PREBIOTIC CHEMISTRY

Reactivity of Alanylalanine Diastereoisomers in Neutral and Acid Aqueous Solutions: a Versatile Stereoselectivity

Raphaël Plasson · Maika Tsuji · Masazumi Kamata · Kouichi Asakura

Abstract A good comprehension of the reactivity of peptides in aqueous solution is fundamental in prebiotic chemistry, namely for understanding their stability and behavior in primitive oceans. Relying on the stereoselectivity of the involved reactions, there is a huge interest in amino acid derivatives for explaining the spontaneous emergence of homochirality on primitive Earth. The corresponding kinetic and thermodynamic parameters are however still poorly known in the literature. We studied the reactivity of alanylalanine in acidic to neutral conditions as a model system. The hydrolysis into amino acids, the epimerization of the N-terminal residue, and the cyclization into diketopiperazine could be successfully identified and studied. This kinetic investigation highlighted interesting behaviors. Complex mechanisms were observed in very acidic conditions. The relative kinetic stability of the diastereoisomers of the dipeptide is highly dependent of the pH, with the possibility to dynamically destabilize the thermodynamically more stable diastereoisomers. The existence of the cyclization of dipeptides adds complexity to the system. On one hand it brings additional stereoselectivities; on the other hand fast racemization of heterochiral dipeptides is obtained.

Keywords Oligopeptide · Diketopiperazine · Kinetics · Homochirality · APED model

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Introduction

Peptides and amino acids derivatives are important chemical compounds for the origin of life (Rode 1999). Such compounds are thought to have been present on primitive Earth. Typically, the amino acids could have been synthesized (Miller 1955; Taillades et al. 1998) and polymerized (Commeyras et al. 2002; Huber et al. 2003; Pascal et al. 2005) in prebiotic conditions. The relevance of such abiotic reactions is bolstered by the detection of amino acids (Kvenvolden et al. 1971), oligopeptides and cyclic peptides (Shimoyama and Ogasawara 2002) in meteorites. More than solely providing building blocks for the onset of life, the chemistry of amino acid derivatives—involving stereoselective reactions (Bartlett and Dittmer 1957; Idelson and Blout 1958; Blocher et al. 2001)—is deeply related to the origin of homochirality (Podlech 2001). Abiotic non racemic amino acids were even detected in meteorites (Cronin and Pizzarello 1997).

However, if most of these studies are concerned about peptide formations, few of them give details about the stability and the behavior of these compounds in prebiotic oceans. The primitive atmosphere was likely to be neutral, containing large quantity of CO_2, rather than reductive with CH_4 (Kasting 1993; Zahnle et al. 2010). This implies that the primitive oceans were probably weakly acidic (Morse and Mackenzie 1998; Cleaves et al. 2008), influencing the reactivity of the amino acid derivatives that can exist in different protonated forms.

The corresponding kinetic and thermodynamic parameters are still only partially known and understood. If several experiments have been performed for the determination of the kinetic parameters concerning the stereoselectivities of polymerization (Bartlett and Dittmer 1957; Idelson and Blout 1958), only few experiments (Saetia et al. 1993) and theoretical calculations (Zhou et al. 2005, 2006), up to our knowledge, were developed concerning the stereoselectivities of peptide hydrolysis and the relative stability of peptide diastereoisomers. Some researches were also involved in the study of biotic homochiral peptides (Goolcharran and Borchardt 1998; Li and Brill 2003), namely focusing on the formation kinetic of cyclic dipeptides (diketopiperazines, or DKP). These compounds are common products of degradation of pharmaceutical peptides, spontaneously occurring in aqueous solution (Gu and Strickley 1987), but they are also of prebiotic interest (Nagayama et al. 1990). Moreover, an important difference of stability between the different diastereoisomers of cyclic peptide has been measured in some cases (Eguchi and Kakuta 1974). A more precise study of these compounds in prebiotic environment is to be performed.

On this basis, models have been developed describing the reactivity of amino acid derivatives in prebiotic conditions, namely for understanding how only one configuration of amino acids could emerge from an initially racemic mixture (Plasson et al. 2007; Plasson and Bersini 2009). The theoretical model of activation, polymerization, epimerization and depolymerization (APED) (Plasson et al. 2004; Brandenburg et al. 2007) is showing that the spontaneous deracemization of amino acids can be obtained, eventually leading to spatial organization (Gleiser and Walker 2009), but requires non-obvious conditions. It is namely based on the possibility of obtaining homochiral peptide that are at the same time *thermodynamically* more stable, and *kinetically* more unstable. In that scope, an earlier work focused on the study of the epimerization and cyclization of oligopeptides (Danger et al.

2010) unexpectedly showed that heterochiral oligopeptides were more stable than homochiral oligopeptides.

The purpose of this new study is to clarify the reactivity of oligopeptides in solution, by simplifying the investigated system to only necessary elements. The work was limited to the simplest chiral oligopeptide, dialanine, in order to get rid of any effect of the side chain besides its sterical hindrance. No other catalysts than inorganic acids were used. We focused the investigations on a full range of pH below 7, in order to quantify the reactivity of both protonated and zwitterionic forms of the oligopeptides, in the three reactions of hydrolysis, epimerization, and cyclization. Specific attention was paid to the stereoselectivity of these reactions.

Experimental Section

Chemicals

Perchloric acid (analytical grade) and distilled water (HPLC grade) were purchased from Wako pure chemical industries, Ltd. L-Alanine, D-Alanine, LL-Alanylalanine, DD-Alanylalanine and D,L-D,L-Alanylalanine were purchased from Sigma-Aldrich Co. L-Alanyl-D-alanine, D-Alanyl-L-alanine, Cyclo-L-Alanyl-D-alanine and Cyclo-L-Alanyl-L-alanine were purchased from Bachem AG.

In this work, alanylalanine was taken as a model compound, and only alanine derivatives were used. Further, L-alanine will thus be simply represented by L, D-alanine by D, L-Ala-L-Ala by LL, D-Ala-D-Ala by DD, D-Ala-L-Ala by DL, L-Ala-D-Ala by LD, and cyclic dipeptides by cyLL, cyDD and cyLD. The mixture of stereoisomers D,L-D,L-Alanylalanine will be noted $(D,L)Ala_2$.

Kinetic Determinations

50 mM of pure dipeptide (either racemic or enantiopure) or enantiopure cyclic dipeptide were dissolved in 10 ml of either pure water, hydrochloric acid solutions (up to 6 M), phosphoric acid solutions (up to 5 M), or phosphoric acid buffer (100 mM in total phosphate, pH=2). The solutions were then thermostated at 80 °C. 500 μl samples were regularly taken and analyzed by chiral HPLC.

Separation Conditions

The samples were analyzed on a Shimadzu HPLC apparatus (auto injector SIL-10A, HPLC pump LC-10AD, column oven CTO-10A, UV detector SPD-10AV, recorder C-R6A). A chiral column Daicel Crownpak CR(+) (particle size 5 μm, column length 150 mm, internal diameter 4 mm) was used for all the separations. The eluent was a perchloric acid solution (adjusted to pH=1.22). 5 μl were injected, and the elution was performed at a flow rate of 0.4 ml/min. Each analyte could be analyzed and calibrated from pure commercial samples.

Two temperatures of analysis were used. At 0 °C, seven peaks can be observed: cyLL + cyDD, cyLD, D, L, DD, DL, LL + LD. At 35 °C, five peaks can be observed:

cyLL + cyDD + cyLD, DD + D, DL + L, LD, LL. As a consequence, measurements were done at 0 °C, with additional measurements at 35 °C when the separation of LL and LD was necessary.

Theoretical

Reactivity of Dipeptides

When dipeptides are in aqueous solution, several reaction can be observed: hydrolysis, cyclization, and epimerization.

