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The opposite effect of K^+ and Na^+ on the hydrolysis of linear and cyclic dipeptides

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ABSTRACT

Potassium and sodium are generally considered inert 'spectator' ions for organic reactions. Here, we report rate constants for the acid-promoted hydrolysis of the seven dipeptides of glycine (G) and alanine (A) and an unexpected pattern in how these rates differ in the presence of K^+ and Na^+. The linear dipeptides hydrolyze 12–18% percent slower in the presence of KCl versus an equal concentration of NaCl, while the cyclic dipeptides hydrolyze 5–13% faster in the presence of KCl (all P-values < 0.025). We believe this is the first report of a general organic reaction—here, amide hydrolysis—for which some substrates react faster in the presence of K^+ and others in Na^+. The results offer a potential reason for life's mysterious universal selection of intracellular potassium over sodium.

Introduction

While all living systems on Earth enrich K^+ from their external environments—and the vast majority of cells have higher intracellular concentrations of K^+ than Na^+—there exists no definitive explanation for this preference.[1–3] Features that are ubiquitous in biology are likely to have evolved early in the development of life, perhaps as early as the first living system.[4] Examples of potential biochemical 'fossils' from life's origin that have been the focus of origin-of-life research include lipid membranes,[5,6] nucleic acids,[7,8] proteins/polypeptides,[9–11] and common metabolic cycles.[4,12,13]

Another ubiquitous feature of life is the maintenance of ion gradients across cellular membranes.[14] In modern organisms, K^+ and Na^+ ion gradients are critical for a number of functions.[14,15] The high concentration of potassium in cells has led some to hypothesize that life may have developed in an environment with high levels of potassium.[3,16] However, the observation that cells can expend up to a third of their energy budget on Na^+/K^+-ATPase suggests the intracellular enrichment of potassium is important itself—not just a vestigial remnant of prebiotic conditions that would have faded as life evolved.[17] Still, the role of intracellular K^+ is not obvious.[18] Why did life choose K^+ when it could seemingly use intracellular Na^+ for the same purpose?

Few non-biological systems exist in which identical concentrations of potassium or sodium considerably change the properties of the system. Salts of K^+ and Na^+ often have different solubilities, and the presence of equal concentrations of KCl and NaCl can affect the solubility of organic compounds in aqueous solutions.[19] The only study we are aware of that reports a notable difference for an organic reaction in water in the presence of K^+ and Na^+ is for the coupling of glutamic acid mediated by carbonyldiimidazole.[20] Natochin and coworkers observed higher yields of oligo(glutamic acid) formation in the presence of K^+ versus Na^+. Motivated by this report and the premise that universal features of biochemistry may have been important to the origin of life, we decided to measure the influence of K^+ and Na^+ on reactions of peptides.

Experimental design

In selecting conditions for model prebiotic reactions, decisions must be made to balance historical relevance with experimental convenience. This section explains our choices regarding the design of experiments and any simplifications or assumptions that went into these decisions.

Selection of reaction for study

We elected to examine amino acids and peptides for their (i) obvious relevance to biology, (ii) historical interest to prebiotic chemistry, and (iii) well-studied structure and reactivity.[9] In aqueous solution, equilibrium favors the hydrolysis of peptides. And if we wish to understand the potential generation of protein biopolymers from the coupling of amino acids in water, we will have to understand the influence of hydrolysis as a competing deleterious side reaction.[21]

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Selection of substrates for study

We chose to focus our initial work on dipeptides in order to begin with the simplest system available. While a single amino acid can dimerize into only two dipeptides—the linear and cyclic forms—there are seven permutations when selection is expanded to two amino acids. The obvious candidates for study were dipeptides of glycine and alanine. Glycine (G) is the simplest amino acid. It is the smallest by mass, has no functional groups on its side chain, and is achiral. Alanine (A) is the next smallest amino acid by mass and has only a relatively inert methyl group for a side chain. G and A also appear to be the amino acids most relevant to prebiotic chemistry. A multi-factor analysis of 60 criteria by Trifonov concluded that G and A were the first two dominant amino acids in the chronology of evolution.[22] G and A are synthesized in the highest yields in most reported prebiotic syntheses of amino acids, including spark-discharge experiments.[9,23] G and A are also routinely the most abundant amino acids found on potential extraterrestrial impactors like meteorites, asteroids, and comets, which may have seeded early Earth from space.[11]

