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Unraveling the sequence and structure of the protein osteocalcin from a 42 ka fossil horse

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Abstract

We report the first complete amino acid sequence and evidence of secondary structure for osteocalcin from a temperate fossil. The osteocalcin derives from a 42 ka equid bone excavated from Juniper Cave, Wyoming. Results were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) and Edman sequencing with independent confirmation of the sequence in two laboratories. The ancient sequence was compared to that of three modern taxa: horse (*Equus caballus*), zebra (*Equus grevyi*), and donkey (*Equus asinus*). Although there was no difference in sequence among modern taxa, MALDI-MS and Edman sequencing show that residues 48 and 49 of our modern horse are Thr, Ala rather than Pro, Val as previously reported (Carstanjen B., Wattiez, R., Armory, H., Lepage, O.M., Remy, B., 2002. Isolation and characterization of equine osteocalcin. *Ann. Med. Vet.* **146**(1), 31–38). MALDI-MS and Edman sequencing data indicate that the osteocalcin sequence of the 42 ka fossil is similar to that of modern horse. Previously inaccessible structural attributes for ancient osteocalcin were observed. Glu_{39} rather than Gln_{39} is consistent with deamidation, a process known to occur during fossilization and aging. Two post-translational modifications were documented: Hyp_9 and a disulfide bridge. The latter suggests at least partial retention of secondary structure. As has been done for ancient DNA research, we recommend standards for preparation and criteria for authenticating results of ancient protein sequencing.

1. Introduction

Studies of ancient DNA have advanced our understanding of phylogenetic relationships among extinct taxa and genetic diversity through space and time (Leonard et al., 2000; Paxinos et al., 2002; Hadley et al., 2004; Weinstock et al., 2005). However, the majority of the samples analyzed are young (<50 ka) and/or from permafrost. Because some proteins have a greater likelihood of survival than DNA (Collins et al., 2000) they may provide genetic information that is not accessible via DNA analysis. We putatively identified the bone protein osteocalcin in fossils via matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) and later demonstrated that it could be sequenced from >53,000 ka permafrost bones (Ostrom et al., 2000; Nielsen-Marsh et al., 2002). Partial amino acid sequences of osteocalcin from ca.

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75 ka Neandertal specimens from Iraq (Nielsen-Marsh et al., 2005) and radioimmunoassay data in 300 ka bones from warm environments (Ostrom et al., 2000) document this protein's survivability.

The recent emergence of proteomics as a molecular tool for evolutionary biology is an exciting prospect. However, efforts to extend ancient biomolecular records in time and space must be increasingly aware of technological limitations and pitfalls. Our challenges are to (1) expand mass spectral databases for proteins from previously uncharacterized taxa, (2) define temporal and spatial limits of survival of osteocalcin, and (3) develop strict criteria for sample handling and analysis and interpretation of mass spectra. Relative to ancient DNA, the science and technology associated with ancient protein sequencing is in its infancy. We should avoid mishaps that befell early ancient DNA research (Wayne et al., 1999). Our efforts to provide the first complete osteocalcin sequence from a non-permafrost fossil speak to the challenges noted above. The sequence was obtained from a ca. 42 ka horse bone from Juniper Cave, Wyoming and compared to that of three phylogenetically related taxa (horse, zebra, and donkey). The ancient osteocalcin sequence was derived by multiple MALDI-MS techniques, confirmed in two laboratories and substantiated by Edman sequencing. The results identify post-translational modifications in the fossil and diage-

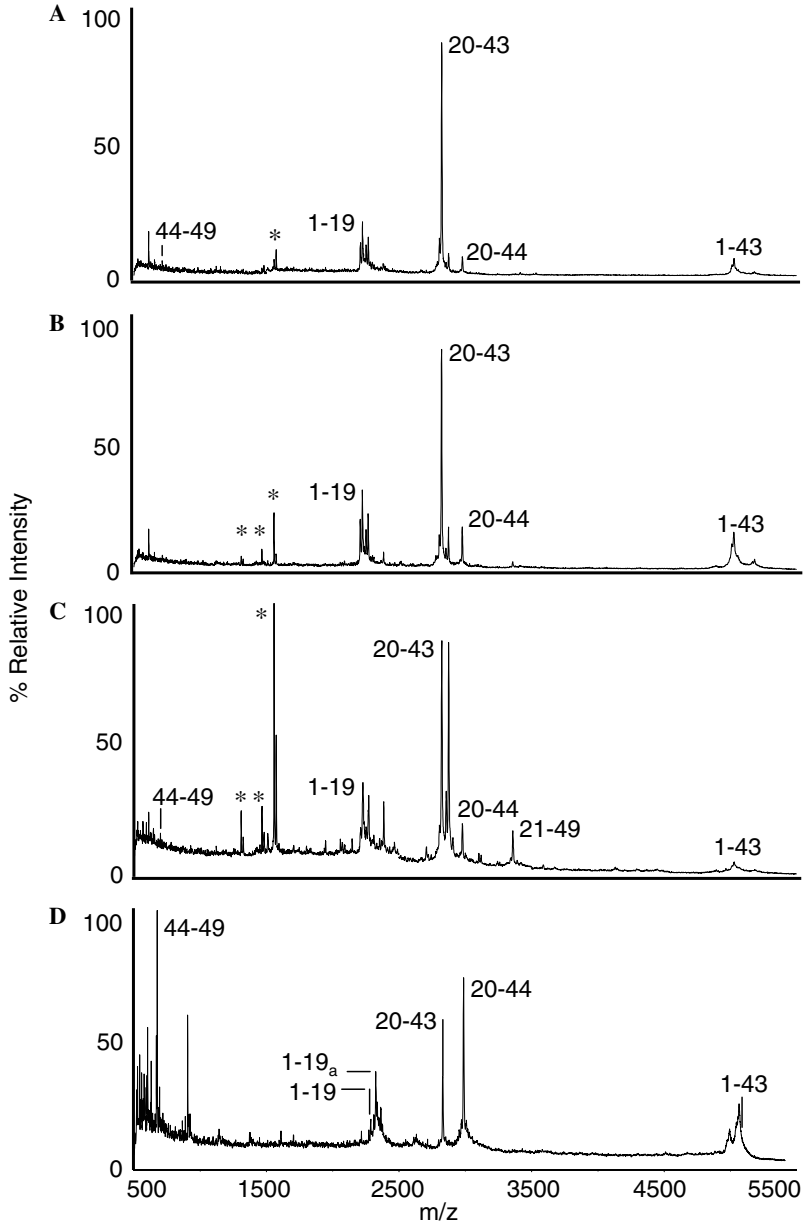

Fig. 1. Peptide mass fingerprint for osteocalcin tryptic digest from: (A) extant horse; (B) zebra; (C) donkey; and (D) partially purified osteocalcin from the 42 ka horse produced from an ABI-4700 (confirmed on an ABI-STR). Peptide 1–19a, 43 Da greater than 1–19, is from a modified form of osteocalcin. *Self-digest products of trypsin.

netic chemistries. Mirroring the stringency now applied to ancient DNA, the work leads us to recommend criteria for authentication (Cooper and Poinar, 2000; Paabo et al., 2004; Willerslev and Cooper, 2005).

