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Emergence of the inflection point on racemization rate constants for D- and L- amino acids in the early stages of terrestrial diagenesis

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Abstract

The racemization rate constants of chiral amino acids such as asparagine and aspartate (Asx, k_{ASX}), glutamine and glutamate (Glx, k_{GLX}) and alanine (Ala, k_{ALA}) were determined for terrestrial sediments from in from Rikubetsu, Hokkaido, Japan. The racemization rate constants, plotted in the form ln [(1+D/L)/(1-D/L)] versus sediment age, demonstrate that the initial rate constant was based on a fast racemization for L-form amino acids in the terrestrial sediment. The racemization reaction for labile organic matter on the surface could be markedly affected by the rapid hydrolysis: for example, the initial racemization rate constant $k_{ASX\,1}$ was 1.1 x 10^{-4} yr^{-1} (r = 0.98) until about 2,200 yrBP. After the inflection point, the rate constant $k_{ASX\,2}$ was 2.4 x 10^{-5} yr^{-1} (r = 0.93) in the refractory organic matter. The racemization rate constant in the oceanic sediment showed a one order of magnitude difference from the terrestrial sediment in time scale to reach the inflection point. This study clearly demonstrates that the rate of increase in the extent of racemization of amino acids in sediments is discontinuous and related to the degree of oxygenation (and by extension, microbial activity).

1. Introduction

The D-isomers of amino acids are far less abundant in nature than the corresponding L-isomers, which are the predominant form occurring in biological molecules. Analysis of the chemical process of amino acid racemization occurring in various geochemical samples in marine environments has been widely applied to geochemical research, particularly for age determination using fossil foraminifera (e.g Wehmiller and Hare, 1971; Bada and Schroeder, 1972; Kvenvolden et al., 1973; Harada and Handa, 1995; Harada et al., 1996), marine mollusk shell (e.g.Wehmiller, 1982), speleothems (e.g. Lauritzen et al., 1994) and corals (Goodfriend, 1991; Goodfriend and Meyer, 1991; Goodfriend et al., 1992) throughout the Quaternary period.

Under physiological conditions, proteins and peptides are subject to a variety of spontaneous degradation reactions that can limit their useful lifetimes (Harding, 1985). Aspartyl and asparaginyl (Asx) residues are particularly susceptible to non-enzymatic degradation, because intramolecular succinimide-forming reactions at these sites lead to their isomerization, racemization, and in the case of asparaginyl residues, deamidation to aspartyl (Geiger and Clarke, 1987; Lura and Schirch, 1988). Clarke's data was adopted to model Asx racemization in collagen in order to predict protein decomposition and aspartic acid kinetics (Collins et al., 1999). The combined signals from these compounds and from glutamine and glutamate are termed Asx and Glx, respectively, to distinguish them from the observed racemization behavior of free aspartic acid (Asp) and Glutamic acid (Glu) in aqueous solution (e.g. Bada, 1971).

The racemization reaction generally proceeds at a constant rate with time, given a constant surrounding temperature (e.g. Sejrup and Haugen, 1992). Of particular interest is the racemization reaction of amino acids, which can be written as,

$$\text{L-amino acid} \underset{k'}{\overset{k}{\rightleftarrows}} \text{D-amino acid} \qquad (1)$$

where k and k' are the first-order rate constants for the interconversion of L- and D-enantiomers of amino acids. The first-order rate constant of Asx (k_{ASX}) for interconversion of the D- and L- enantiomers can be calculated from the following equation (e.g. Schroeder and Bada, 1976):

$$\ln [(1 + D/L) / (1 - D/L)]_t - \ln [(1 + D/L) / (1 - D/L)]_{t=0} = 2 \cdot k_{ASX} \cdot t \qquad (2)$$

where t is the age (in years) and D/L is the enantiomeric ratio of aspartic acid in the sediment. The racemization rate constants of amino acids in marine sediments were shown to be extremely useful in determining the geological age of marine sediment and assessing the quality of sediment core stratigraphy (Harada et al., 1996).

By contrast, little is known about the rate constants for racemization reactions of amino acids in terrestrial sediment samples. In the present study, we determine the racemization rate constants for chiral amino acids and derive a suitable model for racemization in the labile, semi-labile, and refractory states during the diagenetic process in the boreal terrestrial environment of Rikubetsu, Hokkaido, Japan.

2. Experimental

2.1. Samples

Core samples of boreal terrestrial sediments over depths of 0-300 cm were analyzed (Fig. 1). The drilling site was located at 43° 28' 0" N, 143° 44' 5" E, near Rikubetsu, which is one of coldest cities in Japan and is located near the center of Hokkaido (Takano et al., 2004). The altitude of the drilling site was 207 m, and the annual average temperature is 5.8 ºC (Takano et al., 2004). The drilling site was situated in a slightly marshy area that is seasonally frozen down to a depth of 80 cm, and is covered with ice during the winter. Total organic carbon (TOC), total hydrolyzed amino acids (THAA) and microbial cell density were greatest at the surface and drastically decreased with depth: THAA was 62 μmol/g at a depth of 0-5 cm. Microbial cell density in the sediment was determined by fluorescence microscopy (Tsuji et al., 1995). The correlation coefficients (r) for TOC and THAA versus microbial cell density were 0.97 and 0.98, respectively (Takano et al., 2004). The sediment core samples were analyzed for age using ^{14}C radiocarbon dating (Fig. 2; Takano et al., 2004). Because the concentration of organic carbon in sediment was very low, a bulk sample of ca. 200 g was analyzed using an accelerator mass spectrometric system (AMS) after washing with HCl.

2.2. Analytical methods

The freeze-dried and ground sample of 1.00 g was placed in a Teflon tube which had been cleaned by soaking in 6 M HNO_3 overnight and rinsed in Milli-Q-water

(Millipore Corp.). Acid mixture (10 ml of 5 M HF-0.1 M HCl) was poured into the Teflon tube, placed in a metal vessel and continuously heated at 110 ˚C for 16 hours in order to extract organics from the silicate matrix. After HF-HCl digestion, Teflon tubes were placed on a hot plate in a draft chamber to evaporate acids. The organic residues were extracted with pure water by ultra-sonication. The aqueous fraction was filtered with a GF/A 1.6 μm glass filter, and then freeze-dried in a glass test tube. Acid (2 ml of 6 M HCl) was added to each test tube to obtain total hydrolyzed amino acid fraction (THAA). The test tube was sealed, placed in a block heater and heated for 2 hours at 110 ˚C. The hydrolysates were then dried *in vacuo* using a diaphragm pump. After dryness, the portions were adjusted to pH 1 using 0.1 M HCl, followed by desalting with a AG-50W-X8 (200-400 mesh) cation exchange resin column (Bio-Rad Laboratories). Before application of the sample to the column, the resin had been cleaned by passing 1 M HCl, H_2O, 1 M NaOH and H_2O through the column in succession. Just before applying the sample the resin was reactivated with 10 ml of 1 M HCl and rinsed with 10 ml of H_2O. The amino acid fraction was eluted with 10 ml of 10% NH_3. The eluate was freeze-dried and redissolved in 1.0 ml of 0.1 M HCl before liquid chromatograph protocols.