Cyclic dipeptides (2,5-diketopiperazines or DKPs, see Scheme 1) are of special relevance to origin-of-life chemistry as potentially important species in the construction and destruction of oligopeptides. Although conditions have been reported where direct attack of DKPs by the free amine of an amino acid can extend a growing peptide chain by two residues,[24] the formation of DKPs via intramolecular attack of the peptide backbone is a principal degradation route of short oligopeptides.[10,25] The absence of free amino and carboxyl groups in DKPs limit their participation in peptide coupling reactions unless they first hydrolyze, so DKPs are often invoked as "dead ends" or traps for amino acids that remove them from a pool of molecular building blocks.[26] High yields of DKPs—to the detriment of linear peptides—from amino acid precursors have been reported from a variety of experiments, including those with simulated prebiotic conditions.[25,27]

Selection of reaction conditions

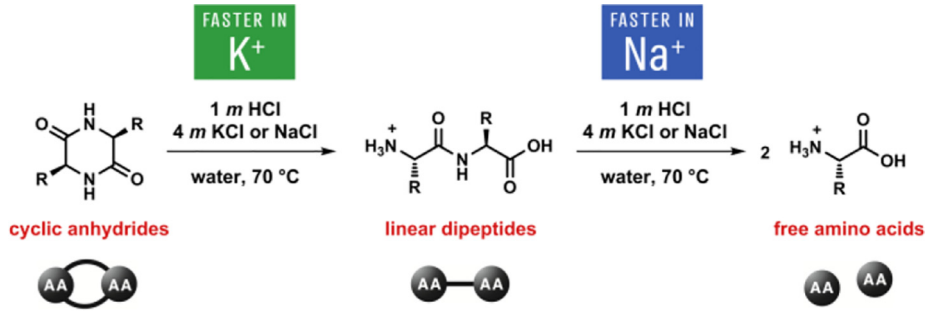
It is always preferable to match experimental conditions to those presumed to exist in the prebiotic landscape, but some concessions must be made for experimental convenience. We selected a temperature of 70 °C for the reactions to match the temperature of the Archean ocean inferred from geochemical analysis of chert minerals that date to ~3.5 Ga,[28] though that analysis and the question of the temperature of the early ocean are the subject of great debate. The concentration of the substrate was set to 50 millimolal (m*m*) and the concentration of salt was set to 4 molal (*m*). This concentration of salt allowed us to observe the maximum effect of the ions on the rate of hydrolysis, as 4 *m* approaches the limit of solubility of KCl and NaCl at 70 °C. We report components with relatively high concentration in molality to maintain consistent ionic strength and concentration (i.e., 4 M NaCl and 4 M KCl are not the same ionic strength, while 4 *m* NaCl and 4 *m* KCl are the same ionic strength).[29] The addition of 1 *m* HCl allowed reasonable reaction rates, resulted in pseudo-first-order kinetics,[30,31] and is more prebiotically relevant than alkaline conditions.[32,33] While high concentrations of acid and salts are of limited pertinence to extant biology, they are directly relevant to the 'drying lagoon' model for prebiotic chemistry in which condensation and hydrolysis reactions transpire in trapped, evaporating bodies of water on Prebiotic Earth.[34] Though the hydrolysis of amides at room temperature and neutral pH is notoriously slow,[31] conveniently, our hydrolysis experiments were complete in hours to days under these conditions.

