CATALYSIS OF DIALANINE FORMATION BY GLYCINE IN THE SALT-INDUCED PEPTIDE FORMATION REACTION

Dedicated to Professor Dr. K. E. Schwarzhans

YUTTANA SUWANNACHOT and BERND M. RODE
Institute for General, Inorganic and Theoretical Chemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria

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Abstract. Mutual catalysis of amino acids in the salt-induced peptide formation (SIPF) reaction is demonstrated for the case of glycine/alanine. The presence of glycine enhances dialanine formation by a factor up to 50 and enables dialanine formation at much lower alanine concentrations. The actual amounts of glycine play an important role for this catalytic effect, the optimal glycine concentration is 1/8 of the alanine concentration. The mechanism appears to be based on the formation of the intermediate Gly-Ala-Ala tripeptide, connected to one coordination site of copper(II) ion, and subsequent hydrolysis to dialanine and glycine.

1. Introduction

Several pathways for peptide formation under possible prebiotic reaction conditions have been discussed (Steinman and Cole, 1967; Rabinowitz *et al*., 1969; Flores and Leckie, 1973; Hulshof and Ponnamperuma, 1976; Fox and Dose, 1977; Lahav *et al*., 1978; Rishpon *et al*., 1982; Yamanaka *et al*., 1988; Yanagawa *et al*., 1990). The recent discovery of a novel peptide formation reaction has established the most simple way so far (Schwendinger and Rode, 1989; Rode and Schwendinger, 1989; Schwendinger and Rode, 1991; Schwendinger and Rode, 1992). This 'Salt-Induced Peptide Formation (SIPF)' reaction needs only high concentrations of NaCl in aqueous solution and Cu(II) ions, the most abundant transition metal ion found in seawater (Cloud, 1973; Hay, 1984), as catalyst for the condensation of amino acids to peptides. Moderate temperatures (ca. 80–90 °C) produce considerable yields of dipeptides with all amino acids investigated so far (Saetia *et al*., 1993). Investigations of the reaction mechanism by potentriometric and spectrophotometric methods (Tauler and Rode, 1990), Monte Carlo simulations (Rode, 1992) and ab initio calculations (Liedl and Rode, 1992), have shown that the active species enabling the reaction is an amino acid-monochlorocuprate complex (Eder and Rode, 1994).

This postulated mechanism gives the explanation for the primary peptide formation; however, in binary or more complicated systems, various peptides are produced apparently by follow-up or side reactions, e.g. by acid-catalyzed sequence inversion (Schwendinger and Rode, 1992). A previous study (Saetia *et al*., 1993)

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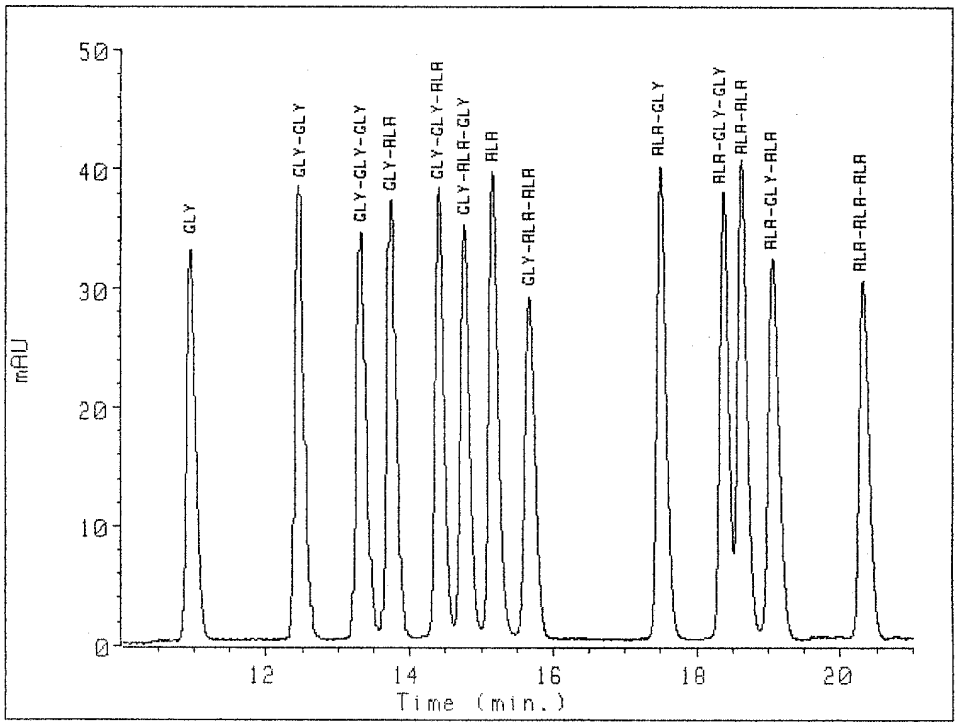

