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Research Article

Cryo-bioorganic chemistry: freezing effect on stereoselection of L- and DL-leucine cooligomerization in aqueous solution

T. Vajda* and M. Hollósi

Department of Organic Chemistry, Eötvös Loránd University, P.O. Box 32, Budapest 112, H-1518 (Hungary), Fax + 36 1 372 2620, e-mail: vajda@szerves.chem.elte.hu

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Abstract. The chirality of L-/DL-leucine (50–50%) cooligomerization was investigated in liquid and frozen aqueous solutions. Cooligomerization was carried out by carbonyldiimidazole activation without initiator at an ambient (+ 22 °C) and frozen (− 18 °C) temperature, respectively. The separated samples obtained after different time intervals of treatment were completely hydrolyzed (HCl) and the diastereomeric L- and D-leucine derivates of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) were then traced and evaluated by RP-HPLC analysis. After 9 days of oligomerization, the L-Leu content was slightly enhanced in the liquid (57%) and somewhat more enhanced in the frozen (64%) samples. After 17 days, however, the L-Leu content had decreased in the liquid (53%) and frozen (56%) conditions. These L-enantiomer amplifications indicate that an L-antipode is preferentially incorporated into the α-helical turn of the oligomer in the earlier stage of cooligomerization, while, later, the D-antipode is also incorporated. The role of ice in the improved stereoselection is discussed. This is the first recorded example of the effect of freezing on stereoselection.

Key words. Freezing; cooligomerization; stereoselection; enantiomeric excess; prebiotic chiral amplification.

The effect of freezing on biomolecular interactions [1], including chirality, is an important and intriguing topic, and chiral asymmetry generation, the generation of enantiomeric excess (ee) from a racemic mixture is of interest for understanding the origin of the homochirality of biomolecules [2–6]. This long-existing puzzle of prebiotic chemistry has attracted the extensive interest of scientists, and several abiotic mechanisms have been proposed for the origin and amplification of optically active biomolecules. Some of these theories have also been proven experimentally [7–11]. Concerning the amino acids, Wald [12] suggested that amplification of a slight excess of an L-enantiomer may occur via formation of an α-helical structure, which provides a sufficient basis for stereoselection out of a mixture of enantiomers. This hypothesis was validated by polymerization of the N-carboxyanhydride (NCA) active intermediate of alanine, leucine, and γ-benzylglutamate. The enantiomeric excess of these L > D mixtures was enhanced in the copolymers to 6–14% [7–11]. These polymerizations were conducted in organic solvents of low polarity. Poly-/oligoamino acid formation in an aqueous environment, however, would more adequately represent the original abiotic conditions on primitive Earth. Prompted by this idea, cooligomerizations of L- and DL-leucine (50–50%) have been investigated in aqueous solution and in ice. Leucine was chosen as a monomer because it is one of the strongest α-helix-forming L-amino acids. Our previous results on peptide synthesis carried out in frozen organic solvents demonstrated that beside the enhanced reaction rate and/or yield, some diminution of racemization could also be

* Corresponding author.

detected [13]. According to this stereochemical effect of freezing, it seemed an attractive challenge to elucidate the effect of ice on the cooligomerization of an L-/DL-amino acid mixture. For this purpose, the NCA-forming 1,1′-carbonyldiimidazole (CDI) [14] was used without initiator. After complete hydrolysis of the oligomer samples, the enantiomeric excess was evaluated by RP-HPLC chromatography of the diastereomeric Marfey derivatives of L-and D-leucines [15–17].

Materials and methods

Chemicals. All chemicals obtained from Sigma were of reagent grade. The optical purity of the L- and DL-leucine were controlled by optical rotation. The RP-HPLC solvents were purchased from Carlo-Erba (Italy) and Romil (UK). The 0.4 M imidazole buffer solution (pH 6.8 ± 0.2), together with the stock solutions of 0.08 M L- and DL-leucine in buffer, was prepared 2–3 days before the experiments and kept in the refrigerator.