2. Materials and methods

Modern horse (*Equus caballus*) and zebra (*Equus grevyi*) bones were obtained from Michigan State University School of Veterinary Medicine. A modern donkey (*Equus asinus*) tooth was obtained from Donkey Rescue (Michigan). The fossil horse from Juniper Cave, Wyoming was a complete, left front, first phalanx (Sample No. 48BH3178/27, University of WY, Department of Anthropology, Archaeological Repository collections). It is classified as a non-stilt legged horse and referred to as *Equus scotti* based on biometrics (maximum length = 50.0 mm, midshaft width = 36.7 mm). The XAD-purified gelatin (Stafford et al., 1991) was dated to 41,770 ± 1060 RC yr (CAMS-90973) by Stafford Research Laboratories. Juniper Cave is a limestone cave located at 1537 m in the Bighorn Mountains near Lovell, WY. Sediments are extremely dry, and consist of wood rat, bighorn sheep feces, and macroflora (prickly pear, sagebrush and juniper wood). Fossils are primarily small mammal bones and include one extralimital taxon, *Ochotona princeps* (*Pika*). The only other dated vertebrate, jackrabbit

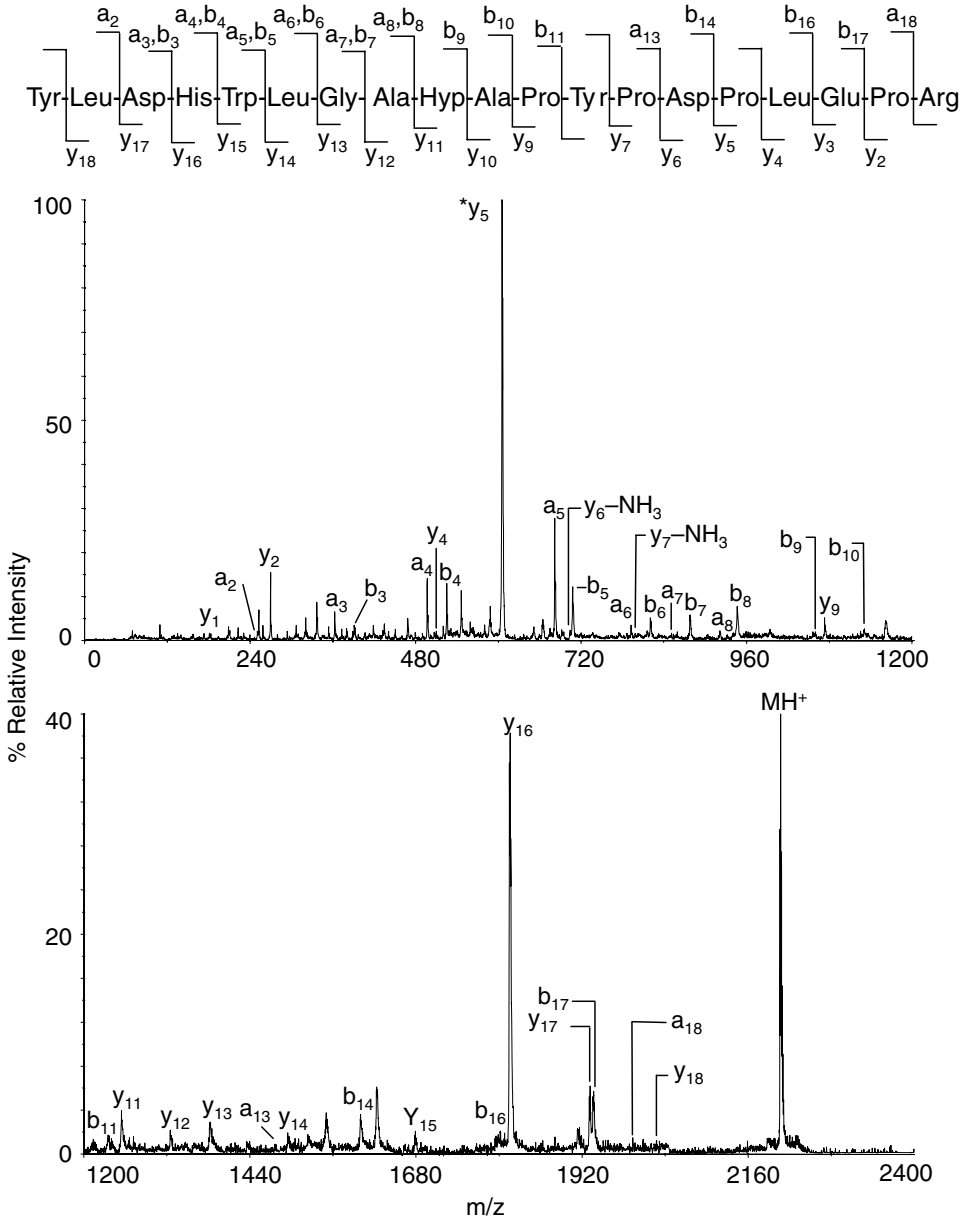

Fig. 2. Post source decay spectrum for osteocalcin tryptic peptide 1–19 from modern horse bone produced on an ABI-STR (complete sequence for residues 1–19). Lower panel of spectrum is scaled to enhance clarity. Scale is relative to the largest fragment ion (y_5) allowing comparison to upper panel. *Largest fragment ion, full scale (i.e., 100%).

(*Lepus sp.* cf. *L. towsendii*) is >45,300 RC yr (CAMS-90972) (Kelly et al., 2002).

Osteocalcin was extracted and purified from bone powder (20–50 mg) using a modification of previous methods (Nielsen-Marsh et al., 2002). Cleaned bone was powdered (SPEX CertiPrep 6750 Freezer/Mill), and demineralized (sodium EDTA, 0.5 M, pH 8.0; 4 h, 25 °C). The extract was centrifuged (14,000 rpm, 10 min) twice and applied to a new (0.5 × 2 cm) C_{18} gravity column (60 Å, Fisher) equilibrated with solvent A (95/4.9/0.1: water/acetonitrile (ACN)/trifluoroacetic acid (TFA), v/v/v). Osteocalcin was eluted with eight, 1 mL aliquots of increasing concentration (20%, 25%, 30%, 32%, 34%, 36%, 38%, 40%) of solvent B (10.0/89.9/0.1: water/ACN/TFA, v/v/v), dried (SpeedVac) and reconstituted in 10 μL of 1% *n*-octyl-β-D-glucopyranoside (Sigma) in 50 mM Tris–HCl, pH 8.0. An aliquot from each of eight fractions was spotted on a MALDI target using 4-hydroxy-α-cyanocinnamic acid (4-HCCA) matrix. MALDI-MS analyses from an Applied Biosystems DE-STR (ABI-STR) identified fractions with a peak at *m/z* consistent with osteocalcin (ca. 5.7 kDa).