The determination of D- and L-amino acid enantiomers was achieved with an RP-HPLC (reversed phase - high performance liquid chromatograph) system, which comprised high performance liquid chromatograph pump (Tosoh CCPM II), a reversed phase column (YMC-pack Pro C18 4.6 mm i.d. × 250 mm), a precolumn derivatization system with OPA and N-AcCys, and a Tosoh UV 8020 detector. An

aliquot of desalted and redissolved amino acid extract was mixed in a glass vial with OPA (*o*-phthalaldehyde) and N-AcCys (N-acetyl-L-cystein), derivatives were extracted by solid phase extraction using a TOYOPACK ODS column, to eliminate hydrophobic impurities. The extract was injected to the RP-HPLC system to separate amino acid enantiomers (Kudo et al., 2003). Gradient elution was applied using the following eluents; A: 40 mM sodium acetic acid buffer (pH 6.5), B: 100% methanol. Gradient elution was performed as follows: 10 min (Eluent B: 0%) – 25 min (Eluent B: 10%) – 65 min (Eluent B: 20%) – 80 min (Eluent B: 20%) – 85 min (Eluent B: 40%) – 115 min (Eluent B: 60%) – 120 min (Eluent B: 80%) – 135 min (Eluent B: 0%).

3. Results and Discussion

3.1. Emergence of inflection point

Separations of chiral amino acid enantiomers in the core samples by RP-HPLC are shown in Fig. 3. The concentrations of acidic amino acids Asx and Glx are given by the composite signal (i.e., Asx = asparagine and aspartate; Glx = glutamine and glutamate), as hydrolysis of the amide group of the amino acids, namely asparagines and glutamine, to their corresponding acids occurs during the acid hydrolysis step. Nonenzymatic intra-molecular reactions can result in the deamidation, isomerization, and racemization of protein and peptide asparaginyl and aspartyl residues via succinimide intermediates (Stephenson and Clarke, 1989).

As shown in Fig. 4, the D/L ratios of Asx, Glx and alanine show gradual

racemization from the L-form to the D-form with increasing depth and sediment age. Regularity in the decrease in the proportions of the L-form and increase in D-form is observed. The racemization reaction rates for the these amino acids differed from each other, with Asx having the highest rate, and Glx, the lowest.

The relationship between a racemization reaction rate and reaction time can be demonstrated by a first-order kinetic model (Bada and Protsch, 1973). However, according to recent reports, other kinetic models, such as the parabolic kinetic model, have also been implemented to improve linearity between the D/L ratios and reference ages (Mitter and Kriausakul, 1989; Goodfriend, 1992; Goodfriend et al., 1992). In order to determine the racemization reaction rate constant (k value) for each amino acid by applying a first-order kinetic model, the regression lines were examined for Asx, Glx, and alanine. Using an accumulation rate of 0.17 mm yr^{-1} and the ^{14}C dating method for the core sample (Takano et al., 2004), racemization rate constants are plotted for ln [(1+D/L)/(1-D/L)] versus sediment age in Fig. 5.

The racemization reaction would be markedly affected by the rapid hydrolysis of labile organic matter in the surface sediment: the initial racemization rate constant $k_{ASX\,1}$ was 1.1 x 10^{-4} yr^{-1} (r = 0.98) until about 2,200 yrBP. A discontinuity at about 2,200 yrBP, near the inflection point in the racemization rate constant profile, was clearly observed. The initial rate constant strongly suggests a fast racemization reaction for labile organic matter, attributed to rapid hydrolysis of L-form amino acids to D-form amino acids in the terrestrial sediment. After the inflection point, the rate constant $k_{ASX\,2}$ was 2.4 x 10^{-5} yr^{-1} (r = 0.93).

The present data suggest that the rate constants $k_{ASX\ 1}$ and $k_{ASX\ 2}$ changed drastically during this geological time. The vertical profiles of the rate constants for alanine (k_{ALA}) and Glx (k_{GLX}) were quite similar to that of Asx (k_{ASX}). The initial racemization rate constants, $k_{ALA\ 1}$ and $k_{GLX\ 1}$ were 9.2 x 10^{-5} yr^{-1} (r = 0.99) and 5.9 x 10^{-5} yr^{-1} (r = 0.97), respectively. After an inflection point around 2,200 yrBP, the racemization rate constants $k_{ALA\ 2}$ and $k_{GLX\ 2}$ were 9.8 x 10^{-6} yr^{-1} (r = 0.94) and 6.0 x 10^{-5} yr^{-1} (r = 0.68), respectively.

In the present study, by separating the curve into two parts each side of the inflection point, two values for k_{ASX} , k_{ALA} and k_{GLX} were assessed from each regression curve. Every k_1 value is approximately one order of magnitude larger than the k_2 values recorded for the period from the present to 2,200 yrBP.

3.2. Racemization reaction in labile (surface) and refractory (sub-surface) organic matter

Non-linearity pattern in Asx racemization was also observed in another report, though little is know about the mechanism. The interpretation of Asp racemization data was reported in terms of the effects of residues C-terminal to Asp and Asn (asparagines), and the extent of surviving secondary and higher order structure (Collins and Riley, 2000). Harada et al. (2002) reported changes in the racemization rate constant k_{ASP} during the period up to several hundred thousand yrBP in the oceanic sediment in the northwestern Pacific Ocean. The k_{ASP} of the oceanic sediment core showed 0.5 x 10^{-5} yr^{-1} until an inflection point ca. 20,000 yrBP, after

which the k_{ASP} was showed 0.58 x 10^{-6} yr^{-1}. In the study of amino acid chronology in the fossil planktonic foraminifera from the Pacific Ocean sediment, an inflection point also occurred in the racemization rate constant of k_{ASP} at 25,000 yrBP while k_{GLU} (k value for glutamic acid) and k_{ILE} (k value for isoleucine) had no inflection points (Harada et al., 1996). The age range of the fossil planktonic foraminifera would be too recent for the occurrence of the inflection point of glutamic acid and isoleucine (Harada et al., 1996).

