LETTER

Isotopic fractionation during peptide bond hydrolysis

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Abstract—Isotopic fractionation of nitrogen and carbon is considered during peptide bond hydrolysis. Theoretical considerations suggest that hydrolysis will enrich residual, unhydrolyzed protein in ¹⁵N while ¹³C should be relatively unaffected. Preliminary experimental results support this conclusion, although further studies are required to quantify the magnitude of this effect as a function of protein degradation, in particular under natural environmental conditions.

INTRODUCTION

During the last decade there has been increasing use of carbon and nitrogen stable isotope ratios in archaeological bone for estimating the diet of prehistoric human populations (see references in Price, 1989). In order to determine the reliability of these dietary reconstructions, investigations have been conducted to ascertain how factors such as trophic level interactions (Schoeninger and Deniro, 1984), environmental conditions (Ambrose and Deniro, 1986), and physiological parameters (Young and Bier, 1981) affect the collagen isotopic signatures. Additional studies have focused on the scrambling of the original signatures by depositional and diagenetic processes (Hare, 1980; Deniro, 1985; Nelson et al., 1986; Masters, 1987; Tuross et al., 1988). It is generally considered that reliable palaeodiетary information can be obtained from the isotopic analyses of collagen, the major protein present in fresh bone. Recent efforts have concentrated on defining the chemical and physical characteristics of collagen in modern bone and assessing the extent that these are preserved in archaeological material (Deniro and Weiner, 1988; Schoeninger et al., 1989). Only organic fractions which exhibit the properties of modern collagen are considered usable for isotopic dietary analyses. “Collagen-like” material, retained in variable amounts in archaeological bone, is believed to have retained its original carbon and nitrogen isotopic signatures.

As we discuss here, peptide bond hydrolysis which is responsible for the degradation of proteins can, in theory, result in significant alterations of the original nitrogen isotopic composition in residual, unhydrolyzed protein. Our preliminary experimental results suggest that if the degree of hydrolysis of the original protein has been substantial, residual collagen isolated from bone can have isotopic ratios enriched in ¹⁵N. In contrast, peptide bond hydrolysis should theoretically have a minimal affect on carbon isotopic ratios, and our preliminary results support this prediction.

THEORETICAL CONSIDERATIONS

The peptide bond hydrolysis reactions can be generalized as follows.

\[
\begin{align*}
\text{H}_2\text{N}-
\end{align*}
\]

where \( k \) is the first-order rate constant for hydrolysis and \( \sim \) denotes continuation of the peptide chain. The value of \( k \) is both pH and temperature dependent. In addition, \( k \) will be a function of the isotopic atoms in the peptide bond.

We will first consider the nitrogen isotopic effect on the hydrolysis rate. Since the zero-point energy for the ¹²C-¹⁵N bond is less than that of the ¹²C-¹⁴N bond, the peptide bond containing ¹⁴N should rupture preferentially (Stacey et al., 1952). Therefore, the rate constant for the hydrolysis of the peptide bonds containing ¹⁴N \( (k_{14}) \) will be greater than those with ¹⁵N \( (k_{15}) \), i.e., \( k_{14} > k_{15} \). As hydrolysis proceeds, the remaining unhydrolyzed protein should become increasingly enriched in ¹⁵N. The magnitude of this isotopic fractionation hydrolysis effect will be a function of the \( k_{14}/k_{15} \) ratio, which, as we discuss here, is poorly known.

Nitrogen isotopic fractionation during peptide bond hydrolysis can be examined theoretically by treating the reactions involving the isotopic bonds as two independent, competing reactions. Thus,

\[
\begin{align*}
¹⁴\text{N} \rightarrow & \text{B + C} \\
¹⁵\text{N} \rightarrow & \text{B + C}
\end{align*}
\]

where \( k_{14} \) and \( k_{15} \) are the hydrolysis rate constants for cleavage of the ¹²C-¹⁴N (¹⁴N) and ¹²C-¹⁵N (¹⁵N) bonds, respectively, in a protein.

The irreversible, first-order rate expressions for these two reactions can be written as
was assumed to be 10 m$^{-3}$ y$^{-1}$. The initial nitrogen isotopic ratio was residual, unhydrolyzed protein as a function of % hydrolysis calculated from Eqn. (3)

$$\ln \left( \frac{^{15}N_t/^{14}N_0}{^{14}N_t/^{14}N_0} \right) = -k_{14} \cdot t - k_{15} \cdot t$$

where $N_t$ and $N_0$ refer to the nitrogen in unhydrolyzed protein at time $t$ and $t = 0$. Combining and rearranging these equations yields

$$\ln \left( \frac{^{15}N_t/^{14}N_0}{^{14}N_t/^{14}N_0} \right) = \ln \left( \frac{^{15}N_t/^{14}N_0}{^{14}N_t/^{14}N_0} \right) + (1 - k_{15}/k_{14}) k_{14} \cdot t$$

where the subscripts of the $^{15}N/^{14}N$ isotopic ratios refer to times $t$ and $t = 0$.