Oligomerization. All oligomerizations were performed in weighed polypropylene (Eppendorf) centrifuge tubes in a total volume of 1.0–1.2 ml. Stock solutions of L- and DL-leucine (1:1) were added to the tubes containing solid CDI (0.16 M final concentration) and kept at 0 °C (ice-water bath) during the distribution of the samples. After rigorous shaking, half of the tubes were kept at room temperature (22 ± 1 °C), while the other half were frozen (solid CO_2-methanol) and then kept in a freezer (-18 ± 1 °C). Each sample was left to stand separately for a given time and the frozen samples were then thawed in a bath (ca 30 °C). All mixtures were centrifuged at the end of treatment and the obtained precipitates were washed with 400 µl of water in a centrifuge and dried in a vacuum desiccator over P_2O_5. The precipitates weighed together with the tubes were dissolved in 300 µl trifluoroacetic acid (TFA) and 100 µl of each solution was then transferred into a weighed small glass cup located in an Eppendorf tube. All TFA solutions were evaporated in the previous manner; over P_2O_5/granular KOH. The residues remaining from the 100-µl solutions were weighed together with the glass cups and completely hydrolyzed with 100 µl 6 M hydrochloric acid at 110 ± 1 °C for 24 h. The hydrolysis was conducted in the cup-containing sealed Eppendorf tubes. After evaporation as previously, the cup contents were washed into the Eppendorf tubes by vigorous shaking twice with 100 µl of water. The cups were removed from the centrifuge tubes and the aqueous solutions evaporated. The remaining hydrolysate was derivatized for HPLC analysis with Marfey's reagent. The other part of the samples in TFA solution (200 µl) was evaporated and saved for mass spectrometry.

Derivatization. Derivatization of the samples followed procedures in the literature [15–17]. The solid hydrolysate was dissolved in an appropriate volume of water (2 µmol/100 µl) and 50 µl (1 µmol) of this stock solution was mixed with 78 µl (1.4 µmol) of a 0.5% solution of Marfey's reagent in acetone and 16 µl (8 µmol) 0.5 M sodium bicarbonate solution (pH 8.0 ± 0.2). This solution was then heated at 40 °C for 60 min and after cooling, 10 µl of 1 M hydrochloric acid was added. After 50-fold dilution with methanol, the mixture was kept in the refrigerator until the RP-HPLC analysis.

HPLC analysis. Analytical RP-HPLC was performed using a Vertex column (250×4 mm) with C_{18}-silica as the stationary phase and methanol-0.02 M sodium acetate buffer (pH 4.0 ± 0.1)-acetonitrile (20:65:15, v/v/v) as the mobile phase, with a flow rate of 1 ml/min at ambient temperature. A 25-µl (3.3 nmol) volume of the diluted sample was injected and the peaks were monitored at 340 nm. The peaks were identified with authentic samples of L- and D-leucine derivatives and all chromatographies of the samples were repeated at least three to four times.

Results and discussion

The RP-HPLC chromatograms demonstrated good resolution of all identified peaks, with the hydolyzed reagent, i.e., the 1-hydroxy-2,4-dinitrophenyl-5-L-alanine amide always present at the very beginning of the chromatograms, and the peaks of the diastereomer L- and D-Leu derivatives appearing with a mean retention time of $t_R = 13.8 \pm 0.2$ (min) and $t_R = 37.9 \pm 0.7$ (min), respectively. The integrated area under the L-Leu derivative peak compared with that of the authentic sample served for a quantitative evaluation of the results (table 1). The integrated peak of the authentic sample is an average value of the L-Leu derivative peaks obtained from hydrochloric-acid-treated L-Leu, DL-Leu, L-/DL-Leu (1:1), and hydrolyzed oligo-L-Leu (after 17 days oligomerization). All procedures with these controls were carried out identically to those of the investigated samples.

As can be seen from table 1, the oligomerizations resulted in some enantiomeric amplification over the original 50% L-Leu content of the mixture. The oligomerizations proceeded quite slowly, significant precipitation being observed only after 6 days of treatment. It is of interest to note that cooligomerization of the L-/DL-Leu NCA-active derivatives in dioxane at ambient temperature resulted in an ee enhancement of 14.3% [11]. Among the enantiomeric amplification experiments conducted in organic solutions, this increase is the largest, and is practically identical with our result of

14.4% found in ice after 9 days of treatment. Nevertheless, our room temperature experiments produced a 7.5% lower ee. Furthermore, after 17 days incubation, the enantiomeric excess decreased in both liquid and frozen condition. The 55.8% L-Leu content obtained after 17 days in ice was also confirmed by optical rotation measurement. As polyamino acids are known to have random conformation in TFA, optical rotation measured in this solvent correlates directly with the L/D composition of the cooligomer. For the measurement of $[\alpha]$, the $\lambda = 436$ nm wavelength was chosen instead of the regular 589 nm because of the larger rotation value and better reproducibility at this wavelength. With this method, the oligomer gives a rotation value of $[\alpha]^{25} = -36.7°$ (0.6, TFA), which means a 59% L-leucine content. The calculation is based on the optical rotation of oligo-L-leucine (product of 17 days oligomerization), which is $-62.1°$ (0.4, TFA). The data of 55.8% versus 59% obtained by quite different methods demonstrate reasonable agreement.

