtain high quality data" (Cleland et al., 2012, p. 1). Firstly, recovery of the bone protein requires removal of the inorganic bone portion known as hydroxyapatite $[Ca_5(PO_4)_3OH]$ with which the tissue is bound. Demineralization is accomplished using dilute strong acid such as HCl or ethylene diamine tetraacetic acid (EDTA). Hydrochloric acid while removing the inorganic matrix has

Peptide	Species	Type	Amino Acid Sequence (NH_2 ….COOH)*	Mass
12	*B. canadensis/T. rex*	Actin	AGFAG***DD***APR	976.448
13	*B. canadensis*	Actin	AVFP***S***IVGR	945.552
14	*T. rex*	Tubulin a -1A	***Q***[-17]LF***H***P***EQ***LI***T***GK	1393.747
15	*B. canadensis/T. rex*	Tubulin a -1A	***E***II***D***LVL***D***R	1085.620
16	*B. canadensis*	Histone H4	VFL***EN***VIR	989.578
17	*B. canadensis*	Histone H4	***T***V***T***AM[+16]***D***VV***Y***ALK	1326.697
18	*B. canadensis*	Histone H4	***T***L***Y***GFGG	714.346
19	*B. canadensis/T. rex*	Histone H4	I***S***GLI***YEET***R	1180.621
20	*B. canadensis*	Histone H4	***D***AV***TY***T***EH***AK	1134.543

Table 2. List of actin, tubulin, and histone peptides found by mass spectrometry in dinosaur bone samples. Reproduced from Schweitzer et al. (2013b). *Amino acids in bold font* are potentially reactive.

the unfortunate side effect of degrading the protein by hydrolysis. This particular reagent for example was not used in the recovery of peptides shown in Table 2. EDTA, which was used, releases the phosphate into solution and thereby frees the protein. The next step requires extraction of the protein into solution for which guanidine hydrochloride was employed. This reagent denatures proteins into random coils increasing their water solubility. The final step is lyophilization, or freeze-drying, leaving the protein as a powder isolate (Cleland et al., 2012). For mass spectrometric work presented in the tables, a tryptic enzyme digest treatment of the powdered protein is lastly applied to cleave the polypeptide at known amino acid locations, arginine (R) or lysine (K) (Schweitzer et al., 2013b). The smaller fragments are then analyzed.

Preservation Motifs

Eleven fossil peptides in Table 1 were identified which comprise less than fifteen percent of the length of the collagen triple helix known in the human/rat database (San Antonio et al., 2011). Some trends were observed. Firstly, the general observation that proteins are intimately bound to the mineral bone matrix or biomineralized is thought to provide a significant defense against potential degradative influences (Schweitzer et al., 2011; Cleland et al., 2012). The collagen I protein-inorganic matrix complex cannot be infiltrated by large postmortem enzymes, for example, thereby reducing digestion of the protein. Secondly, the team also speculates that absorption of biomolecules on minerals may reduce degradation. They also propose that protein-bone matrix fossil sequences were from regions known to be deficient in acidic residues and enriched in hydrophobic residues, which retards hydrolysis. In addition,

> one peptide (Peptide 4) mapped to the Matrix Metalloproteinase-1 (MMP-1) cleavage domain crucial for collagen remodeling, and a site for fibronectin binding. In living tissues, the integrin binding site and MMP-1 cleavage/fibronectin binding sequences are somewhat buried under the surface of the collagen fibril. (San Antonio, et al., 2011, p. 3)

According to San Antonio, et al. (2011), the results imply that deep time preservation is enhanced by these mechanisms. Overall, they suggest that the nonrandom pattern common to both therapods suggests molecular motifs or themes important to survival of the sequences.

The osteocyte peptides (Table 2) were thought to be preserved through other protective mechanisms (Schweitzer et al., 2013b). The fragments represented originating from osteocytes suggest an absence of "necrotic or apoptotic cells which are likely destroyed rapidly by phagocytosis or by microbial attack in the post-mortem environment" (Schweitzer et al., 2013b, p. 423).

> Osteocytes [or peptide fragments] are inaccessible to other live cells, which may, in part, explain their preservation in these ancient tissues…. Osteocyte expression of apoptotic repressor proteins may also contribute to their persistence. The association of actin with alpha-actin and fibrin confers stability to actin over the lifetime of the cell and may also stabilize the protein after death. Finally, osteocytes have

> limited access to oxygen within the bone matrix, and may thus be protected from oxidative damage…. Iron, released post-mortem from hemoglobin and myoglobin through autolysis/degradation of red blood cells and muscle tissue, would act to "fix" both tissues and molecules, a hypothesis also put forth by others…. Iron is a reactive oxygen species (ROS), and this switch triggers the formation of hydroxyl radicals. Through a cascade of events referred to as Fenton chemistry, highly reactive hydroxyl radicals trigger both crosslinking of proteins and peroxidation and crosslinking of the fatty acids making up cell membranes. Because osteocytes are intimately linked through filopodia to the vascular system of bone … and because the iron-binding protein ferritin has been identified in this cell line they would be susceptible to this chain reaction. (Schweitzer et al., 2013b, p. 421).

Schweitzer's group tested this Fenton chemistry proposal in light of detecting iron associated with dinosaurian cellular material using TEM (Schweitzer et al., 2013b).

Fenton Chemistry and Tissue Preservation

The working hypothesis was simple. The iron present in cellular materials available from hemoglobin, myoglobin, cytochromes and non-heme iron proteins such as ribonucleotide reductase, fatty acid desaturase and ferritin served as causative factors in fixing proteins for long term survival. Schweitzer believed that that iron biomaterials intimately associated with surrounding soft tissue produced hydroxyl radicals which penetrate the soft tissue and cross-linked the protein thereby "fixing" the tissue. The process is similar to the fixing action of formaldehyde, which results in increasing resistance to microbial digestion, enzymatic digestion or other degradative processes (Schweitzer et al., 2013a). This study shows additional microscopic evidence that iron is closely associated with still soft tissues and their semitransparent, pliable vessels and microstructures. Additionally the group tested the hypothesis by soaking and incubating postmortem ostrich blood vessels in a red blood cell lysate rich in solubilized hemoglobin (HB). The control specimens included the same ostrich blood vessels in distilled water. Both the controls and test specimens were held under oxygenated or deoxygenated conditions. Hemoglobin was a chosen because of its "preservation properties" according to Schweitzer (Schweitzer et al., 2013a, p. 5).