In Fig. 1, we have used Eqn. (3) to model the change in the initial $^{15}N/^{14}N$ isotopic composition of a protein as a function of the percent hydrolysis using various $k_{14}/k_{15}$ ratios. As can be seen, even if the difference in the isotopic rate constants is only 0.5 to 1.0%, the residual, unhydrolyzed protein is significantly enriched in heavy nitrogen if the percent hydrolysis is greater than 50%.

Although equations similar to those given above would apply to the effect of peptide bond hydrolysis on the carbon isotopic composition of a protein, the magnitude of the carbon effect should differ from that of nitrogen. Carbon is present in protein components (e.g., the amino acid R-groups) other than the peptide bond whereas nitrogen is essentially contained entirely within the peptide bond. Carbon isotopic fractionation during peptide hydrolysis would affect only the isotopic composition of the carbonyl carbon. Since this carbon atom constitutes only part of the total carbon in a protein molecule (it makes up roughly 30% of the total carbon in collagen), the carbon isotopic fractionation effect due to hydrolysis should be less in comparison with that for nitrogen when the total protein carbon is utilized for isotopic analyses.

In order to evaluate the actual magnitude of isotopic modification of a protein during peptide bond hydrolysis, values of the isotopic rate constants given in Eqn. (3), and in the corresponding equations for the carbon isotopes, are required. As can be seen in Fig. 1, the value of $k_{14}/k_{15}$ is critical in determining the enrichment of heavy nitrogen associated with various degrees of protein hydrolysis. However, no experimental data for either the nitrogen or carbon isotopic hydrolysis rate constants in peptides or proteins existing exist.

Limited data are available for reactions somewhat related to peptide bond hydrolysis. The $k_{14}/k_{15}$ ratio for the hydrolysis of amides has been cited as ranging from 1.00-1.01 (O'LEYER and KLUETZ, 1972), although the actual experimental results are unpublished. The $k_{14}/k_{15}$ ratio for the intramolecular hydrolysis of phthalaldehyd and phthalimide and ammonia has been measured to be 1.010-1.014 at 180°C (STACEY et al., 1952). In the case of carbon, studies of the alkaline hydrolysis of esters have yielded $k_{13}/k_{14}$ ratios for the carbonyl carbon of around 1.04 (O'LEYER and MARLER, 1979). However, as discussed above, the overall carbon isotopic fractionation during peptide bond hydrolysis should be significantly less than this $k_{13}/k_{14}$ ratio because of the non-carbonyl carbon in proteins. Although amide and ester hydrolysis may be considered roughly analogous to peptide bond hydrolysis, it is unclear to what extent the isotopic rate constants for these reactions can be used to evaluate nitrogen and carbon isotopic fractionation during protein hydrolysis. It is unknown whether peptide bond hydrolysis in biogeochemical systems is acid or base catalyzed (or perhaps neither). For these reasons, these amide and ester rate constants can be considered as crude indicators, at best, of the magnitude of the kinetic isotopic effect during protein degradation in natural systems.

**EXPERIMENTAL PROCEDURE**

A relatively straightforward experiment was devised as a preliminary investigation of the isotopic fractionation rate constants during peptide bond hydrolysis. A piece (approximately 0.1 g) of bovine Achilles tendon collagen (CALBIOCHEM, La Jolla, CA) was placed in a test tube containing about 10 ml of 0.1 N NaOH. Basic pH was chosen because hydrolysis takes place rapidly under mild temperature conditions. The tube was sealed and then heated at 50°C for 20 h until only a small portion of the initial collagen fragment remained.

