Origins of Life and Evolution of Biospheres (2005) 35: 313–322

SODIUM CHLORIDE ENHANCED OLIGOMERIZATION OF L-GLUTAMIC ACID IN AQUEOUS SOLUTION

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(Received 5 May 2004; accepted 25 May 2004)

Abstract. The presence of NaCl was found to significantly enhance the formation of longer peptides in N,N'-carbonyldiimidazole induced oligomerization of L-glutamic acid in homogeneous aqueous solution. The enhancement was detected in the presence of as low as 0.01-M NaCl and the highest yield of longer oligomers was achieved in the presence of 1-M NaCl. The possible prebiotic relevance is discussed.

Keywords: L-glutamic acid, polyglutamic acid, oligomerization, peptides, sodium chloride, N,N'-carbonyldiimidazol

1. Introduction

Prebiotic polymers with lengths in the range of 30–60 monomers would be needed to get a self-replicating system started (Szostak and Ellington, 1993). However, oligomer formation in homogeneous aqueous solution rarely exceeds 10-mers (Ferris *et al*., 1996; Orgel, 1998). Therefore prebiotic catalysis, especially mineral catalysis, has been emphasized as essential for reactions leading to the origins of life (Lohrmann and Orgel 1973; Ochiai, 1981; Ferris, 1993; Orgel, 1998; de Duve, 2003). Long oligomers have indeed been observed in clay-catalyzed reactions (Ferris *et al*., 1996). Although alkaline metal ions are among the most abundant elements in nature and the major metal ions in living systems, they have not been the regarded as candidates for prebiotic catalysis because of the well established weak coordination of alkaline metal ions to most biological ligands and almost diffusion-controlled substitution rate ($\sim 10^9$–$10^{10} s^{-1}$) (Diebler *et al*., 1969).

The N,N'-carbonyldiimidazol (CDI) induced oligomerization of L-glutamic acid (L-Glu) has been used as a model reaction to simulate peptide formation on the primitive earth (Ferris *et al*. 1996; Hill and Orgel, 1996). This reaction typifies "the condensations that proceed via *N*-carboxyanhydrides and hence is relevant to reactions that involve the activation of an amino acid in the presence of carbon dioxide" (Brack, 1987; Liu and Orgel, 1998). Another advantage of this reaction is that the oligomers of L-Glu are the dominant products, therefore facilitating the characterization of the reactions. Here we present the preliminary account of the

NaCl-enhanced formation of longer oligomers of L-Glu. In view of the inevitable presence of the alkaline metal ions in the primitive oceans, the enhancement of NaCl is believed to be of prebiotic relevance.

2. Materials and Methods

The L-amino acids, N,N'-carbonyldiimidazole, Glu-Glu and the other major compounds were from Sigma. H-Glu(OMe)-OMe.HCl (L) was from Fluka. The oligomerization reactions were based on established procedures (Hill and Orgel, 1996). Unless specified, all reactions were carried out using a solution of L-amino acids at pH 8. The oligomerization was started by adding the solution of the amino acids (pH 8.0, 2°C) to twice excess of solid CDI. After quick vortexing and storage at 2°C for 5 min, the tubes were kept at room temperature (about 20°C) for 24 h. The samples were injected for analysis by HPLC (Hitachi L7100 pump with UV-VIS L7420 detector) on Zorbax Bio-Oligo (6.2 mm × 80 mm, 5 μm) using a NaCl gradient (2–80% B in 60 min; Buffer A: 20 % acetonitrile in 0.02-M NaH_2PO_4 at pH 7; buffer B is 2-M NaCl in buffer A). The pH of the amino acid solutions containing the salts was first adjusted to 8. The oligomerization and analysis of the samples containing the salts were carried out following the same procedures.

