Evolutionary Importance of the Intramolecular Pathways of Hydrolysis of Phosphate Ester Mixed Anhydrides with Amino Acids and Peptides

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Aminoacyl adenylates (aa-AMPs) constitute essential intermediates of protein biosynthesis. Their polymerization in aqueous solution has often been claimed as a potential route to abiotic peptides in spite of a highly efficient CO₂-promoted pathway of hydrolysis. Here we investigate the efficiency and relevance of this frequently overlooked pathway from model amino acid phosphate mixed anhydrides including aa-AMPs. Its predominance was demonstrated at CO₂ concentrations matching that of physiological fluids or that of the present-day ocean, making a direct polymerization pathway unlikely. By contrast, the occurrence of the CO₂-promoted pathway was observed to increase the efficiency of peptide bond formation owing to the high reactivity of the N-carboxyanhydride (NCA) intermediate. Even considering CO₂ concentrations in early Earth liquid environments equivalent to present levels, mixed anhydrides would have polymerized predominantly through NCAs. The issue of a potential involvement of NCAs as biochemical metabolites could even be raised. The formation of peptide–phosphate mixed anhydrides from 5(4H)-oxazolones (transiently formed through prebiotically relevant peptide activation pathways) was also observed as well as the occurrence of the reverse cyclization process in the reactions of these mixed anhydrides. These processes constitute the core of a reaction network that could potentially have evolved towards the emergence of translation.

The biosynthesis of peptides involves aminoacyl adenylates (aa-AMPs), formed through the reaction of ATP with α-amino acids (aas) (Fig. 1), that are subsequently used to aminoacylate tRNA. Their standard free energy of hydrolysis value ΔG°' = ca. −70 kJ mol⁻¹, determined for Tyr-AMP¹, ranks them among the energy-richest biochemicals. Aa-AMPs possess a phosphate group transfer potential much higher than ATP¹ and might then constitute adenylation agents as well as aminoacylating agents²⁻³. The otherwise unfavourable⁴ reaction of ATP with α-amino acids (K = 3.5 × 10⁻¹⁷) is driven towards completion by selective stabilization of aa-AMPs in the active sites of aminoacyl tRNA synthetases (aaRSs). They usually remain sequestered by the enzyme and are not released in solution before reacting with tRNA. The importance of this process can be appreciated by considering that the set of aaRS enzymes, responsible for the association of amino acids with their cognate tRNAs, actually holds the key of the genetic code. The evolutionary path through which adenylates were introduced in the process remains unidentified. In addition of being thermodynamically unfavourable, the spontaneous reaction is indeed very slow in the absence of enzyme⁴⁻⁶, so that the emergence of the biochemical amino acid activation pathway remains unexplained before a set of catalysts (very probably ribozymes) could lead to an embryo of the genetic code for prebiotically available amino acids⁸. In spite of this obstacle, the evolution of this pathway from an abiotic process of random peptide formation via the polymerization of α-amino acid mixed anhydrides with phosphate (aa-PMAs) or phosphate esters (aa-PEMAs) and adenylates (aa-AMPs) has prompted much work⁹⁻¹⁰. However, the abiotic formation of adenylates or their analogues from phosphate anhydrides did not receive any experimental support. As a matter of fact, the claim¹¹ that ATP is capable of driving the polymerization of α-amino acids on clays through aa-AMP intermediates turned out to be non-reproducible¹². Though the genetic code might have evolved late in the hypothesis of an “RNA world” without needing ATP activation as shown by the successful selection of ribozymes capable of aminoacylating RNAs using either amino acid esters¹³ or activated RNAs¹⁴, an early co-evolution involving the chemistries of nucleotides and amino acids is consistent with the comparatively higher abundance of the latter as the products of abiotic processes. Therefore, selecting the co-
evolutionary option, the elucidation of the potential evolutionary process through which aa-AMPs could have been introduced requires the identification of simple pathways capable of leading to these intermediates. A likely possibility is the reaction of α-amino acid N-carboxy anhydrides (NCAs) with inorganic phosphate and its esters including adenylates that takes place spontaneously at moderate pH20,21. This possibility is supported by the role of NCAs deduced from the literature2 and the disclosure of realistic abiotic pathways for their formation during the last decade18,19. Since the activation of the C-terminus in peptides has recently been identified as a plausible prebiotic pathway and involves the formation of 5(4H)-oxazolone intermediates20, it is reasonable that similar mixed anhydrides with phosphates involving acylated amino acids (acyl-aa-PEMAs) or peptides (peptidyl-PEMAs) could be formed by reaction of the energy-rich cyclic intermediate (Fig. 2b). The occurrence of abiotic pathways leading to aa-PEMA or peptidyl-PEMA must have preceded their involvement in chemical evolution. However, the low stability of these mixed anhydrides and the availability of highly reactive cyclic intermediates prone to polymerize more easily renders their role in early abiotic processes of peptide formation highly questionable.