Four amino acid fractions were prepared for isotopic analysis. Fraction #1: a piece of the original, unheated Achilles tendon collagen was acid hydrolyzed in 6 M HCl for 24 h. Following acid hydrolysis, the HCl was evaporated to dryness, the residue dissolved in doubly distilled water, and the solution desalted using cation exchange chromatography to isolate the amino acids. Fraction #2: the heated NaOH solution from the sealed glass tube was filtered through a glass fiber filter. The solution was adjusted to neutrality by HCl addition, and a portion of it was dialyzed against water in cellulose dialysis tubing with a molecular weight cut off of >1000 daltons. The solution retained in the tubing (peptides with more than 8 amino acid residues) was dried, acid hydrolyzed and desalted to obtain amino acids as described above. Fraction #2: the residual, unhydrolyzed component retained on the glass fiber filter during filtration of the heated NaOH solution was also acid hydrolyzed and desalted to obtain the component amino acids. Fraction #4: the remaining portion of the heated, filtered NaOH solution (i.e., the portion not placed in dialysis tubing) was evaporated to dryness, the residue dissolved in doubly distilled water, the pH adjusted to neutrality with HCl, and the solution desalted to obtain the component amino acids.

Before combustion of the amino acid extracts for carbon and nitrogen isotopic analyses, any NH$_3$ which perhaps remained from
RESULTS AND DISCUSSION

The four amino acid fractions described above represent different stages of peptide bond hydrolysis. The relative amounts of carbon in each component were estimated from the quantity of CO₂ generated during combustion of each fraction and were used to estimate the relative percent of each component (see Table 1). Fraction #1 represents the amino acids in the initial intact starting collagen and thus corresponds to 0% hydrolysis. Fraction #2 represents large protein fragments first liberated during hydrolysis. This soluble protein fraction constitutes approximately 70% of the original collagen and thus represents peptide bonds which remain intact after approximately 30% hydrolysis. Fraction #3, the residue component, is that material most resistant to hydrolysis. It constitutes approximately 5% of the starting material. Thus, this fraction represents the material remaining after the initial collagen has been degraded by more than 90%. Fraction #4 is composed of free amino acids liberated during hydrolysis. They total approximately 25% of the starting collagen and correspond to the end-products of complete protein hydrolysis.

The isotopic compositions of the various components analyzed in our collagen NaOH hydrolysis experiment are shown in Table 1. The carbon isotopic ratio of the soluble protein (the second fraction) is virtually identical with that of the starting collagen. The nitrogen isotopic ratio, on the other hand, is noticeably more positive than that of the original collagen. Even more obvious, however, the amino acids in the residue (the third fraction) are significantly enriched in 15N and slightly depleted in 13C when compared to the ratios of the initial collagen. The nitrogen and carbon isotopic compositions of the liberated amino acids differ very slightly in comparison with those of the initial collagen amino acids.

The elemental ratio C:N of the soluble protein component suggests an increased relative contribution of glycine (C:N ratio = 2). As discussed by Tuross et al. (1988), however, an increase in glycine should result in a depletion in 15N rather than an enrichment as observed here. These results suggest that the observed enrichment in 15N cannot be explained by a change in amino acid composition. Similarly, the elemental ratio of the residue (6±2) is much higher than that observed in collagen extracted from fresh bone (Schoeninger and DeNiro, 1981), although there is a high degree of uncertainty in this number given the measurement.

Although not consistent with a change in amino acid composition, the nitrogen isotopic changes observed in both the unhydrolyzed material and the soluble peptides are consistent with theoretical predictions of the kinetic isotope effect during protein hydrolysis. The soluble peptides which represent a point at which the initial protein has undergone roughly 30% hydrolysis show an enrichment in 15N of +7%. The residue

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percent of starting material</th>
<th>C:N</th>
<th>δ¹⁵Nair (%)</th>
<th>δ¹³Cproj (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1: starting collagen</td>
<td>100%</td>
<td>3.4 ± 0.1</td>
<td>+6.2 ± 0.2</td>
<td>−15.5 ± 0.1</td>
</tr>
<tr>
<td>#2: soluble protein</td>
<td>70%</td>
<td>2.7 ± 0.1</td>
<td>+12.5 ± 0.2</td>
<td>−15.6 ± 0.1</td>
</tr>
<tr>
<td>#3: residual unhydrolyzed collagen</td>
<td>&lt;5%</td>
<td>6 ± 2</td>
<td>+25.4 ± 1.0</td>
<td>−20.1 ± 0.2</td>
</tr>
<tr>
<td>#4: free amino acids</td>
<td>25%</td>
<td>3.0 ± 0.2</td>
<td>+7.8 ± 0.3</td>
<td>−17.9 ± 0.1</td>
</tr>
</tbody>
</table>

* An isotopic mass balance for carbon indicates we have examined most of the components in the system. A nitrogen isotope mass balance, on the other hand, indicates that some of the 14N has been lost. The components of this missing fraction are not known but most likely are small peptides (2-8 residues) not isolated during the processing steps. These small peptides would diffuse through the dialysis tubing, and would thus not be components of the soluble fraction. Nor would they be isolated along with the free amino acids; because the pH of their free amino group is significantly less than that of free amino acids, these small peptides would not be retained on the cation exchange column.