Since the deep sea floor constantly keeps a low temperature, approximately 277 K (Kvenvolden et al., 1973), the terrestrial racemization rate constants obtained from the Rikubetsu core samples here might be higher than those from submarine-collected sediments. In addition, the significant difference in the inflection point between the oceanic sediment and terrestrial sediment could be examined in the present study.

The inflection point in the oceanic sediment showed one order of magnitude difference from the terrestrial sediment. The crucial reason for such differences might be derived from the oxidation-reduction boundary, between the oxidized and reduced layer. Oxidation potential of organic matter in the terrestrial sediment is far greater than in submarine sediment. The degradation indices between the oxidized layer and reduced layer were drastically changed during the transition boundary (Dauwe et al., 1999). Consequently, this study clearly demonstrates that prolonged exposure of labile organic compounds to O_2 can lead to rapid racemization of chiral amino acids, and this oxidation environment could control the distribution and the rate constant values.

3.3. Origins of D-amino acids in the sub-surface environment

The present D/L ratio for Asx, Glx, and alanine in the vertical profile may reflect two origins of D-form amino acids: i) D-amino acids formed from protein L-form analogs by racemization, ii) D-amino acids derived from microbial constituents such as bacterial cell walls (e.g. Nagata et al., 1998). Thus, D-amino acids may also originate from peptidoglycans in bacterial cell walls (e.g. Nyberg et al., 2001). Peptidoglycans are products of bacterial metabolism and the principal biochemical sources of D-amino acids. D-Ala and D-Glu are among the most common D-amino acids found in bacterial cell walls (e.g. Friedman, 1999).

From the point of view of early diagenesis, it was reported that there was a negative relationship between the total microbial population and the percentage of uncharacterized organic matter in sediments from the Peru margin, suggesting that a portion of the uncharacterized organic matter may have come from dead bacterial cells (Parkes et al., 1993). Estimates of bacterial production from rates of thymidine incorporation were significantly correlated with increases in uncharacterized organic matter (Parkes et al., 1993). Generally, labile components degrade to semi-labile components in the stage of early diagenesis during sedimentation and to biologically inactive refractory components in the next step. In the present study, the sub-surface zone of the upper 40 cm represents labile component stage up to around 2,200 yrBP. Around 40 cm semi-labile components are present, representing a transition state between labile and refractory components.

We previously reported the vertical distribution of microbial cell density, TOC, and hydrolyzed amino acids in the sediment (Takano et al., 2004). The apparent degradation rate constant (*k* value) for microbial cell density, ln (CEL_0/CEL_t) versus sediment age (yrBP) of *t* was plotted as,

$$k_{CEL} = t^{-1} \cdot \ln (CEL_0/CEL_t) \quad (3)$$

where CEL_0 is initial microbial cell density (t = 0) and CEL_t is the microbial cell density at the corresponding sediment age (yrBP). A parabolic relationship between ln (CEL_0/CEL_t) and age was observed, as shown in Fig. 6. Apparently, the initial and later degradation rate constants $k_{CEL\,1}$ and $k_{CEL\,2}$ were 2.1 x 10^{-3} yr^{-1} (r = 0.97) and 3.9 x 10^{-5} yr^{-1} (r = 0.20), respectively. The inflection point in apparent microbial degradation at around 2,200 yrBP corresponds with that seen in the discontinuity of amino acid racemization behavior.

Evidence of early diagenetic processing to refractory organic matter was also obtained from the molar ratio of β-alanine and γ-aminobutyric acid versus total hydrolyzed amino acid (Takano et al., 2004). In the samples from the upper part of the sediment column, the combined relative abundance of β-alanine and γ-aminobutyric acid is less than 4 mole % of all amino acids. With depth, β-alanine and γ-aminobutyric acid increased in relative abundance and in the deepest sample they reached up to 28% of total hydrolyzed amino acid. According to the literature (e.g. Cowie and Hedges, 1994), the mole percentages of β-alanine and γ-aminobutyric acid are sensitive biogeochemical indicators of diagenetic alteration in a natural organic matter mixture. The relationships here strongly indicate that the β-alanine and the

γ-aminobutyric acid in the terrestrial sediment at Rikubetsu are of diagenetic origin.

The asymptotic decrease below approximately 40 cm indicates very poor biological activity to produce D-amino acids and the natural constraint of the amino acid racemization reaction. Consequently, the present study reveals that the racemization rate constants of Asx, Glx, and Ala were controlled by terrestrial diagenesis and not by the biological production rate of D-amino acids.

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Figure Legends

Fig. 1 Geographical location of drilling site at Rikubetsu, Hokkaido, Japan.

Fig 2. Age profile of core sediment from ^{14}C radiocarbon dating by accelerator mass spectrometric system (Partial data set taken from Takano et al., 2004).

Fig 3. Separation of enantiomers by RP-HPLC technique for the sediment core samples at Rikubetsu, Hokkaido, Japan at depth of (a) 5-10 cm, (b) 250-300 cm. Abbreviations; D, L-Asp: D, L-aspartic acid, D,L-Glu: D,L-glutamic acid, Gly: glycine, βAla: β-alanine, γ-ABA: γ-aminobutyric acid, D, L-Ala: D, L-alanine, L-Val: L-valine, L-Leu: L-leucine.

Fig. 4 Vertical ^{14}C age profiles of D/L ratio of Asx, Ala, Glx in the sediment core samples at Rikubetsu, Hokkaido, Japan.

