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Amino Acids

5(4*H*)-Oxazolones as Intermediates in the Carbodiimide- and Cyanamide-Promoted Peptide Activations in Aqueous Solution**

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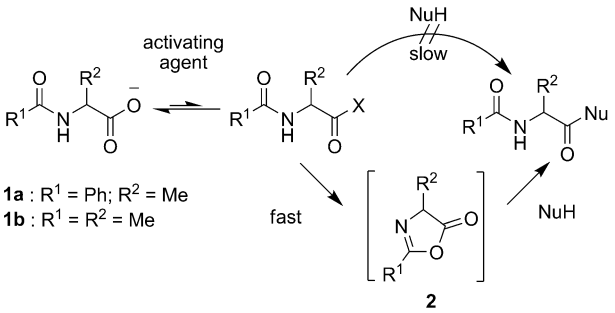
The formation of biopolymers in prebiotic environments is still unresolved regarding nucleic acids and functional peptides. Peptide function especially requires proper folding and hence sufficient lengths. In 1969, Cavadore and Previero[1] observed that the EDC-mediated (EDC = 1-ethyl, 3-(3-dimethylaminopropyl)carbodiimide hydrochloride) α-amino acid polymerization in water is significantly improved when N-acylated amino acids are introduced as initiators. Obviously, EDC is highly unlikely to have been abiotically formed but there are indications in the literature that underivatized carbodiimide HN=C=NH is involved as an intermediate in reactions of cyanamide,[2] a prebiotically plausible reagent, and dicyandiamide is reported to behave similarly as carbodiimides.[3] The behavior of N-acylamino acids as polymerization initiators was explained in the original work[1] by an inhibition of activation owing to the greater acidity of free α-amino acids ($pK_A \approx 2.3$) compared to C-terminal carboxy groups in peptides ($pK_A \approx 3.7$). However, alternative explanations could be proposed. For example, 1) the blockade of elongation by diketopiperazine formation at the dipeptide level,[4] or 2) an increased efficiency of the activation process resulting from formation of the 5(4*H*)-oxazolone **2** (Scheme 1).[5a]

The formation of 5(4*H*)-oxazolones has exhaustively been studied owing to its importance for the chiral integrity of synthetic peptides,[7] a result of the fast proton exchange at the α-carbon atom in the presence of bases. Peptide chemists tend therefore to avoid the formation of 5(4*H*)-oxazolones during α-amino acid activation. But, in the context of prebiotic peptide formation starting from racemic mixtures, epimerization may, on the contrary, constitute a prerequisite for symmetry breaking as a result of appropriate processes involving an autocatalytic reproduction of chirality.[5] The formation of 5(4*H*)-oxazolones is additionally likely to increase reaction rates since it corresponds to the principle of overactivation through cyclization (Scheme 1), thus expressing that the presence of a conveniently positioned intramolecular group can allow the formation of a highly reactive intermediate which would not have been formed intermolecularly.[8a] The cyanate-mediated activation of peptides with a C-terminal aspartyl residue[9] proceeds similarly by cyclization, and amino acid N-carboxyanhydrides are involved when activated α-amino acids are coupled at the N terminus.[8] Herein we report the first results of studies undertaken with the aims of 1) clearly identifying the mechanism of the EDC-promoted activation of N-acylamino acids, 2) demonstrating the possibility of activating peptides with cyanamide in a similar way, and 3) addressing the issue of chirality in these processes suspected to proceed through a chirally unstable 5(4*H*)-oxazolone.[7]



Scheme 1. Overactivation through cyclization by fast intramolecular conversion of activated *N*-acyl-α-amino acids into 5(4*H*)-oxazolones. The presence of the amide oxygen nucleophile at a convenient position for reaction allows the fast intramolecular[6] formation of a reactive 5(4*H*)-oxazolone from the instable activating agent adduct.

