Preservation of peptide moieties in three Spanish sulfur-rich Tertiary kerogens

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Abstract

Thermochemolysis with tetramethylammonium hydroxide (TMAH) and solid-state ¹⁵N NMR were utilized for the characterization of refractory organic nitrogen in Tertiary Spanish kerogens. The samples included sulfur-rich oil shales from the Ribesalbes (Serravallian), Libros (Tortonian) and Cerdanya (Tortonian) basins. Analysis using solid state ¹⁵N NMR showed that part of the refractory nitrogen in the kerogens corresponds to amide groups. Moreover, the release of amino acid derivatives after pyrolysis in the presence of TMAH indicated that this amide-N arose from peptide moieties. The amino acids released from the kerogens were dominated by high amounts of glycine and alanine. Minor amounts of aspartic acid, serine, α-aminobutyric acid and other unidentified amino acids were also detected. Because proteinaceous structures, including small peptides, are generally considered as being highly sensitive to diagenetic degradation, encapsulation of labile peptide material into aliphatic structures in S-rich kerogens (probably via lipid sulfuration) has been proposed to explain the survival of these moieties. Substantial amounts of fatty acids (as methyl esters) were also released from all the kerogens after pyrolysis/TMAH, indicating their highly aliphatic character. The production of both fatty acids and amino acids from the kerogens supports the encapsulation process.

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1. Introduction

Nitrogen is an important component that influences the cycling of organic matter in the biosphere. However, the nature of nitrogen in geochemical samples is far from clear. Nitrogenous compounds in living organisms exist mainly as complex molecular structures known as protein, made up of various combinations of 20 amino acids. Proteins are highly sensitive to degradation and until recently, the fate of peptides during diagenesis was considered to be either total remineralization or random recombination with other degraded biomolecules to yield heterocyclic moieties (Patience et al., 1992; Schulten and Schnitzer, 1997). However, recent studies using solid-state ¹⁵N NMR and thermochemolysis with tetramethylammonium hydroxide (TMAH) have shown that part of the nitrogen-containing materials in refractory organic matter from several sediments corresponds to peptide material that survived microbial degradation (Knicker and Hatcher, 1997; Zang et al., 2000; Knicker,

2000; Garcette-Lepecq et al., 2001; Knicker et al., 2001). Some authors have suggested that encapsulation of labile nitrogen-containing materials into refractory organic matter can partly explain the fate of proteinaceous materials in biogeochemical deposits (Knicker and Hatcher, 1997; Zang et al., 2000; Knicker et al., 2001; Nguyen and Harvey, 2003; Nguyen et al., 2003). Recently, Riboulleau et al. (2002) also suggested that encapsulation into aliphatic sulfurized material was probably the major process for amino acid preservation in kerogens with a high sulfur content.

Traditionally, wet chemical methods have been applied to the characterization of nitrogenous materials in natural organic matter (Huang et al., 1993). More recently, the use of solid-state ^{15}N NMR for studying different types of organic matter in geochemical samples has shown the importance of amide functional groups, probably from peptides and proteins, among the refractory organic nitrogen in sediments (Knicker and Hatcher, 1997; Derenne et al., 1998; Knicker et al., 2001). Pyrolysis techniques have also proven valuable for obtaining detailed molecular information about macromolecular organic matter. The pyrolysis of polar macromolecular materials is known to produce volatile polar products, only some of which can be chromatographed; polar products remain attached to the column, undetected and unquantified. Pyrolysis in the presence of TMAH derivatizes polar compounds to become less polar products, which are more amenable to chromatographic separation (Challinor, 1989; del Río et al., 1996). This technique, and the off-line version have been widely used in the analysis of different biopolymers and geomacromolecules (de Leeuw and Baas, 1993; Saiz-Jiménez, 1994; Clifford et al., 1995; Martín et al., 1994; del Río et al., 1995, 1996, 1998; del Río and Hatcher, 1998). This method has also been shown to cleave peptide bonds efficiently (Knicker et al., 2001; Knicker and Hatcher, 1997; Zang et al., 2000). The technique has been successfully applied to the analysis of protein-derived materials in biopolymers and geomacromolecules (Knicker and Hatcher, 1997; Zang et al., 2000; Knicker et al., 2001; Mongenot et al., 2001; Riboulleau et al., 2002).

Recently, the release of amino acids upon TMAH treatment of the methanol-eluted, non-acid fraction of the pyrolysate of a 140 Ma old S-rich kerogen has been reported (Mongenot et al., 2001; Riboulleau et al., 2002). These papers confirmed a major role of encapsulation into aliphatic structures for the survival of proteinaceous materials in highly aliphatic S-rich kerogens, probably via lipid sulfurization.