Figure 1. The elution profile of standard amino acids and peptides in the system of glycine/alanine using derivatisation method.

has shown that from optically pure alanine (160 mM), besides L-L or D-D dialanine, also 1.97 and 1.65% of L-D/D-L dialanine are obtained starting from L-Ala and D-Ala, respectively. Better overall dialanine yields were generally achieved with L-Ala (4.04% at the best) than with D-Ala (3.51% at the best).

Glycine is the simplest amino acid and in many aspects the most reactive one. Due to its easy formation from simple molecules in the gas phase, glycine should have been present, wherever alanine was formed. The main idea of the current study was to investigate the role of glycine in the reactivity of alanine in the SIPF reaction. It has been observed that dialanine is formed only in low yields (1.6% at the best) from an equimolar 490 mM glycine/alanine system with constant volume (Schwendinger and Rode, 1991), a 40 mM equimolar Gly/Ala mixture subjected to evaporation cycles gave 1.27% of dialanine (Saetia *et al.*, 1993). Some recent investigations with mixed amino acid system have indicated, however, that homodipeptides can be formed in better yields in the presence of some other amino acids (Yongyai, 1995).

In order to obtain a clearer picture of the mutual influence of amino acids on homodipeptide formation it seemed necessary, therefore, to investigate the behaviour of mixed systems with varying concentrations in more detail.

Table I
The effect of glycine on dialanine formation in the Salt-Induced Peptide Formation reaction

Experiment number	Alanine (mM)	Glycine (mM)	Percent yield Ala_2	Gly-Ala (mM)	Ala-Gly (mM)
1	40.0	0.0	0.14	–	–
		0.1	0.16	nf	nf
		0.5	0.23	nf	nf
		1.0	0.54	nf	nf
		3.0	4.67	trace	nf
		5.0	6.90	0.14	0.08
		10.0	5.69	0.10	0.10
		20.0	2.70	0.17	0.10
		30.0	1.90	0.32	0.36
		40.0	1.21	0.26	0.34
		60.0	0.95	0.34	0.52
		80.0	0.87	0.34	1.34
2	20.0	0.0	nf	–	–
		5.0	5.67	trace	0.07
3	10.0	0.0	nf	–	–
		2.0	5.70	0.03	0.03
		5.0	4.17	0.14	0.07
4	5.0	0.0	nf	–	–
		5.0	3.75	0.08	0.06

Yields are given in % initial amino acid concentration.
nf = Not found.

2. Experimental

2.1. Reaction system

Amino acids and peptides were obtained from Sigma Chemical Company and Senn Chemical Company, other reagents from Fluka Chemical Company in *pro analysi* quality and used without further purification. Experiments were performed as wetting-drying cycles attempting to mimic tidal or laguna processes in a primitive earth environment, with aqueous solutions containing alanine (40 mM), copper(II) chloride (40 mM) and NaCl (500 mM). A varying amount of glycine was added to this solution (from 0.1 mM up to 80 mM). The conditions for all experiments are given in Table I. 1.0 mL of the solutions was heated in glass vials at 85 °C to complete evaporation within 24 hr (one cycle) and the residue was then taken up with 1.0 mL distilled water and subjected to the next cycle. Analysis of the systems