Four notable properties of solubilized hemoglobin include (i) bacteriostatic properties, (ii) oxygen + HB produces free radicals, (iii) after death large vertebrates release large amounts of HB which lyse, (iv) degraded HB releases heme and iron contents to surrounding cells. Using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) the group observed the condition of the cells over a two-year period. The stability of the tissue was judged by the relative appearance, which demonstrated the following: After nearly 2 years at room temperature in the presence or absence of oxygen the HB vessels showed almost "no change" and were "intact" compared with fresh specimens. The comparable controls lacking HB and soaked in water were "significantly degraded in 3 days" (Schweitzer et al., 2013a, p. 5). The control specimens were judged to have "extensive tissue degradation." Some of the signs of degradation included vessel wall thinning, bacilliform infection, fungal invasion, and vessel content reduction.

The overall judgment was that the HB-oxygen combination led to the greatest tissue stability over the two years. The deoxygenated-HB ostrich tissue specimens also appeared stable. The general conclusion was that "the hypothesis that iron contributes to preservation in deep time, perhaps by both free-radical-mediated fixation and antimicrobial activity, is supported by data presented" (Schweitzer et al., 2013a, p. 7). The group presents several possible additional routes of stabilization. The iron blocks active sites on proteins preventing enzymatic action or oxygen binding (antioxidant) or "outcompeting bacterial mechanisms" (Schweitzer et al., 2013a, p. 7). The preferred if not more discussed model involved deep time preservation through "protein and lipid cross-linking" via hydroxyl radical. They additionally speculated that other metals may contribute to hydroxyl radical production and subsequent "exceptional fossil preservation" (Schweitzer et al., 2013a, p. 8).

Analysis of Preservation Motifs

Schweitzer et al. (2013a, 2013b) have proposed numerous possible explanations for the survival of recovered collagen and other soft tissue materials found in *T. rex* and *B. canadensis* including for example molecular sheltering, hydrophobic enrichment, iron-protein blocking, cross-linking, etc. Undoubtedly all if not most of the proposals have some merit. However, in our view, the matter of these mechanisms explaining deep time survival has not been adequately supported either empirically or by literature review. We begin by merely pointing out that the Fenton chemistry hypothesis supported by the ostrich tissue preservation experiment over 2 years, using hemoglobin as a preservative is simply inadequate to extrapolate and infer stabilization over 68 million years. It is unknown if environmental factors like high-low temperature cycling or dehydration might radically alter the test specimens appearance. Is a tissue specimen soaked in blood kept in a laboratory an adequate experiment to model the environmental weathering of postmortem tissue? In this vein, it is

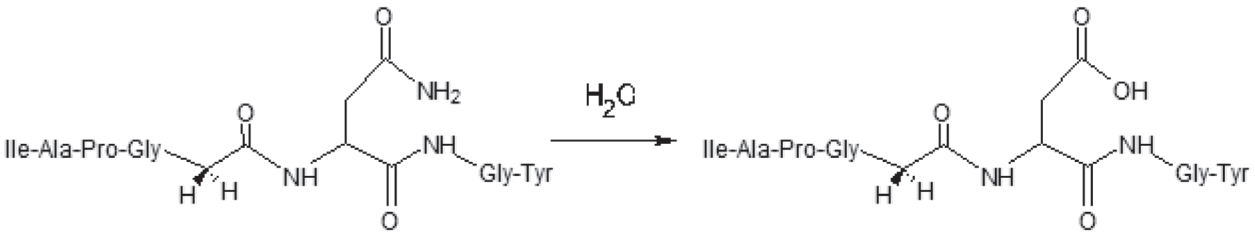


Figure 1. Deamidation of a model octapeptide.

again trivially pointed out that the visual inspection method of tissue analysis is woefully inadequate to draw any conclusions concerning a molecular mechanism of stabilization. The group has access to mass spectra evaluation which could have identified footprints of hydroxyl radical presence. As we shall see, a more careful analysis of the mass spectral data related to the particular peptides and sequences shows that some doubt, if not complete rejection, of several preservation motifs is warranted. The particular motif that introduces more problems than it purportedly solves concerns Fenton chemistry iron fixation of the peptides.

The proposal is essentially hydroxyl free-radical infiltration into soft tissue. The free radicals are generated by iron-biominerals with which the tissue is combined. Success of this mechanism depends upon deployment of the free radicals through an aqueous medium in contact with the polypeptide. We hold that "chemical fingerprints" of this activity should be registered upon the peptides themselves. For example, hydrolytically sensitive amino acids (asparagine, glutamine) should have degraded and free-radical sensitive compounds (tyrosine) should have reacted. These observations may seem like an unnecessary if not insignificant detail to observe but recall that the specimens have been in the ground for some 68 million years. If a chemical mechanism (Fenton chemistry or iron mediated hydroxyl radical fixation) is to be believed, its entire consequent (fugitive water and hydroxyl radicals dosing the peptide remnants) ought to have occurred. Below we set out upon an inspection of two general ideas concerning the presence of water and hydroxyl free radicals and their potential signature upon peptide chemistry.

Peptide Chemical Sensitivity

First, we note that Schweitzer et al. (2013a, 2013b) rightly identified molecular sheltering and hydrophobic character as likely mechanistic candidates for soft tissue survival. However, the proposal seems to run into trouble when one considers the eons of time where the specimens are thought to have laid exposed to elements such as oceanic water. The literature is particularly clear that peptide bonds on exposure to water are hydrolyzed; certain amino acids degrade very rapidly upon contact or shortly afterward with water. These studies potentially have bearing on the likelihood of proposed mechanisms to account for postmortem soft-tissue survival over 68 million years. We are not aware that Schweitzer and her coworkers have discussed the effects of these reactive amino acids, which we shall analyze below.

Asparagine and Glutamine

The reported dinosaurian peptide sequences contain asparagine (Asn, N) and glutamine (Gln, Q) which are chemically reactive amides, unlike the relatively unreactive nonpolar residues highlighted by San Antonio et al. (2011). These chemically reactive species apparently survived some 68 million years variously in the presence of Fe^{2+}, Fe^{3+}, water and hydroxyl radicals, according to Schweitzer (Schweitzer et al., 2013a, 2013b). Numerous studies show the instability of these amino acids in the presence of water. Amino acids will follow three letter shorthand abbreviations in the text and one letter symbols in the tables following below.

Asparagine: Hydrolytically Stable?

Firstly, asparagine (Asn) is hydrolytically unstable. Klotz and Thomas have studied the kinetics of deamidation of Asn for a model octapeptide (Ile-Ala-Pro-Gly-Gly-Asn-Gly-Tyr) (Klotz and Thomas, 1993). The group reports deamidation occurs under fairly mild conditions (60°C in 0.1M NaPO4, pH 7.4) to form Ile-Ala-Pro-Gly-Gly-**isoAsp**/Asp-Gly-Tyr. The group proposes that the water can attack the side chain (shown in the reaction) or the "main" chain amide group can attack the side chain (shown below). The half-life for this octapeptide was 2.17 h with an activation energy of 18.6 kcals (Payne and Manning, 2009) (Figure 1).