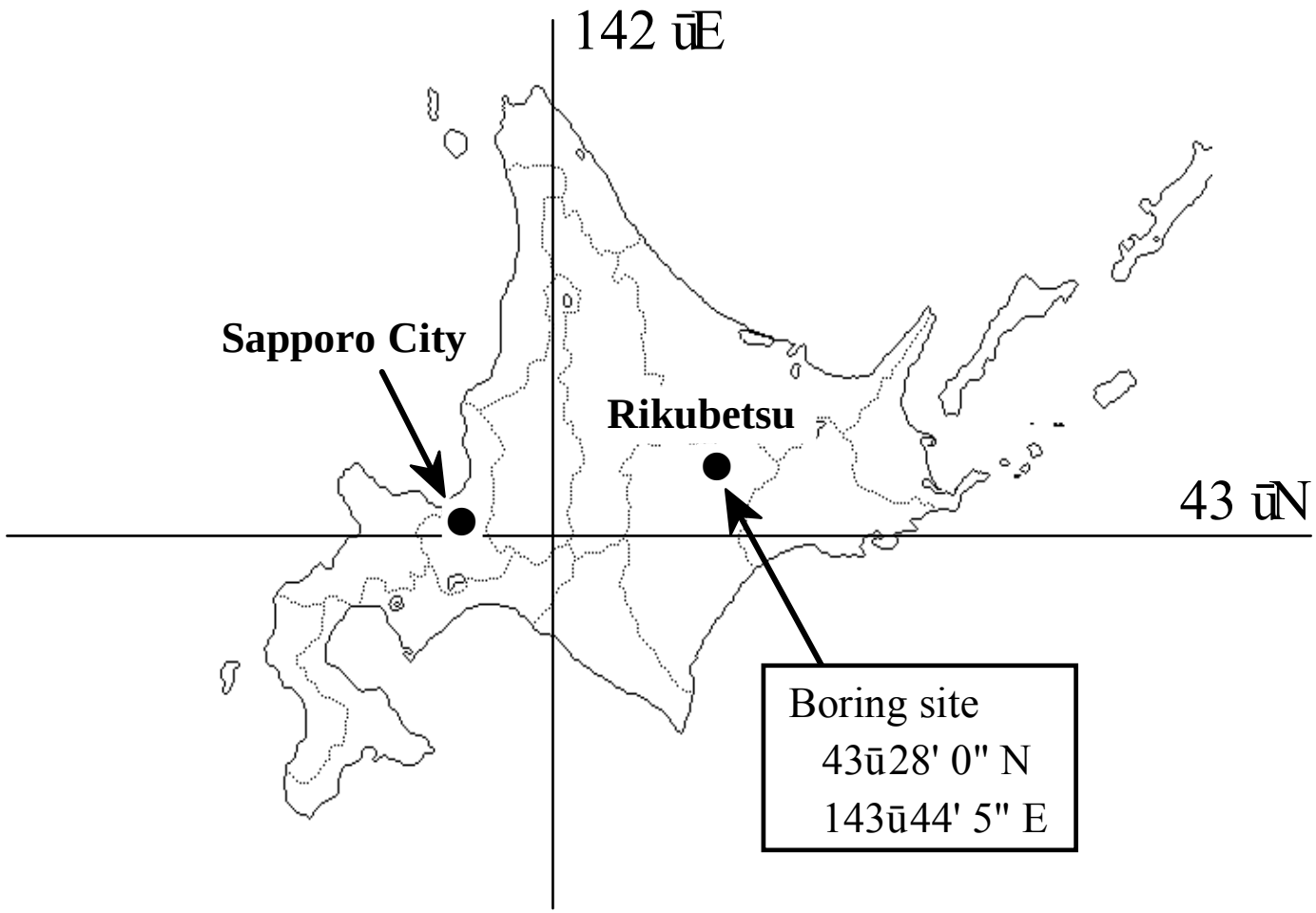
Fig. 5 Racemization rate constant (*k*) profiles for (a) Asx, (b) Glx and (c) alanine in the terrestrial sediment from Rikubetsu, Hokkaido, Japan.

Fig. 6 Apparent microbial cell density degradation rate constant as a function of sediment ^{14}C age (Primary data taken from Takano et al., 2004). Counting cell number was performed by the method of Tsuji et al., 1995.

Table Legends

Table 1 Radiocarbon age determination by accelerator mass spectrometry (AMS) of partial sediment core sequences.

Table 2 Cross section of vertical profiles for surface and sub-surface microbial activity related with organic matter and racemization rate constant of chiral amino acids in the terrestrial sediment from Rikubetsu, Hokkaido, Japan.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Sapporo City
Rikubetsu
43 ūN
Boring site
43ū28' 0" N
143ū44' 5" E