As a model of the activation of C-terminal residues in peptides, the behavior of *N*-benzoyl-alanine (**1a**) and *N*-acetyl-alanine (**1b**; 10 mM) in the presence of EDC (20 mM) was monitored in buffered D_2O (pD 5–7) by NMR spectroscopy. From Bz-Ala-OH (**1a**), the observation of an NMR signal at $\delta = 1.47$ ppm (Figures 1 B–D) is consistent with the formation of an intermediate, which is predominantly present in a deuterated form, and consistent with the observation of a fast H/D isotope exchange at the α-carbon atom from a pure sample of 2-phenyl-4-methyl-5(4*H*)-oxazolone (**2a**) under

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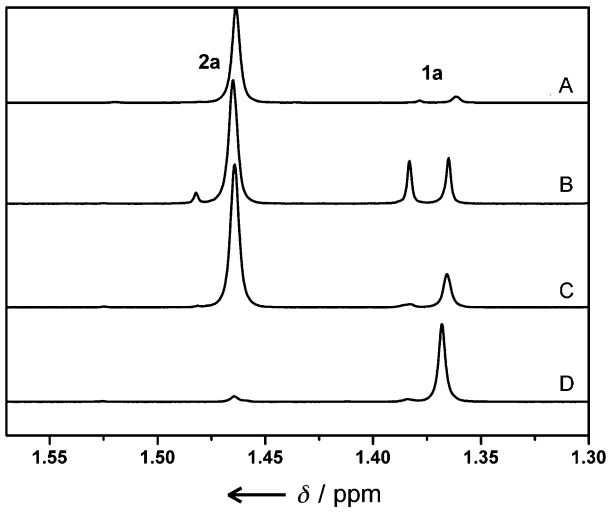
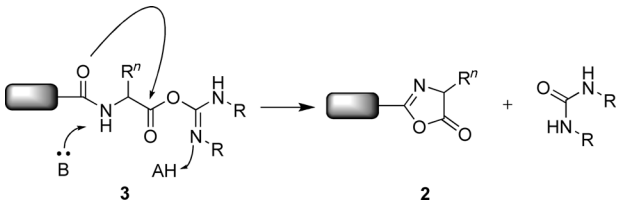


Figure 1. Deuteration experiments in D_2O solutions buffered with MES (100 mM, pD 5.5). ^{1}H NMR spectra (400 MHz) of methyl protons of **1a** and **2a**. A) Fast deuteration of **2a** (10 mM) in MES buffer (after 10 min at RT). B) Activation of **1a** (10 mM) with EDC (20 mM); reaction progress after 10 min. C) Reaction progress after 65 min. D) Reaction progress after 369 min.

similar reaction conditions (Figure 1 A). Structure **2a** was confirmed after isolation of the intermediate by extraction (see the Supporting Information). A similar behavior was observed for Ac-Ala-OH (**1b**), except that H/D exchange was slower so that the appearance of the ^{1}H NMR signal for 2,4-dimethyl-5(4*H*)-oxazolone (**2b**) and the subsequent deuteration could be observed to occur independently (see Figure S2 in the Supporting Information). These observations establish that a chirally unstable 5(4*H*)-oxazolone is transiently formed when activating **1a** and **1b** with EDC. The exchange of a proton at the C-terminal residue was also observed during the activation of the dipeptide Ac-Tyr-Ala-OH at pH 6.5, thus leading to a moderate excess in favor of the L,L-diastereomer (see Figure S3 in the Supporting Information) and indicating that elongation into longer peptides also proceeds through 5(4*H*)-oxazolones.

To demonstrate that the process is driven by overactivation through cyclization, we monitored, by HPLC, the progress of the coupling reaction of **1b** ($k_{rel} = 1$, $pK_A = 3.72$[10]) with glycine *p*-nitroanilide (H-Gly-pNA) in the presence of EDC at room temperature (see the Supporting Information). The initial reaction rate was compared with those of carboxylic acids unable to react intramolecularly: acetic acid (k_{rel} = ca. 0.1, $pK_A = 4.76$[10]) and lactic acid (k_{rel} = ca. 0.01, $pK_A = 3.86$[10]), with the latter bearing a hydroxy substituent having electron-withdrawing properties similar to that of the acetamido group of **1b**. The approximate 100-fold difference in kinetic rates (and the higher coupling yields) demonstrates that the reaction of **1a** takes place predominantly via the 5(4*H*)-oxazolone (ca. 99%; Scheme 1). In contrast, carboxylic acids lacking any possibility of assistance by a neighboring group would proceed through the unstable O-acylisourea intermediate **3** which rapidly reverts to the reactants by expelling the carboxylate nucleophile and does not accumulate to an extent sufficient enough to allow a fast reaction with nucleophiles. The presence of an amide oxygen atom at a convenient position for reaction enables the irreversible cyclization into 5(4*H*)-oxazolone in spite of a nucleophilic power that is not likely to exceed that of the leaving urea group of the O-acylisourea intermediate **3** (Scheme 2). 5(4*H*)-Oxazolones have been considered as