Robinson and Robinson (1991, p. 8882) call the sequence Asn-Gly "the most rapidly deamidation sequence known" and the triplet sequence SNG

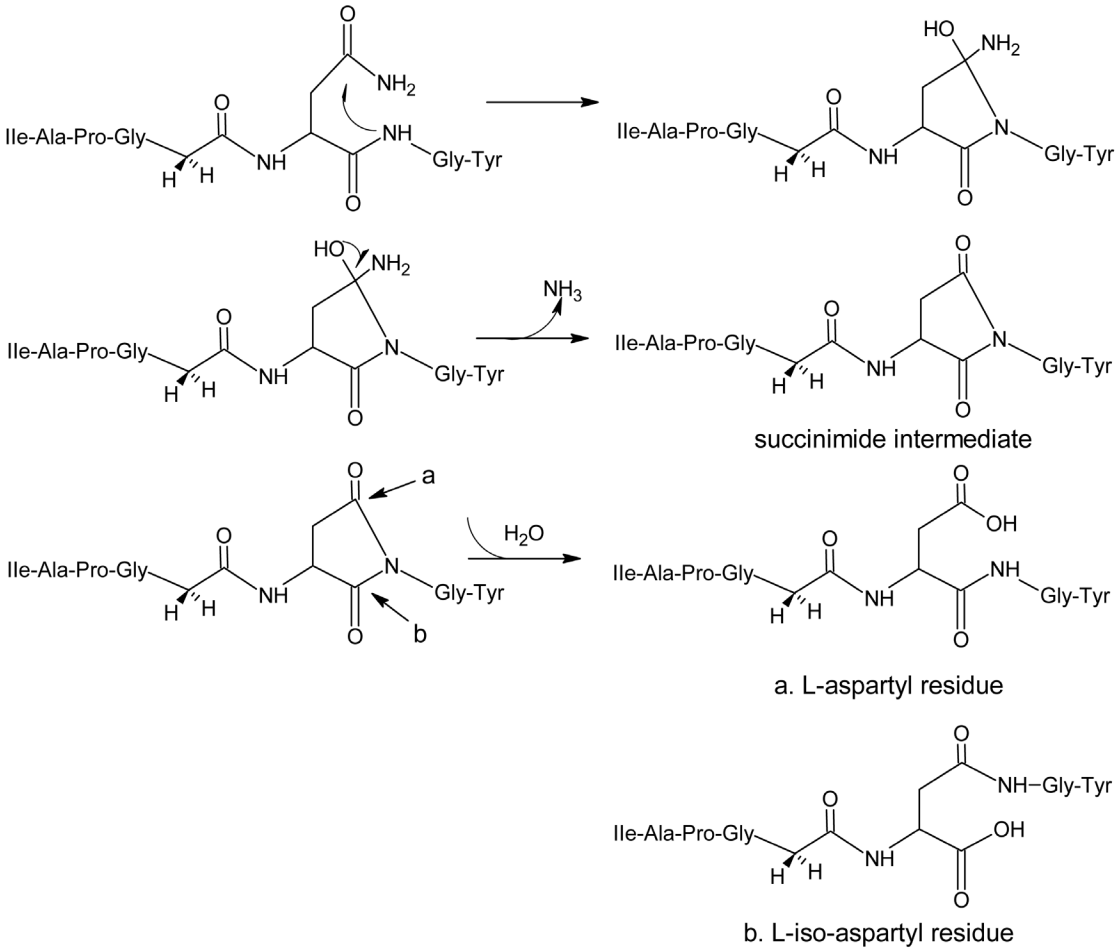


Figure 2. Deamidation of Asn-Gly through a succinimide intermediate.

among the least probable occurring, due to its instability. SNG was found in *B. canadensis* (peptide 10, Table 1).

Catak et al. (2009) suggests that the pathway of deamidation proceeds through direct hydrolysis of the Asn side chain or hydrolysis of a succinimide intermediate as shown in the diagram. Sang-aroon and Ruangpornvisuti (2013) have demonstrated in more recent work that the reaction likely involves a succinimide intermediate followed by hydrolysis (Figure 2).

Given the great hydrolytic instability of Asn, found in *B. canadensis* b-2, how did this unstable amide avoid hydrolytic or intramolecular attack for tens of millions of years? Perhaps, it might be assumed, the fragment was sheltered in some way from waters of hydration, thereby preventing hydrolysis. There undoubtedly was some sheltering, given that peptide fragments were recovered, but were these fragments also sheltered from "protective" agents for 68 million years? Schweitzer et al. (2013a, 2013b) proposes that Fenton chemistry actively cross-linked polypeptides via hydroxyl free radicals. This assumption rests upon the efficient delivery of hydroxyl species through an aqueous medium and it would seem within the peptide matrix. The dilemma is this: how did the fragment successfully become cross-linked through aqueous hydroxyl free radical attack apparently explaining peptide survival while hydrolytically unstable moieties such as Asn avoid contact with the aqueous medium—for 68 million years? If we are to accept the benefits of random aqueous hydroxyl radicals cross-linking the peptide matrix in an undefined chemical bonding, we should also accept the cost—peptide and amino acid hydrolysis.

Aspartic Acid: Out-of-place Remnant?

What can be said about the intramolecular reaction? In fact, about 25% of the reported polypeptide fragments contain L-aspartyl residue (Table 1 peptide 5; Table 2 peptides 12, 15, 17, 20), but L-*iso*-apartyl residue was not found. Assuming the presence of both products indicates the action of the intramolecular process above, we might assume the intramolecular process did not proceed, and that the L-aspartyl residue is native to the peptide sequence. This is a reasonable enough hypothesis, but the presence of the L-aspartyl residue *alone* is again problematic in light of its further potential reactions, the purported great age of the specimen, and the need for Fenton chemistry to operate.

Cournoyer et al. (2005) report the isomerization of aspartic acid residues to isoaspartic acid under mild conditions studying short peptide model systems (e.g. RAAAGADGDGAGADAR). Their work was done in an effort to understand protein aging. The side chain aspartic acid participates in a chain transfer event whereby the -NH-R_2- group on the C_1 "side" attacks the carboxylic acid on the C_2 "side" resulting in an iso-aspartyl residue. In Figure 3 the change can perhaps easily be seen by marking the changes as C_1 and C_2, or carbons alpha and beta, respectively, to the amide nitrogen.

The group reports, "the common, and largely accepted, model for this nonenzymatic, post-translational modification is that it spontaneously occurs under physiological conditions through a succinimide intermediate whose rate is affected by both its amino acid sequence and three-dimensional structure" (Cournoyer et al., 2005, p. 453).