2.1. Separation and characterization of 3-mer, 4-mer, 5-mer, and 6-mer of L-Glu

The trimer, 4-mer, 5-mer, and 6-mer of L-Glu were separated by Bio-Oligo HPLC using an NH_4Ac gradient (Buffer A: 20% acetonitrile in 0.1-M NH_4HCO_3 at pH 7.4; buffer B 20% acetonitrile in 1-M NH_4HCO_3 at pH 8.3, Gradient 2– 80% B in 40 min). The concentrated fractions were lyophilized and then dialyzed for 8 h using Spectra/Por MWCO 1000. Satisfactory spectra were obtained in both $[M + H]^+$ and $[M - H]^-$ modes for the 3-mer and the 4-mer, and in $[M - H]^-$ mode for the 5-mer and 6-mer; 3-mer of L-Glu: calcd. for $C_{15}H_{23}N_3O_{10}$ MW 405, detected 428$[M + Na]^+$, 450$[M + 2Na - H]^+$, 472$[M + 3Na - 2H]^+$, 494$[M + 4Na - 3H]^+$, 404$[M - H]^-$, 426$[M + Na - 2H]^-$, 448$[M + 2Na - 3H]^-$; 4-mer of L-Glu: calcd. for $C_{20}H_{30}N_4O_{13}$ MW 534, detected 535$[M + H]^+$, 557$[M + Na]^+$, 579$[M + 2Na - H]^+$, 601$[M + 3Na - 2H]^+$, 623$[M + 4Na - 3H]^+$, 645$[M + 5Na - 4H]^+$, 533$[M - H]^-$, 555$[M + Na - 2H]^-$ and 577$[M + 2Na - 3H]^-$.

2.2. Determination of L-Glu after the oligomerization

The concentration of free L-Glu left after the oligomerization was determined by HPLC on Zorbax Bio-Oligo using an isocratic 2% buffer B (buffer A: 20% acetonitrile in 0.01-M NaH_2PO_4 at pH 5; buffer B is 0.5-M NaCl in buffer A). The elution was monitored at 200 nm. The relative error was 4.6%.

2.3. THE RATES OF PEPTIDE FORMATION WITH AND WITHOUT NaCl

The time course of peptide formation of 50-mM L-Glu with 100-mM CDI with and without 1-M NaCl at 20 °C was determined by Bio-Oligo HPLC. The products in 50-mM L-Glu with 100 CDI are short successive oligomers. Therefore the total peak areas of the 3-mers and longer oligomers detected by HPLC were used as the indication of the reaction rate. At the set time interval, the solution was analyzed for peptide formation by HPLC using Zorbax Bio-Oligo column.

2.4. THE IDENTIFICATION OF IMIDAZOLIDES OF L-Glu AND H-Glu(OMe)-OMe· · · HCl (L)

Previously, it has been demonstrated that *N*-[imidazolyl-(1)-carbonyl]-L-amino acids are relatively stable intermediates in CDI-induced peptide formation of amino acids in aqueous solution (Ehler and Orgel, 1976). In this reaction, Zorbax Oligo ion exchange HPLC detected a new peak after the addition of L-Glu solution into CDI, which decreased with time and finally disappeared after the completion of the oligomerization. The formation of *N*-[imidazolyl-(1)-carbonyl]-L-Glu was confirmed by ESI MS (*N*-[imidazolyl-(1)-carbonyl]-L-Glu; calculated for $C_9H_{11}N_3O_4$ MW 241, detected: 308$[M+3Na-2H]^+$, 593$[2M+5Na-4H]^+$ and 878$[3M+7Na-6H]^+$). The quantification of formation of imidazolylcarbonyl-L-Glu was achieved by HPLC using Zorbax Bio-Oligo column.