Our data show that the obtained L-enantiomer amplification is the result of a preferential incorporation of the L-Leu residue into some oligopeptide chains at the earlier stage of treatment. Because of the poor water solubility of the leucine peptides, however, the oligomerizations resulted in peptides of only two to six residues, as demonstrated by mass spectrometry (table 1). This low number of leucine residues suggests that the tetrapeptides may form the α-helical turn. One to two L-Leu molecules could then be incorporated into this structure. The average residue numbers in table 1 were calculated for oligomer fractions containing four to six residues. Di- and tripeptides were not considered, because of their inability to form an α-helical turn. Comparison of the 9 versus 17 days data in table 1 indicates that subsequently the D-antipode also starts to incorporate into some peptides. Alternatively, after a longer treatment, the D-enantiomer may start to oligomerize separately, building its own left-handed helical turn. In this latter case, the competition between the two enantiomeric peptide turns seems similar to the crystallization of racemic compounds which crystallize in chiral forms, if the slow nucleation is combined with rapid crystal growth [18, 19]. In oligoamino acid formation, the α helix structure can be considered as a crystal seed, the great difference, however, being that there is a significant stability difference between the more stable right-handed α helix and its enantiomeric counterpart.

Table 1. Stereoselection of L-/DL-leucine cooligomerization in liquid (+22 °C) and frozen (−18 °C) aqueous solutions.

Time (days)	RP-HPLC		Mass spectrometry	
	L-Leu (%)*		Average residue number†	
	liquid	frozen	liquid	frozen
9	56.9	64.4	4.8	4.6
17	53.5	55.8	4.9	4.8

Oligomerization of L- and DL-monomer (50–50%) with CDI activation in aqueous imidazole buffer solution at pH 6.8 (no initiator).

* The completely hydrolyzed oligomer samples derivatized with Marfey's reagent were traced and evaluated with RP-HPLC analysis. Eluent: methanol-0.02 M sodium acetate buffer (pH 4.0)-acetonitrile (20:65:15, v/v/v), with 1 ml/min flow rate at ambient temperature. Twenty-five microliters (3.3 nmol) injected of the 50-fold-diluted sample. Peaks were monitored at 340 nm and the integrated areas of the identified peaks served for evaluation of the L-leucine yield. Enantiomeric excess (ee) = [the given L-Leu (%) data] − 50%.

† Based on molecular mass determination with FAB mass spectrometry.

Table 2. Relative permittivity (ε) data for water ice [20] and organic solvents [21].

Temperature (°C)	Solvent	ε
0	water	88
−10	ice	3.3
20	1,4-dioxane	2.2
	tetrahydrofuran	7.5
	N,N-dimethylformamide	38.2

Our results indicate an enhanced ee amplification in ice, which may be due to diverse effects of freezing [see ref. 1]. (i) The influence of the decreased temperature on energy and diffusion rate of the molecules can be compensated to a large extent by the freeze-concentration of the solutes in the diminished liquid phase of the ice cavities. This means that the bimolecular reaction rate between the leucine-NCA intermediate and amino-terminal of the growing peptide chain may be significantly enhanced. (ii) Freezing as partial drying will cause some dehydration of the solvation shells, i.e., an activation (destabilization) of the reactants. (iii) Beside these effects of freezing, the ice crystals themselves can influence the interactions between the molecules adsorbed onto their surface. This type of contribution can be attributed to the dramatically decreased relative permittivity (dielectric constant) of the ice crystals compared to liquid water [20]. As table 2 demonstrates, the relative permittivities of ice versus dioxane are reasonably close. The liquids of table 2 served as solvents in the ee amplification experiments. (iv) Some catalytic role of the ice surface [1] may have helped the incorporation of the L-enantiomer into the oligomer by ligating the CDI-formed NCA-derivative to the growing chain.

Given these suggested roles of ice in ee amplification, speculating on the relevance of ice to the prebiotic chemistry of chirality on early Earth seems very worthwhile.

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