What kinds of three-dimensional structural types discouraged hydrolysis for the dinosaurian proteins? Schweitzer and coworkers observe that at least one peptide (Peptide 4, Table 1) mapped to a "somewhat buried" location under the surface of the collagen fibril from which the peptide came (San Antonio et al., 2011, p. 3). They speculate that the "'sheltered' environment required to protect crucial biological function may also account for enhanced survival of those protein regions in fossils" (San Antonio et al., 2011, p. 3). They also mention biomineralization or intimate binding between the protein matter and inorganic hydroxyapatite discouraged hydrolysis or proteolytic degradation by blocking access routes to the protein. From this it can be inferred that the surviving peptides were either not accessible to water molecules or very minimally so. There is support for the sheltering model in Cournoyer et al. (2005), which teaches that hydrolytic deamidation depends upon the accessibility of water molecules to the amino acid residues without which hydrolysis proceeds at a slower rate. Cournoyer et al. (2005, p. 453) specifically cite an earlier work by Robinson and Robinson (1991) where "deamidation of an asparaginyl residue within the a-helix of rabbit muscle aldolase experienced a 15-fold slower half-life than that of its linear tetrapeptide model." The former polypeptide (aldolase) positions asparaginyl residue in a larger protein structure than the linear tetrapeptide, thus inhibiting access of water molecules to the residue. Yet it should be understood that while some support for delayed hydrolysis might be inferred from the work of Cournoyer, it is also wise to remember that a "slower half-life" deamidation reaction does not mean an arrested deamidation reaction. For Schweitzer's proposal to work, water would seemingly have to be absent.

Cournoyer et al. (2005) also teaches that less bulky side chain elements such as histidine and glycine favor hydrolysis. Two peptide sequences (Peptide 5 and 12, Tables 1 and 2 respectively) were found to contain glycine (G) where G immediately preceded aspartic (D) in both cases. Histidine (H) while present on one of the peptide sequences was at a distance that would have not have an influence on the hydrolysis mechanism (Peptide 20, Table 2). Cournoyer et al. (2005, p. 453) report that the presence of G causes a "100 fold increase in hydrolysis."

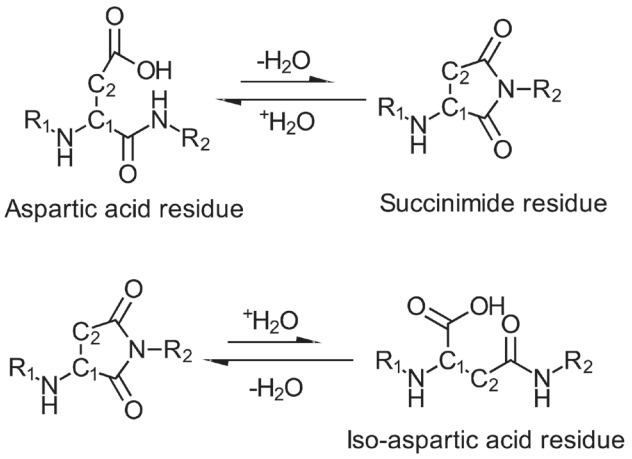


Figure 3. Isomerization of aspartic acid to isoaspartic acid via a succinimide intermediate.

The larger point is that this particular intramolecular transformation (aspartyl residue deamidation) occurs under mild conditions without enzymatic intervention to form the iso-aspartyl acid residue. It is a neighboring group reaction facilitated by the presence of water. Again, given Schweitzer's Fenton chemistry proposal, which requires mild aqueous conditions, and according to the proposal, acting over 68 million years, is it not surprising that isoaspartic acid did not form? Note that this is an intramolecular transformation and needs only mild aqueous conditions. It would seem we need to be stronger in our requirements to allow the proposal to stand. Anhydrous conditions are required to account for the absence of isoaspartic acid—so it would seem—to shut off deamidation, but where does that leave the Fenton chemistry hypothesis? Schweitzer's model must balance molecular sheltering, Fenton chemistry, and the rigorous exclusion of water, which it does not.

Neighboring Group Destabilization

Robinson and Robinson (1991, p. 8883) report that, "in pentapeptide measurements under physiological solvent conditions, deamidation half-times ranging from 1 day to more than 9 years have been measured." Further they observe that steric hindrance plays a role whereby

> the side chain of the residue immediately preceding the amide residue was shown in the series of deamidation half-times Gly < Ala < Leu < Phe < Ile < Val with a range of 418–3278 days for Gln peptides and Gly < Ala < Val < Leu < Ile with a range of 87–507 days for Asn peptides. By contrast, a charged side chain in the residue immediately preceding the amide residues increases the rate of deamidation as was shown by the series of half-times His < Asp < Glu < Lys < Arg with a range of 96–389 days for Gln peptides and Arg < Asp < His < Glu < Lys with a range of 18–61 days for Asn peptides. Also, there are catalytic effects of neighboring hydroxyl groups from Thr (T) and Ser (S). (Robinson and Robinson, 1991, p. 8883)

Given the instability of certain triplet sequences, the group also reports that certain triplet couplings are so unstable that their frequency of occurrence becomes greatly reduced. "The five most rejected neighbors to Asn and Gln in order of the most to least rejected are Asp, Thr, Ser, Lys, and Glu" (Robinson and Robinson, 1991, p. 8882).

The general observation is that a variety of residues accelerate deamidation of Gln (Q) and Asn (N) including Thr (T), Ser (S), Val (V), Ile (I), Arg (R), His (H), Lys (K), Asp (D), and Glu (E). Inspection of the bolded amino acids in Tables 1 and 2 shows the presence of these amino adjacent to or near Gln and Asn. In Table 1, Gln is adjacent to V (peptides 3, 8), or R (peptide 4) or G (peptide 8, 9) and the SN combination is found in peptide 10. In Table 2, the triplet sequence EQL is found (peptide 14).

Regarding the unstable nature of the pairs, Robinson and Robinson (1991, p. 8883) offer the following remarks:

> Why should these unstable residues (Asn, Gln) be included in most protein molecules? Our hypothesis is that their instability is in fact their principal biological function—that deamidation serves as a molecular clock for biological processes.

Arrested Molecular Clocks?