The solution of 100-mM H-Glu(OMe)-OMe (L) (pH 7.0) was prepared by adding adequate quantity of 1 M NaOH. After adding 1000-μL 50 mM H-Glu(OMe)-OMe (L) (2 °C) into 16.4 mg solid CDI and then kept at 2 °C for 5 min, a highly hydrophobic peak relative to H-Glu(OMe)-OMe.HCl (L) was detected using a reverse phase HPLC analysis (Alltech, Alltima 5μm, 4.4 mm × 250 mm; Buffer A: 0.02-M NaH_2PO_4 at pH 6.0, buffer B: 30% acetonitrile in buffer A, Gradient: 50% B to 100% B at 15 min. at flow rate of 1 mL/min); *N*-[imidazolyl-(1)-carbonyl-H-Glu(OMe)-OMe (L), calculated for $C_{11}H_{14}N_3O_4$ MW 269, ESI MS detected: 270$[M + H]^+$ and 292$[M+Na]^+$. The quantification of the formation and the hydrolysis of *N*-[imidazolyl-(1)-carbonyl]-H-Glu(OMe)-OMe (L) with and without 1 M NaCl was determined using reverse phase HPLC. The elution was monitored at 214 nm.

2.5. THE RATE OF THE DIMERIZATION OF 10 mM L-Glu WITH AND WITHOUT 1 M NaCl

50-mM L-Glu was activated first by adding 1000-μL 50-mM L-Glu into 16.4 mg of solid CDI, after standing at 2 ^{0}C for 5 min, 200-μL solution was transferred into 800-μL 125-mM L-Glu (pH 8) or 800-μL 125-mM L-Glu (pH 8) containing 1.25 M NaCl, the samples were then kept in a 20 °C water bath, every 15 minutes the samples were taken for analysis by Bio-Oligo HPLC.

3. Results and Discussion

The CDI-induced peptide formation in aqueous solution is a reaction which proceeds presumably through mixed anhydride intermediates (Ehler and Orgel, 1976; Hill and Orgel 1996). The typical experiments carried out at 20 °C using 50-mM L-Glu and 100-mM CDI give rise to a series of successive oligomers with 10-mer as the longest oligomer (Figure 1A). The assignment of the oligomers was based on coinjecting of the authentic glu-glu and the ESI-MS identified 3-mer, 4-mer, 5-mer and 6-mers with the oligomerization samples respectively. The assignment of the other oligomers was based on the one-charge increasing of oligomers on HPLC traces.

It was found that the presence of as low as 0.01-M NaCl increases the yield of the 6-mer and the longer oligomers slightly, as estimated from the peak areas of HPLC detectable traces of trimer and the longer peptides (Figure 1B). Further increasing the concentration of NaCl to 0.05-M, 0.1-M (Figure 1C) and 0.5-M gives increasing yields of 6-mer and longer oligomers, and decreasing yields of oligomers shorter