The kinetic stability of aa-AMPs and of other aa-PEMAs has been studied in aqueous solution leading to contradictory results in the literature21–23. Of particular interest with regard to an evolutionary context is the description of a highly efficient CO2-catalyzed path of hydrolysis21–23. No definitive mechanism has been proposed but the intermediacy of NCAs is highly probable21,22,25 since other activated amino acids (nitrophenyl esters, thioesters) proved to undergo conversion into NCAs in hydrogen carbonate buffers25. This analysis casts doubts on the possibility that aa-AMPs constitute efficient monomers for the abiotic formation of peptides in aqueous solutions2,3,26 since most early Earth aqueous environments are likely to have contained CO2 or HCO3–. The present investigations were aimed at providing data on the efficiency of the CO2-promoted pathway (Fig. 3a) in aqueous solution at neutral pH and in the presence of CO2 concentrations compatible with early Earth environments and at clearly identifying the NCA as an intermediate. They address both the issues of the stability of aa-AMPs and of other aa-PEMAs and that of the path of peptide formation. They demonstrate the prevalence of the CO2-promoted pathway in the hydrolysis of adenylates. More importantly, using model amino amide reactants, they additionally demonstrate that peptide bond formation takes place predominantly from the cyclic intermediates rather than directly from the mixed anhydrides ruling out any possibility of considering the latter as direct peptide precursors at early stages of chemical or bio-

Figure 1 | The aaRS-catalyzed reaction of α-amino acids with ATP.

Figure 2 | Potential pathways for the abiotic formation of mixed anhydrides of α-amino acids and peptides with phosphate (PMA) and phosphate esters (PEMA) including adenylates (AMP). (a) Reaction of NCAs with phosphate esters; (b) The hypothesized similar reaction of 5(4H)-oxazolones.

Figure 3 | Intramolecular pathways competing with direct nucleophilic reactions for the conversion of mixed phosphate anhydrides. (a) The efficient hydrolytic pathway and conversion into peptides of amino acid phosphate ester mixed anhydrides promoted by carbon dioxide through NCAs. (b) Cyclization into 5(4H)-oxazolone competing with direct nucleophilic reaction of acyl-aa-PEMA and peptidyl-PEMA.

Figure 4 | Structure of the reactants and products related to O-methyl-tyrosine (H-Tyr(Me)-OH) 5 studied in this work.
chemical evolution. Lastly, considering NCAs as likely precursors of aa-AMPs and aa-PEMAs, the hypothesis of an abiotic formation of non-coded peptides through these mixed anhydrides becomes unnecessary. The evolution of translation must then have proceeded through a pathway independent from abiotic polymerization. This work also addresses the more general goal of understanding the stability of phosphate mixed anhydrides of amino acids and peptides in aqueous media at moderate pH. As a matter of fact, though N-acylation is an obvious way to prevent CO₂ participation, another intramolecular path of breakdown through 5(4H)-oxazolones is possible in the case of acyl-aa-PEMAs (Fig. 3b). Therefore, the issues of the importance of the NCA and 5(4H)-oxazolone pathways in the reactions of the corresponding mixed anhydrides (Fig. 3) are raised as well as that of the potential role of these cyclic intermediates as potential abiotic precursors of these mixed anhydrides (Fig. 2). The consequences of these chemical pathways as factors determining early biological evolution of amino acid activation processes and their constraints on the contemporary biochemistry of adenylates will also be discussed.