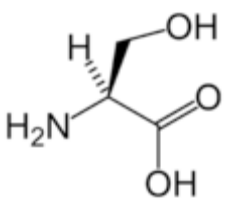
While it is reasonable to draw from Robinson that sensitive or unstable protein sequences do not survive short term physiological conditions, do these studies tutor our understanding of proteins encapsulated in buried bone fragments purportedly 68 million years old? Further are these studies helpful in light of environmental conditions consisting of some combination of heat, light, hydration, oxidation, dehydration, or microbial presence? Robinson and Robinson (1991, p. 8883, emphasis added) provide the note of caution:

> A nearest neighbor effect on deamidation can be diminished or enhanced by preferences in bond angles impressed upon the amide and its neighbors, by the availability of solvent molecules to the amide residue, by the presence of specific peptide or solvent ions, by side chain groups that sterically hinder or enhance reaction intermediates or reaction products and by association of the neighbor groups with other molecules or organelles in the system. These effects depend upon three dimensional as well as primary structures. Therefore, even if we focus our attention on the residues immediately adjacent to Gln and Asn in the peptide chains, *numerous higher-order factors can affect deamidation* rate.

Is molecular sheltering one of the "higher-order factors" that has affected deamidation for Asn and Gln? Undoubtedly the presence of these amino acids (Gln, Asn) requires some combination of complexation, structural folding and sheltering to protect them from hydrolysis whatever age is assigned to relic dinosaurian protein. Yet the studies cited above suggest that neighboring group affects and common hydrolysis are not favorable to deep time survival. The obvious question is what complexation, protein folding or sheltering effectively shut off these degradation motifs for tens of millions of years?

Serine Mediated Degradation

Another residue that destabilizes peptides in aqueous media is serine (Ser, S).



The potential problem for protein survival posed by the side group of this amino acid is demonstrated by the synthetic protein Gonadorelin. "In the pH range 5–6, the peptide backbones of gonadorelin and triptorelin are hydrolyzed at the *N*-terminal side of the serine residue probably involving nucleophilic addition of the serine hydroxyl group to the neighboring amide bond forming a cyclic intermediate resulting in *fragmentation*" (Avanti, 2012, p. 19, emphasis added). Gonadorelin is shown. The proposed cleavage apparently occurs at the carbonyl to the left of the serine residue (arrow) (Figure 4).

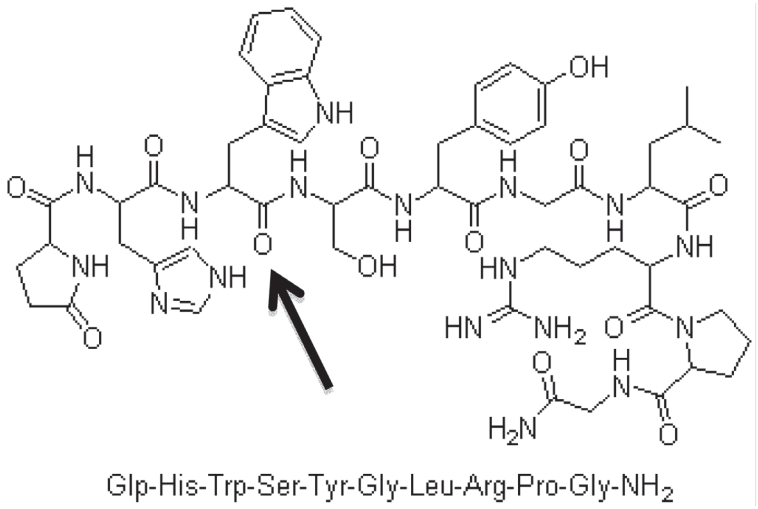


Figure 4. Gonadorelin is sensitive to hydrolysis at the carbonyl shown (arrow). The reaction is facilitated by the adjacent serine residue appearing to the left of the arrow.

Avanti's findings suggest that serine residues may destabilize peptide chain segments of great age. Five of the reported eleven peptides have serine residues (5-B-Can a-1; 6-B-Can a-1; 9- B-Can a-1; 10-B-Can a-2; 11-B-Can a-2). If the pH values in the postmortem collagen fibrils were slightly basic, it would seem serine would destabilize peptide sequences also. The stabilization mechanism proposed by San Antonio et al. (2011) presumably is that serine too was complexed or shielded sufficiently to suppress neighboring group reactions for the entire burial period. But what agent is responsible for this? For example, hydrophobic residues are not expected to stop side serine from interacting with adjacent carbonyl groups. Again it is indeed unclear how basic reactions and in this case neighboring group interactions were suspended for epochs of time.

Fenton Reaction

Finally, we turn our attention to the prospects for iron stabilization of dinosaur soft tissue, as proposed by Schweitzer et al. (2013a). A brief review of Fenton chemistry is necessary to understand Schweitzer's proposed mechanism of preservation.

In his original procedure Fenton oxidized tartaric acid by reacting it in aqueous acetic solution containing Fe^{2+} ions, plus H_2O_2, thus producing •OH (hydroxyl radicals), H_2O and Fe^{3+} ions. The •OH radicals were the active oxidizing agents in the process, which completely decompose the reactant to CO_2 and H_2O. However, excess H_2O_2 reacts to form more HO radical and HO^-, which react with the Fe^{3+} reducing it back to Fe^{2+}, $H^+_{(aq)}$ and $HOO\bullet_{(aq)}$ (peroxyl radical), another powerful oxidizing agent. If these two reactions occur simultaneously, the net result is

$$2H_2O_2\,(aq) \rightarrow HO\bullet + HOO\bullet + H_2O \quad (1)$$

Since both the hydroxyl and peroxyl radicals are available, the likelihood is that any organic substrate will be completely decomposed to its oxide products plus water.

If the initial Fe^{2+} lacks sufficient acid and /or hydrogen peroxide (one mole H^+ and H_2O_2 per mole Fe^{2+}) the oxidation to Fe^{3+} together with the HO• and H_2O will be incomplete. Consequently, the amount of HOO• and H^+ will be limited as well. Barb et al. (1951) subsequently showed that Fenton like oxidations were critically dependent on the ratios of Fe^{2+} to H_2O.

Biomolecular systems containing iron are primarily confined to the cytochromes, ferredoxin, hemoglobin and myogolbin. These are symbolically represented as L-Fe^{2+} and L-Fe^{3+}, where L is the biomolecular ligand. In such systems, the redox mechanism is believed to be initiated via a superoxide ion, $\bullet O_2^-$, which is generated in the decomposition of organic matter and functions either as an oxidant or reductant, depending on what it reacts with. According to Prousek (2007) the pertinent equations are

$$L\text{-}Fe^{3+} + \bullet O_2^- \rightarrow L\text{-}Fe^{2+} + O_2 \quad (2)$$

$$L\text{-}Fe^{2+} + H_2O_2 \rightarrow L\text{-}Fe^{3+} + HO\bullet + HO^- \quad (3)$$

$$\text{Or } L\text{-}Fe^{2+} + H_2O_2 \rightarrow L\text{-}FeO^{2+} + H_2O \quad (4)$$

However, we do not believe that equation (4) is a viable option, since Fe in the 4+ valence state is not readily stabilized. Also, in equation (4) the L-FeO^{2+} is also a very powerful oxidizing agent.