FIG. 1

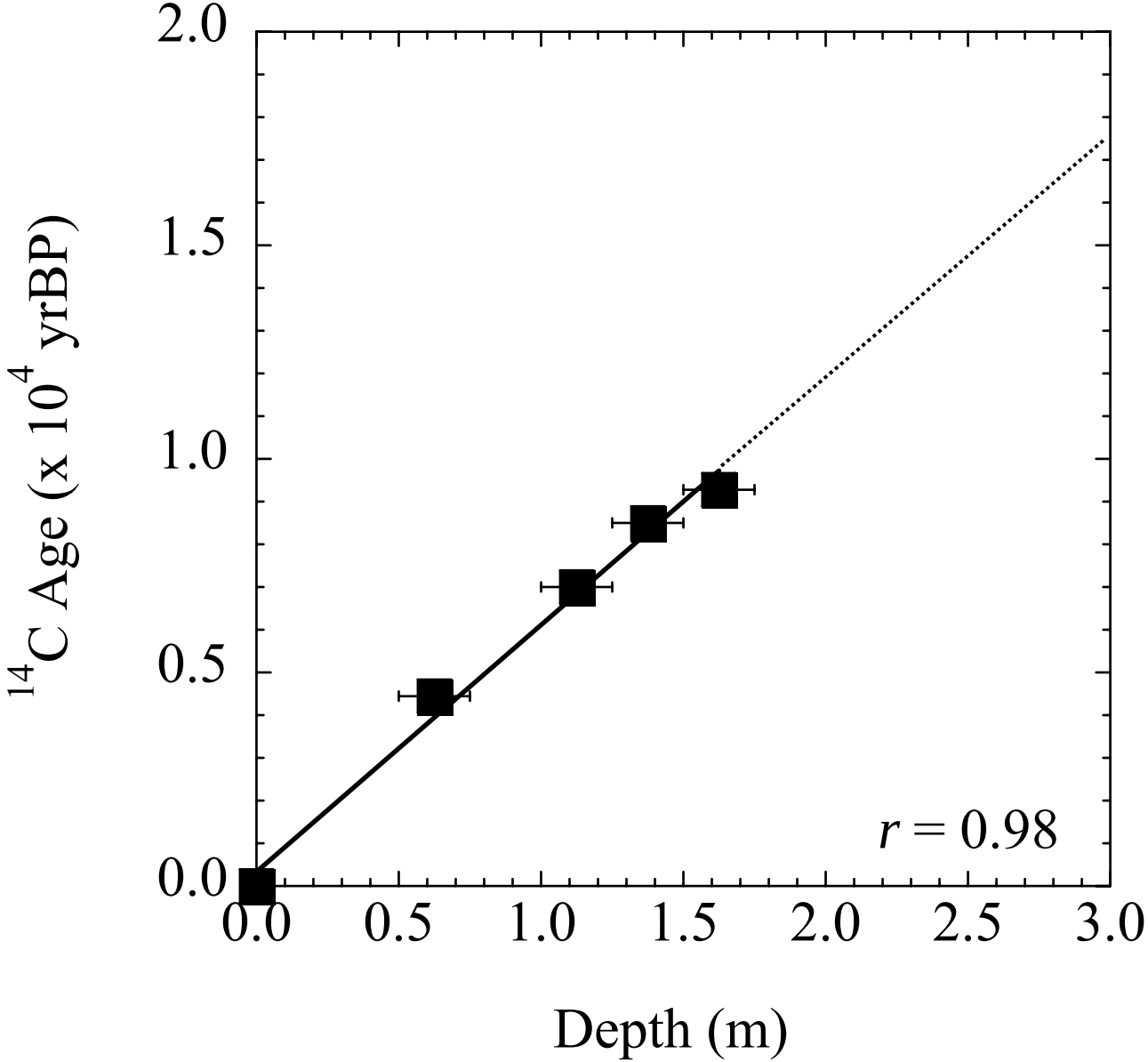

FIG. 2

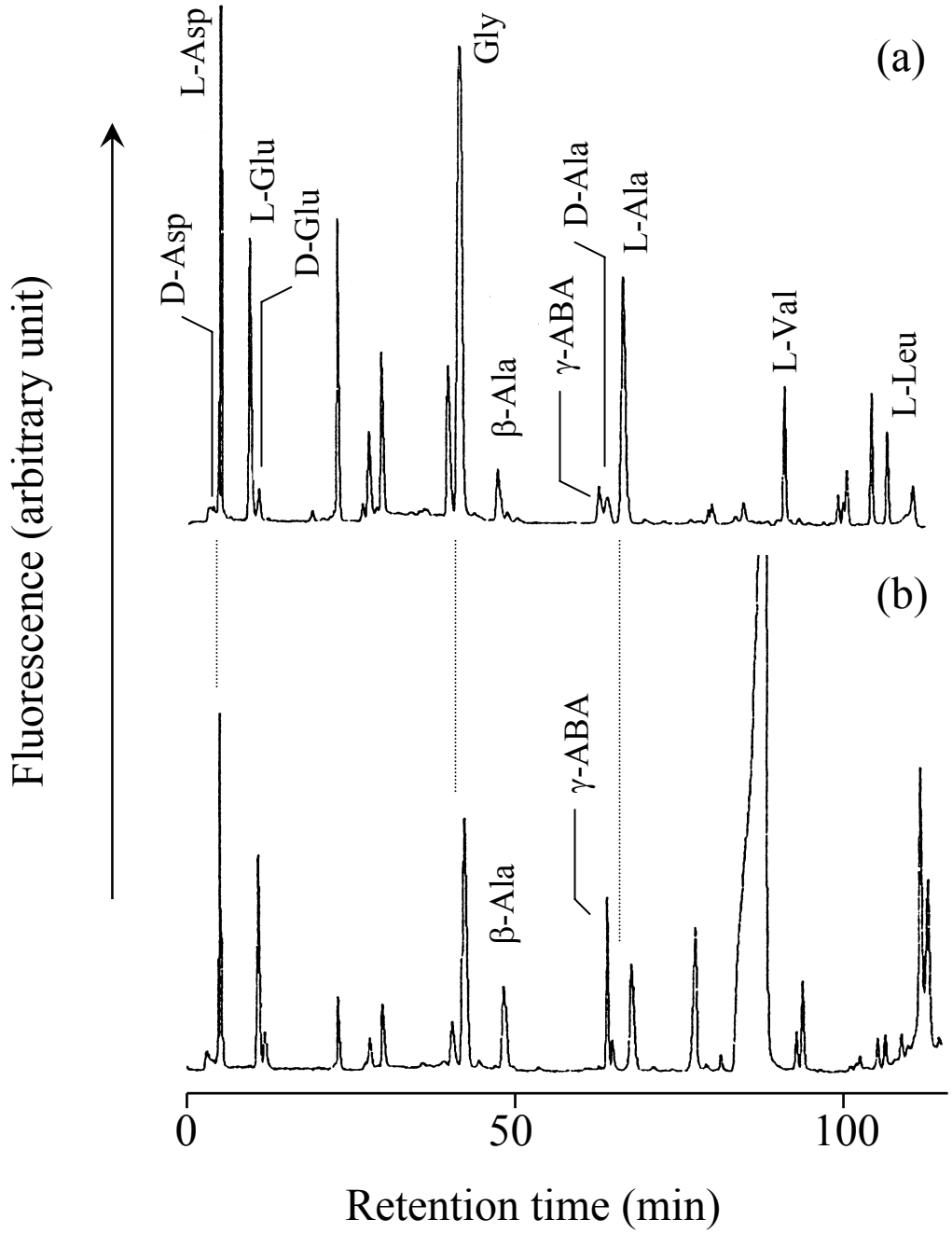
Fluorescence (arbitrary unit)
L-Asp
D-Asp
L-Glu
D-Glu
Gly
β-Ala
γ-ABA
D-Ala
L-Ala
L-Val
L-Leu
(a)
(b)
β-Ala
γ-ABA
0
50
100
Retention time (min)