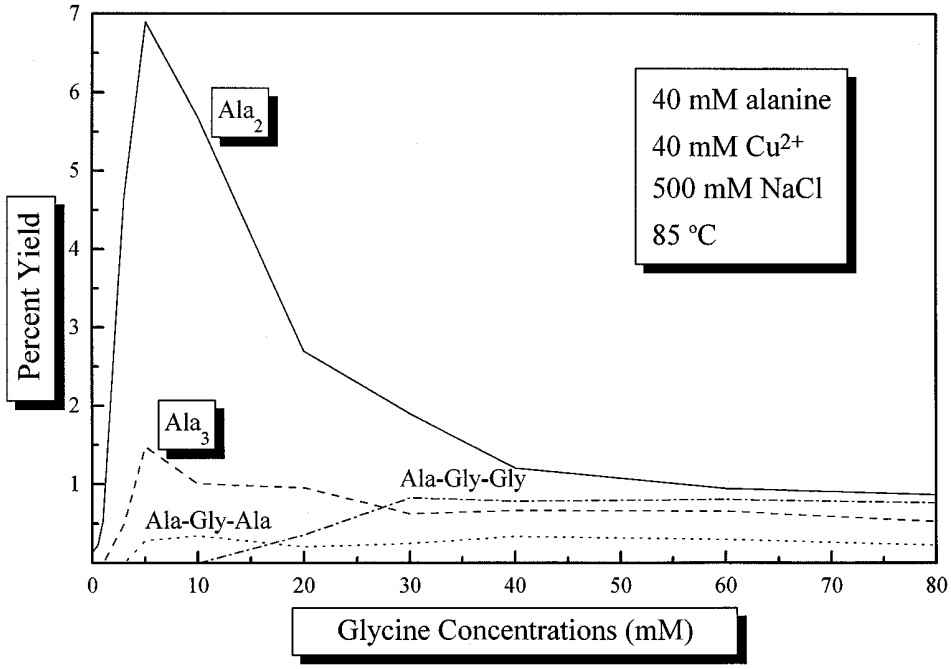

Figure 2. The effect of glycine on dialanine and tripeptide formation at constant 40 mM alanine concentration. Each experimental point is the average result after 7 heating/drying cycles of at least two experiments.

was performed after 7 cycles. At least 2 independent experiments were carried out and the reported results are the average values.

2.2. ANALYTICAL METHODS

High Performance Liquid Chromatography (HPLC) using a Hewlett Packard 1090 LC apparatus with diode array detector was used to analyze all samples by means of comparison of peak retention times to those of authentic samples and by UV-VIS spectroscopic identification. Both direct method and derivatisation method with OPA/MPA were applied according to Schuster (Schuster, 1988).

The best conditions to analyse samples containing di- and tripeptides in the Gly/Ala system were determined as following: derivatives with *ortho*-phthaladehyde/3-mercaptopropionic acid reagent (OPA/MPA) were injected into an AminoQuant column (5 μm/200 $\times$ 2.1 mM) and detection was performed at 338 nm. The mobile phase consisted of **solvent A**; 30 mM NaOAc·$3H_2O$ + 0.1 mM EDTA + 0.2% THF, **solvent B**; 20 mM NaOAc·$3H_2O$ + 0.1 mM EDTA + 80% acetonitrile – at a flow rate of 0.55 mL min^{-1} and a column temperature of 35 °C. Gradients were applied with 0–3 min: 0% B, 3–21 min: 0–15% B, 21–22 min: 15% B, 22–25 min: 15–0%