Hydrolysis (Rate Constant k_h)

In the example of the hydrolysis of LL, the chemical reaction is:

$$LL + H_2O \xrightarrow{k_h} 2\,L \tag{1}$$

The rate of this reaction is

$$v_h = k_h \cdot [LL]_{tot} \tag{2}$$

The reverse reaction, that is the spontaneous coupling of two amino acids into a dipeptide, is actually very slow, the equilibrium being almost totally displaced towards the sole amino acids (Lambert 2008). Thus, it can be safely neglected.

Cyclisation Equilibrium (Rate Constants k_c and k_{-c}, and Equilibrium Constant K_{app})

In the example of the cyclization of LL, the chemical reaction is:

$$LL \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} cyLL + H_2O \tag{3}$$

The rates of this reaction are:

$$v_c = k_c \cdot [LL]_{tot} \tag{4}$$

$$v_{-c} = k_{-c} \cdot [cyLL]_{tot} \tag{5}$$

The apparent equilibrium constant is:

$$K_{app} = \frac{k_{-c}}{k_c} \tag{6}$$

$$= \frac{[LL]_{tot}}{[cyLL]_{tot}} \tag{7}$$

This apparent constant should not to be mistaken with the thermodynamic equilibrium constant $K = \frac{[LL]}{[cyLL]}$. The numerical value of K_{app} will actually depend on relative proportion of the different acido-basic forms of the compounds in solution, that is on the pH.

Epimerization (Rate Constant k_e)

In the example of the epimerization of LL, the chemical reaction is:

$$LL \xrightarrow{k_e} DL \tag{8}$$

The rate of this reaction is:

$$v_e = k_e \cdot [LL]_{tot} \tag{9}$$

The epimerization is known to be faster on the N-terminal residue (Kriausakul and Mitterer [1978], [1980]). This is consistent with what was experimentally observed in this study. No epimerization of the C-terminal residues nor racemization of free amino acid was detected. Traces of direct epimerization of the cyclic dipeptide could however be observed, but always at a rate too low to enable an accurate determination of the rate constants, or to influence notably the system behavior.

It is important to note that these constants are actually apparent constants. Namely, they are not taking into account the different acido-basic forms of the different compounds. They are calculated from the total concentrations in compounds (as indicated by tot in index) as directly observed by HPLC. The intrinsic constants will be further determined from these empiric values.

Ionic Strength Correction

Large variations of the ionic strength exist, from 50 mM to 6 M; corrections thus have to be taken into account. As described by Brönsted and Livingston ([1927]), the activity coefficient must actually be taken into account as following:

$$k_{app} = k_{corr} \cdot \frac{\prod_i \gamma_i}{\gamma_t} \tag{10}$$

where γ_i are the activity coefficients of the reactants, and γ_t the activity coefficient of the transition state. These coefficient can be written:

$$\gamma_i = 10^{-z_i^2 g} \tag{11}$$

$$\text{with} \quad g = \frac{0.51\sqrt{I}}{1 + 1.64\sqrt{I}} \tag{12}$$

where z_i is the number of charge of the compound i, and I is the solution ionic strength. g is coefficient of the extended Debye–Hückel law (Gluck and Cleveland [1994]), depending solely on the ionic strength. Assuming that the studied reaction is elementary, the number of charge of the transition state is the sum of the number of charges of the reactants, so that:

$$k_{app} = k_{corr} \cdot 10^{-g \cdot \left(\sum_i z_i^2 - \left(\sum_i z_i\right)^2\right)} \tag{13}$$

In the more common case, with only two reactants, we thus have:

$$k_{app} = k_{corr} \cdot 10^{2z_1 z_2 g} \tag{14}$$

This ionic strength correction will be systematically taken into account in the following developments, when relevant.

Influence of the pH

At 80 °C, the acidity constant of the amine function $pK_{a,0}$ is 8.66 and the acidity constant of the carboxylic acid function $pK_{a,1}$ is 3.18 (Plasson and Cottet 2006). The maximum pH for all the experiments being 7, the amine function will always be considered as fully protonated. As highly concentrated solutions of hydrochloric acids are used, we also have to take into account the protonation of the peptide bonds (Olah et al. 1970). Typically, the acidity constant of the acetamide is zero (Olah and White 1970). The acidity constant of the peptide bonds will be noted $pK_{a,2}$ for the dipeptide, and $pK_{a,c}$ for the cyclic dipeptide.

During the study of acid catalyses, the pH values were kept under 2. We can thus assume that in these conditions, the dipeptide only existed in monocharged and dicharged form:

$$LL^+ + H^+ \overset{K_{a,2}}{\rightleftharpoons} LL^{++} \tag{15}$$

The thermodynamic equilibrium is:

$$K_{a,2} = \frac{a_{LL^+} a_{H^+}}{a_{LL^{++}}} \tag{16}$$

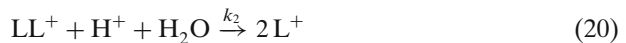$$= \frac{[LL^+]}{[LL^{++}]} h \cdot 10^{2g} \tag{17}$$

where $h = [H^+]$. In the case of the hydrolysis reaction, we can have the uncatalyzed reaction (order 0 in h), that is the direct hydrolysis by a molecule of water:

$$LL^+ + H_2O \overset{k_1}{\rightarrow} L + L^+ \tag{18}$$

The rate of this reaction is:

$$v_1 = \frac{k_1}{1 + \frac{h}{K_{a,2}} 10^{2g}} [LL]_{tot} \tag{19}$$

There are also the reactions of order 1 in h:

$$LL^+ + H^+ + H_2O \overset{k_2}{\rightarrow} 2\,L^+ \tag{20}$$

$$LL^{++} + H_2O \overset{k_2'}{\rightarrow} 2\,L^+ \tag{21}$$

The rates of these reactions are, respectively:

$$v_2 = \frac{k_2 \cdot h \cdot 10^{2g}}{1 + \frac{h}{K_{a,2}} 10^{2g}} [LL]_{tot} \tag{22}$$

$$v_2' = \frac{\frac{k_2'}{K_{a,2}} h \cdot 10^{2g}}{1 + \frac{h}{K_{a,2}} 10^{2g}} [LL]_{tot} \tag{23}$$

These two reactions are actually equivalent, as it is impossible to distinguish the two mechanisms from the kinetic behavior. Globally, only a first order reaction of rate $k_2'' = k_2 + k_2'/K_{a,2}$ can be observed. The second order reaction is:

$$LL^{++} + H_2O + H^+ \xrightarrow{k_3} 2L^+ + H^+ \tag{24}$$
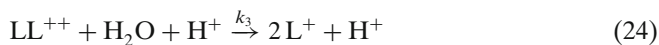
The rate of this reaction is:

$$v_3 = \frac{\frac{k_3}{K_{a,2}} h^2 \cdot 10^{6g}}{1 + \frac{h}{K_{a,2}} 10^{2g}} [LL]_{tot} \tag{25}$$

The general equation, obtained from the combination of the preceding reactions, and taking into account the ionic strength correction, is:

$$k_h = \frac{k_1 + k_2'' \cdot h \cdot 10^{2g} + k_3' \cdot h^2 \cdot 10^{6g}}{1 + \frac{h}{K_{a,2}} 10^{2g}} \tag{26}$$

where $k_3' = k_3/K_{a,2}$. For cyclization reaction, similar reasoning can be used to obtain the equations used further (Eqs. 69 and 70).