In the present paper, we use solid-state ^{15}N NMR and pyrolysis in the presence of TMAH in order to characterize the nature of the refractory organic nitrogen in three Tertiary Spanish S-rich kerogens. Until now, amino acids have been found in a polar off-line

pyrolysate of a kerogen (Riboulleau et al., 2002). However, to the best of our knowledge, identification of amino acids derivatives by direct TMAH treatment of the kerogen has not been reported.

2. Material and methods

2.1. Description of samples

Three kerogens from Tertiary lacustrine systems, corresponding to the Ribesalbes (Serravallian), Cerdanya (Tortonian) and Libros (Tortonian) basins, were selected. Ribesalbes oil shales were deposited during the Middle Miocene (Serravallian; Anadón et al., 1989); they are immature and calcareous in origin. The Ribesalbes lacustrine system evolved in an intermediate sized, extensional basin (up to 150 km²) in the SE Iberian Range (Anadón et al., 1989; Anadón and Roca, 1996). Mesozoic limestones, red bed units and evaporites (mainly Upper Triassic gypsum) make up the source areas of the basin. The Cerdanya oil shale was deposited during the Late Miocene (Tortonian). It is a mudstone which is considered an anapaite vein because of the high amount of phosphorous (15% P_2O_5) that appears in the diatomaceous lacustrine unit. Rare bioturbation and fine laminations as well as good fossil preservation are also the main characteristics of this sedimentary unit, which is similar to the Eocene Messel Oil Shale (de las Heras et al., 1989). The Late Miocene Libros lacustrine system evolved within the large (up to 2500 km²) Early Miocene–Early Pleistocene Teruel basin in the SE Iberian Range (Anadón and Roca, 1996). The source areas of the basin consisted mainly of Mesozoic limestones and Triassic gypsum, with minor siliciclastics (mudstones and sandstones).

2.2. Kerogen preparation and elemental analysis

The kerogen concentrates were prepared by treatment of the powdered, dichloromethane pre-extracted, oil shales with HCl/HF (1:1). The kerogens were subsequently washed four times with double-distilled water and re-extracted (3:1) with dichloromethane:methanol (3:1) by sonication, according to Simoneit et al. (1986).

Elemental analysis of the isolated kerogens was carried out to determine total organic carbon and hydrogen, using a Fisons 1108 elemental analyzer. Organic sulfur was measured according to the ASTM D2492-90 method where wet treatment was used to determine pyritic and sulfate forms directly. Total sulfur was measured using elemental analysis with an elementary analyzer (Carlo Erba 1106) and organic sulfur was obtained as the difference between total sulfur minus pyritic and sulfate sulfur. The results revealed that

Table 1
Bulk features of isolated kerogens

Kerogen	%C	%H	%N	%S	H/C	N/C	S _{org} /C	(Gly + Ala)/FaC ₁₆ ^a
Ribesalbes	58.6	8.4	0.47	7.9	1.76	0.007	0.052	2.0
Cerdanya	45.3	6.0	1.27	9.1	1.58	0.023	0.071	6.1
Libros	30.6	3.84	1.49	1.32	1.51	0.042	0.016	12.6

^a Estimated from pyrolysis in the presence of TMAH.

Ribesalbes kerogen is extremely rich in organic sulfur (atomic S_{org}/C ratio > 0.04) and hydrogen (atomic ratio H/C ratio > 1.5) and is classified as a Type I-S kerogen (Table 1). The Cerdanya kerogen, although with a lower atomic H/C ratio, is still considered an immature Type I kerogen in the van Krevelen diagram. Libros kerogen, on the other hand, has the lowest atomic ratio H/C ¼ 1.51, and is therefore classified as a Type I kerogen. Although its S_{org}/C ratio (0.016) is the lowest, the total sulfur content gives an atomic ratio (S_{tot}/C ¼ 0.071) for the whole rock oil shale and the soluble fraction contains high amounts of organosulfur compounds.

2.3. Pyrolysis in the presence of TMAH

Pyrolysis in the presence of TMAH was performed with approximately 100 µg sample in finely powdered form, deposited on to a ferromagnetic wire and mixed with 0.5 µl TMAH (25% w/w aqueous solution). The wire then was inserted into a glass liner, which was placed in the pyrolyser. Pyrolysis was carried out with a Varian Saturn 2000 GC-MS, using a 30 m × 0.25 mm DB-5MS column (film thickness 0.25 µm), coupled to a Curie-point pyrolyser (Horizon instruments Ltd.). Pyrolysis was carried out at 610 °C. The temperature of the chromatograph oven was programmed to rise from 40 °C (1 min) to 300 °C at a rate of 6 °C min⁻¹; the final temperature was held for 20 min. The injector, equipped with a liquid carbon dioxide cryogenic unit, was programmed from -30 °C (1 min) to 300 °C at 200 °C min⁻¹, while the GC-MS interface was kept at 300 °C. The amino acids were identified by comparing the mass spectra (obtained at 70 eV) with those of the Wiley and NIST computer libraries and with previously published mass spectra (Knicker et al., 2001). Blank experiments were performed to rule out the possibility of contamination.