Fractions containing putative osteocalcin were further purified (rpHPLC) as previously described (Nielsen-Marsh

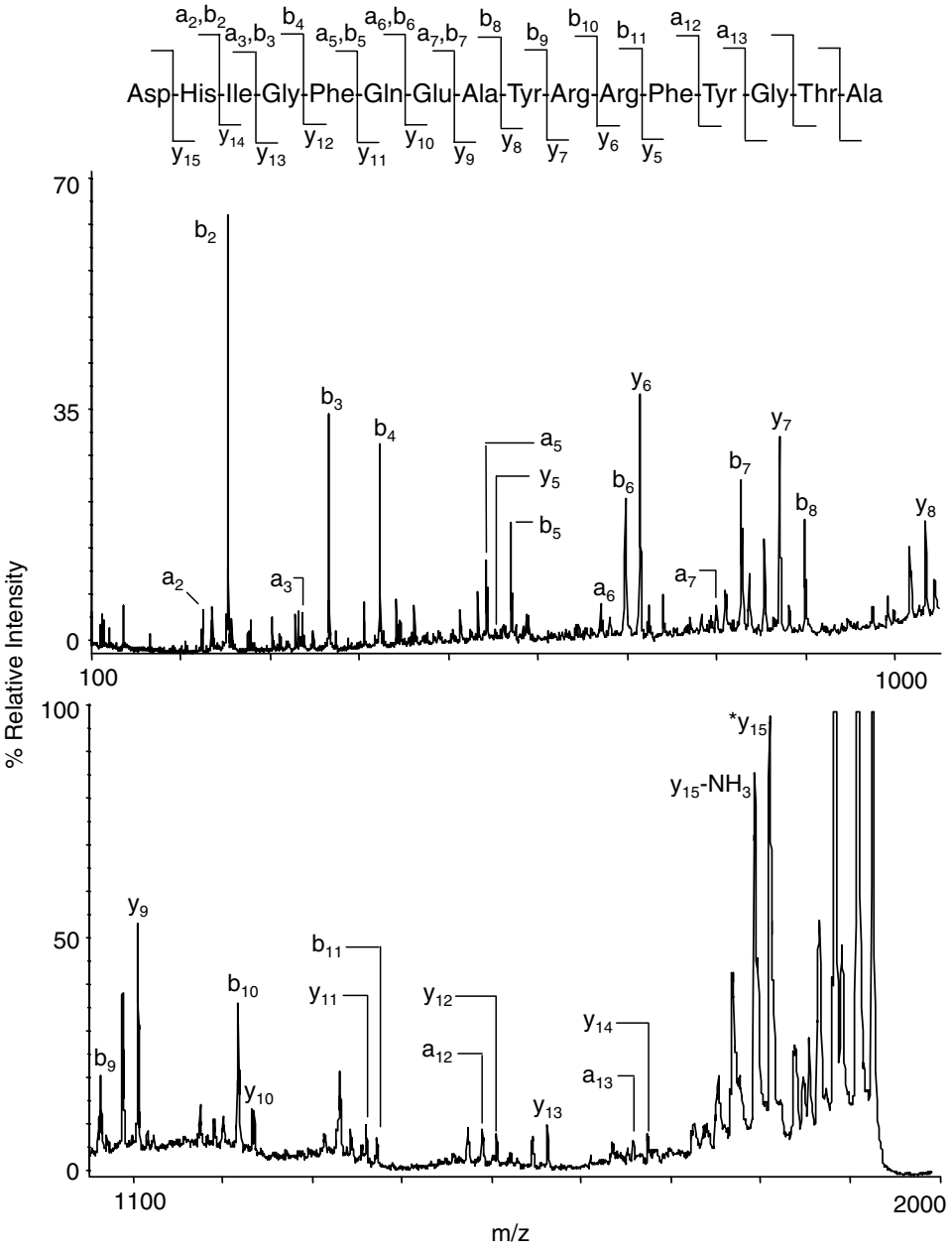

Fig. 3. MS/MS spectrum for ASP-N peptide 34–49 (*m/z* 1931) from osteocalcin of modern horse produced on an ABI-4700 (sequence for residues 34–46). Upper panel is scaled for clarity. Scale is relative to the largest fragment ion (y_{15}) allowing comparison to lower panel. *Largest fragment ion, full scale (i.e., 100%).

et al., 2002). One column was used for separations of protein from extant taxa and a separate column was dedicated to fossil osteocalcin and associated peptides. Putative osteocalcin was digested with modified trypsin (Promega) or ASP-N (Sigma). Resulting peptides were purified (rpHPLC) and analyzed by MALDI-MS. Spectra for the $[M + H]^+$ ion of intact osteocalcin and the peptide mass fingerprint acquired on an Applied Biosystems DE STR (ABI-STR) were externally calibrated using the 1+ and 2+ peaks for bovine insulin (average mass 5734.6 Da; Sigma). Resolution was ca. 530 and mass accuracy at m/z of 2800 was better than 630 ppm (range 92–630 ppm). Post source decay (PSD) spectra were acquired on an ABI-STR at Michigan State University and data are average mass. The fragment ion masses in the PSD spectrum were externally calibrated using the fragmentation for angiotensin I (average mass 1297.51; Sigma). Resolution was ca. 650 and mass accuracy was better than 640 ppm (range 8–640 ppm).

MALDI-MS and tandem mass spectrometry (MS/MS) were conducted on an ABI-4700 at the University of Michigan and results are monoisotopic mass. The MALDI-MS scans were acquired using 1500–3000 laser shots. MS/MS spectra of peptides were externally calibrated using the Mass Standards Kit for the 4700 Proteomics Analyzer (Applied Biosystems). Spectra of intact osteocalcin were externally calibrated using the 1+, 2+, and β-chain peaks for bovine insulin (monoisotopic mass 5730.6; Sigma). Resolution was ca. 13,000 and mass accuracy was better than 34 ppm (range 9–34 ppm). For MS/MS spectra, atmosphere was used as the collision gas with a pressure of 6×10^{-7} torr and collision energy of 1 kV. Spectra were acquired using 8000–10,000 laser shots. For MS/MS spectra calibration, frag-