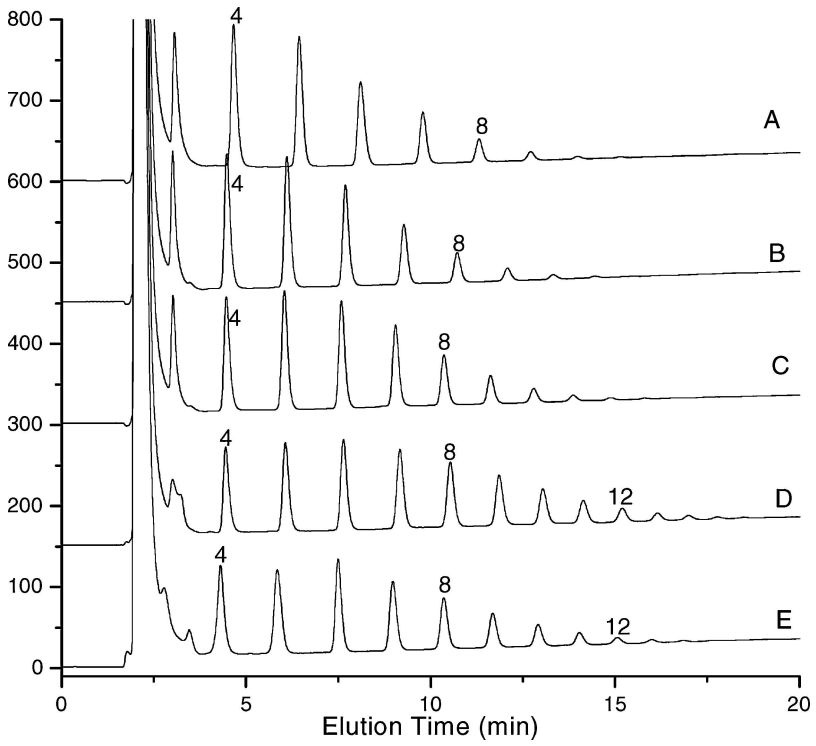

Figure 1. HPLC elution profiles of products from CDI-induced oligomerization of 50 mM L-Glu with different concentration of NaCl. A, Control reaction; B to E, eluates in the presence of 0.01, 0.1 M, 1 M and 3 M NaCl.

than 6-mer. The yields of longer peptides are optimal when the concentration of NaCl is 1-M (Figure 1D), where the longest oligomer detected is 15-mer, while 3-mer and 4-mer are about one-quarter and one-half, respectively, of the amounts in the absence of NaCl. The percentage yields of 8-mers and longer oligomers to total peak areas of the trimer and longer oligomers without NaCl was increased from 7.03% to 40.1% with 1 M NaCl. The yields of 10-mer and the longer oligomers are less than 0.2% of the total peak areas of 3-mer and longer oligomers without NaCl. However, in the presence of 1 M NaCl, the corresponding yield is 10.8%, 54 times more than that without NaCl. Increasing the concentration of NaCl to 2-M, 3-M (Figure 1E) and 4 M gives rise to slightly inhibited longer oligomer formation compared to that with 1M NaCl.

The enhancement is more evident when the concentration of L-Glu is low. When the concentration is reduced to 5 mM and 10 mM, the length of the longest oligomer is 4-mer and 6-mer respectively (Figures 2A and 2C). Following the similar pattern of 50-mM L-Glu oligomerization with NaCl, increasing the concentration of NaCl results in the formation of significantly more long peptides and less short peptides. The reaction of 5 mM and 10 mM L-Glu in the presence of 1-M NaCl gives 8-mer and 10-mer as the longest product (Figures 2B and 2D).

The presence of 0.1-M to 1-M Na_2SO_4, $NaNO_3$ and $NaClO_4$ gives rise to a similar pattern of enhancement as that with NaCl, suggesting the essential role of Na^+ in the enhancement. We also observed a similar pattern of enhancement by chlorides of Li^+, K^+ and Cs^+ to that of NaCl.

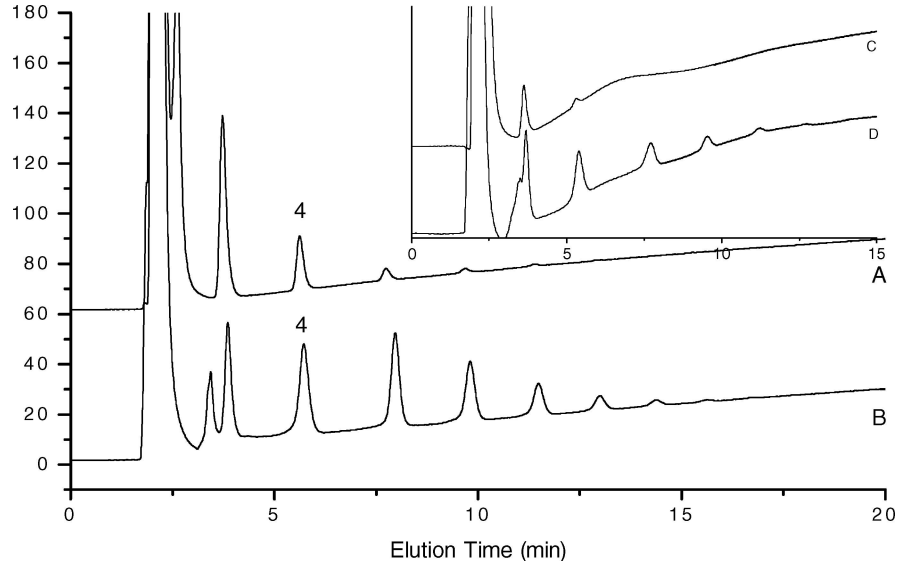

Figure 2. HPLC elution profiles of products from CDI-induced oligomerization of 10 mM L-Glu (A) and that in the presence of 1 M NaCl (B). Inset: Oligomerization of 5 mM L-Glu (C) and that in the presence of 1 M NaCl (D).