Such redox processes, as those listed above, are known to induce extensive oxidative damage to biological systems (Prousek, 2007). Furthermore, oxy radicals and the Fe^{2+} cation (equation (4) are so destructive to biological systems that Prousek (2007, pp. 2328–2329) said, "Due to its strong reactivity with biomolecules, hydroxyl radical is capable of doing more damage to biological systems than any other reactive organic species."

HO• Cross Linking Reactions

The work of Dizdaroglu and Gajewski (1989) cite a number of publications involving HO• as a cross-linking reagent in biomolecules, but these studies all relate to controlled irradiative techniques for generating the hydroxyl radicals within samples that were carefully prepared and buffered. But as we have already noted, given unregulated conditions, the destructive consequences of hydroxyl radical reactions are paramount and will certainly take precedence over any controlled cross-linking reactions.

It is interesting, that there do not appear to be any published studies in which nonliving tissue was observed to produce cross-linking within biomolecules, under simulated conditions of the natural environment. Nonetheless, Schweitzer et al. (2013, p. 2) explicitly state, "oxy radicals facilitate protein cross-linking in a manner analogous to the action of tissue fixatives (e.g. formaldehyde), thus inducing resistance of these "fixed" biomolecules to enzymatic or microbial digestion." However, the reference citation given in the Schweitzer et al. (2013a) paper is that of Hawkins and Davies (2001), which cites 240 publications all relating to bioenergetic deleterious reactions of radicals primarily on proteins. This is hardly an endorsement to the statement quoted above.

The work of Schweitzer et al. (2013a) hinges upon a natural, uncontrolled production of hydroxyl radicals involving iron containing molecules, via reactions (2) - (4) above, which will selectively promote cross-linking, thereby affording stabilization of dinosaur tissue toward degradation. But it has been shown that the bulk of all relevant evidence strongly suggests that it is highly unlikely for such cross-linking to occur. On the contrary, it is most likely that the involvement of such reactive radicals would lead to greater decomposition, rather than stabilization. Whichever the situation, neither direct evidence nor a model study have been offered to support this cross-linking claim.

Hydroxyl Radical Markers

Perhaps the most questionable aspect of the Fenton chemistry hypothesis of cross-linking or protein fixing arises from the data itself. If hydroxyl radical randomly reacted with peptides, it would seem that various amino acids should have registered the change. In short, these amino acids serve as markers for hydroxyl radical activity.

Inspection of Table 2 shows various peptide fragments that contain amino acids which are known to react with hydroxyl radicals. We present the following brief discussion of several amino acids.

Tyrosine

The amino acid tyrosine (Y) is present in both the *T. rex* and *B. canadensis* histone protein fragments (Table 2). This particular amino acid has been the subject of extensive study and found to readily react with active oxygen species. Bartesaghi et al. (2010) show that tyrosine is sensitive to oxidative alteration and C-C bond formation resulting in cross-linking:

> Tyrosine dimerization and nitration to 3,3´-dityrosine and 3-nitrotyrosine (3-nitro-Tyr), respectively, represent biologically-relevant oxidative post-translational modifications in proteins generated by the reactions with reactive oxygen and nitrogen intermediates both in vitro and in vivo. These tyrosine oxidation processes depend on the intermediate formation of tyrosyl radical (•Tyr), a transient species formed by the one-electron oxidation of tyrosine. For instance, 3,3´-dityrosine formation results from the termination reaction between two •Tyr radicals with the formation of a new C-C bond; 3,3´-dityrosine participates in protein cross-linking (1) and also serves as a marker for oxidatively damaged proteins. Indeed, elevated levels of 3,3´-dityrosine can be found as a product of aging, inflammation, exposure to UV and γ-radiation and other oxidative stress conditions. (Bartesaghi et al., 2010, p. 822)

If iron as Fe^{2+} is driving formation of HO• and fixing or cross-linking peptide fragments, it is reasonable to assume that the tyrosine marker would have registered the presence of HO• perhaps in the form of a cross-linked species. Tyrosine was unaltered in all of the histone fragments (Table 2). It would seem if a significant amount of hydroxyl radical was present tyrosine would have reacted as well.

Schweitzer's model hypothesizes that hydroxyl radical formed by Fe^{2+} fixes proteins through cross-linking, but fails to account for the presence of unaltered tyrosine, which is sensitive to oxidative stress. The point is that even if Fenton chemistry had prevailed, it is unlikely that all of the tyrosine side chains would have survived for 68 million years.

Methionine

One register that is sensitive to the presence hydroxyl radical is methionine. Schweitzer found the sulfur containing amino acid in a histone peptide (M[+16]) (Peptide 17, Histone 4, TVTAM[+16]DVVYALK, *B. canadensis*,

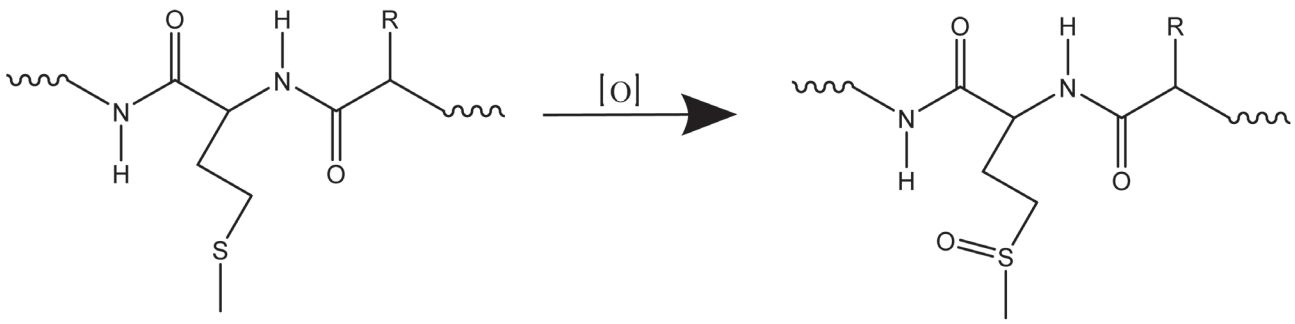


Figure 5. Oxidation of methionine residue to methionine sulfoxide.

Table 2). Methionine was lightly oxidized which Schweitzer et al reported was an "*in vitro* artifact." This is generally taken to mean that oxidation happened *upon handling* of the sample during work up. From this we can infer that the thioether group remained unoxidized during its long entombment while other parts of the same polypeptide were cross-linked—if the Fenton chemistry proposal is assumed. A closer inspection of this proposal reveals problems concerning the oxidative stability of the methionine residue (Figure 5).