Results

Experiments were carried out from model systems derived from O-methylated tyrosine 5 (Fig. 4) likely to be representative of the reactivity of usual amino acid derivatives. The UV-absorption of the tyrosine side chain (λ_max = 273 nm) was selected to monitor reactions by HPLC at a reasonably low (0.05–1 mM) concentration range in which activated intermediates have a lifetime sufficient for their behaviour to be determined. Furthermore, phenol methylation was introduced to simplify analyses by avoiding any side-reaction of this group. Reactions were carried out in non-nucleophilic MES or MOPS buffers at pH values of 6.5 or 7.5, respectively, whereas 50 mM phosphate or methyl phosphate buffers were used for studying the transient formation of mixed anhydrides. Analyses were performed to monitor the reaction progress of samples stored in the HPLC systems located in a room maintained at the temperature of 20°C. Fast reactions were monitored by withdrawing 1 mL samples from the reaction medium and the reaction was blocked by addition of a formic acid solution to bring the pH to a value below 4 (Supplementary information).

NCAs as intermediates of aa-PEMA reactions promoted by CO₂

The hydrolysis of methyl phosphate mixed anhydride 1b was studied in buffered solutions in the presence of varying contents of CO₂/HCO₃⁻. The reaction rates were observed to strongly depend on the presence of CO₂ as shown by a c.a. 4 fold increase in rate using pH 6.5 MES buffers previously equilibrated with air as compared with a solution flushed with N₂ for 60 min (Fig. 5, panel A). The rates could be reduced by further c.a. 35% by extensive degasification through cycles of freezing at −95°C/gas removal under vacuum/melting in a closed vessel. Under the conditions of the experiment displayed in the panel A of Fig. 5, the starting material 1b (HPLC retention time, r.t. 4.6 min, method A) disappeared slowly and several species containing the methoxyphenyl moiety (λ_max 273 nm) were observed, namely the free amino acid 5 (r.t. 8.4 min) representing the main product of hydrolysis but also several peaks corresponding to the dideptide H-Tyr(Me)-Tyr(Me)-OH (r.t. 22.7 min) and the diketopiperazine cyclo-Tyr(Me)-Tyr(Me) (r.t. 23.6 min), very probably resulting of the cyclization of the mixed anhydride H-Tyr(Me)-Tyr(Me)-OPO₃Me, which has not been properly identified. The presence of these two products was confirmed by HPLC-MS analysis ([M + H] = 373.2 at r.t. 1.52 min and 355.2 at r.t. 1.88 min, method C). By contrast, the addition of 2 or 10 mM NaHCO₃ to the buffer led to the fast disappearance (≤1% after 3 min) of the mixed anhydride 1b as monitored by HPLC analysis (Fig. 5, panel B). An intermediate (r.t. 23.1 min, method A) formed in proportion yields as high as