† Because of the small amount of N₂ obtained from this fraction, this ratio has a high degree of uncertainty.
which corresponds to more than 90% hydrolysis is even more enriched in $\delta^{15}N$, i.e., $+20\%$. These changes are expected in the nitrogen isotopic composition of unhydrolyzed protein if the $k_{14}/k_{15}$ ratio was roughly 1.01 (see Fig. 1). The free amino acids, which are the end-products of complete protein hydrolysis, have nitrogen and carbon isotopic ratios similar (within 1–2%) to that of the starting material. This is also consistent with the theoretical expectation in that free amino acids liberated during the early part of protein hydrolysis should approximately reflect the isotopic composition of the initial protein. The small differences in carbon and nitrogen isotopic ratios we have measured between the starting collagen amino acids and the liberated free amino acids are likely due to differences in amino acid composition of these two components.

The carbon isotopic composition of the soluble protein fraction is also consistent with our prediction that there is less carbon isotopic fractionation during hydrolysis in comparison to that for nitrogen. The carbon isotopic shift between the initial and the residue fraction cannot be the result of isotopic fractionation during hydrolysis. A shift due to hydrolysis would result in an enrichment in the heavier isotope rather than a depletion as observed here. As discussed above, this carbon isotopic modification probably reflects a change in the amino acid composition of the unhydrolyzed component, rather than carbon isotope effect from hydrolysis.

Both the soluble protein and residue fractions have significant enrichments in $^{15}N$ which are likely due to isotopic fractionation during peptide bond hydrolysis. These fractions, however, have C:N ratios which are considered out of the range for meaningful isotopic analysis of archaeological bone collagen (DENIRO, 1985). Isotopic analyses, however, are carried out on archaeological bones which contain little collagen but which nevertheless have acceptable C:N ratios (SCHOENINGER et al., 1989). One example is the report by MASTERS (1987) for a human skeleton (SDM 16709) from a coastal Southern California midden burial. The skeleton had a collagenous amino acid composition and a C:N ratio of 3.14, but the percent remaining collagen was only a few percent of modern bone. The $\delta^{15}N$ of $+21.5\%$ for this skeleton is considerably more positive than the $\delta^{15}N$ values (range $+14$ to $+15\%$) reported for other human skeletons from coastal Southern California and offshore island sites (WALKER and DENIRO, 1986). In contrast, the $\delta^{13}C$ of the SDM 16709 skeleton ($-13.8\%$) was nearly as large as the $\delta^{13}C$ values (range $-14$ to $-15\%$) of the other skeletons. These results are consistent with the predicted changes in collagen stable isotope composition arising from isotopic fractionation during peptide bond hydrolysis.

The theoretical treatment of peptide bond hydrolysis (see Fig. 1) indicates that even at 50% hydrolysis, the remaining intact collagen may be enriched in $^{15}N$. Further studies are needed to ascertain the magnitude of this nitrogen fractionation effect as a function of collagen degradation and changes in C:N ratios.

* Even though there have been extensive isotopic analyses of collagen in archaeological bone, the percent remaining collagen is rarely reported.

CONCLUSIONS

As we have discussed here, isotopic fractionation should take place during the process of peptide bond hydrolysis. This fractionation should result in the isotopic enrichment of the nitrogen and carbon atoms in those peptide bonds which remain intact. Because nitrogen in proteins is primarily present in the peptide bond whereas carbon is present in other protein components, this isotopic enrichment should be manifested mainly in nitrogen. This effect will be increasingly important as the degree of protein hydrolysis increases. Thus, the isotopic analysis of collagen extracted from archaeological bone where there has been extensive organic depletion may give misleadingly heavy nitrogen isotopic ratios. These, in turn, would complicate the use of these isotopic results in palaeodietary reconstructions. Such an effect may occur when the C:N ratio is similar to that of intact collagen.

Clearly, extensive studies of peptides and proteins are required in order to establish the isotopic hydrolysis rate constants for nitrogen and carbon. In addition, a systematic study of how stable nitrogen and carbon isotopic ratios are affected by the degree of collagen preservation is needed. These results will help ascertain to what extent isotopic fractionation during peptide hydrolysis affects the original isotopic composition of the residual collagen remaining in archaeological and fossil bone.

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REFERENCES


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