The total peak areas of 3-mer and the longer oligomers in the oligomerization of 5-mM, 10-mM and 50-mM L-Glu with 1-M NaCl are 4.1, 2.1 and 1.12 times, respectively of those without NaCl, indicating higher yields of peptide formation. Since carboxyl-activated dipeptides are not formed, the formation of 2,5-diketopiperazine does not occur to a significant extent (Ehler and Orgel, 1976). Therefore L-Glu left after the oligomerization is the other indication of the transition rate of L-Glu to peptides. The L-Glu left after the oligomerization was found to decrease with the increase of NaCl concentration. From Figure 3, 91% of the input 50-mM L-Glu was converted to peptides without NaCl. The presence of 1 M NaCl results in 95% transformation. In the presence of 1-M NaCl, about 86% of the input 10-mM L-Glu was converted to the peptides. However only 61% of the input 10-mM L-Glu is converted without NaCl.

The enhancement of NaCl was found to be fully applicable to the oligomerization of L-aspartic acid. The rate of peptide formation was also determined by HPLC. From Figure 4 it is clear that the rate of peptide formation with 1-M NaCl was about two times of that without NaCl.

The pH of the solution of 50-mM L-Glu (pH 8.0) without salts determined at 10 min after the CDI was added, was 7.71. The pHs of the solutions in presence of 0.1 – 4 M NaCl determined at 10 min after the CDI was added were found to be 7.81 – 8.03. We eliminated the effect of the slight pH difference of the solutions with NaCl as the cause of the longer peptide formation by showing that the yields

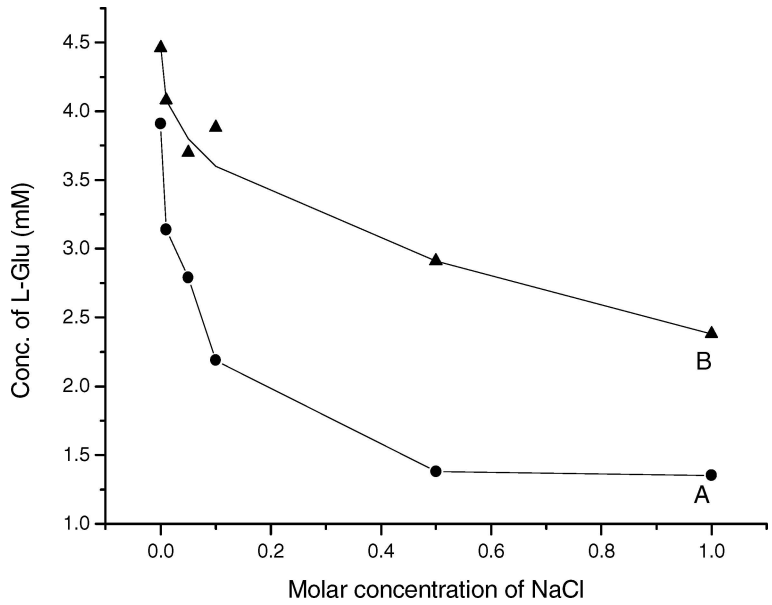

Figure 3. The molar concentration of L-Glu left after the oligomerization of 10 mM L-Glu with CDI (A) and 50 mM L-Glu (B) in the presence of different concentrations of NaCl.