2.4. Solid-state ¹⁵N NMR spectroscopy

The solid-state CPMAS ¹⁵N NMR spectra were obtained using a Bruker DMX 400 operating at 40.56 MHz. The contact time was 1 ms and a 90° pulse width of 6.8 µs, a pulse delay of 150 ms and a line broadening between 200 and 400 Hz were applied. Between 1 and 3 × 10⁶ scans were accumulated at a magic-angle spinning speed of 5.5 kHz. A ramped ¹H-pulse starting at

100% and decreasing to 50% of the maximal power was used during contact time (1 ms) in order to circumvent Hartmann-Hahn mismatches (Cook et al., 1996; Peer- sen et al., 1993). The chemical shift was standardized to the nitromethane scale (0 ppm) and adjusted using ¹⁵N-labelled glycine (δ 347.6 ppm). The assignment of the different integrals was performed according to Wit- nowski et al. (1993).

Fig. 1. Solid state ¹⁵N NMR spectra of kerogens from: (a) Ribesalbes; (b) Cerdanya; (c) Libros.

3. Results and discussion

The CPMAS ^{15}N NMR spectra of the selected Tertiary S-rich kerogens are shown in Fig. 1. Although the signal to noise ratio is very low, due to the low N concentration in the samples (N/C from 0.007 to 0.042; Table 1), it is clear that they exhibit intensity in the

chemical shift region between $\delta 250$ and $\delta 285$ ppm, characteristic of amide structures. A shoulder is seen in the region of pyrrolic N ($\delta 145$ to $\delta 240$ ppm) in the spectra. Therefore, the spectra indicate that amide functional groups, which are normally highly susceptible to chemical and microbiological degradation, are part of the organic nitrogen in the kerogens. It has been previ-

Fig. 2. TIC chromatogram of products released after pyrolysis in the presence of TMAH from the Ribesalbes, Cerdanya and Libros kerogens. Gly: glycine; Ala: alanine; α-Aba: α-aminobutyric acid; Ser: serine; Asp: aspartic acid; AA116 amino acid derivatives with base peak at m/z 116 similar to those detected by Riboulleau et al. (2002); C_n refers to the series of fatty acids (as methyl esters); *: unknown.

ously proposed that the amide N found in kerogens may derive from a refractory biopolymer composed of long chain alkyl amides (Derenne et al., 1993). Refractory chitinous residues (Stankiewicz et al., 1997) and peptidoglycans, which are a product of bacterial metabolism (Nyberg et al., 2001), might also explain the existence of amide N in these samples. Alternatively, such amides may derive from protein-derived peptide structures entrapped within the kerogen network. However, the survival of proteinaceous moieties in geological materials is surprising since they would be expected to be rapidly mineralized by microorganisms during the early stages of sediment formation.

In order to reveal the nature of the amide N revealed by ^{15}N NMR and to know whether it could be assigned to peptide-like material entrapped within the kerogen network, the samples were analysed using pyrolysis in the presence of TMAH.

Fig. 2 shows the chromatogram of the compounds released from the kerogens after pyrolysis in the presence of TMAH. The chromatograms are dominated by peaks corresponding to fatty acids (as methyl esters) and amino acid derivatives. The fatty acid series consists mainly of C_{10} – C_{26} monocarboxylic acids with an even-over-odd carbon atom number predominance and a bimodal distribution with maxima at C_{16} and C_{22} or C_{24} , and contains unsaturated compounds ($\text{C}_{16:1}$ and $\text{C}_{18:1}$), reflecting a contribution from living organisms and its highly aliphatic nature. The aliphatic acids may be chemically bound to the macromolecular network via sterically-protected, and hence, non-hydrolysable ester bonds (del Río et al., 1995; Gonzalez-Vila et al., 2001) and may be inherited directly from living organisms at an early stage of sedimentation (Kawamura and Ishiwatari, 1985).