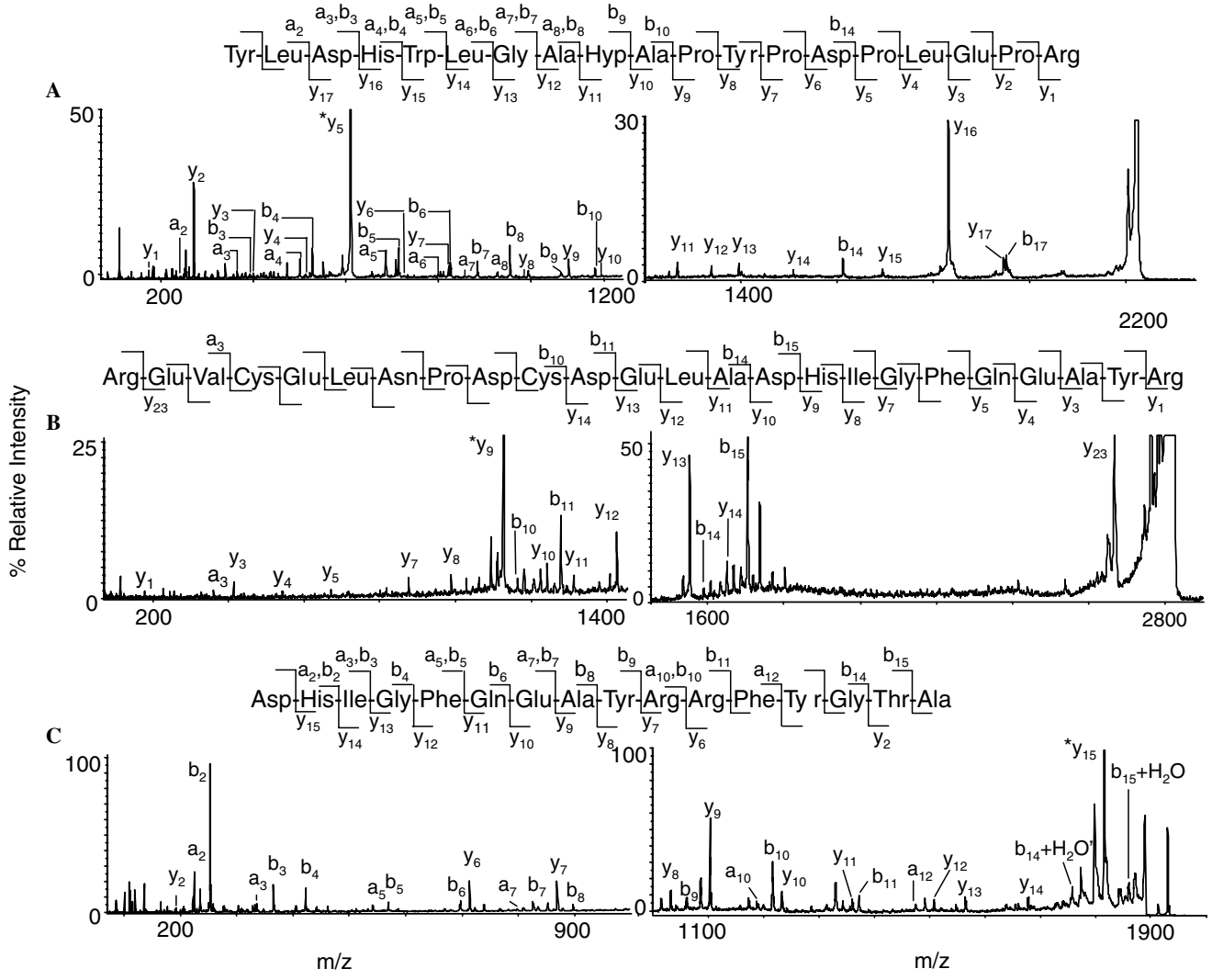

Fig. 4. MS/MS spectrum for tryptic peptides (A) 1–19 and (B) 20–43, and (C) ASP-N peptide 34–49 of zebra. Spectra produced on the ABI-4700. Peptide 1–19 shows complete coverage and together peptides 20–43 and 34–49 provide sequence for residues 20 and 29–49. For each spectrum, right and left panels of each are scaled for clarity. To allow comparison, both panels are scaled relative to the largest fragment ion: (A) y_5, (B) y_9, and (C) y_{15}. *Largest fragment ion, full scale (i.e., 100%).

mentation of angiotensin II was used. Resolution was ca. 3000 and mass accuracy was 65 ppm or better, based on peptide fragment assignments. Edman sequencing was performed on an Applied Biosystems 494 CLc.

3. Results

Mass spectra were obtained from an ABI-STR and ABI-4700. Ions from the ABI-STR are average m/z and are reported as nominal mass and those obtained from ABI-4700 are monoisotopic m/z and are reported to one decimal place. After rpHPLC purification osteocalcin from modern horse shows an $[M + H]^+$ ion with m/z 5719.7 and 5723. Because HCCA matrix results in decarboxylation of γ-carboxyglutamic acid (Gla) of the intact protein during MALDI-MS (Nielsen-Marsh et al., 2002), the observed mass reflects the fully decarboxylated molecule. The observed m/z for modern horse differs from the monoisotopic m/z 5743.7 predicted from the published sequence (Carstanjen et al., 2002) (YLDHWLGAPAPYPDPLEPRREVCELNPDCDELADHIGFQEAYRRFYGPV) by 24 Da (with decarboxylation of Gla_{17}, Gla_{21}, and Gla_{24}; Hyp_9; disulfide bond between Cys_{23} and Cys_{29}). The peptide mass fingerprint (PMF) of tryptic digest products (Fig. 1) for putative osteocalcin of modern horse contains peaks whose m/z are consistent with those predicted from the published sequence (confirmation on ABI-STR and ABI-4700). However, the m/z of the putative C-terminal peptide, 44–49 (m/z 714.4) was 24 Da less than that predicted from the published sequence. PSD of tryptic peptide 1–19 confirms that the first 19 residues of our sequence and the published sequence are the same (Fig. 2). The MS/MS spectra for