Results

Preliminary Study

In order to evaluate the difference of stability between the diastereoisomers of dialanine, a mixture of commercial (D,L)Ala$_2$ was diluted in different solvents. The enantiomeric excess (*ee*) of this commercial sample was measured to zero, that is [LL] = [DD] and [LD] = [DL], but an excess of homochiral dipeptide was observed. The diastereoisomeric excess *de* was measured to about 10%, computed by:

$$de = \frac{[LL] + [DD] - [LD] - [DL]}{[LL] + [DD] + [LD] + [DL]} \tag{27}$$

The evolution of the concentration was studied at 80 °C, in 2 M hydrochloric acid, 2 M phosphoric acid, acid phosphate buffer (pH=2, 100 mM in total phosphate), and in pure water. In these conditions, initially only first order reactions of degradations of dipeptides can be observed, so that the following profile of concentrations could be empirically observed:

$$[DD] = [LL] = [LL]_0 \cdot e^{-k_h^= t} \tag{28}$$

$$[LD] = [DL] = [LD]_0 \cdot e^{-k_h^{\neq} t} \tag{29}$$

An example of such an evolution is given in Fig. 1a.

The rate constants of degradation of homochiral dipeptides ($k_h^=$) and heterochiral dipeptides ($k_h^{\neq}$) were determined by curve fitting of the experimental concentration
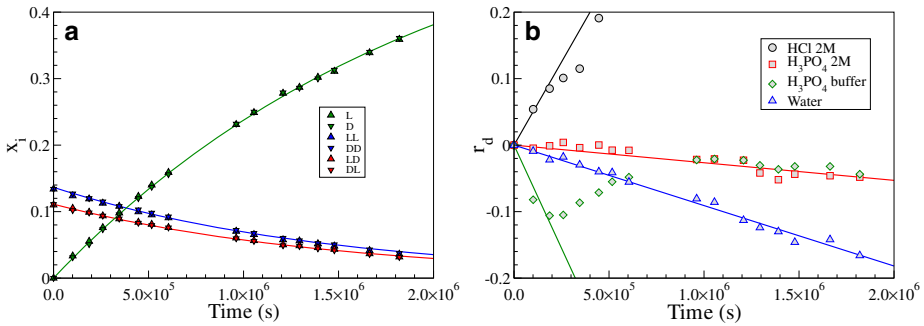
Fig. 1 Hydrolysis of a mixture of $(D,L)Ala_2$ stereoisomers. **a** Evolution of the ratio concentration/total amino acid residue concentration (x_i) in $2M\,H_3PO_4$. **b** Evolution of r_d (as defined in Eq. 31) in 2 M hydrochloric acid, 2 M phosphoric acid, 100 mM phosphoric buffer (pH 2), and water. Linear evolution is observed on the whole timescale in H_3PO_4 and water, and for $2 \cdot 10^5$ s in HCl and H_3PO_4 buffer

evolution. In order to check if the observed difference between $k_h^=$ and $k_h^{\neq}$ is not due to experimental errors, the difference $k_h^{\neq} - k_h^=$ can also be directly determined by:

$$\frac{[LL] + [DD]}{[DL] + [LD]} = \frac{[LL]_0}{[DD]_0}e^{\left(k_h^{\neq} - k_h^=\right)t} \tag{30}$$

$$r_d = \ln\frac{[LD]_0([LL] + [DD])}{[LL]_0([LD] + [DL])} \tag{31}$$

$$= \left(k_h^{\neq} - k_h^=\right)t \tag{32}$$

As long as r_d is linear, only the first order degradation of peptides occurs. In this case $k_h^{\neq} - k_h^=$ could be directly determined by linear regression of the experimental values of r_d. When non-linear evolution was observed, the slope at the origin was measured (see Fig. 1b), in order to evaluate the global stereoselectivity that is observed at the very beginning of the degradation. The results are summarized in Table 1.

Table 1 Apparent rate constants of dialanine degradation in acidic and neutral conditions (see text for experimental details)

Solution	pH	$k_h^=$	$k_h^{\neq}$	$k_h^{\neq} - k_h^=$	$\dfrac{k_h^{\neq}}{k_h^=}$
HCl (2M)	−0.3	$9.3 \cdot 10^{-6}$ (0.9993)	$9.8 \cdot 10^{-6}$ (0.9984)	$+5.0 \cdot 10^{-7}$ (⋆)	1.05
H_3PO_4 (2M)	0.9	$6.0 \cdot 10^{-7}$ (0.9989)	$5.8 \cdot 10^{-7}$ (0.9987)	$-2.7 \cdot 10^{-8}$ (0.946)	0.96
H_3PO_4 buffer	2.0	$1.3 \cdot 10^{-6}$ (0.986)	$7.3 \cdot 10^{-7}$ (0.998)	$-6.2 \cdot 10^{-7}$ (⋆)	0.56
Water	7.0	$1.2 \cdot 10^{-7}$ (0.997)	$4.2 \cdot 10^{-8}$ (0.983)	$-7.7 \cdot 10^{-8}$ (0.998)	0.35

The correlation coefficients are given into parenthesis. Values indicated by a star were not linear, and corresponds to the the slope at the curve origin. The pH is calculated by $-\log a_{H^+}$

Acid Catalyses

General Evolution

We investigated the evolution of pure LL or cyLL in acidic solutions, at different pH. The experiments were realized in hydrochloric and phosphoric acid solutions, ranging in a pH scale from −1 to 3. Several behavior can be observed, summarized in four categories indicated by letters from **A** to **D**. Example evolutions are represented in Fig. 2

Case A   In very acidic solutions, mainly the hydrolysis of LL is observed. Traces of DL—coming from epimerization—and cyLL—coming from cyclization—
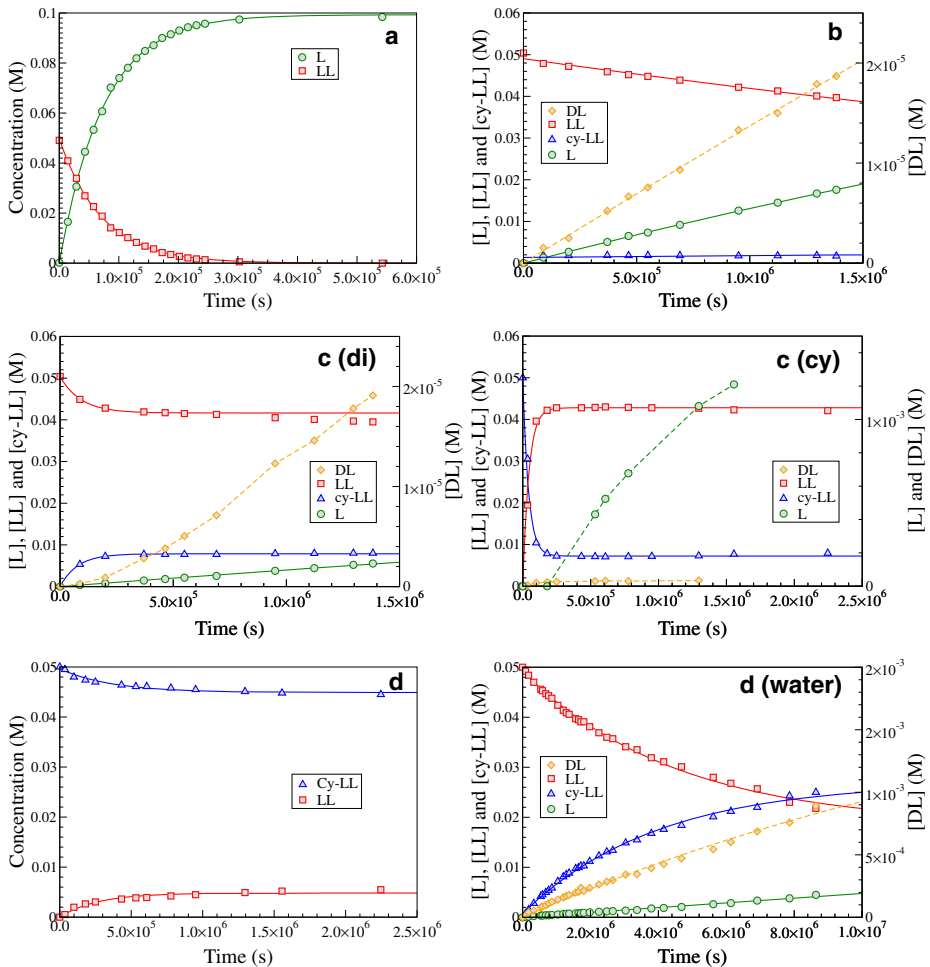