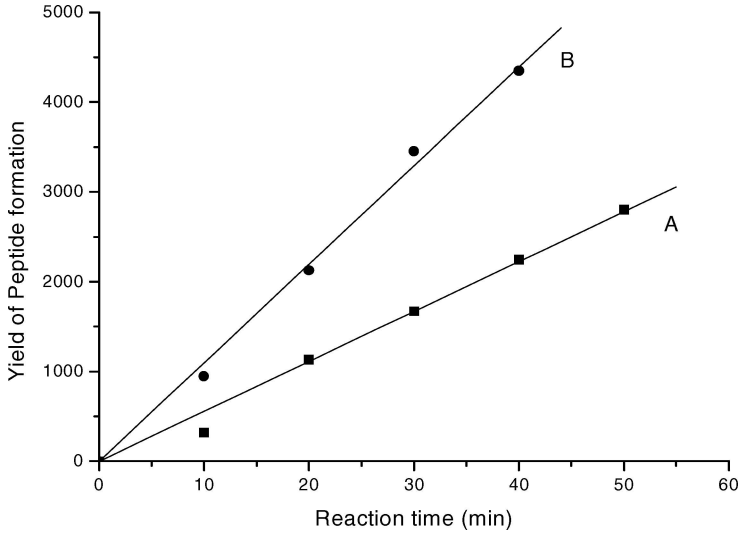

Figure 4. The time course of the formation of the trimers and longer oligomers of 50 mM L-Glu with CDI (A) and that with 1 M NaCl (B).

of peptides of 50-mM L-Glu at pHs from pH 6 to pH 9 are quite similar in both the yield and the pattern of the oligomer formation.

The enhancement of NaCl on the oligomerization of L-Glu is dependent on the concentration of NaCl. It was noted that the presence of 0.1-M $MgCl_2$ results in similar pattern of slight enhancement of oligomer formation to that without $MgCl_2$, with 11-mer as the longest peptide, one unit longer than that without $MgCl_2$ in 50-mM L-Glu oligomerization, in accordance to the previous report (Hill and Orgel, 1996). However increasing the ionic strengths(to 0.25M and 0.5-M) results in the slight inhibition of peptide formation compared to that with 0.1-M $MgCl_2$. The presence of 0.5-M $NaHCO_3$ or 0.5-M NaH_2PO_4at pH 8 gives rise to trivial yields of 3-mer or 5-mer as the longest oligomers, respectively. However, the corresponding oligomerization in the presence of 0.1-M $NaHCO_3$ and 0.1-M NaH_2PO_4produced 8-mer as the longest peptide. The oligomer formation was eliminated by the presence of 1-M NH_4Ac and 1-M NH_4Cl. Therefore the ionic strength of the solutions appeared not to be the major factor responsible for the NaCl enhancement of the CDI-induced oligomerization of L-Glu either.

The oligomerization of 50-mM L-Glu gives the same results whether the addition of NaCl is before or after the activation of L-Glu by CDI. By comparing the HPLC peak areas of *N*-[imidazolyl-(1)-carbonyl]-L-Glu in 50-mM L-Glu with 100-mM CDI reaction with and without 1-M NaCl, we did not observe significant difference in the yield of *N*-[imidazolyl-(1)-carbonyl]-L-Glu. This is strengthened by the results from the reaction of 50-mM H-Glu(OMe)-OMe (L) with 100 CDI. Based on the peak areas of *N*-[imidazolyl-(1)-carbonyl]-Glu(OMe)-OMe (L) formed in 50-mM

H-Glu(OMe)-OMe (L) with 100-mM CDI reaction, the yields of *N*-[imidazolyl-(1)-carbonyl]-Glu(OMe)-OMe (L) with and without 1-M NaCl was found to be very similar. Also, by monitoring the hydrolysis of *N*-[imidazolyl-(1)-carbonyl]-Glu(OMe)-OMe (L) with and without 1-M NaCl with time, no significant difference was detected. Therefore the formation and the hydrolysis of *N*-[imidazolyl-(1)-carbonyl]-L-Glu, the intermediate form of L-Glu, are not obviously affected by the presence of 1-M NaCl.