FIG. 3

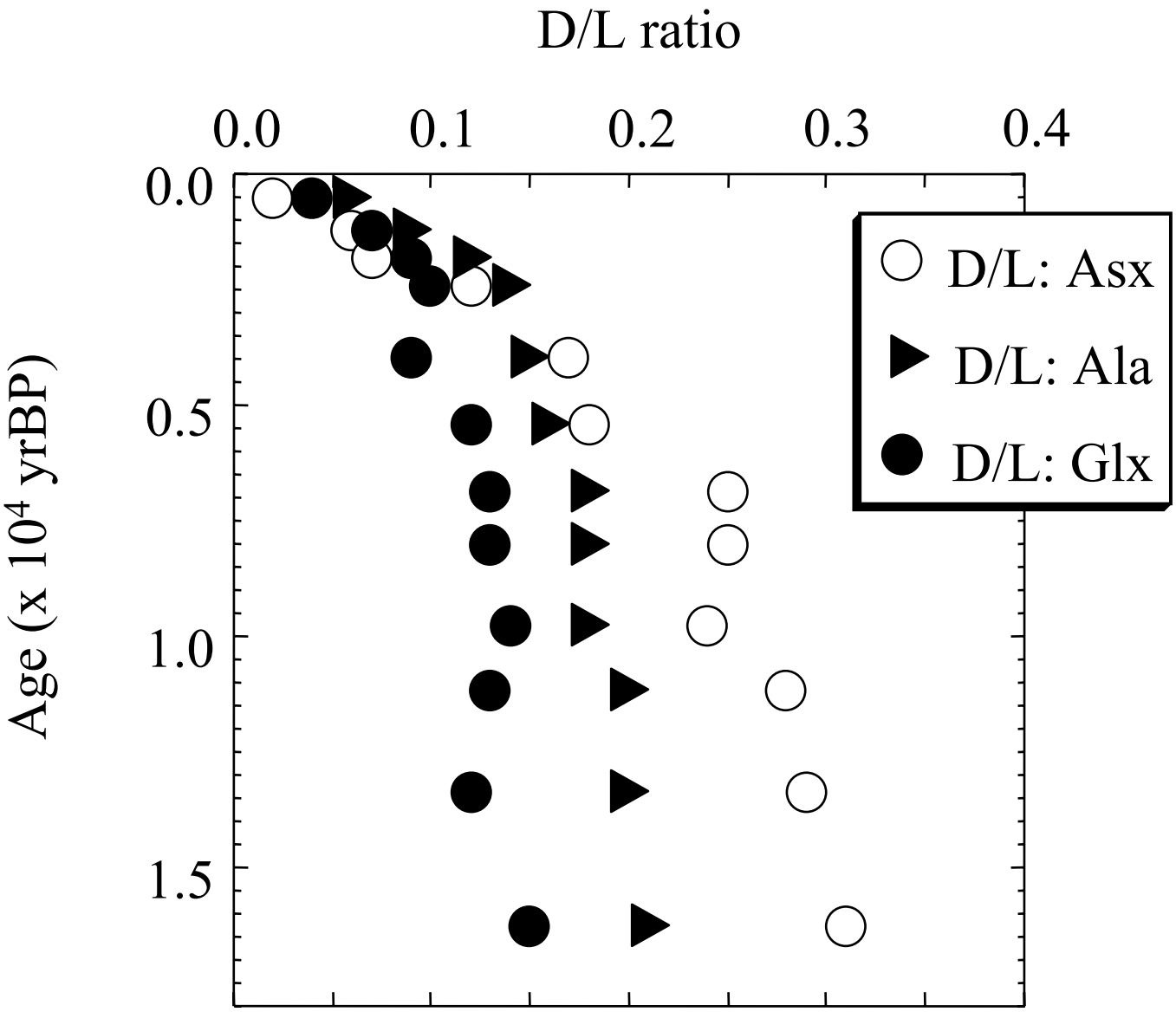
D/L ratio
0.0
0.1
0.2
0.3
0.4
0.0
0.5
1.0
1.5
Age (x 10⁴ yrBP)
D/L: Asx
D/L: Ala
D/L: Glx

FIG. 4

a)

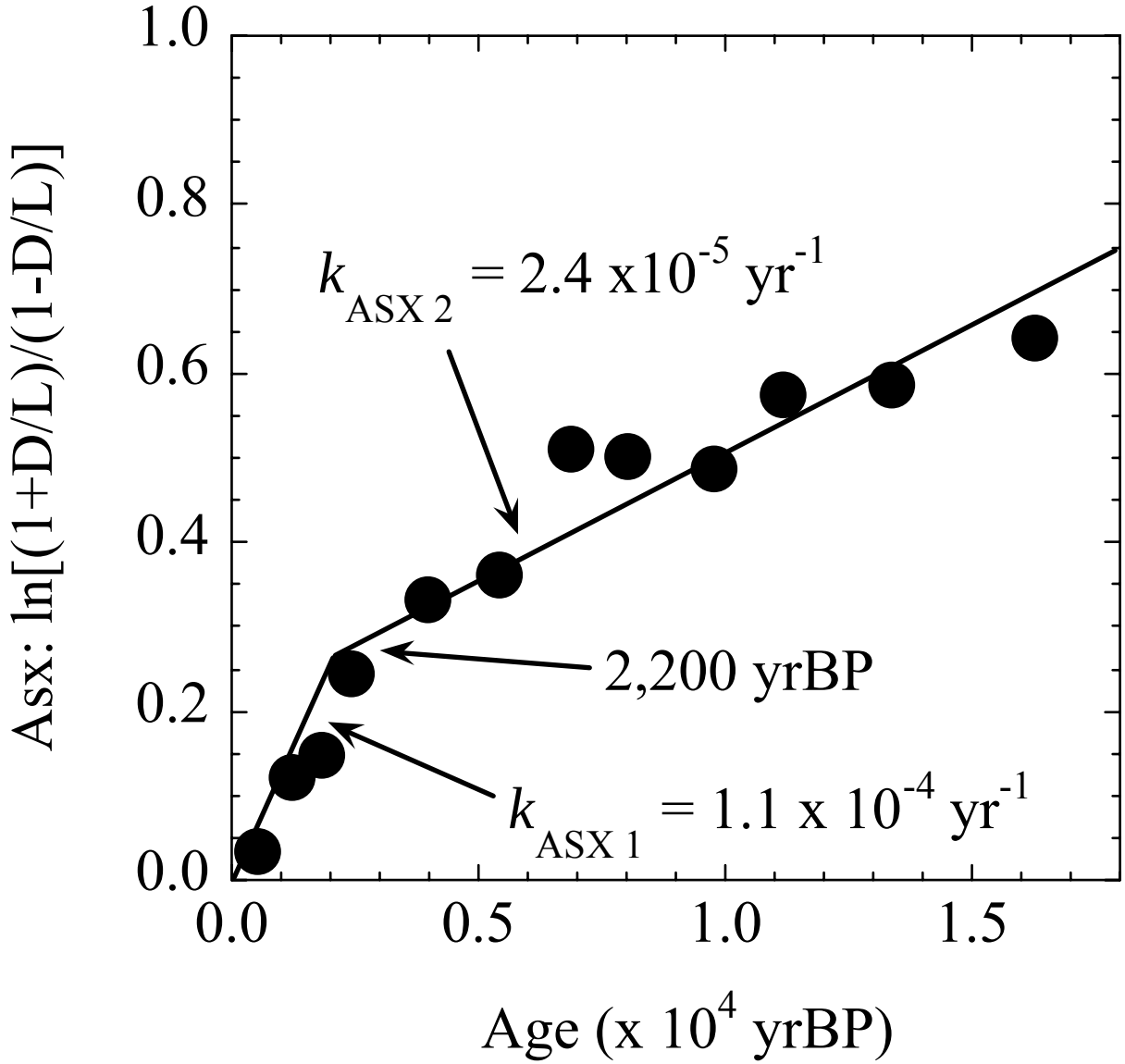

FIG. 5-(a)