Firstly, it is acknowledged that methionine does oxidize upon handling. For example, Hongcheng et al. (2013) discusses the oxidation sensitivity of methionine during laboratory handling.

> Methionine (Met) oxidation is a major modification of proteins, which converts Met to Met sulfoxide as the common product. It is challenging to detect the level of Met sulfoxide, because it can be generated *during sample preparation* and analysis as an artifact. (Hongcheng et al., 2013, p. 11705; emphasis added)

Schweitzer is correct to suggest that the methionine residue might suffer *in vitro* alteration. Yet the consequence of this suggestion is hardly insignificant. Did methionine survive 68 million years in the ground unaltered while in the presence of hydroxyl radicals? Given the great proposed antiquity of this peptide fragment and the proposed presence of hydroxyl radicals, it is surprising that the fully oxidized product was not detected.

Researchers have observed that larger suites of oxidized products are to be expected even for younger specimens (Hiller et al., 1981; Bobrowski et al., 1987; Bobrowski and Holcman, 1989; Bobrowski et al., 1991; Schöneich et al., 1994). Parker et al. (2011, p. 212) report, "methionine decomposes readily in the presence of oxygen and produces methionine sulfoxide, methionine sulfone, and various sulfides and thiols."

Bern et al. (2010) studied the reaction of a model polypeptide (Gly-Met-Gly) in the presence of flash photo generated *hydroxyl radical*. The group reports that "this oxidation method produces diffusing hydroxyl radicals"(Bern et al., 2010, p. 3). The method altered the methionine residue producing oxidized side chains (Figure 6).

Methionine is not, it would seem stable in the presence of hydroxyl free radicals; yet to be clear, Schweitzer suggests,

> Through a cascade of events referred to as Fenton chemistry, highly reactive hydroxyl radicals triggered both cross-linking of proteins and peroxidation and crosslinking of proteins and peroxidation and crosslinking of fatty acids making up cell membranes. (Schweitzer, 2013b, p. 421)

Did methionine avoid oxidative assault by the "highly reactive hydroxyl radical" for some fraction or all of 68 million years while other parts of the peptide fragments were successfully and selectively cross-linked by the same hydroxyl radicals? The proposal that the oxidation-prone methionine residue can survive 68 million years through aggressive environmental weather changes and fugitive free radical assault and then end its career through *laboratory* handling in its mildly oxidized form (sulfoxide), appears to be a just so story. A reasonable expectation is that methionine should have been oxidized in the presence of fugitive hydroxyl radicals resulting in its homocysteine form or at least its sulfone form at some time in its long burial period. Homocysteine or methionine sulfone was undetected.

Histidine

Another amino acid that is a "litmus test" for hydroxyl radical presence is histidine. This residue has been described as an effective *antioxidant*.

> Experimental data show that the antioxidant effect of these endogenous substances is likely to be provided by histidine residue. In fact, in its free state this amino-acid is a HO-radical catcher and one of the most effective "suppressors" of singlet oxygen. …Indeed oxidation of histidine to 2-oxohistidine is a good marker of

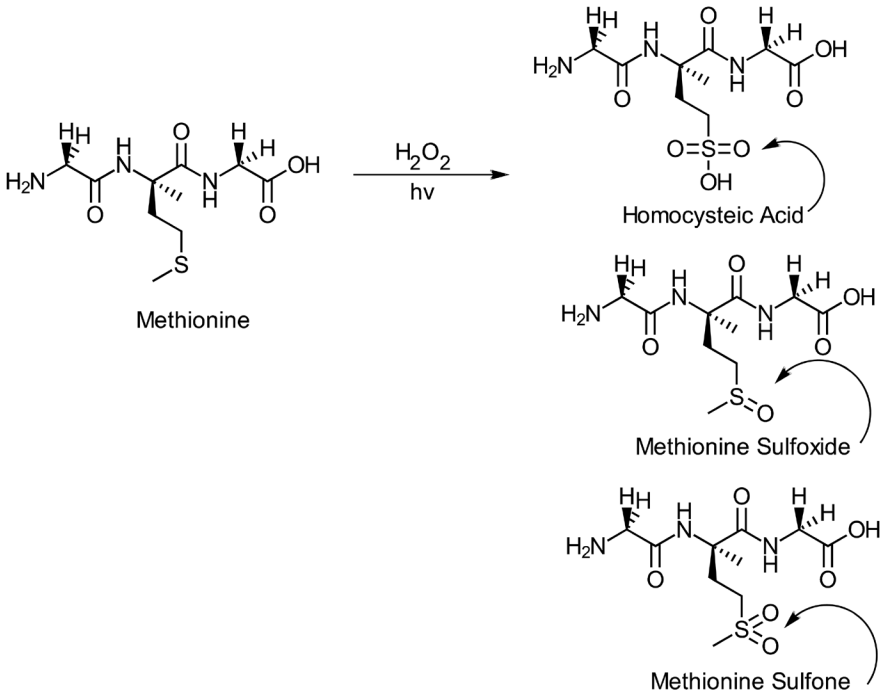


Figure 6. Flash photo-generated hydroxyl radical treatment of Gly-Met-Gly.

> oxidative stress. (Shleikin and Medvedev, 2014, p. 125)

Farber and Levine (1986, p. 4577), more specifically report:

> Oxidative modification of susceptible enzymes proceeds under anaerobic conditions in the presence of Fe^{2+} and H_2O_2. Each mixed function oxidation system examined thus far can reduce Fe^{3+} to Fe^{2+} and, in the presence of oxygen, can generate H_2O_2. A simple summary of the reaction is as follows.

$$Fe^{2+} + \text{enzyme} \rightarrow Fe^{2+}\text{- enzyme}$$

$$Fe^{2+}\text{- enzyme} + H_2O_2 \rightarrow \text{enzyme-[O]} + Fe^{3+}$$

The ferric iron can again be reduced to the ferrous form and can catalyze the oxidation of additional protein molecules.

The group notes that oxidation of an enzyme proceeds in a site-specific fashion resulting in the oxidation of particular histidine residues.

Zhao et al. (1997, p. 9028) studying a copper oxidizing system analogous to the ferrous/ferric oxidizing system notes the following:

> We note that the formation of 2-oxo-His via the displayed pathway requires the net addition of HO· at C-2 of the imidazole ring whereas ESR investigations on the addition of radiation chemically produced freely diffusable hydroxyl radicals to imidazole derivatives not complexed to transition metals have provided evidence for the addition of HO· at C-4 and C-5.

Figure 7 provides an illustration of this histidine oxidation.