Fig. 2 Different evolutions of hydrolysis of 50 mM of LL or cyLL in acidic and neutral solutions at 80 °C. **a** LL in 3 M HCl; **b** LL in 100 mM HCl; **c**(di) LL in 50 mM HCl; **c**(cy) cyLL in 50 mM HCl; **d** cyLL in 1 mM HCl, **d**(water) LL in neutral water

were sometime detected, but with too low concentrations for any reliable measurement. Such an evolution is noted **A** in Table 2. k_h can be directly measured by:

$$[LL] = [LL]_0 \cdot e^{-k_h t} \tag{33}$$

$$[L] = 2[LL]_0 \left(1 - e^{-k_h t}\right) \tag{34}$$

Case B When the pH increases, the peptide hydrolysis slows down. The epimerization reaction is observed—only on the N-terminal residue—and is important enough to be evaluated by:

$$\frac{d[DL]}{dt} = k_e [LL] \tag{35}$$

$$k_e = \frac{d[DL]}{dt} \cdot \frac{1}{[LL]} \tag{36}$$

$\frac{d[DL]}{dt}$ can be measured directly from the slope of the variation of [DL] at a given time. This equation is true as long as DL is not also hydrolyzed, which is empirically true as the concentration of DL remains extremely low during the length of the experiments.

Table 2 Experimental rate constants of hydrolysis and cyclization of LL in hydrochloric acid and phosphoric acid solutions

	Init.	Type	$[H^+]$	K_{app}	Rate constant (s^{-1})			
					k_c	k_{-c}	k_h	k_e
[HCl]								
6 M	di	A	6 M	—	—	—	$3.6 \cdot 10^{-5}$	—
3 M	di	A	3 M	—	—	—	$1.4 \cdot 10^{-5}$	—
1 M	di	B	1 M	600	—	—	$3.3 \cdot 10^{-6}$	—
0.2 M	di	B	0.2 M	68	—	—	$3.8 \cdot 10^{-7}$	$3.0 \cdot 10^{-10}$
0.1 M	di	B	0.1 M	24	—	—	$1.5 \cdot 10^{-7}$	$3.1 \cdot 10^{-10}$
50 mM	di	C	50 mM	*5.29	*$1.9 \cdot 10^{-6}$	* $1.0 \cdot 10^{-5}$	$4.8 \cdot 10^{-8}$	$4.0 \cdot 10^{-10}$
50 mM	cy	C	50 mM	6.06	$3.3 \cdot 10^{-6}$	$2.0 \cdot 10^{-5}$	$4.0 \cdot 10^{-8}$	$4.0 \cdot 10^{-10}$
20 mM	cy	C	20 mM	1.09	$8.1 \cdot 10^{-6}$	$8.9 \cdot 10^{-6}$	$9.0 \cdot 10^{-9}$	$7.0 \cdot 10^{-10}$
10 mM	cy	C	10 mM	0.558	$8.4 \cdot 10^{-6}$	$4.7 \cdot 10^{-6}$	$5.5 \cdot 10^{-9}$	$6.7 \cdot 10^{-10}$
5 mM	cy	C	5 mM	0.322	$6.7 \cdot 10^{-6}$	$2.2 \cdot 10^{-6}$	$4.0 \cdot 10^{-9}$	$5.0 \cdot 10^{-10}$
2 mM	cy	D	2 mM	0.163	$4.3 \cdot 10^{-6}$	$7.0 \cdot 10^{-7}$	—	—
1 mM	cy	D	1 mM	0.111	$3.0 \cdot 10^{-6}$	$3.3 \cdot 10^{-7}$	—	—
$[H_3PO_4]_{tot}$								
5 M	di	B	0.22 M	150	—	—	$3.8 \cdot 10^{-6}$	$8.0 \cdot 10^{-9}$
2 M	di	B	0.14 M	85	—	—	$1.0 \cdot 10^{-6}$	$4.7 \cdot 10^{-9}$
0.2 M	di	C	40 mM	*8.6	*$2.1 \cdot 10^{-6}$	*$1.8 \cdot 10^{-5}$	$1.1 \cdot 10^{-7}$	$2.3 \cdot 10^{-9}$
0.2 M	cy	C	40 mM	9.9	$1.7 \cdot 10^{-6}$	$1.7 \cdot 10^{-5}$	—	—
0.1 M	di	C	27 mM	4.4	$2.3 \cdot 10^{-6}$	$1.0 \cdot 10^{-5}$	$7.4 \cdot 10^{-8}$	$1.7 \cdot 10^{-9}$
0.1 M	cy	C	27 mM	4.7	$2.6 \cdot 10^{-6}$	$1.2 \cdot 10^{-5}$	—	—
50 mM	cy	D	18 mM	2.1	$3.8 \cdot 10^{-6}$	$8.1 \cdot 10^{-6}$	—	—
10 mM	cy	D	6.2 mM	0.56	$5.4 \cdot 10^{-6}$	$3.0 \cdot 10^{-6}$	—	—

The column "Init." indicate that the system initially contained 50 mM of either LL (noted "di") or cyLL (noted "cy"). The column "type" indicate the kind of evolution that is observed, following the classification detailed in Section "General Evolution"

Cyclic peptides are formed almost instantly, so that no rate constants can be determined. However, the apparent equilibrium constant can be measured by:

$$K_{\text{app}} = \frac{[\text{LL}]}{[\text{cyLL}]} \tag{37}$$

The equilibrium being faster than other reactions, it can be assumed that the thermodynamic equilibrium is reached at any moment. Such an evolution is noted **B** in Table 2).

Case C At higher pH, reactions are sufficiently slowed down to clearly distinguish the cyclization of dipeptides. During the equilibration process, remaining the faster process, very few dipeptides are hydrolyzed or epimerized. Thus, k_c, k_{-c} and K_{app} can be determined during the initial moments of the experiments, until the cyclization equilibrium is reached, by:

$$[\text{LL}] + [\text{cyLL}] = c_0 \tag{38}$$

$$\frac{d[\text{LL}]}{dt} = -k_c[\text{LL}] + k_{-c}[\text{cyLL}] \tag{39}$$

$$= -(k_c + k_{-c})[\text{LL}] + k_{-c} \cdot c_0 \tag{40}$$

$$[\text{LL}] = \lambda_0 e^{-(k_c+k_{-c})t} + \frac{c_0}{K_{\text{app}}^{-1} + 1} \tag{41}$$

When starting from pure dipeptide, we have:

$$\left[LL\right]_0 = c_0 \tag{42}$$

$$\left[cyLL\right]_0 = 0 \tag{43}$$

$$\left[LL\right] = c_0 \frac{K_{\text{app}} + e^{-(k_c+k_{-c})t}}{K_{\text{app}} + 1} \tag{44}$$

$$\left[cyLL\right] = c_0 \frac{1 - e^{-(k_c+k_{-c})t}}{K_{\text{app}} + 1} \tag{45}$$

And when starting from pure cyclic dipeptide, we have:

$$\left[LL\right]_0 = 0 \tag{46}$$

$$\left[cyLL\right]_0 = c_0 \tag{47}$$

$$\left[LL\right] = c_0 \frac{1 - e^{-(k_c+k_{-c})t}}{K_{\text{app}}^{-1} + 1} \tag{48}$$

$$\left[cyLL\right] = c_0 \frac{K_{\text{app}}^{-1} + e^{-(k_c+k_{-c})t}}{K_{\text{app}}^{-1} + 1} \tag{49}$$

These relations are exact as long as the hydrolysis of the dipeptide can be neglected. This is true when starting from pure cyclic dipeptide: dipeptide exists in low concentration at the very beginning, so that no hydrolysis can be detected. When starting with pure dipeptide, this is only an approximation as hydrolysis readily happens. If significant difference were detected

when comparing the values measured from cyclic and linear dipeptides in identical conditions, the values corresponding to the linear dipeptide were thus rejected. Such approximate results are indicated for information by a star in Table 2, and were not used further.