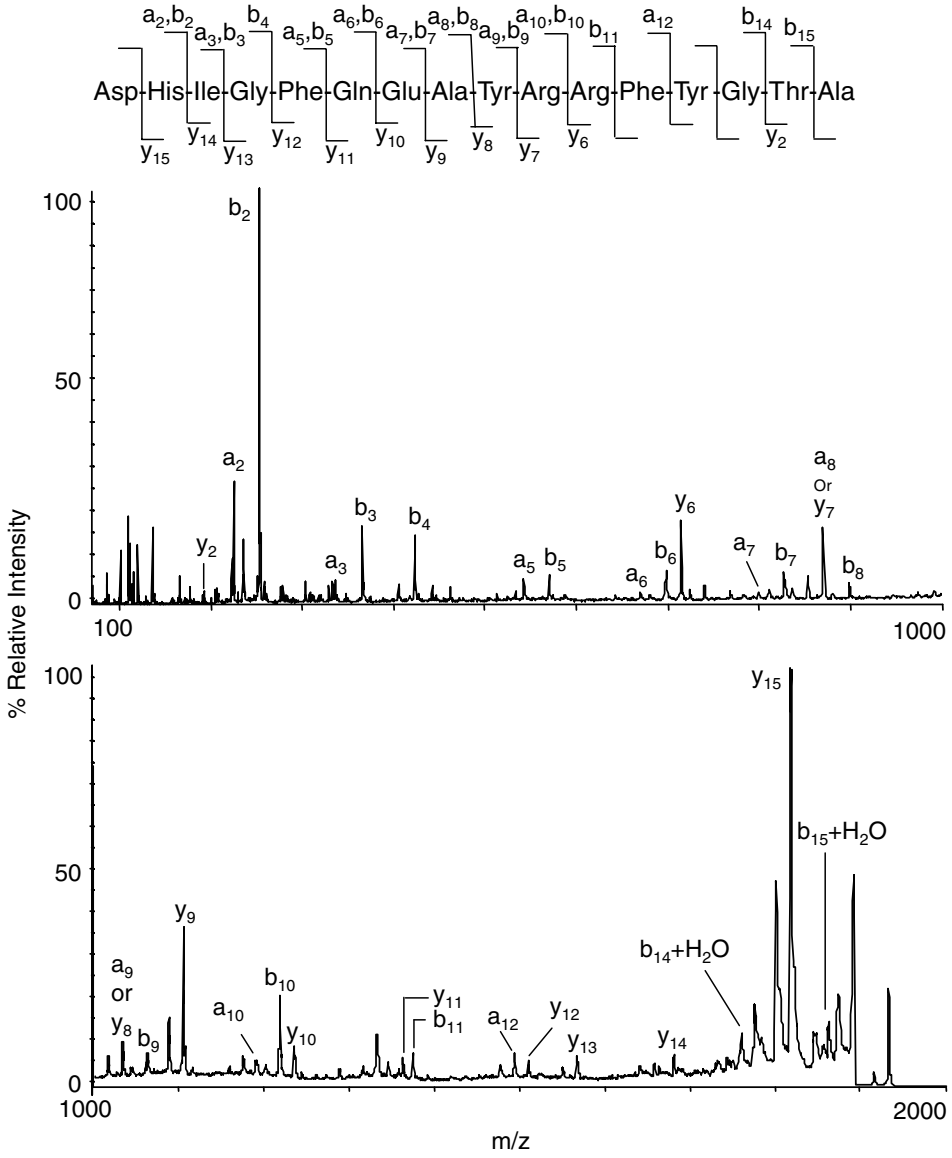

Fig. 5. MS/MS spectrum for ASP-N peptide 34–49 (m/z 1931) from osteocalcin of modern donkey produced on the ABI-4700 (sequence for residues 34–49).

ASP-N peptide 34–49 of extant horse confirms that residues 34–46 are the same as the published sequence (Fig. 3). Informative fragmentation was incomplete and residues 47–49 could not be assigned (i.e., y_1, y_2, y_3 are not observed). The complete sequence of this peptide, DHIGFQEAYRRFYGTA was obtained by Edman sequencing. It differs from the published sequence at positions 48 and 49. The residue changes account for the 24 Da decrease in the *m*/*z* for osteocalcin relative to that predicted from the published sequence. Thus, the observed *m*/*z* (5719.7) for modern horse reflects Thr_{48}, Ala_{49}; decarboxylation of Gla_{17}, Gla_{21}, and Gla_{24}; Hyp_9 and a disulfide bond between Cys_{23} and Cys_{29}. While C-terminal Pro, Val occurs in the majority of species (Hauschka et al., 1989), Thr_{48} was observed in cat and rat (Linde et al., 1980; Shimomura et al., 1984), Ala_{49} in pig (Hoang et al., 2003) and C-terminal Thr, Ala in wallaby (Huq et al., 1984).

Like modern horse, the $(M + H)^+$ ion of osteocalcin from both zebra and donkey shows a peak at 5723 for osteocalcin (average mass). The PMF of the tryptic products are also the same (Fig. 1). Sequence similarity between zebra and horse was confirmed by MS/MS for tryptic peptides 1–19 and 20–43, and ASP-N peptide 34–49 (Fig. 4). We scrutinized peptide 34–49 from extant donkey by MS/MS (Fig. 5) to confirm that its C-terminus is the same as horse and zebra (Figs. 3 and 4).

The ancient horse extract contained two analytes that sometimes partially co-eluted by rpHPLC: osteocalcin and an analyte 43 Da greater in mass (confirmed on ABI-STR and ABI-4700). The latter appears to be modified osteocalcin and a mixture of the two analytes is termed partially purified osteocalcin. The Edman sequence for residues 1–22 derived from partially purified osteocalcin, -LDHWLGAUAPYPDPL-PRR-V (- = unknown and U = hydroxyproline), was the same as that of modern horse.

The PMF of tryptic products of partially purified osteocalcin from the 42 ka horse showed peaks with the same *m*/*z* as modern horse. In addition, a peak at *m*/*z* 2266, 43 Da greater than the *m*/*z* of peptide 1–19 (Fig. 1), likely reflects a modification of peptide 1–19 in the second analyte. Carbamylation of osteocalcin has been proposed (Gundberg and Weinstein, 1986) and could account for the 43 Da disparity.

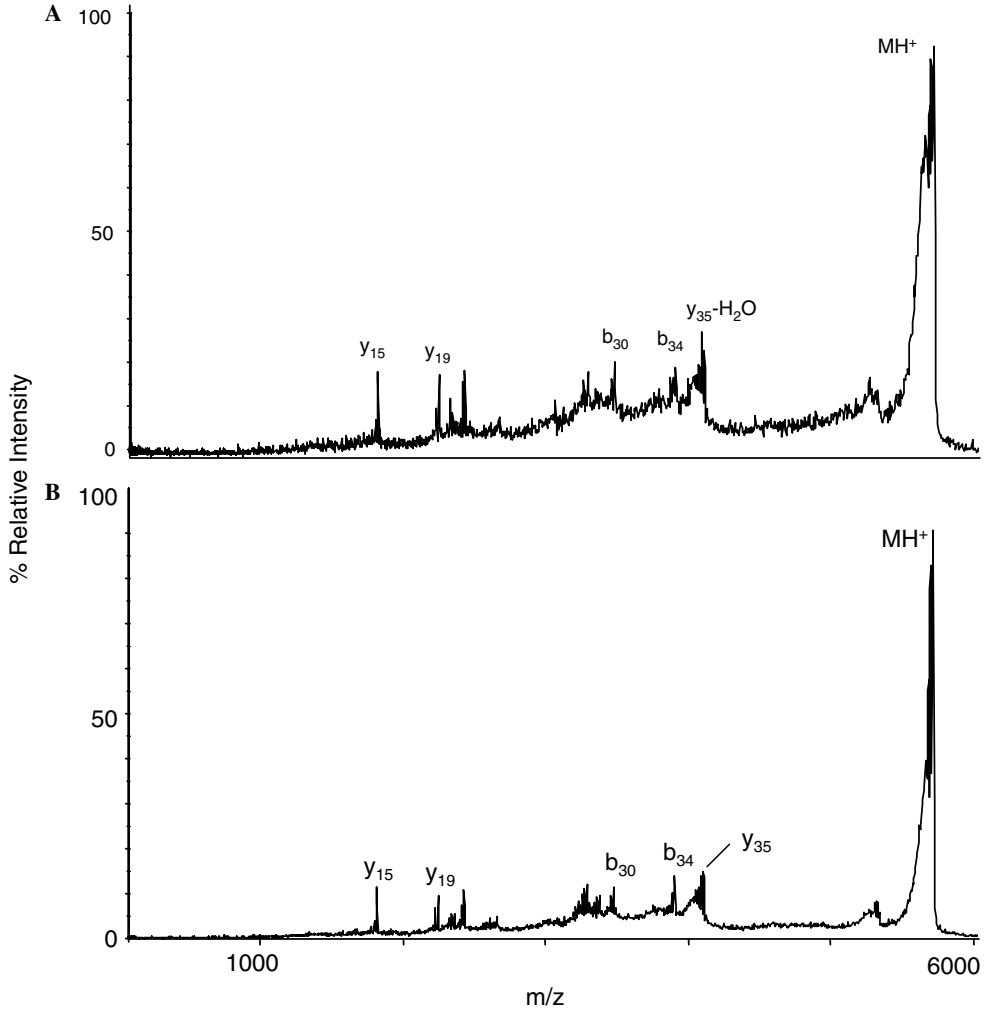

Fig. 6. MS/MS spectrum for the $(M + H)^+$ ion of intact osteocalcin from: (A) modern and (B) 42 ka horse produced on the ABI-4700.