Figure 5 | Hydrolysis of aa-PEMA 1b. Panel A - Monitoring by the evolution of its HPLC peak area (% method A): (a) in a 100 mM pH 6.5 MES buffer flushed with N₂ for 60 min (half-life c.a. 80 min, filled squares); (b) in a similar buffer equilibrated with air (half-life c.a. 18 min, open squares); (c) in a similar buffer to which was added 10 mM NaHCO₃ (half-life c.a. 0.3 min, filled diamonds, 1 mL samples were withdrawn and acidified with 20 µL of 2 M formic acid before analysis); the expanded time scale in the inset shows that reaction (c) is completed within 2 min. Panel B - HPLC traces (method A) of samples withdrawn at 3 min from experiments carried out (a) in a 100 mM pH 6.5 MES buffer flushed with N₂ for 60 min indicating the presence of unreacted mixed anhydride 1b and a minor conversion to α-amino acid 5 and (b) in a 100 mM pH 6.5 MES buffer to which was added 2 mM NaHCO₃ demonstrating a complete conversion of the starting material into NCA 3. Panel C - HPLC traces corresponding to the range of retention times of the peptide products (method A, H-(Tyr(Me))ₙ-OH with n = 2 to 5, r.t. 22.7, 24.9 min for n = 2, 3, respectively and diketopiperazine, cyclo-Tyr(Me)-Tyr(Me), DPK, r.t. 23.6 min) of the reactions after completion (a) in a 100 mM pH 6.5 MES buffer flushed with N₂ for 60 min and (b) in a 100 mM pH 6.5 MES buffer to which was added 10 mM NaHCO₃. HPLC peaks were identified by both injections of authentic samples and HPLC-ESI-MS analyses (method C) from similar experiments.
60% and was identified as the NCA 3 by a retention time identical to the authentic product and by HPLC-ESI-MS (negative mode [M+H] = 220.07, r.t. 1.96 min, method C). This intermediate was rapidly converted into the product 5 accompanied by the dipeptide H-Tyr(Me)-Tyr(Me)-OH. The presence of the dipeptide was confirmed by HPLC-ESI-MS (positive mode [M + H] = 373.2) as well as that of higher oligomers H-[Tyr(Me)]_n-OH (with n = 3 to 5, [M + H] = 550.2, 727.3, 904.4 for peaks at r.t. 1.76 min, 1.93 min, 2.06 min, respectively, method C). By contrast reduced amounts of diketopiperazine cyclo-Tyr(Me)-Tyr(Me) formed confirming that the starting material lifetime was not sufficient for it to behave as a polymerization initiator leading to a dipeptide mixed anhydride prone to cyclization. Under these conditions involving the presence of HCO_3^-, the polymerization into peptides thus proceeds through the NCA rather than directly from the starting material. An NCA intermediate was also observed to form rapidly at pH 7.5 in 100 mM MOPS buffers in the presence of added HCO_3^- (Supplementary Information, Fig. S1). This behaviour indicates that the formation of long peptides from adenylates reported in the literature results probably from the polymerization of NCAs rather than from that of adenylates. The conversion of aminoacyl adenylates into NCA in the presence of CO_2/HCO_3^- was investigated starting from the Tyr(Me) derivative 1c (Supplementary Information, Fig. S2). The conversion of 1c into NCA was observed to proceed with rates similar to that observed for mixed anhydride 1b. The release of AMP (r.t. 1.5 min, method A) accompanying the formation of NCA 3 could be detected by HPLC allowing the reaction to be monitored at 50 μM concentrations of reactant 1c (r.t. 6.8 min, method A). The lifetime of the adenylate decreased with increasing concentrations of CO_2/HCO_3^- (t_{1/2} = 80 min, ~25 min, and <2 min at pH 6.5 in N_2-flushed buffer, air equilibrated buffer and in the presence of 500 μM HCO_3^-, respectively). At pH 7.5 the lifetime of adenylate 1c was reduced to less than 1 min in the presence of 500 μM HCO_3^-, which means that this mixed anhydride is likely to be converted into NCA within a few seconds at concentrations of CO_2/HCO_3^- above 2 mM and at pH 4.5 value close to neutrality, which are representative of the present day ocean or physiological fluids. It is worth noting that this lifetime is not sufficient for peptides to be significantly formed by a direct reaction with adenylate so that any observation of peptide products under these conditions results for the most part from the intermediacy of NCAs.

At pH 4, the hydrolysis of mixed anhydride 1b was much slower (t_{1/2} = ca. 550 min) and CO_2 catalysis was not observed (Supplementary Information, Fig. S3). This result is consistent with the results obtained by Kluger from alanyl ethyl phosphate. The protonation of the amino group of 1b increases the electrophilic character of its acyl group and then the rates of nucleophilic attack, but it also prevents any possibility of reaction with CO_2 according the pathway of Fig. 3a. The hydrolysis of the acetylated mixed anhydride 2b was indeed observed to be slower (t_{1/2} ~ 950 min at pH 6.5) and was not affected by addition of 10 mM NaHCO_3 (Fig. 6) in a way consistent with this explanation and with previously reported analyses. However, it is important to emphasize that the CO_2-catalyzed pathway does not only constitute a process leading to the deactivation and the hydrolysis of mixed anhydrides since peptide formation can be improved significantly by this means. As a matter of fact, with regard to peptide formation, the prevalence of the NCA pathway was demonstrated by studying the model reaction of 1 mM mixed anhydride 1b with 5 mM glycaminode either in a nitrogen-flushed sample or in the presence of 2 mM NaHCO_3 (Fig. 7). Importantly, less than 2 min were sufficient for the starting material to be exhausted in the presence of carbonate, whereas CO_2 removal increased the reaction times to much higher values (t_{1/2} ~ 50 min) and reduced the final yield in dipeptide (Fig. 7). This reaction remained faster than that observed for the acetylated mixed anhydride 2b (t_{1/2} ~ 260 min) unable to undergo the conversion into NCA, but that will be demonstrated below to partly undergo cyclization into 5(4H)-oxazolones. These experiments carried out using glycynamide for mimicking a growing peptide chain show that the polymerization of adenylates and other aa-PEMA is improved in the presence of CO_2 by the occurrence of the NCA pathway owing to both the higher reactivity of the latter intermediate and its ability to suppress diketopiperazine formation.