Scheme 2. Conversion of the O-acylisourea intermediate **3** of the carbodiimide-promoted peptide activation into 5(4*H*)-oxazolone **2**: the strength of the amide oxygen nucleophile and that of urea oxygen leaving group are likely to be similar.

potential intermediates of peptide activation,[11] the present results indeed demonstrate that in diluted aqueous solution, the corresponding pathway is predominant.

The nonreversible conversion into 5(4*H*)-oxazolone increases the concentration of species able to react with nucleophiles with rates which must not be very different from that of the O-acylurea, and results in an increase in rate by a factor of several orders of magnitude. The kinetic advantage of α-acylamino acid derivatives is likely to be the source of the efficiency in the formation of peptide chains in the experiment reported by Cavadore and Previero.[1] We determined the length of the peptides formed by EDC activation in a CO_2/HCO_3^- buffer. Bz-Ala-OH (**1a**; 5 mM) and glycine (50 mM) were reacted with ten portions of EDC (5 mM each) over three days. The peptide products were separated using a column with a cation-exchange resin and analyzed by NMR spectroscopy to assess a mean value of about three for the degree of polymerization (see Figure S4, in the Supporting Information). The presence of peptides Bz-Ala-$(Gly)_n$-OH (with $n = 1$ to 11) was confirmed by ESI/MS and MALDI/MS (see Figures S5 and S6 in the Supporting Information). A control experiment carried out in a similar way but in which the Bz-Ala-OH initiator was omitted demonstrated the absence of diketopiperazine which should have been formed from any polymerization process in the absence of an initiator.

The prebiotic activation of peptides according to a similar scheme requires that a prebiotically available reagent could replace EDC. The hydration of cyanamide into urea is a slow reaction at neutral pH and is subject to acid catalysis.[12] Evidence that the reaction involves a prior conversion into carbodiimide has been reported.[2,13] To determine if cyanamide could act as a carbodiimide equivalent or precursor for the activation of carboxy groups in peptides, **1a** was reacted for periods of time ranging from 48 hours to 30 days in D_2O in the presence of NH_2CN. To increase the reaction rates, samples were heated to 80 °C. An exchange of the α-hydrogen atom was detected by ^{1}H NMR spectroscopy (Figure 2), whereas no reaction occurred in a control experiment in

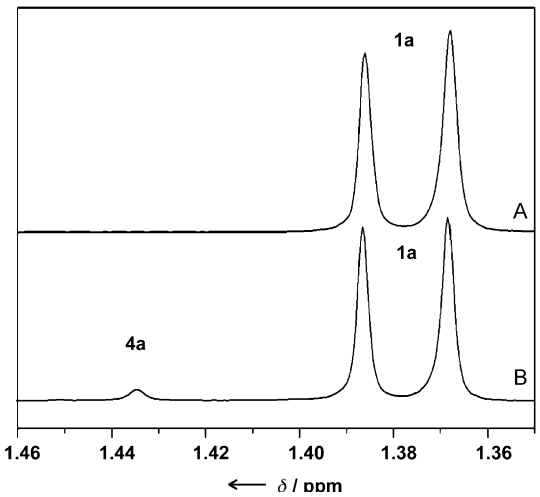


Figure 2. Activation of 10 mM **1a** with 20 mM cyanamide in buffered D_2O (100 mM MES buffer, pD 5.5) at 80 °C for 72 h, monitored by ^{1}H NMR spectroscopy (400 MHz). A) The dissymmetry of the methyl doublet of **1a** reveals the formation of a singlet at $\delta = 1.37$ ppm resulting from H/D exchange at C_α. B) Same reaction in the presence of Gly (50 mM) after 72 h: the resonance at $\delta = 1.43$ ppm, consistent with the formation of **4a** in a deuterated form and the symmetry of the methyl doublet show that the 5(4*H*)-oxazolone reacts with glycine faster than it is reverted into **1a**.