Further, relatively high amounts of amino acid derivatives were released after Py-TMAH. Most of the identified amino acid derivatives originate from cleavage of peptide bonds and subsequent methylation of the carboxylic and the amino groups (Knicker et al., 2001). The release of amino acids provides evidence complementary to the ^{15}N NMR results shown above, which indicate the presence of peptide materials in the samples. The released amino acids are dominated by glycine and alanine (as the N,N-dimethyl, methyl esters), which are especially abundant in the Cerdanya and Libros kerogens. Additionally, minor amounts of aspartic acid and serine, plus a non-protein amino acid, tentatively identified as α -amino-n-butyric acid and two compounds with a base peak at m/z 116, probably α -amino acid derivatives (Mongenot et al., 2001; Riboulleau et al., 2002), were detected in the Cerdanya kerogen. The kerogen from Libros released the highest amounts of amino acid derivatives compared to fatty acids while the kerogen from Ribesalbes released the lowest proportion, as indicated by the semiquantitative parameter $(\text{Gly} + \text{Ala})/\text{FaC}_{16}$ (Table 1). Previous studies (Monge-

not et al., 2001; Riboulleau et al., 2002) have shown release of amino acids from TMAH thermochemolysis of the methanol-eluted, non-acid fraction of the pyrolysate of a kerogen. However, this is the first time that such high quantities of amino acids have been released directly from kerogens upon Py-TMAH. It is interesting to note that the composition of amino acids released from the kerogens studied here is very similar to that found in other S-rich kerogens (Riboulleau et al., 2002) which also showed glycine and alanine as the most abundant amino acids. Glycine and alanine were also found to be the most abundant amino acids liberated after hydrolysis of the Pula kerogen (Nguyen and Harvey, 2003; Nguyen et al., 2003), which was consistent with the relative enrichment seen for these two non-polar amino acids during the early diagenesis of the alga *Botryococcus braunii*. The high abundance seen for glycine and alanine in these and other kerogens can be explained as they are common diagenetic degradation products of other amino acids (Riboulleau et al., 2002).

Several mechanisms have been proposed to explain the survival of labile organic compounds such as peptide-like moieties in geological materials. It is widely accepted that labile compounds can be protected from microbiological decay via absorption to clay minerals (Hedges and Keil, 1995; Keil et al., 1994). According to this mechanism, labile materials are adsorbed on to mineral surfaces and stabilized by incorporation into mineral pores (mesopores) that are too small to allow entry and/or effective enzyme function. On the other hand, hydrophobic and hydrogen-bond interactions with other refractory organic materials in the sediment have also been proposed as major forces for the stabilization of nitrogen in sediments that offers protection from degradation. Peptide-like moieties may be effectively protected from degradation by encapsulation in the network of refractory biopolymers, such as the highly aliphatic biopolymer known as algaenan (Knicker and Hatcher, 1997; Knicker et al., 2001; Nguyen et al., 2003; Nguyen and Harvey, 2003). This encapsulation process may sterically protect peptides from enzymatic attack via intimate association with refractory, macromolecular material and can partly explain the fate of generally labile nitrogen in geological deposits. A preservation mechanism for the survival of protein-derived materials in humic substances, based on physical protection from highly aliphatic refractory substances in soil has also been proposed (Knicker and Hatcher, 1997; Zang et al., 2000; Knicker et al., 2001).

The present work supports the previous studies (Mongenot et al., 2001; Riboulleau et al., 2002; Nguyen and Harvey, 2003) that suggested that the survival of amino acids within an organic matrix is probably rather common in kerogen, especially glycine and alanine (part of which may arise from degradation of other amino acids, as stated above). Then, it is likely that these

non-polar aliphatic amino acid components have stronger hydrophobic interactions with the paraffinic network than the fragments that have a higher content on polar amino acids. During kerogen formation, the paraffinic hydrocarbon moieties in the kerogen network may encapsulate the already closely associated aliphatic amino acid fragments and subsequently protect them from chemical and/or microbiological degradation. In addition, Mongenot et al. (2001) considered that protection might also be afforded by encapsulation into S-rich macromolecules and that the efficiency of encapsulation might be increased by the presence of sulfur links. The identification of relatively high amounts of amino acids released from the high sulfur Ribesalbes, Cerdanya and Libros kerogens seems to suggest that encapsulation into aliphatic structures in S-rich kerogens may at least partly explain the survival of peptide moieties in these geopolymers, as suggested by Riboulleau et al. (2002) for similar highly aliphatic S-rich kerogens.

4. Conclusions

Analysis using solid-state ^{15}N NMR has shown that part of the refractory organic nitrogen in a series of Tertiary lacustrine S-rich kerogens corresponds to amide groups. Moreover, the release of amino acids after pyrolysis in the presence of TMAH demonstrated that the refractory organic nitrogen in these kerogens corresponds mostly to peptide-like moieties. Because peptide structures are generally considered as being highly sensitive to diagenetic degradation, an encapsulation mechanism within the refractory organic matter has been proposed to explain the survival of these structures, although the influence of other mechanisms such as interaction with the mineral matter cannot be completely excluded. However, the highly aliphatic character of the selected kerogens, as well as the release of both fatty acids and amino acids after Py/TMAH, supports the encapsulation process. The efficiency of encapsulation might also be increased by the presence of sulfur links in these S-rich kerogens.

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