The interconversion of 5(4H)-oxazolones and acyl-aa-PEMA and peptidyl-PEMA. The reaction of Ac-Tyr(Me)-OH-derived oxazolone 4 in methyl phosphate-buffered aqueous solution (pH 6.5) at 20°C was monitored by HPLC and compared with the hydrolysis of mixed anhydride 2b in MES buffers (Fig. 6). Comparable rates were observed and the intermediate of the 5(4H)-oxazolone 4 was identified in situ by HPLC-ESI-HRMS (negative mode, calcd for C_{13}H_{12}NO_3P+, 330.0743; found 330.0747) as the mixed anhydride 2b. A similar behaviour was observed from a reaction of inorganic phosphate (Supplementary Information, Fig. S5). The hydrolysis of mixed anhydride 2b was monitored by HPLC at 20°C in buffered solutions (Fig. 6). The reaction was also carried out in D_2O to detect any hydrogen/deuterium exchange resulting from the transient formation of 5(4H)-oxazolone and compared to the product of a similar reaction of pure oxazolone 4 (Table 1). The values obtained demonstrate the occurrence of an intramolecular pathway already suspected from the higher rate of conversion of acylated aa-AMPS compared to simple acyl-adenylates. At pH values below 5, the hydrolysis of anhydride 2b (Supplementary Information, Fig. S4) has been observed to become faster in a way similar to the observation made by Lacey’s group for Ac-Phe-AMP. The identification of an intramolecular pathway made in the present work strongly suggests that the acid catalysis of acyl-aa-PEMA hydrolysis is the consequence of a facilitated cyclization from a good neutral phosphate leaving group. However, the absence of H/D exchange from the reaction of neither acyl-aa-PEMA 2b nor 5(4H)-oxazolone 4 at this pH (Table 1) prevented any
before the subsequent reaction of the 5(4-
As regards aa-PEMA reactions, it is noteworthy that CO2 catalysis
Discussion
2b
anhydride
determination of the actual pathway of hydrolysis of mixed
were identified by ESI-MS.

Figure 7 | Formation of the dipeptides Ac-Tyr(Me)-Gly-NH2 or H-

Similarly, we analyzed the degree of D/H exchange during the
reaction of 2b with L-Ala-NH2 in D2O at pH 6.5 (Table 1). The
observation of a partial deuteration of the two diastereoisomers of
the dipeptide product demonstrates that even when a better nucleo-
phile is present, the α-proton is exchanged to a significant extent
before the subsequent reaction of the 5(4H)-oxazolone takes place.
The fast reaction of acyl-aa-AMP25 and other acyl-aa-PEMA results
therefore, at least for a noticeable part, from a transient conversion
into 5(4H)-oxazolones. Interestingly, the different degrees of deu-
teration of the two diastereomers indicate that the intramolecular
path of Fig. 3b has a higher stereoselectivity as compared to the direct
path (the reactants 2b and 4 were prepared under a racemic form26).