While essentially no data exist regarding microenvironments on early Earth—including the possibility of environments that mimic the high acidity and heat of our study—there exists both geological and biological evidence that the early Earth was generally hotter and more acidic than today.[32,33] Several thermoacidophilic microorganisms are known to thrive in very acidic microenvironments on modern Earth, including those of the genus *Picrophilus*, which can grow near pH 0 and up to 65 °C.[35] The ability of these archaea to thrive in inhospitable environments thought reminiscent of the hot, acidic, volcanic settings on the early Earth suggests these organisms may be "primordial relics from which more complex life evolved."[35] There is also a theory that life evolved in an acidic, highly saline environment because the presumed "prebiotic set" of amino acids lacked amino acids with aromatic and basic side chains, and peptides composed of residues from this set likely restricted the possibility of folding to acidic and saline environments.[11,36]

We previously used NMR spectroscopy to measure rate constants for the hydrolysis of thioesters as a function of pH.[37] Here, we measure pseudo-first-order rate constants for the acid-promoted hydrolysis (k_a) of each substrate at [HCl] = 1 *m* and [KCl] or [NaCl] = 4 *m*. We define the starting material to have hydrolyzed after any net hydrolysis of peptide bonds is observed. A typical kinetics experiment involved removing aliquots of the reaction mixtures at various time points, diluting them with an equal volume of deuterium oxide, and collecting an ^{1}H NMR spectrum of the sample. The relative concentration of hydrolyzed substrate was determined by comparing the integration values of signals corresponding to the reactants and products (Fig. 1). Experimental details are provided as Supporting Information.

Results and discussion

The histograms in Fig. 2a and b depict the rates of hydrolysis for the seven dipeptides of G and A in 1 *m* HCl at 70 °C in the presence of 4 *m* KCl, 4 *m* NaCl, or no added alkali salt. We compared the rates



Scheme 1. Cyclic dipeptides—thought to be "dead ends" in the prebiotic synthesis of polypeptides—hydrolyse to form linear dipeptides. In turn, these acyclic compounds hydrolyse to form free amino acids. In our experiments, we examined the hydrolysis of the seven dipeptides formed from glycine (G, R = H) and alanine (A, R = CH_3) at 70 °C in 4 *m* KCl or NaCl, promoted by 1 *m* HCl.