MS/MS of intact ancient osteocalcin and modern horse show similar fragment ions (Fig. 6). Fragment ion pairs y_{19}/b_{30} and y_{15}/b_{34} are consistent with fragmentation at the C-terminus of Asp_{30} and Asp_{34}, respectively (Wysocki et al., 2000). MS/MS of tryptic peptide 1–19 and 20–43 and ASP-N peptide 34–49 from purified osteocalcin provided nearly complete sequence coverage for the 42 ka horse (Fig. 7). The fragment ions are similar to those observed in modern horse. However, a change from Gln to Glu at position 39 is observed for both tryptic peptide 20–43 and ASP-N peptide 34–49 from ancient osteocalcin. This is consistent with deamidation, a process known to occur during fossilization and aging (Robbins et al., 1993; Clarke, 2003). The sequence of partially purified osteocalcin from a different aliquot of bone powder was identical to modern horse and did not show deamidation (data not shown). Deamidation could be a diagenetic process or an artifact of sample handling. The lack of deamidation in MALDI-MS of modern osteocalcin argues against the latter possibility. In either case, mass shifts associated with hydrolytic and oxidative processes should be considered in future interpretations of ancient protein sequences.

The peak for the tryptic peptide 20–43 from one preparation of ancient horse osteocalcin was at m/z 2820.4 and is 3 Da lower than expected if this peptide were deamidated (Glu_{39}) as described above. The mass difference could exist if a disulfide bridge exists between Cys_{23} and Cys_{29} and position 39 is Gln (Fig. 8). An internal Cys_{23}-Cys_{29} disulfide is a characteristic feature of modern osteocalcin (Hoang et al., 2003). A reduction and alkylation (iodoacetamide) confirm a disulfide bridge in the ancient sample. As predicted, reduction increases the m/z of peptide 20–43 by 2 Da (m/z 2822.8) and alkylation results in an additional 114 Da increase (2936.7), consistent with a shift of 57 Da associated with alkylation of both Cys residues (Fig. 8).

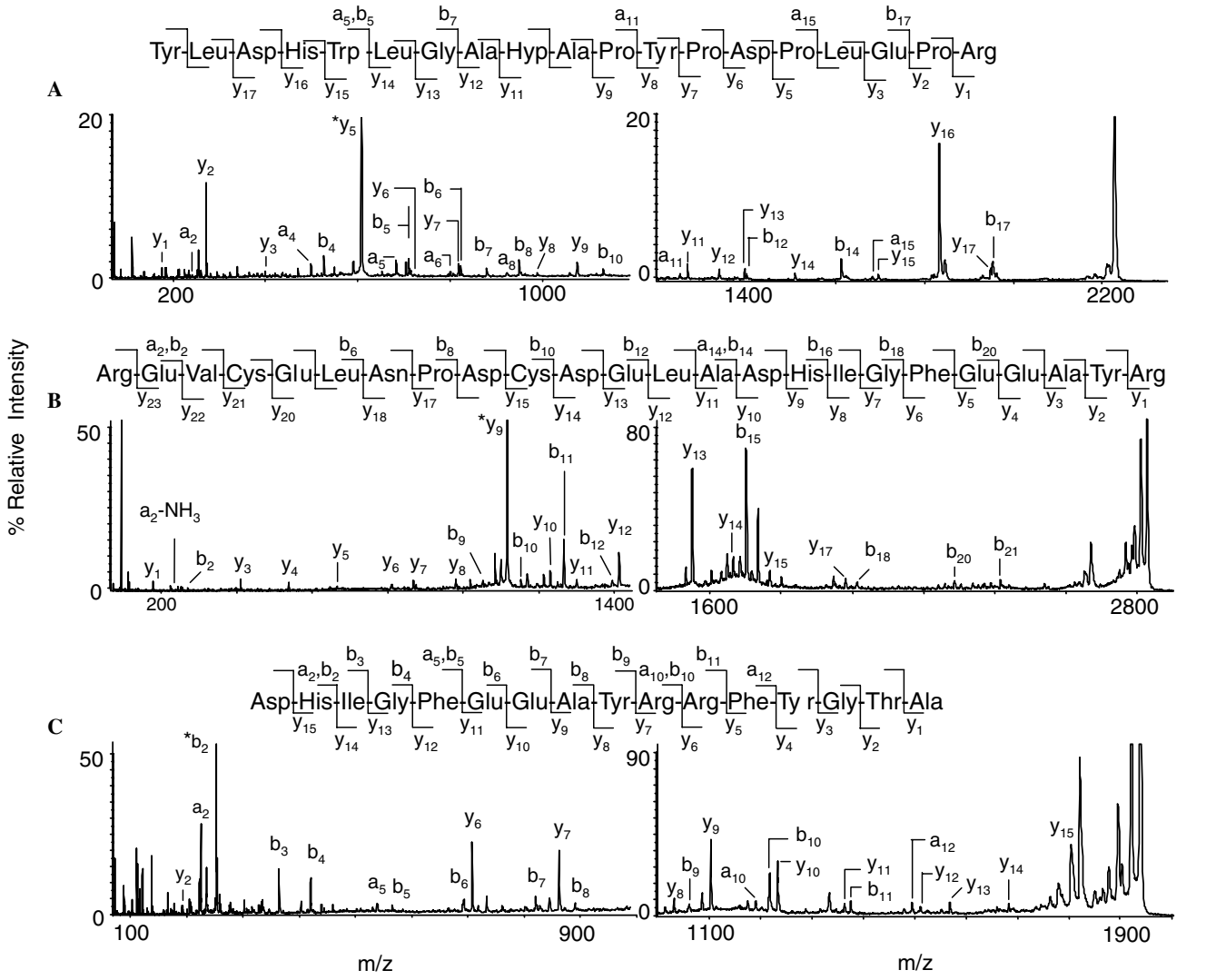

Fig. 7. MS/MS spectra of tryptic peptide (A) 1–19 and (B) 20–43; and (C) ASP-N peptide 34–49 from the 42 ka horse. Spectra produced on the ABI-4700. Complete coverage for peptide 34–49. Coverage for 1–19 and 20–43 provide all residues except 15 and 24 (P and E, respectively). Fragment ions in bold are from a separate aliquot of partially purified OC. For each spectrum, right and left panels are scaled for clarity. To allow comparison, the panels are scaled relative to the largest fragment ion: (A) y_5, (B) y_9, and (C) b_2. *Largest fragment ion, full scale (i.e., 100%).