Table 1 | Results of H/D exchange experiments carried out from either acetylated mixed anhydride 2b or oxazolone 4 under various
conditions

| Nucleophile | Buffer | Ratio of [M + 1] MS peak in the product corrected from natural abundancea |
|-------------|--------|--------------------------------------------------|
| H2O         | 100 mM formateb | <1% | <1% |
| H2O         | 100 mM MES5  | 55% | ≥93% |
| H-Ala-NH2   | 100 mM MES5  | 27% [L, L-isomer] | 99% [L, L-isomer] |
|             |         | 16% [D, L-isomer] | 98% [D, L-isomer] |

*a Determination by ESI-MS: after the reaction in D2O the residue was freeze-dried and then submitted three times to cycles of addition of H2O and freeze-drying to convert any easily exchangeable hydrons and finally analyzed by MS;
*Buffer ratio equivalent to that of a pH 4 buffer in H2O.

These experiments demonstrating that the NCA path is prevailing
at pH values close to neutrality in solutions equilibrated with air at
present atmospheric levels of CO2 (ca. 0.04%) suggest that the path-
way must be overwhelming in natural environments with higher
contents. The experiments at 2 mM HCO3 are representative of
present day ocean total concentration of dissolved carbonate39 show-
ing that the lifetime of aa-PEMA is expressed in tens of seconds in
these media at pH 7.5. In biological media, with total carbonate
concentrations approaching or exceeding 10 mM, the lifetime of
mixed anhydrides would be even shorter. The early atmosphere
had a CO2 content that remains poorly constrained40 but values
similar to the present atmospheric levels41, or representing up to
hundred times this value42,43, are often considered. Under these con-
ditions, aa-PEMA would be rapidly converted into NCA before any
direct conversion into peptides could take place, which discards the
earlier proposed contribution of aa-AMPs in the formation of pre-
biotic peptides7–11. Moreover, a less efficient polymerization ability
of aa-PEMA and the diketopiperazine side-reaction make them
improbable peptide precursors. The possibility that a very low con-
tent of CO2 in the atmosphere could have transiently permitted
mixed anhydrides to be stabilized26 is made unlikely because it would
have also required a very efficient removal of the most part of CO2 in
the whole ocean (≈2 mM in HCO3). On the contrary, the develop-
ment of the activation pathway leading to translation must have
occurred in an environment in which the role of NCA was unavoid-
able rather than in a local environment in which the mixed anhy-
drides were preserved from the presence of CO2 and HCO3 by any
kind of geochemical processes. NCA can be considered not only as
intermediates of the degradation pathway of adenylates but also as
precursors of any kind of aa-PEMA mixed anhydrides including
adenylates as well as precursors of peptides through a pathway sup-
pressing diketopiperazine side-reaction. From this point of view, the
catalysis by carbon dioxide may lead to a fast exchange among dif-
ferent energy-rich species capable of linking activated amino acids to
phosphorylating species. This distribution of energy in a reaction
network, that may have anticipated the role of ATP as an energy
currency, ensured a global far from equilibrium situation that was
essential even at early stages of chemical evolution44. Considered
from the point of view of a co-evolutionary development of peptide
necessary for enzymes to exceed a physical limit45. Induced intramo-
lecularity has also been used to drive highly stereoselective catalysis
in organic synthesis46,47. The efficiency of this kind of catalysis relies
on the rates of intramolecular reactions48. Carbon dioxide present at
total concentrations of ca. 30–40 µM in pH 6.5 solutions equili-
brated with air (as deduced from the Henry’s coefficient of CO249
and the pKa of carbonic acid) brings about a rate increase sufficient
to render the catalytic pathway largely predominating, which is remark-
able by considering a simple three-atom molecule compared to the
efficiency of enzymes48. The ease of formation of 5-membered cycles
from α-amino acid mixed anhydrides is also demonstrated by the
conversion of acyl-aa-PEMA into 5(4H)-oxazolones.

As regards aa-PEMA reactions, it is noteworthy that CO2 catalysis
proceeds through a pathway involving induced intramolecularity26.
This kind of process shares some of the most important components
of enzymatic activity, which corresponds to the utilization of binding
energy to non-reacting portions of the substrate to bring about cata-
lysis48. It was also proposed to constitute the easiest path for enzyme
evolution under the name of uniform binding26 and is moreover

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|             |         | 16% [D, L-isomer] | 98% [D, L-isomer] |
and nucleotide chemistries the CO₂-catalyzed pathway may then constitute a key-element in the systemic integration of the two sub-systems.