The epimerization can be determined as in the precedent case. Similarly, as the only origin of the amino acid is the hydrolysis of dipeptide, we have:

$$\frac{d[L]}{dt} = 2k_h \left[LL \right] \tag{50}$$

$$k_h = \frac{d[L]}{dt} \cdot \frac{1}{2[LL]} \tag{51}$$

$\frac{d[L]}{dt}$ can be measured from the slope of the variation of L at a given time.

Case D When the solution is weakly acid or neutral, the cyclization becomes the major reaction. A much longer time of reaction must be realized in order to observe and determine the reaction rates k_e and k_h. This was only realized on the last part, aiming the stereoselectivity determinations, where total reaction times of about 3 months where used, while reaction times of about 2 weeks were used in the other experiments.

Variations of the Rates of Dipeptide Hydrolysis in Hydrochloric Acid Solutions

General Analysis The values of k_h were measured either directly from the consumption of LL in cases **A** and **B** as described in Eq. 33, or from the formation of L in case **C**, as described in Eq. 51. These rate constants are summarized in Table 2. The rate of hydrolysis is monotonously increasing with the acidity of the solution. Thus, acidic catalysis of hydrolysis is observed. When plotting the rate constants as a function of the concentration in H^+ with logarithmic scales, the slope gives the order of catalysis by hydronium ions (see Fig. 3). An intermediate slope between 0 and 1 should be expected, depending on the contribution of the direct hydrolysis by water (order 0 in $[H^+]$) or catalyzed by one hydronium ion (order 1). However, the slope quickly increases and reaches the value of 2, sign of a second order reaction.

Result The experimental data were fitted with Eq. 26:

$$k_1 = 4.09 \cdot 10^{-9} \text{ s}^{-1} \tag{52}$$

$$k_2'' = 0 \text{ s}^{-1}.\text{M}^{-1} \tag{53}$$

$$k_3' = 1.38 \cdot 10^{-5} \text{ s}^{-1}.\text{M}^{-2} \tag{54}$$

$$pK_{a,2} = 1.4 \tag{55}$$

$$R = 0.9997 \tag{56}$$

At first, it was impossible to fit correctly the experimental data while taking into account the first order reactions. k_2'' was then set to zero. A good correlation could then be obtained. However, an abnormal value was obtained for $pK_{a,2}$. According to the literature, a value close to 0 should be expected (Olah and White 1970).

Fig. 3 Variations of the hydrolysis constant k_h for LL in water, as a function of $[H^+]$ in log-log scale. The experimental values are represented by squares. The straight curve was obtained by fitting the experimental data with Eq. 26. The rate increases as a function of $[H^+]$ are due to the catalytic effects, a second order being reached at about pH 1.5; above this value, the curve slope is about 2, with a slight curvature due to ionic strength effects
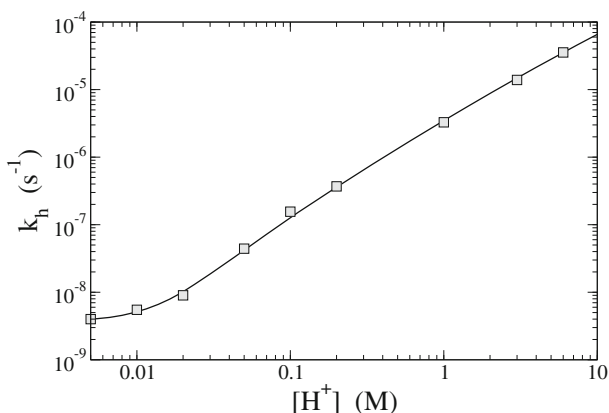
Moreover, it is clearly reported in the literature that the acid-catalyzed hydrolysis of an amid bond is only first order, the exact mechanism involving only the attack of two molecule of water on the protonated peptide bond (Brown et al. 1992). No explanation for this second order catalysis could be found.

Variations of the Rates of Dipeptide Hydrolysis in Phosphoric Acid Solutions

The hydrolysis in phoshoric acid solutions is about ten times faster than in hydrochloric acid solutions of same pH (see Table 2). There is thus a strong catalysis by phosphoric acids. In order to determine the rate constant relative to the activity of H_3PO_4, the contribution of water and water ions on the hydrolysis was subtracted to the experimental value of k_h, according to the preceding equations:

$$k_{h,\text{corr}} = k_h - \frac{k_1 + k_3' \cdot h^2 \cdot 10^{6g}}{1 + \frac{h}{K_{a,2}} 10^{2g}} \tag{57}$$

The value are summarized in Table 3. It can be seen that in all the cases, the hydrolysis is mostly due to the catalysis by phosphoric acid.

A linear relation between $[H_3PO_4]$ and this corrected value is observed. There is thus a generalized catalysis by phosphoric acid on hydrolysis. We obtain $k_{h,p} = 6.78 \cdot 10^{-7}$ s^{-1}.M^{-1} ($R = 0.986$), so that in phosphoric solution, we have:

$$k_h = k_{h,\text{H}_2\text{O}} + k_{h,p}[H_3PO_4] \tag{58}$$

where $k_{h,\text{H}_2\text{O}}$ is the rate constant due to the sole action of water and H^+.

Table 3 Rate constants of hydrolysis of LL catalyzed by phosphoric acid

$[H_3PO_4]_{\text{tot}}$	$[H_3PO_4]$	$[H^+]$	k_h (s^{-1})	$k_{h,\text{corr}}$ (s^{-1})
5 M	4.78 M	0.22 M	$3.8 \cdot 10^{-6}$	$3.4 \cdot 10^{-6}$
2 M	1.86 M	0.14 M	$1.0 \cdot 10^{-6}$	$8.0 \cdot 10^{-7}$
0.2 M	0.16 M	40 mM	$1.1 \cdot 10^{-7}$	$8.2 \cdot 10^{-8}$
0.1 M	73 mM	22 mM	$7.4 \cdot 10^{-8}$	$5.9 \cdot 10^{-8}$

Variations of the Rates of Dipeptide Epimerization

The epimerization is only observed on the N-terminal residue. The rate constant k_e was measured from the formation of DL, as described in Eq. 36. It is a very slow reaction, but it is sufficient to be detected and measured in many conditions. In hydrochloric acid solutions, values between $3 \cdot 10^{-10}$ s^{-1} and $7 \cdot 10^{-10}$ s^{-1} are determined (see Table 2). However, no clear regular variation could be observed, probably due to the low precision related to the low intensity of the reaction.

In phosphoric acid solutions, epimerization reactions are about ten time faster. This time, a linear variation is observed with the concentration in $H_2PO_4^-$ (and not with H_3PO_4), sign of a generalized basic catalysis. The rate constant of epimerization can then be expressed as:

$$k_e = k_{e,H_2O} + k_{e,p}[H_2PO_4^-] \tag{59}$$

with $k_{e,H_2O} = 8.5 \cdot 10^{-10}$ s^{-1} and $k_{e,p} = 3.19 \cdot 10^{-8}$ s^{-1}.M^{-1} (R=0.997), obtained by the linear regression of the experimental data. The value of k_{e,H_2O} is noticeably larger than the epimerization rates determined directly in hydrochloric acid, but is of the same order of magnitude.