which cyanamide was replaced by its hydrolysis product, urea. The selective deuteration at a single site was confirmed by MS analysis of **1a** recovered from the reaction medium (see Figure S7 in the Supporting Information).

A similar experiment was carried out in D_2O with **1a** in the presence of 50 mM glycine and 20 mM cyanamide (Figure 2), thus leading to the observation of a singlet at $\delta =$ 1.43 ppm, which is consistent with the presence of an additional deuterated product in significant concentrations and with the chemical shift determined independently for Bz-Ala-Gly-OH (**4a**). The dipeptide **4a**, formed in a 7.5 % yield from a 10 mM solution of **1a** in a MES buffer at pH 5.5 in the presence of 50 mM glycine and 33 mM cyanamide heated to 80 °C for 17 days, was subsequently identified by HPLC/ESI/MS (see Figure S8 in the Supporting Information). No peptide was observed from similar experiments carried out at a higher pH value (6.5), which is consistent with the acid catalysis of cyanamide reaction.[12]

These results show that cyanamide behaves as an activating agent for N-acyl α-amino acids and strongly suggest the formation of a 5(4*H*)-oxazolone undergoing a fast exchange of the proton bound to the α-carbon atom. They are consistent with the fact that the reaction of cyanamide with water and other nucleophiles proceeds through a carbodiimide[2,12,13] and is subject to acid catalysis.[12] This slow reaction may at first glance be considered as a drawback, thus rendering its participation to the formation of prebiotic peptides unlikely. However, kinetic barriers have been considered essential to hold chemical environments far from equilibrium and therefore to bring about the development of protometabolic pathways based on catalysis and autocatalysis.[14] Hydrolytic processes and side reactions must then proceed with time scales consistent with the migration of activated species from the location of their formation to that of the self-organizing system.[15] For these reasons, cyanamide could be considered a prototype of a prebiotic activating agent which is able to transfer energy to an environment distant from that of its formation, and could be exhausted by very specific dissipative structures possibly using catalytic pathways and potentially capable of growing at its expense. This simple five-atom molecule has been identified in interstellar mediums[16] in which it can also be converted into carbodiimide.[17] We found no indication that it could be delivered as such in meteorites but processes, such as the UV or sunlight irradiation of NH_4CN solutions, leading to its abiotic formation on Earth have been proposed.[18] Cyanamide, most often studied in the field of prebiotic chemistry as a peptide condensing agent,[19] turned out to be very useful for the abiotic synthesis of nucleotides[20] and phosphorylation[21] as well. The involvement of a 5(4*H*)-oxazolone during peptide activation may allow the coupling of hindered α-amino acids[22,23] such as the C^α-methylated amino acids which are present in enantiomeric excess in certain meteorites.[23,24] The issues of epimerization of acyl amino acids and of C-terminal residues in peptides during the EDC- and cyanamide-promoted coupling of peptides is under investigation in our group. However, since considering it as a drawback for peptide coupling is not relevant in prebiotic chemistry,[5] epimerization, associated with stereoselectivity in the elongation pathway, may lead to the formation of substantial amounts of homochiral domains in peptides which may be essential for their activity. Protometabolic peptide systems,[5] in which cyanamide-promoted peptide formation is associated with depolymerization by hydrolytic processes, could potentially enable it to reach states far from equilibrium with respect to chirality.[25] Such states are ruled by a dynamic kinetic stability[26] rather than evolving towards the equilibrium state. They may have contributed to symmetry breaking in a way, thus presenting analogies to the APED model proposed earlier.[25]

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