(Continued to next page)

b)

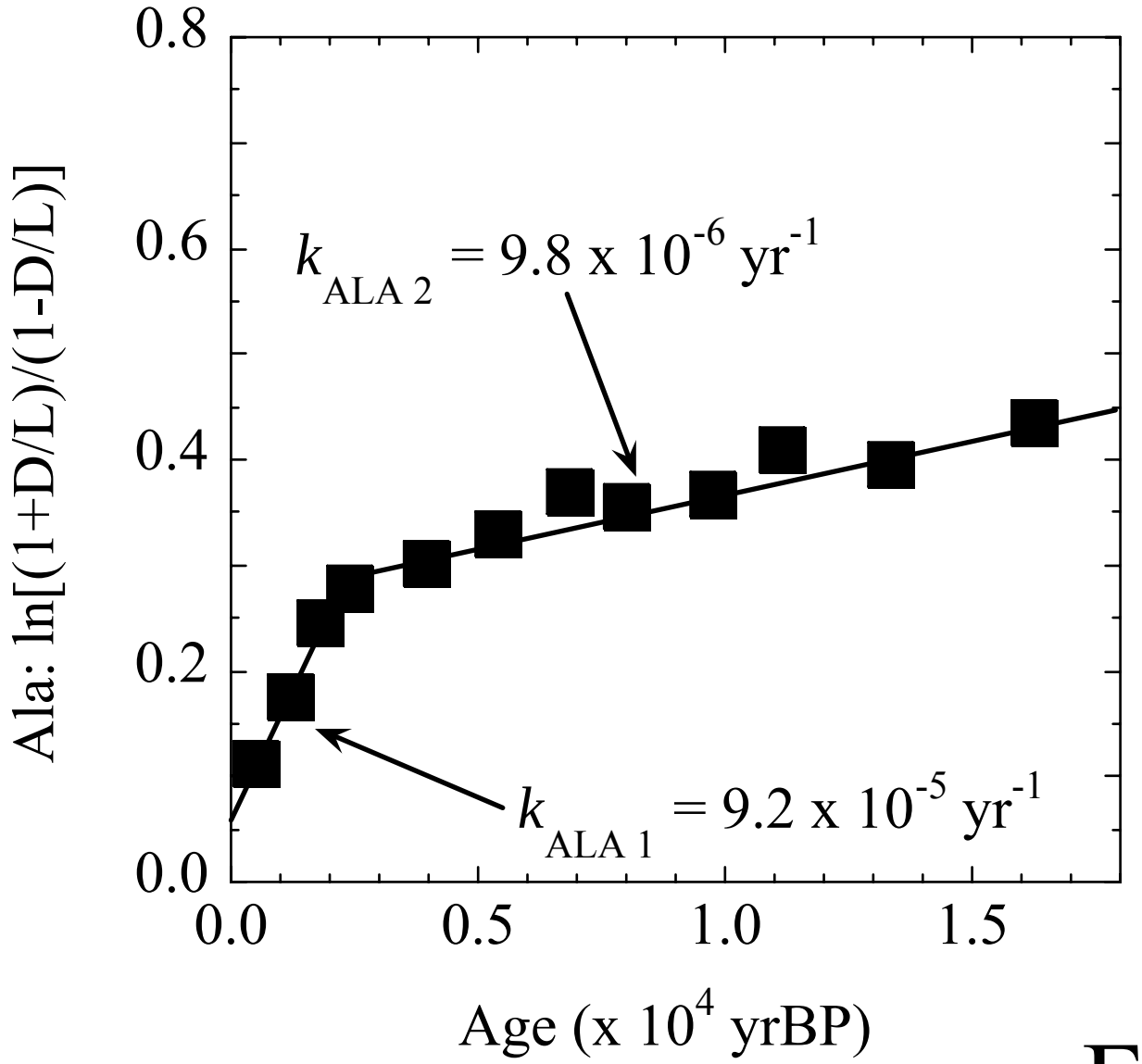

FIG. 5-(b)

(Continued to next page)

c)