Variations of the Rates of Dipeptide Cyclisation

Evolution of the Equilibrium Constant   The apparent equilibrium constant K_{app} was measured either directly from the relative ratio between LL and cyLL, as described in Eq. 37, when the equilibrium could be reached in reasonable time, or indirectly by fitting the time evolution of LL and cyLL by Eqs. 48 and 49. In hydrochloric acid solutions, there is first a slow evolution, of slope 1 in a log/log diagram, with an inflection around $[H^+] = 20$ mM (pH=1.7), switching to a slope 2. The constant in phosphoric acid solutions follows a similar evolution, all the values being greater than the values in hydrochloric acid solutions of the same pH (see Fig. 4a).
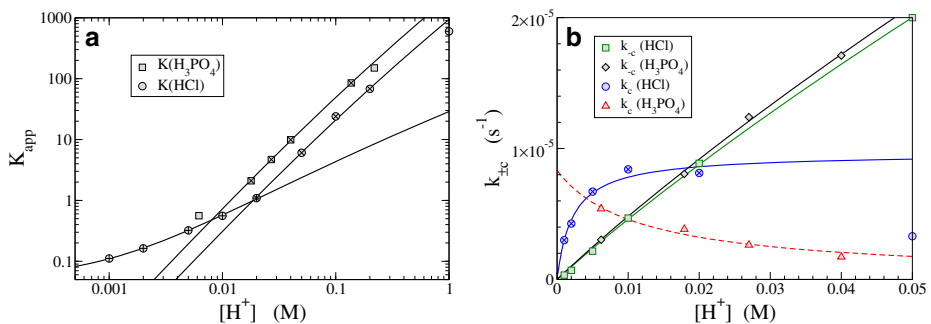

Fig. 4 Variations of the apparent equilibrium constant K_{app} (graph **a**) and of the rate constants of cyclization $k_{\pm c}$ (graph **b**) of LL as a function of $[H^+]$ in log-log scale, measured in acid chloride and phosphoric acid solutions. **a** The curves were obtained by fitting the experimental data with either Eq. 62 (data indicated with a +) or Eq. 63 (data indicated by a ×). **b** the straight curves were obtained by fitting the experimental data with Eqs. 69 and 70

In the pH range where K_{app} can be observed (down to pH=1), we only have LL, LL^+ and cyLL in solution, so that:

$$K_{app} = \frac{[LL]_{tot}}{[cyLL]} \tag{60}$$

$$= \frac{[LL] + [LL^+]}{[cyLL]} \tag{61}$$

$$= K + \frac{K}{K_{a,1}} h \cdot 10^{-g} \tag{62}$$

where $K = \frac{[LL]}{[cyLL]}$ is the thermodynamic equilibrium constant of cyclisation of the neutral dipeptide. By non-linear curve fitting of the experimental data in hydrochloric acid, before the inflexion point, we obtain $K = 0.054$ and $pK_{a,1} = 3.0$ ($R = 0.9993$). This is compatible with the theoretical value of $pK_{a,1} = 3.18$ (Plasson and Cottet 2006).

The experimental data in hydrochloric acid for $[H^+] \geq 0.2$ M and in phosphoric can be *empirically* modelled by a second order regression:

$$K_{app} = 3.8 \cdot 10^3 \cdot a_{H^+}^2 \quad (R = 0.9985) \quad (HCl) \tag{63}$$

$$K_{app} = 9.0 \cdot 10^3 \cdot a_{H^+}^2 \quad (R = 0.9999) \quad (H_3PO_4) \tag{64}$$

This result is difficult to interpret. A second order in hydronium would appear when LL^{++} compounds are formed, which should occur at much lower pH values than observed.

Evolution of Rate Constants The rate constants k_c and k_{-c} were indirectly measured by fitting the time evolution of LL and cyLL by Eqs. 48 and 49. The evolution of k_{-c} is very similar in HCl and H_3PO_4, with a linear relation as a function of H^+ (see Fig. 4b). This is compatible with the hypothesis of an acid-catalyzed equilibrium of cyclization:

$$cyLL + H^+ \overset{K_{a,c}}{\rightleftharpoons} cyLL^+ \tag{65}$$

$$LL + H^+ \overset{K_{a,1}}{\rightleftharpoons} LL^+ \tag{66}$$

$$LL \underset{k'_{-c}}{\overset{k'_c}{\rightleftharpoons}} cyLL + H_2O \tag{67}$$

$$LL^+ \underset{k''_{-c}}{\overset{k''_c}{\rightleftharpoons}} cyLL^+ + H_2O \tag{68}$$

The apparent rate constants will thus have the following variation as a function of $[H^+]$:

$$k_c = \frac{k'_c + \frac{k''_c}{K_{a,1}} h \cdot 10^{-g}}{1 + \frac{h}{K_{a,1}} 10^{-g}} \tag{69}$$

$$k_{-c} = \frac{k'_{-c} + \frac{k''_{-c}}{K_{a,c}} h \cdot 10^{-g}}{1 + \frac{h}{K_{a,c}} 10^{-g}} \tag{70}$$

The fitting of experimental data for k_{-c} gives $pK_{a,c} = 0.2$ for the two sets of data in either hydrochloric acid and phosphoric acid, $k'_{-c} \approx 0 \text{ s}^{-1}$ (i.e. only the hydrolysis of the protonated form cyLL is observed), and $k''_{-c} = 3.26 \cdot 10^{-4} \text{ s}^{-1}$ in HCl ($R = 0.9998$), and $k''_{-c} = 3.41 \cdot 10^{-4} \text{ s}^{-1}$ in H_3PO_4 ($R = 0.9998$). The constant of protonation of the peptide bond of the cyclic dipeptide is conform to the pK_a of an amide. The difference between the values of k''_{-c} in hydrochloric acid and phosphoric acid solutions may be the sign of a slight generalized catalysis by H_3PO_4.

This model is also compatible with the cyclization in HCl, up to 20 mM concentrations, giving $pK_{a,1} = 2.7$, $k'_c \approx 0 \text{ s}^{-1}$ and $k''_c = 9.67 \cdot 10^{-7} \text{ s}^{-1}$ ($R = 0.985$). Only the cyclization of the protonated form LL^+ is observed. However, this model doesn't work for both hydrochloric acid solutions of higher concentrations and phosphoric acid solutions, where a decrease of k_c as a function of $[H^+]$ is observed. The cyclization is slower in phosphoric acid solutions than in hydrochloric acid solutions of same pH. This globally corresponds to an inhibition of the reactivity by acid species. The first idea would be to invoke the protonation of the peptide bond of the dipeptide. However, the observed decrease in reactivity shouldn't be observed in such high values of pH. Moreover, the inhibition of the cyclization by phosphoric acid is so strong that a monotonous decrease is observed in the whole scale of pH investigated in phosphoric acid solutions. No explanation for this acid inhibition of the cyclization could be found.