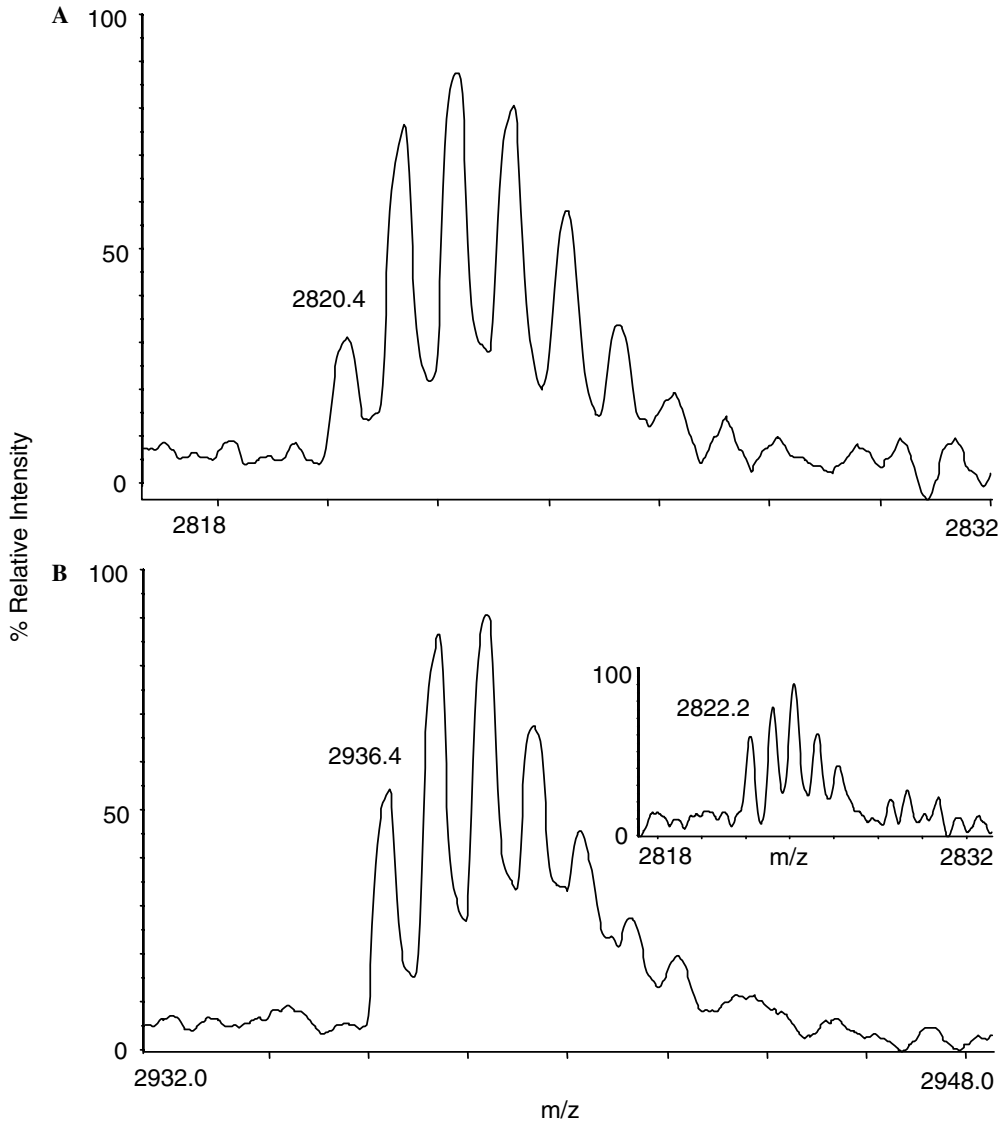

Fig. 8. Mass spectra for tryptic peptide 20–43 from the 42 ka horse showing (A) monoisotopic *m/z* and isotope distribution; (B) the reduced and alkylated (iodoacetamide) peptide; and (B) (inset) reduction alone produces a 2 Da shift. Data produced on the ABI-4700.

The PMF provides complete sequence information for the modern and ancient horse and indicates sequence similarity for the two taxa. MS/MS and Edman data confirm the PMF (Fig. 7). Together the MS/MS and Edman data provide 96% of the sequence (residues 24 and 25 were not given by either technique).

4. Discussion and conclusions

We demonstrate that extant horse, zebra, and donkey, and the 42 ka horse have the same osteocalcin sequence. Oakenfull and Clegg (1998) estimate the divergence of the horse lineage from the zebra-ass lineage of 1.2 Ma and zebra-ass from donkey-hemione of 0.4 Ma. Thus, within the genus *Equus*, amino acid substitutions for osteocalcin did not occur within the past 1.2 Ma. The absence of sequence variation over this time frame is consistent with the highly conserved nature of osteocalcin (Hauschka et al., 1989). Although probing Pleistocene phylogenetic divergences may require a protein with greater sequence elasticity (e.g., albumin, type-1 collagen), deeper evolutionary divergences may be tractable with osteocalcin. Capturing the more variable and genetically informative, N-terminus of osteocalcin (Hauschka et al., 1989) is an important strategy for phylogenetic applications.

In addition to complete coverage, we document a disulfide bridge in osteocalcin from the 42 ka horse. This is a prominent characteristic of the molecule and is an important determinant of its tertiary structure. In modern osteocalcin, this bridge stabilizes the type III turn structure that connects the two N-terminal α-helices (Hoang et al., 2003). Previously, the persistence of a hydrophobic core in ancient osteocalcin has been proposed from a partial primary sequence (Nielsen-Marsh et al., 2005). Because this core is hydrophobic and coordinates Ca^{2+} at the hydroxyapatite surface, it may play a key role in osteocalcin survival. Spe-

cifically, the core is composed of three α-helices whose hydrophobicity is promoted by conserved residues Leu_{16}, Leu_{32}, Phe_{38}, Ala_{41}, Tyr_{42}, Phe_{45}, and Tyr_{46} (Hoang et al., 2003). We suggest that, in addition to the primary sequence, the interhelix disulfide bridge reflects an intact tertiary structure that is a key element to the hydrophobic core and, potentially, preservation of osteocalcin during diagenesis.

Although small and highly conserved in its α-helical region, osteocalcin is a useful candidate for developing approaches for protein sequencing and extending temporal limits to biomolecular records. Contamination issues are largely alleviated because osteocalcin is exclusive to vertebrates and absent in common contaminators (e.g., bacteria, plants). Enhanced survivability is afforded by osteocalcin's affinity for bone mineral (Hoang et al., 2003; Oakenfull and Clegg, 1998) and unlike ancient DNA, protein sequencing does not rely on amplification, a step that can magnify contaminants.

While attributes of osteocalcin limit contamination they do not eliminate the need to standardize research protocols and develop criteria for authenticity, nor do they address numerous issues inherent in MALDI-MS sequencing approaches. Cross contamination and false positives result from improperly cleaned sample plates or spill over between close (<2 mm) wells where samples are spotted. Suppression resulting from inadequate purification results in false negatives or reduced analyte signal. Peaks resulting from the matrix or self-digestion of an enzyme can be confused with those resulting from an enzymatic digest. Because masses alone are not diagnostic, a partial PMF, especially from an incompletely purified analyte, may reflect a set of diagenetic products rather than enzyme specific cleavage products. However, MS/MS results are diagnostic if a complete set of ion fragments for the protein or peptide is present. Because MS/MS results produce complex mass spectra, interpretations can be difficult. Here, independent Edman sequencing was valuable to confirm disparities from expected sequences.