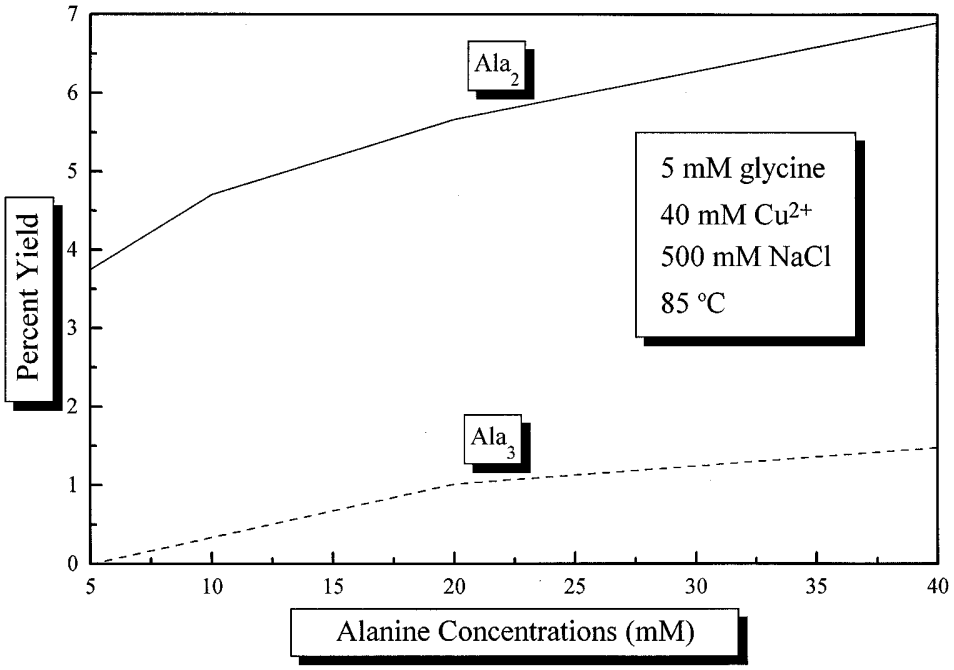

Figure 3. The effect of glycine on di- and trialanine formation at varying alanine concentration. Each experimental point is the average result after 7 heating/drying cycles of at least two experiments.

B. An illustration of a chromatogram using these conditions for the Gly/Ala system is provided in Figure 1.

3. Results and Discussion

Table I shows dialanine (Ala_2) yields (together with Gly-Ala and Ala-Gly) resulting from 40 mM alanine in the presence of different glycine concentrations (experiment number 1) and the yields obtained at constant glycine concentration of 5 mM decreasing the alanine content from 20 to 10 and 5 mM (experiments 2, 3 and 4, respectively).

3.1. 40 mM ALANINE

The results obtained from 40 mM alanine in the absence of glycine confirm that alanine itself hardly dimerizes to Ala_2 (0.14%). $(L\text{-}Ala)_2$ is generated exclusively at this concentration. The comparison with previous results from 160 mM pure alanine (Saetia *et al.*, 1993) indicates that although 40 mM of starting alanine produces a relatively lower yield, it preserves the L conformation in the assembly of peptide. The low yield of Ala_2 indicates that this direct formation in the SIPF reaction is rather difficult, e.g. compared to Gly_2 formation.

Table II

Percent yields of tripeptide products obtained from glycine/alanine system in the presence of glycine in the Salt-Induced Peptide Formation reaction

Exp.	Starting AA (mM)		Tripeptide amount in % of AA				
No.	Ala	Gly	Gly-Gly-Gly	Gly-Gly-Ala	Ala-Gly-Gly	Ala-Gly-Ala	Ala-Ala-Ala
1	40.0	0.1	nf	nf	nf	nf	nf
		0.5	nf	nf	nf	nf	nf
		1.0	nf	nf	nf	nf	nf
		3.0	nf	nf	nf	nf	0.50
		5.0	nf	nf	trace	0.29	1.48
		10.0	nf	nf	trace	0.35	1.01
		20.0	nf	trace	0.36	0.21	0.96
		30.0	trace	trace	0.83	0.25	0.63
		40.0	trace	trace	0.79	0.34	0.67
		60.0	0.15	trace	0.81	0.30	0.66
		80.0	0.12	trace	0.77	0.23	0.53
2	20.0	5.0	nf	trace	trace	trace	1.02
3	10.0	2.0	nf	trace	nf	trace	0.50
		5.0	nf	trace	trace	0.25	0.34
4	5.0	5.0	nf	nf	nf	nf	trace

Yields are given in % initial amino acid concentration.
nf = Not found.