The fast conversion of adenylates, and more generally mixed anhydrides aa-PEMAs, into NCAs at low concentrations of CO₂ in water questions the way through which the biochemical amino acid activation evolved. As a matter of fact, aa-AMPs, possibly produced from ATP through ribosome activity, would rapidly be converted into NCAs impeding the evolution of translation. Conversely, the catalytic activity of aaRSs might have evolved by acting on the thermodynamically favourable reverse reaction of aa-AMPs (formed spontaneously from NCAs) as a primitive pathway to produce ATP. One could argue that the NCA pathway of Fig. 3a is still active in living cells but this speculation is not supported by any experimental data. However, the mechanism of pretransfer editing of misactivated aaRSs (through which adenylates are hydrolyzed) remains uncertain. Any possible release of adenylates from the active site to solution during this step would lead to the formation of the corresponding NCA within seconds. Whatever NCA is actually or not a biochemical metabolite, the present results indicate that living organisms probably had to limit the importance of the release of adenylates into solution after translation evolved since a conversion into NCA would certainly lead to random aminoacylation of pending amino groups likely to be harmful to protein functional integrity. From this point of view, the N-formylation of methionine needed to initiate ribosomal peptide synthesis in bacteria might be considered as a remnant of a period in which NCA could be released in the cytoplasm. Therefore, we conclude that the potential formation of NCAs at least influenced the development of the translation apparatus and that of the aaRS family of enzymes in order to avoid random aminoacylation and that the NCA pathway must be taken into account in evolutionary studies.

Our analyses confirm the observations made by Lacey that CO₂ is a very efficient catalyst for the conversion of adenylates. However, taking into account the probable role of NCAs and the diversity of processes made available through their intermediary leads us to the very different conclusion that the process could be favourable to the development and evolution of life rather than solely detrimental to the role of adenylates as intermediates of peptide formation. It is also worth noting that acyl-aa-PEMA that were considered by Lacey as blocked equivalents of aa-AMP5,22,23 does actually not constitute models of the reactivity of their parent compounds since they also undergo a spontaneous cyclization into 5(4H)-oxazolone. The transient formation of 5(4H)-oxazolone intermediates may be responsible for their efficiency in peptide formation.20 The mixed anhydrides formed from free amino acids as well as peptide segments turn out to constitute unlikely precursors of peptides since their reactions are actually preceded by a very efficient cyclization into uncharged intermediates that thus constitute better electrophilic agents. This observation can be related to the evolutionary advantage of phosphate derivatives that is partly related to their negative charge reducing spontaneous hydrolytic degradation with respect to their enzyme-promoted reactions. From this perspective, their involvement required specific and efficient catalysts. However, the fact that NCA and 5(4H)-oxazolone also constitute precursors of mixed anhydrides through spontaneous processes provides a potential path through which these intermediates may have led for example to aminooxy esters of RNA at predisposed locations.25,30,36

**Methods**

Reagents and solvents were purchased from Bachem, Sigma-Aldrich, or Euriso-Top and used without further purification. Starting materials and products samples were prepared according to standard procedures and characterized by H, C and 31P NMR spectrometry and HRMS (Supplementary Information). NMR analyses were performed on a Bruker Avance 300 apparatus. HPLC analyses were performed on a Waters Alliance 2690 system with a photodiode array detector 996 using a Thermo Scientific BDS Hypersil C18 5 μm 2.1 × 50 mm column; mobile phase: A: H₂O + 0.1% TFA, B: CH₃CN + 0.1% TFA; flow rate: 0.2 ml/min and two different gradients; method A: 0 min (5% B), to 15 min (15% B), 25 min (60% B) and 26 min (100% B); method B: 0 min (5% B), to 10 min (20% B), 10 min (100% B). HPLC-ESI-MS analyses were carried out on a Waters Synapt G2-S system connected to a Waters Acquity UPLC H-Class apparatus equipped with an Acquity UPLC BEH C18, 1.7 μm 2.1 × 50 mm column; method C: A: H₂O + 0.01% formic acid, B: acetonitrile + 0.01% formic acid; flow rate: 0.5 ml/min; linear gradient 0% to 100% B over 3 min.
27. Brack, A., Ehler, K. W. & Orgel, L. E. N'-carbonylimidazole-induced diketopiperazine formation in aqueous solution in the presence of adenosine-5'-monophosphate. J. Mol. Evol. 8, 307–310 (1976).