Stereoselectivity

In order to investigate the stereoselectivity of reactions, pure LL, LD or DL were dissolved in either 2 M hydrochloric acid solution (noted **H** in Table 4), 2 M phosphoric acid phosphate buffer (noted **P**), phosphoric buffer (100 mM in total phosphate adjusted to pH 2, noted **B**) or pure water (noted **W**). When starting from LL compounds, the rate constants were obtained as described in the previous part. When starting from LD or DL, the reaction network to be considered is a little more complex. For symmetry reason, cyLD and cyDL are actually the same achiral

Table 4 Experimental rate constants of hydrolysis and cyclization of LL, LD and DL in water (**W**), phosphoric acid buffer (**B**), H_3PO_4 2M (**P**) and HCl 2M (**H**)

	pH	Init.	K_{app}	Rate constant (s^{-1})				α	β	γ	$\dfrac{k_h^{\neq}}{k_h^{=}}$
				k_c	k_{-c}	k_h	k_e				
		LL	0.65	$1.3 \cdot 10^{-7}$	$8.5 \cdot 10^{-8}$	$6.6 \cdot 10^{-9}$	$2.9 \cdot 10^{-9}$				
W	7.0	LD	0.44	$6.2 \cdot 10^{-8}$	$2.7 \cdot 10^{-8}$	$3.3 \cdot 10^{-9}$	$2.5 \cdot 10^{-9}$	0.74	0.51	0.88	0.35
		DL	0.44	$6.8 \cdot 10^{-8}$	$3.0 \cdot 10^{-8}$	$3.4 \cdot 10^{-9}$	$2.6 \cdot 10^{-9}$				
		LL	3.6	$2.5 \cdot 10^{-6}$	$8.9 \cdot 10^{-6}$	$1.0 \cdot 10^{-7}$	$5.0 \cdot 10^{-9}$				
B	2.0	LD	2.1	$1.3 \cdot 10^{-6}$	$2.7 \cdot 10^{-6}$	$7.3 \cdot 10^{-8}$	$9.3 \cdot 10^{-9}$	0.85	0.72	1.82	0.56
		DL	2.1	$1.3 \cdot 10^{-6}$	$2.7 \cdot 10^{-6}$	$7.2 \cdot 10^{-8}$	$9.0 \cdot 10^{-9}$				
P	0.9	LL/DD	–	–	–	$6.0 \cdot 10^{-7}$	–	–	0.96	–	0.96
		LD/DL	–	–	–	$5.8 \cdot 10^{-7}$	–				
H	-0.3	LL/DD	–	–	–	$9.3 \cdot 10^{-6}$	–	–	1.05	–	1.05
		LD/DL	–	–	–	$9.8 \cdot 10^{-6}$	–				

compound. As a consequence, cyLD can be either hydrolyzed in LD or in DL. This formally comes down to the total racemization of LD into DL via cyLD. It does not involve the inversion of any residue, but leads to exchange the position of the two residues in the peptidic chain. The different involved reactions are:

$$\text{LD} \xrightarrow{k_c} \text{cyLD} \tag{71}$$

$$\text{cyLD} \xrightarrow{k_{-c}} \text{LD} \tag{72}$$

$$\text{cyLD} \xrightarrow{k_{-c}} \text{DL} \tag{73}$$

$$\text{DL} \xrightarrow{k_c} \text{cyLD} \tag{74}$$

The evolution of this system can be studied by setting $x = [\text{LD}] + [\text{DL}]$:

$$\frac{dx}{dt} = -k_c \cdot x + 2k_{-c} \cdot [\text{cyLD}] \tag{75}$$

The evolution of $[\text{LD}] + [\text{DL}]$ is thus similar to the evolution of $[\text{LL}]$ when cyclization is the major reaction, with the same cyclization rate, but with a doubled decyclization rate. We thus have, when starting from pure dipeptide:

$$\left[LD\right]_0 + \left[DL\right]_0 = c_0 \tag{76}$$

$$\left[cyLD\right]_0 = 0 \tag{77}$$

$$\left[LD\right] + \left[DL\right] = c_0 \frac{K'_{\text{app}} + e^{-(k_c + 2k_{-c})t}}{K'_{\text{app}} + 1} \tag{78}$$

$$\left[cyLL\right] = c_0 \frac{1 - e^{-(k_c + 2k_{-c})t}}{K'_{\text{app}} + 1} \tag{79}$$

with

$$K'_{\text{app}} = \frac{2k_{-c}}{k_c} \tag{80}$$

$$= \frac{[\text{DL}] + [\text{LD}]}{[\text{cyLD}]} \tag{81}$$

$$= 2K_{\text{app}} \tag{82}$$

K_{app}, k_c and k_{-c} were then determined from the fit of:

$$\frac{[\text{cyLL}]}{[\text{LL}]} = \frac{1 - e^{-(k_c + k_{-c})t}}{K_{\text{app}} + e^{-(k_c + k_{-c})t}} \tag{83}$$

$$\frac{[\text{cyLD}]}{[\text{LD}] + [\text{DL}]} = \frac{1 - e^{-(k_c + 2k_{-c})t}}{2K_{\text{app}} + e^{-(k_c + 2k_{-c})t}} \tag{84}$$

k_h and k_e were determined from the slopes of the formation of amino acids and diastereoisomers of initial dipeptide, as in the previous part.

The difference in reactivity between homochiral and heterochiral compounds was evaluated by computing the parameters α, β and γ as following:

$$\alpha = \frac{K_{\text{app}}^{\text{LL}}}{2K_{\text{app}}^{\text{LD}}} \tag{85}$$

$$\beta = \frac{k_h^{\text{LD}}}{k_h^{\text{LL}}} \tag{86}$$

$$\gamma = \frac{k_e^{\text{LD}}}{k_e^{\text{LL}}} \tag{87}$$

The constants noted LL correspond to the values relative to homochiral compounds, and the one noted LD correspond to the values relative to heterochiral compounds. Values for α, β and γ larger than one correspond to reactions that tend to stabilize the homochiral dipeptide, while values smaller than one correspond to reactions that tend to stabilize the heterochiral dipeptide . More precisely, a value $\beta > 1$ indicates a faster hydrolysis for heterochiral compounds. In this case, LL and DD are *kinetically* more stable than LD and DL towards hydrolysis. A value $\gamma > 1$ indicates an epimerization equilibrium displaced towards the homochiral compound. In this case, the heterochiral compounds are *thermodynamically* less stable than the homochiral ones. A value $\alpha > 1$ indicates a better stability of heterochiral cyclic dipeptides (i.e. more cyLD would be obtained than cyLL at equilibrium when starting from the same quantity of LL and LD). The factor 2 in Eq. 85 comes from the fact that cyLD is in equilibrium with both LD and DL, so that the parameter K'_{app} as defined in Eq. 80 rather than K_{app} must be considered.

The measures realized in water (**W**), phosphoric acid buffer (**B**), phosphoric acid 2M (**P**) and hydrochloric acid 2M (**H**) are summarized in Table 4. In the cases **P** and **H**, only the hydrolysis reaction was observed, so that the kinetic parameters could be directly measured from the hydrolysis of the (D,L)Ala$_2$ mixture (see Table 1).

Discussion

General Behaviour

As expected, the reactivity of dipeptide in aqueous solution is highly dependent on the pH. Strong acid catalysis is effective for both hydrolysis and cyclization. Hydrolysis rates are multiplied about hundred fold at pH 1 compared to neutral conditions, and can be multiplied up to ten thousand fold in concentrated acid. Moreover, phosphoric acid was shown to act as an effective general acid catalyst.

Epimerization reactions are very slow, but nonetheless detectable. As reported in the literature (Kriausakul and Mitterer 1978, 1980), only the N-terminal position of the peptide was affected. This reaction kinetics was surprisingly weakly modified by pH changes. The only measurable catalysis that could be observed was a general basic catalysis by $H_2PO_4^-$.

The cyclization reactions are leading quickly to an equilibrium, displaced towards DKP in neutral conditions and towards linear dipeptide in acid conditions. This is

characterized by the increase of the rate of degradation of the cyclic dipeptides, while the cyclization of the dipeptide is inhibited in acid conditions. This observed behavior is totally different from the behavior observed for the cyclization of Phe-Pro (Goolcharran and Borchardt 1998). Typically, a decrease of k_c with the pH—when moving from acid towards neutral solutions—is observed in this study, while the opposite was observed by Goolcharran *et al.*. This corresponds to the fact that we observe a faster cyclization of alanylalanine from the protonated form, while a faster cyclization of phenylproline was observed from the zwitterionic form. This difference of behavior may be due to the proline residue, which induces a faster cyclization (Moss and Bundgaard 1990).

In this context, the dipeptides behave very differently depending on the pH context: only their fast hydrolysis into amino acid is obtained in acid conditions, while a fast cyclization followed by slower degradations is observed in neutral conditions.