In order to further clarify, if the stability of the intermediates is affected by the presence of NaCl, the dimer formation from a system composed of 800-μL 125-mM L-Glu (pH 8) and 200 μL solution from 50 mM L-Glu with 100 mM CDI reaction was determined to see if the rate constant of the dimerization with 1 M NaCl is different from that without NaCl. Figure 5 demonstrated that the rate of the dimerization with and without 1-M NaCl was actually the same. Therefore, once again it is demonstrated that the enhancement in peptide formation of L-glutamic acid due to NaCl, is not related to the formation of the *N*-[imidazolyl-(1)-carbonyl]-L-Glu, nor to the stability of the intermediates. It is hypothesized that the weak interaction of Na^+ with the short peptides is responsible for the longer peptide formation.

It is known that Na^+ mainly binds to oxygen ligands and the binding is electrostatic in origin. Because of the weak coordination of Na^+ on most biological ligands and the almost diffusion-controlled substitution rate of water ($\sim 10^9$–10^{10} s^{-1}) (Diebler et al., 1969), such a mechanism would seem to be unlikely. However,

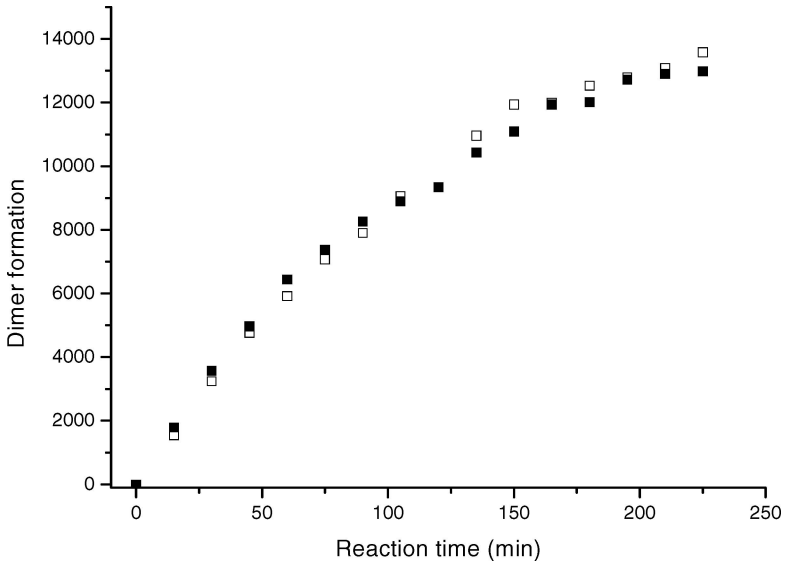

Figure 5. The dimerization of 10 mM L-Glu with (■) and without (□) 1 M NaCl.

the characteristic prerequisite for high concentrations of Na^+ for the optimal enhancement is probably the factor facilitating the binding of Na^+ and the observed effect.

It should be noted that the "salt effect" of $CuCl_2$ and NaCl on peptide formation in solutions below pH 3 and at concentrations above 3 M is essentially owing to complex formation of the amino acid with Cu^{2+}, Cl^- and H_2O (Rode, 1999; Rode and Suwannachot, 1999). The Na^+ was assumed to be the enhancing factor for peptide formation because in the presence of high concentrations of Na^+ (above 4 M), the unsaturated coordination positions around Na^+ could enhance the peptide formation. We found that the oligomerization of L-glutamic acid at pH 2.8 both with and without 1 M NaCl is very limited compared to that at pH 8.0.

Although the alkaline metal ions have not been thought to have played a role in prebiotic catalysis, the NaCl enhanced oligomerization of L-Glu presented here, combined with the inevitable presence of Na^+ and K^+ in the primitive ocean, as well as perhaps even higher concentrations in some localized areas, suggests the possible prebiotic relevance of this phenomenon.

Acknowledgments

We thank the helpful suggestions of my colleague Prof. Pan XM and the reviewers. The financial support from the Presidential Foundation of the Institute of Biophysics (01CA32), Chinese Academy of Sciences is also acknowledged.

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