While some distinction is made by Zhao et al. (1997) concerning the source and subsequent position of hydroxyl radical addition, it is clear that the presence of hydroxyl radicals leads to ring oxidation. Assuming, freely diffusible hydroxyl radicals have saturated the various protein fragments resulting in, according to Schweitzer, fixing or cross-linking the protein, one would expect oxidized histidine, yet none was reported. The absence of oxidative alteration of these sensitive markers Tyr (Y), Met (M), His (H)) is not easily explainable, assuming Fenton chemistry operated during the deep time burial period of the dinosaurian specimens.

Radiocarbon Dating

We lastly note perhaps the most disappointing absence in Dr. Schweitzer's analysis. Assuming that the protein substances discussed are indeed dinosaurian soft tissue, it is also true that the C,H,N,O elemental makeup of the proteins are endogenous. As such, the samples are highly qualified for a carbon-14 analysis. C-14 should help further the discussion on the possible age of the organic material. If this genuine protein matter is 68 million years old, C-14 should be absent. If the tissue is 50,000 years or younger, C-14 will be detected. We also note in passing that C-14 presence if found and not interpreted as a young age must be interpreted as contamination making unreliable the mass spec data presented by Schweitzer.

Radiocarbon dates that have been reported on various dinosaur samples, which all consistently fall within ranges of about 18,000–30,000 years before present are not regarded as reliable, because dinosaurs are believed to be extinct for some tens of millions of years (see Thomas and Nelson, 2015, for a detailed analysis of C-14 in dinosaur fossils). It is equally interesting that Dr. Schweitzer has not subjected her soft

tissue dinosaur samples to radiocarbon dating. To our knowledge, Schweitzer's writings have not offered any explanation for this omission.

Conclusion

Schweitzer has hypothesized that molecular sheltering and lipophilic amino acid character impart deep time survival to protein fragments recovered in *T. rex* and *B. canadensis*. She also invokes Fenton type chemistry to explain protein cross-linking and consequently deep time survival. Some of these motifs perhaps played a role to account for soft-tissue survival, but they seem inadequate to explain deep-time survival—if model studies are to be trusted at all. These studies predict that hydrolytically unstable residues such as asparagine, and glutamine perhaps metaphorically referred to as molecular clocks should have "run down" to their carboxylic acid analogues or altered their native peptide chains long ago. Tantalizingly, the presence of aspartic acid suggests hydrolysis of asparagines, but the absence of isoaspartic acid suggests the aspartic acid residues may be native to the sequence and not the product of asparagines hydrolysis. Glutamine also appears in the peptide fragments in addition to its hydrolyzed analogue (glutamic acid). In one instance the two appear side by side, again frustrating a simple trend or pattern of hydrolysis.

While degradation may not be uniform in a field setting and we may well see advanced decomposition for some remains and lagging decomposition in other remains, it is difficult to explain how hydrolytically sensitive moieties escaped aqueous and intramolecular transformations over vast epochs of time. In this same way, if free radical generation proceeded as suggested through aqueous Fenton type chemistry to cross-link peptides, it would seem tyrosine, methionine and histidine would have been oxidized sometime during the long burial period. Is it reasonable to invoke hydroxyl radical fixation for the bulk polypeptide matrix while having no registry of this phenomenon in several sensitive hydroxyl radical markers? It is readily admitted that a stabilization mechanism is at work buffering hydrolysis, suppressing neighboring group attack and quenching free radical degradation. It is not so clear that Schweitzer has unambiguously identified those mechanisms since chemical markers are present that militate against her immediate proposal.

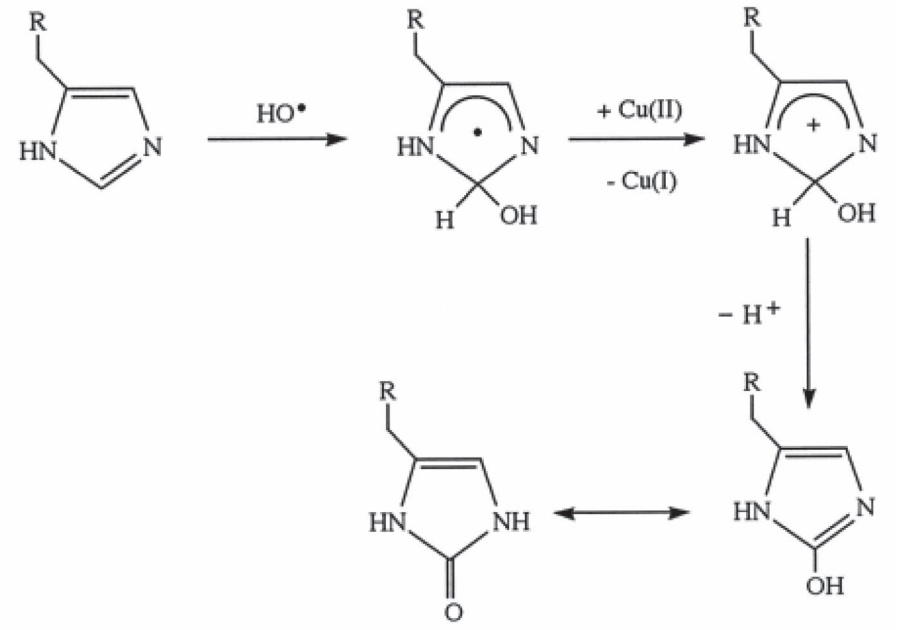


Figure 7. Oxidation of His residue to 2-oxo-His via Cu(II) (Zhao et al. 1997)

We propose that one preservative mechanism has been overlooked and may be the most obvious of all. It is perhaps found in a helpful maxim coined here; soft tissue survival is tied to soft tissue age. We believe that the soft tissue elucidated by Schweitzer et al. (2005a, 2007, 2013a, 2013b) was not buried for 68 million years but some vastly shorter period. This surprisingly simple explanation when combined with some of the other preservation motifs, potentially favors survival of asparagine, glutamine, tyrosine, methionine and histidine. This hypothesis is also favorable to model studies that predict short age survival of peptides, polynucleic acids and as cited within this text the discovery of C-14 in fossil specimens—which might be present in Schweitzer's specimens. *If in fact the dinosaurian specimens are not old, soft tissue remains may even be expected to be provided a kind of short term temporal sheltering in contradistinction to long term temporal assault from prolonged exposure to degradative elements such hydrolysis, free radical attack, and elevated temperature.*

Thus, we raise the possibility here that the survival of collagen and osteocyte peptide fragments is parsimoniously explained by a young age hypothesis which envisages far fewer years of degradative stress and improves the chances of survival of sensitive chemical markers. The young age hypothesis, while counterintuitive to conventional thought concerning the age of the dinosaurs, is

unavoidably visible for all to consider. Indeed it may well turn out that soft tissue survival is tied to soft tissue age, and these dinosaur remnants are among the first of many more and even better preserved specimens that lay undiscovered in the museums and laboratories across the world.

Acknowledgments

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