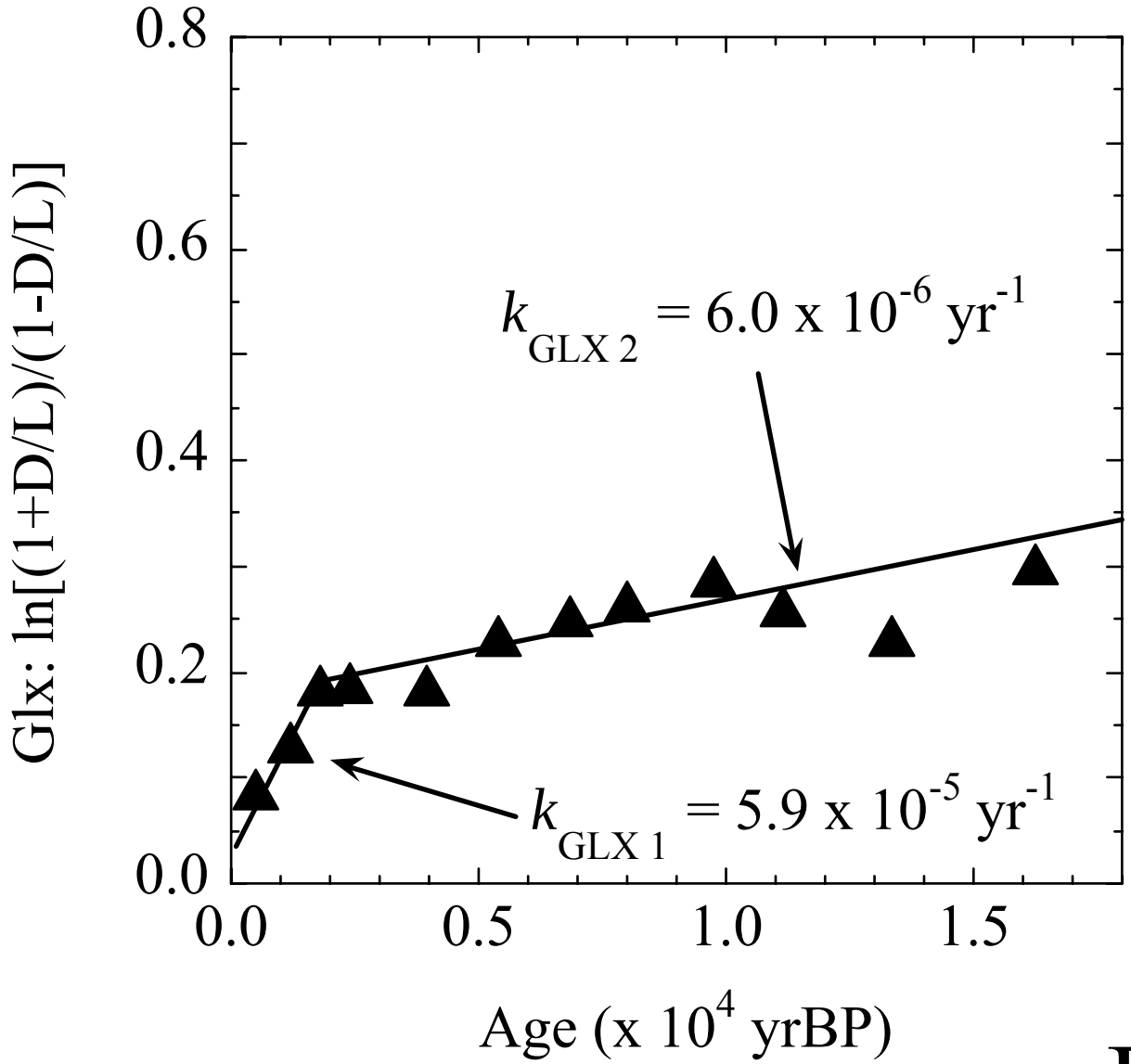

FIG. 5-(c)

(Continued from previous page)

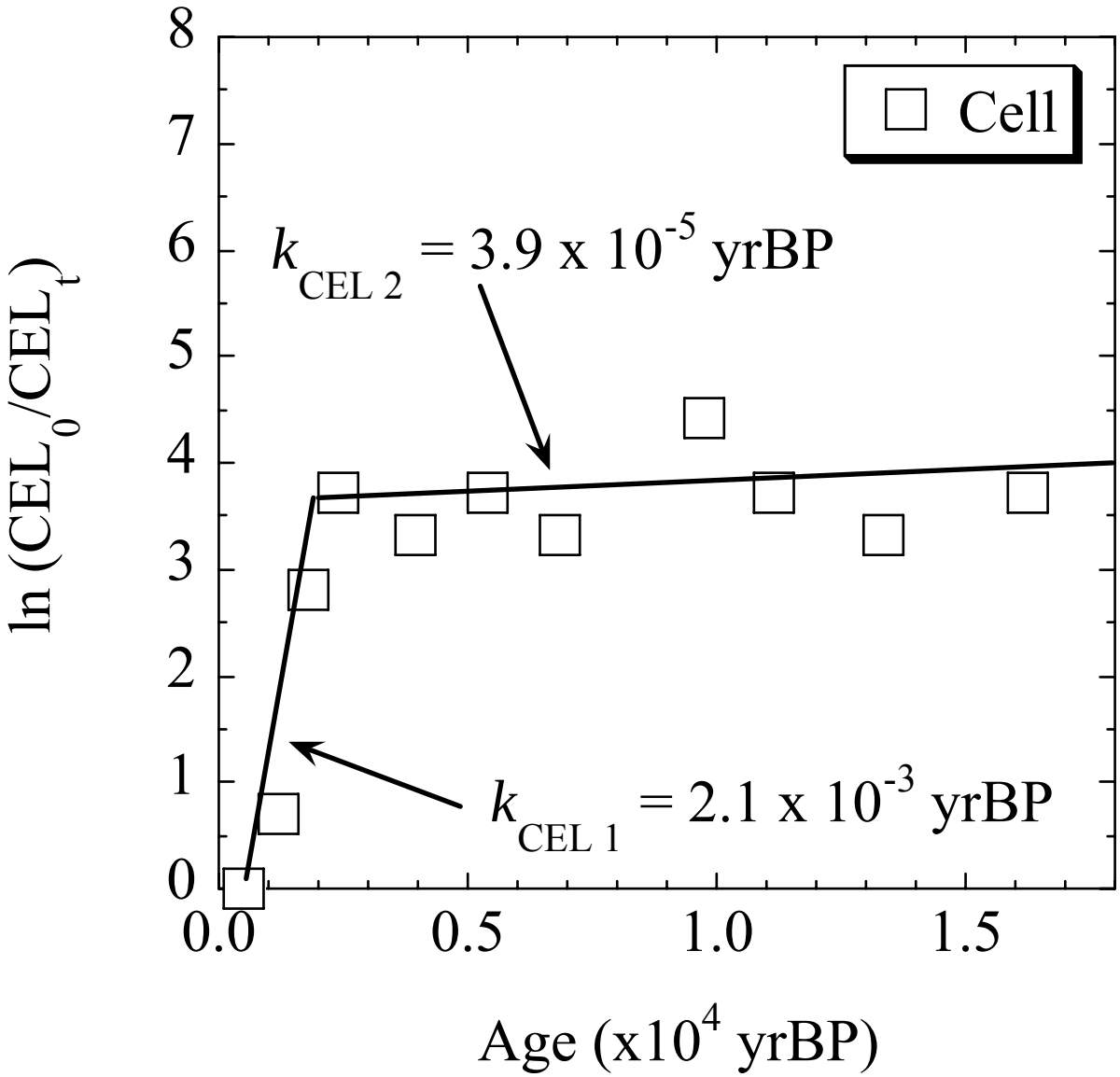
Cell
$k_{CEL\ 2}$ = 3.9 x 10^{-5} yrBP
$k_{CEL\ 1}$ = 2.1 x 10^{-3} yrBP
ln (CEL_0/CEL_t)
Age (x10^4 yrBP)
0
1
2
3
4
5
6
7
8
0.0
0.5
1.0
1.5

FIG. 6

Table 1

depth	*t* (yrBP)	±
50-75	4,420	40
100-125	7,030	40
125-150	8,510	50
150-175	9,290	50

Table 2

Vertical location	Redox state	Microbial population	Enzymatic activity	Organic matter*	Racemization rate constant***
surface	oxidation state	large	rich	labile	fast
sub-surface *(shallow)*		small	poor	semi-labile	inflection point
sub-surface *(deep)*	reduction state	very small	very poor	refractory	slow

* stands for the data from Takano et al., 2004. **stands for the data from Takano et al., 2003. *** This study.