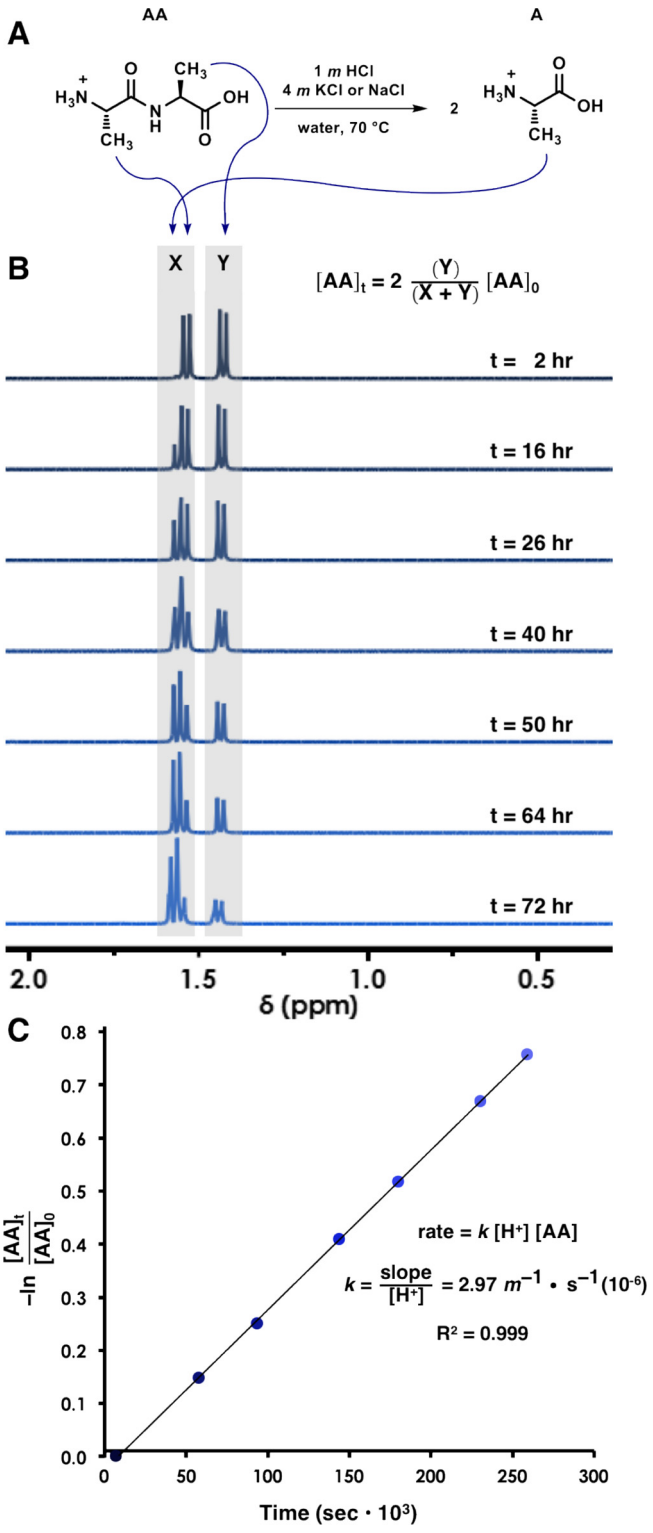


Fig. 1. Example measurement of the rate constant for the acid-promoted hydrolysis (k_a) of a dipeptide. The data shown correspond to the hydrolysis of alanylalanine (AA) under pseudo-first-order conditions, where $[H^+]$ is roughly constant. Aliquots of the reaction (A) are sampled at timed intervals and analyzed by NMR (B). The rate constant is determined from the slope of plot (C). Seven replicates of this experiment were completed for each of the seven dipeptide substrates.

for each substrate in 4 *m* KCl vs. 4 *m* NaCl with two-tailed *t*-test hypothesis testing and found all seven differences to be statistically significant with $P < 0.025$. The linear dipeptides all hydrolyzed slower in KCl (vs. NaCl), while the reverse was observed for the cyclic dipeptides. This stark 'flip' in relative rates based on structure is distinctly apparent in Fig. 2c.

The hydrolysis of peptides and other amides has been studied extensively, especially in strongly acidic solutions at elevated temperatures, as is the case in our study.[30,31,38] The rate-determining step of the acid-promoted mechanism is the water-assisted attack of water on the protonated carbonyl group of the amide undergoing hydrolysis.[38] The most straightforward explanation for the observed differences in rate would arise from differences of the thermodynamic activities of one or more of the molecules involved in the rate-determining step (water, proton, and substrate) caused by K^+ and Na^+.

For equal concentrations of chloride salts in aqueous solution, the activity of water is lower in NaCl vs. KCl (a_w = 0.85 in 4 *m* NaCl vs. 0.87 in 4 *m* KCl at 25 °C).[39] While this difference in water activity could ostensibly explain the faster hydrolysis of the cyclic substrates in KCl vs. NaCl, such an explanation is at odds with the observation that the linear dipeptides hydrolyzed slower in KCl.

In fact, this "flip" in the relative rates is a useful observation for several reasons with regard to elucidation of the mechanism(s) for dipeptide hydrolysis in the presence of the salts. The flip rules out that the difference is simply due to effects of the cations on the reactivity of water alone. While the activity of water must contribute to the observed differences in rate, there must be at least one other effect at play, else all the peptides—cyclic *and* linear—would hydrolyze faster in the presence of the same cation.

Proton/hydronium activities are particularly important, and they are known to vary significantly in aqueous solutions of different salts. The mean activity coefficient of HCl in 2 *m* KCl is 0.8 and in 2 *m* NaCl is 1.4.[40] So, proton activity is higher in concentrated NaCl vs. KCl, while water activity is higher in concentrated KCl vs. NaCl.

If the rate-determining step for the hydrolysis in the present system (at 1 *m* HCl, 4 *m* MCl, and 70 °C) happened to be in a regime where water activity were dominant for one substrate and proton activity were dominant for the other, the results could be explained. But kinetics studies showed both the linear and cyclic dimers to have rates first order in proton for hydrolysis in KCl and NaCl (Figs. S12 and S13).