In the presence of glycine, however, considerably higher Ala_2 yields are achieved in all cases reported here. Glycine apparently plays a catalytic role in Ala_2 formation in the framework of the SIPF reaction. As little as 0.1 mM glycine seems to be effective to induce some yield increase, at 0.5 mM the effect becomes strong. Both heteropeptides were monitored in order to get possible indications towards the pathway (Gly-Ala and Ala-Gly yields are given in the last 2 columns of Table I). Gly_2 is also observed at all glycine concentrations above 10 mM, but not reported, as it should not have any influence on Ala_2 formation. According to the formation constant of Cu(II) with Gly-Ala (Kittl and Rode, 1981, 1983), any Gly-Ala formed by the SIPF reaction should be bound to Cu(II) at the lowest glycine concentrations, explaining why free Gly-Ala is not detected.

3.0 mM glycine drastically increases the yield of Ala_2 to nearly 30 times the amount of Ala_2 obtained from pure alanine. Gly-Ala appears in traces only in the first cycle, after which it completely disappears, indicating that Gly-Ala may be related to the production of Ala_2 through an intermediate compound.

The yield of Ala_2 reaches the maximum (6.90%) when 5 mM glycine (1/8 of alanine) is added, and in the first cycle, Gly-Ala is found. In all further cycles

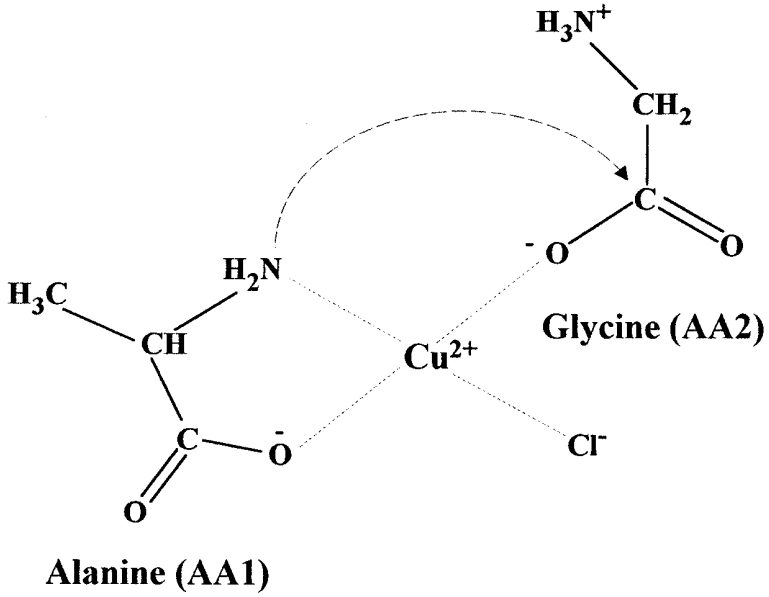

Figure 4. Binding of alanine and glycine in the Salt-Induced Peptide Formation reaction.

Ala-Gly dominates, probably due to the sequence inversion process (Schwendinger and Rode, 1992). Since Ala-Gly is rather stable against hydrolysis, it is present throughout the observation period. Gly_2 occurs in trace amounts in a few cycles.

Ala_2 is decreasing relatively at glycine concentrations above 5 mM, as glycine seems to compete then with alanine molecules in Cu(II) complexation. At 10 mM, Ala_2 yield is still high (5.69%), whereas 20 mM reduces the yield considerably (2.70%). Upon further increase of the glycine content to 30, 40, 60 and 80 mM, the excess glycine inhibits Ala_2 formation. In addition, Gly-Ala seems to persist for longer times and in higher amounts, but always disappears after the fourth cycle. Ala-Gly is increasing at the same time due to the aforementioned inversion reaction process. The overall effect of glycine on Ala_2 formation is illustrated in Figure 2.