A Versatile Stereoselectivity

The general analysis of the dipeptide degradation, without distinguishing the different reaction contributions, shows a reversal of the stereoselectivity as a function of the pH of the solution: faster degradation of homochiral peptide in neutral conditions, and faster degradation of heterochiral peptide in acid conditions (see $k_h^= / k_h^{\neq}$ in Table 4). This general stereoselectivity actually reflects the additive effects of several contributions, mostly hydrolysis and cyclization. This can be explained by the existence of the several acido-basic forms of the compounds, and by the several potential acido-basic catalysis. In the most acid conditions, only peptide hydrolysis is observed, and the general stereoselectivity is solely due to this reaction. But in moderate conditions (typically above pH 1), all the reactions occurs concurrently: the stereoselective effects of both hydrolysis and cyclization (α and β in Table 4) are combined and leads to an increase in the stereoselectivity.

Cyclization

The parameter α is lower than one, and change weakly with the pH (0.74 at pH 7, and 0.85 at pH 2). This implies that the homopeptides possess a better tendency for cyclization than the heterochiral ones in all the studied conditions. For example, a mixture of LL, DD, LD and DL (initially 25% of each) in water would contain at the cyclization equilibrium (and if the other reactions were absent) 19.7% of LL plus DD, 23.4% of LD plus DL, 30.3% of cyLL plus cyDD, and 26.6% of cyLD, that is a diastereoisomeric excess *de* of −8.6% for linear dipeptides, and +6.5% for cyclic dipeptides. Similarly, in the phosphate buffer, at the cyclization equilibrium a *de* of −1.6% is reached for linear dipeptides, and 6.1% for cyclic dipeptides. As a consequence, in such a system, the slower epimerization equilibrium will tend to be more displaced towards the formation of homochiral peptides.

Moreover, the kinetic of cyclization of the homochiral peptides is about two times faster than the cyclization of the heterochiral peptides. This implies a dynamic effect, with an temporarily increased *de* before reaching the equilibrium. Typically, during the degradation of (D,L)Ala$_2$ in water, when starting from a *de* of −10% (excess of the commercial sample), the solution evolves quickly towards an excess of about 20%.

Hydrolysis

The stereoselectivity of peptide hydrolysis is high in neutral conditions, with an hydrolysis of the homochiral peptide that is about two time faster than the heterochiral peptide. This stereoselectivity decreases with the pH, up to the point where an inversion occurs, around pH=0. In very acid solutions, the heterochiral peptide are hydrolyzed faster. As different mechanisms of hydrolysis are involved depending on the pH, we can assume that this evolution is related to the different mechanisms involved, direct hydrolysis being very stereoselective and favoring the cleavage of homochiral peptide bond, while acid-catalyzed is far less stereoselective, slightly favoring the cleavage of heterochiral peptide bond.

Epimerization

The different rates of epimerization are related to the relative thermodynamic stability of peptides. γ is the apparent thermodynamic equilibrium constant for the epimerization constant. At pH = 7, heterochiral peptides are slightly more stable than homochiral peptides, while at pH=2, homochiral peptides are twice more stable than heterochiral peptides. This corresponds to equilibrium *de* values of -6% at pH 7, and 29% at pH 2. This is the sign of a difference of relative stability between the zwitterionic forms (majority form at pH=7) and the protonated form (majority form at pH=2).

Compared with the data available in the literature, this study provides data with more details about the respective contributions of the different acido-basic forms of alanylalanine. Theoretical models (Zhou et al. 2005, 2006) give a difference of energy of 0.2 ± 0.1 kJ/mol between the two diastereoisomers in their zwitterionic form, which corresponds to $\gamma \approx 1.25 \pm 0.1$ at 80 °C. Some experimental data on dialanine are also invoked (Saetia et al. 1993), giving a ratio $\frac{[LD]+[DL]}{[LL]+[DD]} = 0.9$, that is a *de* of 5%. These compounds are obtained from enantiopure alanine after several cycle of evaporation and dilution, in concentrated solutions of NaCl and copper salts, at 80–95 °C. Due to the evaporation cycles, the pH largely fluctuates, but is acid on average. It is however difficult to know if the final states really corresponds to a thermodynamic equilibrium, or if it only results from the stereoselectivity of the dimerizations, in addition with a non-total racemization.

Consequence for the APED Model

The stereoselectivities of these reactions are of great interest for prebiotic chemistry. Theoretical works have shown that activated system of polymerization and depolymerization of amino acids can lead to the spontaneous onset of stable non-racemic state (Plasson et al. 2004; Plasson and Bersini 2009). This is possible when the homochiral peptides are thermodynamically more stable than heterochiral peptides, while being kinetically destabilized towards hydrolysis.

Previous kinetic measurement had cast doubts on the chemical relevance of such conditions, showing namely that several heterochiral peptides were actually more stable than their homochiral diastereoisomer (Danger et al. 2010). This work on alanylalanine actually showed similarly that the heterochiral dipeptide is more stable than the homochiral diastereoisomer in neutral solutions. However, a reversal of stability is observed in acid solutions: when protonated, the homochiral

dipeptides becomes two times more stable than the heterochiral compound. More interestingly, the stereoselectivity of hydrolysis is kept. This means that at pH 2, when the dipeptide is protonated, homochiral alanylalanine is at the same time thermodynamically more stable and kinetically more reactive than the heterochiral compounds.

Moreover, the presence of stereoselective cyclization reactions increases the efficiency of the hydrolysis. Taken into account in APED model, it can potentially bring some benefits, but may also be destructive. The conversion from heterochiral towards homochiral peptides by epimerization may be dynamically increased, using the fact that homochiral peptides can be cyclized faster into more stable compounds, which should increase the efficiency of the APED system. On the other hand, the possibility of cyclization of heterochiral peptides implies the direct and rapid racemization of LD into DL via cyLD, which may destroy the efficiency of the network autocatalysis of the system, that is heavily relying on the specific inversion of N-terminal residues. The global impact of cyclization in APED models is thus complex, with different contributions of opposite effects, and is actually difficult to predict. Further theoretical study, extending the previous model, shall be performed in this direction.

Conclusion

Far from the common conception of "stable non-reactive compounds", the chemistry of dipeptides in acid aqueous solution is rich and complex, due to the large number of possible reactions and compounds they can be involved in. This study has moreover raised some poorly understood questions, especially about the mechanisms in very acidic conditions, that should be further studied. Within this network of reactions involving dipeptides, the competition between thermodynamic and kinetic processes is active. At pH 2, it namely enables the kinetic destabilization of the thermodynamically more stable dipeptide diastereoisomer. This is of great interest for leading to the spontaneous offset of stable non-racemic states in non-equilibrium systems of polymerization/depolymerization of amino acid (Plasson et al. 2004; Plasson and Bersini 2009).

This change of reactivity of amino acid derivatives as a function of the pH may have played an important role for the evolution of amino acid derivatives on primitive Earth. Because of the evolution variation of primitive ocean acidity (from initially weakly acid to neutral conditions (Kasting 1993; Morse and Mackenzie 1998). This acid catalysis could have also been greatly increase by the presence of phosphates in primitive oceans (Pasek 2008; Albarède and Blichert-Toft 2009). With the addition of the cooling of Earth, this may indicates the evolution from an active chemistry, with fast reactions, to a slower chemical system.

Moreover, this work enlightened in which direction the study of such prebiotic systems shall be developed. If the measured stereoselectivities are qualitatively interesting, their intensity may be too low for generating a bifurcation towards homochirality in far from equilibrium systems of amino acids. The possibility to increase their efficiency by the catalysis of hydrolysis using clays (Bujdák et al. 2006) or metallic salts (Bonomo et al. 1986), based on the differential reactivities of the diastereoisomers, is to be studied. Moreover, the cyclization of dipeptides has

been shown to play an important role. More control on this reaction can be gained, typically by the introduction of cobalt complexes (Takarada et al. 2000).

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