It would be remarkable if the K^+ and Na^+ ions were directly involved in the mechanism(s) through binding to the substrates, as alkali cations are generally considered unreactive. But there are precedents for differential binding interactions between alkali ions and dipeptides.[41] We were not able to observe any such interactions by changes in chemical shift ($\Delta\delta$) or relaxation delay times (ΔT_1) of α-protons and methyl protons in the presence of KCl vs. NaCl (see Supporting Information). Specific ion interactions and effects on biological molecules have been extensively studied and are notorious for being poorly understood. This problem has existed since the 19th century, when Hofmeister described the impact of salts on the solubility of proteins.[42] Current models are generally inadequate at rationalizing the observed phenomena, and our results follow in this longstanding, frustrating tradition.[43,44] Experiments to uncover the mechanistic underpinnings of these initial results are ongoing.

Conclusion

In summary, we report a general organic reaction—here, peptide hydrolysis—for which the relative rates not only differ in the presence of K^+ vs. Na^+, but straddle unity, as cyclic substrates react faster in the presence of K^+ but linear substrates are faster in the presence of Na^+. The observed linear/cyclic flip raises a possible explanation for why the earliest living systems may have selected K^+ over Na^+: to assist the synthesis of oligopeptide chains by favor-

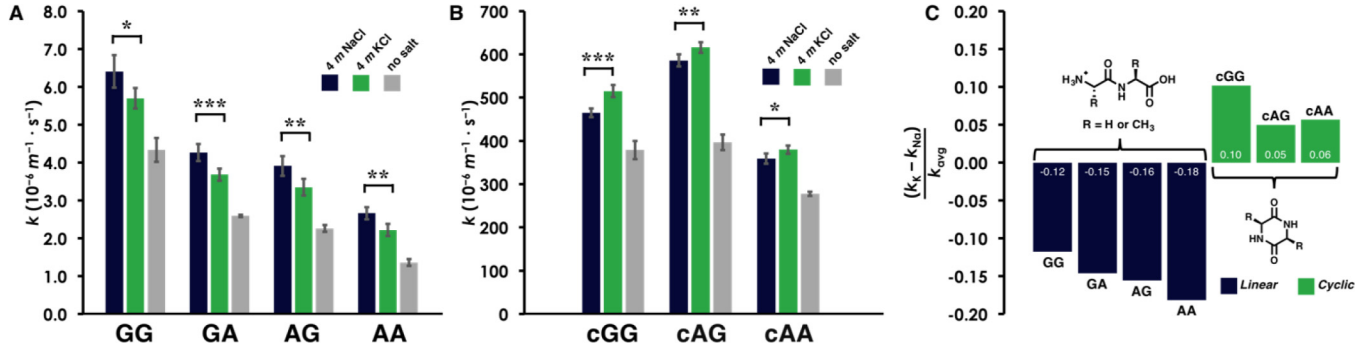


Fig. 2. (A) Histograms of the measured rate constants (k) for the acid-promoted hydrolysis of each linear dipeptide in the presence NaCl (navy), KCl (green), and the absence of additional salt (grey). (B) Analogous histograms for the cyclic dipeptides. (C) Histogram of the difference in k-values in KCl (k_K) vs. NaCl (k_{Na}) divided by their average for each substrate. Upward bars correspond to substrates that hydrolyse faster in KCl than NaCl. Downward bars correspond to substrates that hydrolyse faster in NaCl than KCl. The error bars in the first two histograms represent 90% CIs based on seven measurements. P-values are indicated with asterisks (* – $P \leq 0.025$; ** – $P \leq 0.01$, *** – $P \leq 0.001$).

ing increased rates of hydrolysis of DKPs and decreased rates of hydrolysis of linear peptides. In the presence of potassium versus sodium, dead-end DKPs are more rapidly returned to the pool of reactive linear dipeptides, while linear dipeptides are more slowly broken down into monomers. The coupling of amino acids into dipeptides and longer polypeptides represents an increase in molecular complexity. While the differences in rate in the presence of K^+ versus Na^+ are modest, they are statistically significant. And when given millions of years, small differences in rates of hydrolysis could have had a profound influence on the development of complexity on the Prebiotic Earth.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tetlet.2018.04.073.

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