28. Beaufils, D., Danger, G., Boiteau, L., Rossi, J.-C. & Pascal, R. Diastereoselectivity in prebiotically relevant 5(4H)-oxazolone-mediated peptide couplings. Chem. Commun. 50, 3100–3102 (2014).

29. Schall, O., Suzuki, I., Murray, C., Gordon, J. & Gokel, G. Characterization of Acyl Adenyl Anhydrides: Differences in the Hydrolytic Rates of Fatty Acyl-AMP and Aminoacyl-AMP Derivatives. J. Org. Chem. 63, 8661–8667 (1998).

30. Pascal, R. Catalysis through Induced Intramolecularly: What Can Be Learned by Mimicking Enzymes with Carbonyl Compounds that Covalently Bind Substrates? Eur. J. Org. Chem. 1813–1824 (2003).

31. Jencks, W. P. Binding energy, specificity, and enzymatic catalysis: the CIRCE effect. Adv. Enzymol. Relat. Areas Mol. Biol. 43, 219–410 (1975).

32. Albery, W. J. & Knowles, J. R. Evolution of enzyme function and the development of catalytic efficiency. Biochemistry 15, 5631–5640 (1976).

33. Pascal, R. Do enzymes bind their substrates in the ground state because of a physico-chemical requirement? Bioorg. Chem. 31, 485–493 (2003).

34. Tan, K. L. Catalysis: Temporary intramolecularly. Nat. Chem. 4, 253–254 (2012).

35. Beauchemin, A. M. Site-selective reactions: Exploiting intramolecularity. Nat. Chem. 5, 731–732 (2013).

36. Page, M. I. & Jencks, W. P. Entropic contribution to rate accelerations in enzymatic and intramolecular reactions and the chelate effect. Proc. Natl. Acad. Sci. U.S.A. 68, 1678–1683 (1971).

37. Lide, D. R. Handbook of Chemistry and Physics, 88th edition, 8–84 (CRC Press, Boca Raton, , FL, 2008).

38. Wollfenden, R. & Snider, M. J. The Depth of Chemical Time and the Power of Enzymes as Catalysts. Acc. Chem. Res. 34, 938–945 (2001).

39. Miller, F. J., Feistel, R., Wright, D. G. & McDougall, T. J. The composition of Standard Seawater and the definition of the Reference-Composition Salinity Scale 17. Deep-Sea Res. I 55, 50–72 (2008).

40. Reinhard, C. T. & Planavsky, N. J. Mineralogical constraints on Precambrian pCO2. Nature 474, E1–E2 (2011).

41. Rosing, M. T., Bird, D. K., Rossi, J.-C. & Pascal, R. Diastereoselectivity in prebiotically relevant 5(4H)-oxazolone-mediated peptide couplings. Chem. Commun. 50, 3100–3102 (2014).

42. Ruíz-Mirazo, K., Briones, C. & de la Escosura, A. Prebiotic systems chemistry: new perspectives for the origins of life. Chem. Rev. 114, 285–366 (2014).

43. Pascal, R. K. & Yarus, M. RNA-Catalyzed Amino Acid Activation. Biochemistry 40, 6998–7004 (2001).

44. Nordin, B. E. & Schimmel, P. Transiently Misacylated tRNA Is a Primer for Editing of Misactivated Adenylates by Class I Aminoacyl-tRNA Synthetases. Biochemistry 42, 12989–12997 (2003).

45. Westheimer, F. H. Why nature chose phosphate. Science 235, 1173–1178 (1987).

46. Her, S. & Kluger, R. Biomimetic protecting-group-free 2′,3′-selective aminocyaclation of nucleosides and nucleotides. Org. Biomol. Chem. 9, 676–678 (2011).

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Author contributions
Z.L., J.-C.R. and R.P. designed research; Z.L. performed research; D.B. contributed new reagents and analytic tools; Z.L., J.-C.R. and R.P. analyzed data and wrote the paper.

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