Our purification and sequencing approach has addressed contamination and instrumental issues. Following practices for ancient DNA (Cooper and Poinar, 2000) we make recommendations for preparation of samples and authentication of results: (1) use separate reagents, tools, and rpHPLC columns for modern and fossil samples to avoid contamination, (2) validate lack of contamination by demonstrating that the molecular ion can be obtained from a partial purification using a new gravity column, (3) use separate plates for modern and ancient osteocalcin (4) purify and sequence one or more peptides in a PMF (5) use multiple sequencing approaches and (6) provide independent verification of, at least, part of the sequence in a second independent laboratory.

This paper demonstrates that analysis of fossil proteins provides data previously inaccessible, including fossil post-translational modifications, diagenetic chemistries and sequence information. Careful attention to authentication procedures will be important for validating future efforts to unravel sequence, structural and phylogenetic information in the fossil record.

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References

Carstanjen, B., Wattiez, R., Armory, H., Lepage, O.M., Remy, B., 2002. Isolation and characterization of equine osteocalcin. *Ann. Med. Vet.* **146** (1), 31–38.

Clarke, S., 2003. Aging as war between chemical and biochemical processes: protein methylation and the recognition of age damaged proteins for repair. *Ageing Res. Rev.* **2**, 263–285.

Collins, M.J., Gernaey, A.M., Nielsen-Marsh, C.M., Vermeer, C., Westbroek, P., 2000. Osteocalcin in fossil bones: evidence of very slow rates of decomposition from laboratory studies. *Geology* **28**, 1139–1142.

Cooper, A., Poinar, H.N., 2000. Ancient DNA: do it right or not at all. *Science* **289**, 1139.

Gundberg, C.M., Weinstein, R.S., 1986. Multiple immunoreactive forms of osteocalcin in uremic serum. *J. Clin. Invest.* **77**, 1762–1767.

Hadley, E.A., Ramakrishnan, U., Chan, Y.L., van Tuinen, M., O'Keefe, K., Spaeth, P.A., Conroy, C.J., 2004. Genetic response to climatic change: Insights from ancient DNA and phylochronology. *PLoS Biol.* **2** (10), 1600–1609.

Hauschka, P.V., Lian, J.B., Cole, D.E., Gundberb, C.M., 1989. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. *Physiol. Rev.* **69**, 990–1047.

Hoang, Q.Q., Sicheri, F., Howard, A.J., Yang, D.S.C., 2003. Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature* **425**, 977–980.

Huq, N.L., Ten, L.C., Christie, D.L., Chapman, G.E., 1984. The amino acid sequences of goat, pig, and wallaby osteocalcins. *Biochem. Int.* **8**, 521–527.

Kelly, R.L., Prasciunas, M., Walker, D.N., Finley, J., Weathermon, R., 2002. Report on 2002 activities at Juniper Cave, 48BH3178. Report submitted to the BLM, Cody District in fulfillment of BLM Task Order TO-5, agreement number KAA990012, pp. 39.

Leonard, J.A., Wayne, R.K., Cooper, A., 2000. Population genetics of ice age brown bears. *Proc. Natl. Acad. Sci. USA* **97**, 1651–1654.

Linde, J.B., Bhown, M., Butler, W.T., 1980. Noncollagenous proteins of dentin: A re-examination of proteins from rat incisor dentin utilizing techniques to avoid artifacts. *J. Biol. Chem.* **255**, 5931–5942.

Nielsen-Marsh, C.M., Ostrom, P.H., Gandhi, H., Shapiro, B., Cooper, A., Hauschka, P.V., Collins, M.J., 2002. Exceptional preservation of bison

bones older than 55 ka as demonstrated by protein and DNA sequences. *Geology* **20**, 1099–1102.

Nielsen-Marsh, C.M., Richards, M.P., Hauschka, P.V., Thomas-Oates, J.E., Trinkaus, E., Pettitt, P.B., Karavanic, I., Poinar, H., Collins, M.J., 2005. Osteocalcin protein sequences of Neanderthals and modern primates. *Proc. Natl Acad. Sci. USA* **102**, 4409–4413.

Oakenfull, E.A., Clegg, J.B., 1998. Phylogenetic relationships within the genus *Equus* and the evolution of the α and θ globin genes. *J. Mol. Evol.* **47**, 772–778.

Ostrom, P.H., Schall, M., Gandhi, H., Shen, T.-L., Hauschka, P.V., Strahler, J.R., Gage, D.A., 2000. New strategies for characterizing ancient proteins using matrix-assisted laser desorption ionization mass spectrometry. *Geochim. Cosmochim. Acta* **64**, 1043–1050.

Paabo, S., Poinar, H., Serre, D., Jaenicke-Despres, V., Hebler, J., Rohland, N., Kuch, M., Krause, J., Vigilant, L., Hofreiter, M., 2004. Genetic analysis from ancient DNA. *Annu. Rev. Genet.* **38**, 645–679.

Paxinos, E.E., James, H.F., Olson, S.L., Sorenson, M.D., Jackson, J., Fleischer, R.C., 2002. MtDNA from fossils reveals a radiation of Hawaiian geese recently derived from the Canada goose (*Branta Canadensis*). *Proc. Natl. Acad. Sci. USA* **99**, 1399–1404.

Robbins, L.L., Muyzer, G., Brew, K., 1993. Macromolecules from living and fossil biominerals: Implications for the establishment of molecular phylogenies. In: Engel, M.H., Macko, S.A. (Eds.), *Organic Geochemistry: Principles and Applications*. Plenum Press, New York, pp. 799–816.

Shimomura, H., Kanai, Y., Sanada, K., 1984. Primary structure of cat osteocalcin. *J. Biochem. Tokyo* **96**, 405–411.

Stafford Jr., T.W., Hare, P.E., Currie, L.A., Jull, A.J.T., Donahue, D., 1991. Accelerator radiocarbon dating at the molecular level. *J. Arch. Sci.* **18**, 35–72.

Wayne, R.K., Leonard, J.A., Cooper, A., 1999. Full of sound and fury: The recent history of ancient DNA. *Annu. Rev. Ecolog. Syst.* **30**, 457–477.

Weinstock, J., Willerslev, E., Sher, A., Tong, W., Ho, S.Y.W., Rubenstein, D., Storer, J., Burns, J., Martin, L., Bravi, C., Prieto, A., Froese, D., Scott, E., Xulong, L., Cooper, A., 2005. Evolution, systematics, and phylogeography of Pleistocene horses in the new world: a molecular perspective. *PLoS Biol.* **3** (8), 0001–0007.

Willerslev, E., Cooper, A., 2005. Ancient DNA. *Proc. R. Soc. Lond. Ser. B., Biol. Sci.* **272** (1558), 3–16.

Wysocki, V.H., Tsaprailis, G., Smith, L.L., Breci, L.A., 2000. Mobile and localized protons: A framework for understanding peptide dissociation. *J. Mass Spectrom.* **35**, 1399–1406.