3.2. VARYING ALANINE CONCENTRATION IN THE PRESENCE OF 5 MM GLYCINE

Alanine concentrations lower than 40 mM produce no Ala_2 at all without the addition of glycine. 5 mM glycine was added to solutions containing 20, 10 and 5 mM alanine (experiments 2, 3 and 4, respectively), as 5 mM glycine gave the best yield for 40 mM alanine solution. The yield obtained with 20 mM alanine is very high and similar to 40 mM alanine solution with the same Gly/Ala ratio. Ala-Gly appears from the second cycle on, indicating that glycine seems to be more than sufficient to produce the catalytic effect. For 10 mM alanine, an additional experiment with 2 mM glycine was done in order to observe differences in Ala_2 yields compared with 5 mM glycine. The results confirm again that the production

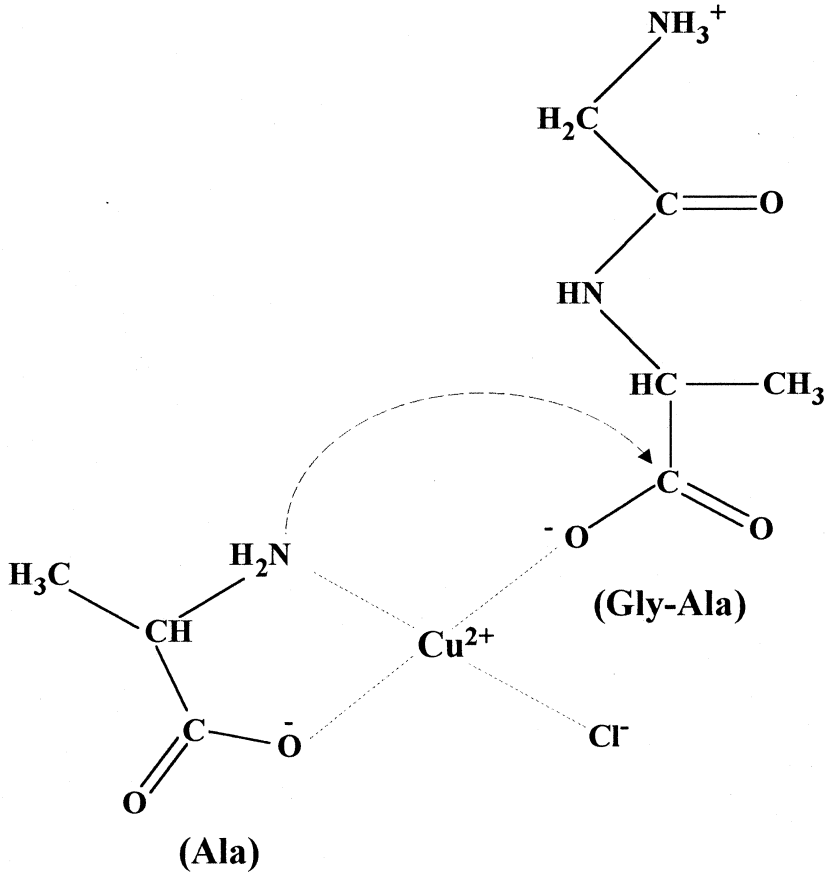

Figure 5. Binding of glycylalanine and alanine in the Salt-Induced Peptide Formation reaction.

of Ala_2 is most favoured by a ratio 1:5. However, a ratio of 1:1 (5 mM Ala/5 mM Gly) still shows considerable enhancement to 3.75% yield. Ala_2 formation at varying alanine concentrations is shown in Figure 3.

3.3. TRIPEPTIDE FORMATION

Table II shows the yields of tripeptides in all investigated Gly/Ala systems. At 40 mM alanine concentration (experiment 1), no tripeptides are formed when the glycine concentration is below 3.0 mM. Trialanine (Ala_3) is produced exclusively in considerable yield (0.50%) at 3.0 mM glycine concentration, most likely due to high Ala_2 presence. The maximum Ala_3 yield (1.48%) is observed at 5.0 mM glycine (as for Ala_2) and in addition, Ala-Gly-Ala is formed in good yield, whereas Ala-Gly-Gly is detected only in trace amounts. Figure 2 displays the formation of Ala_3, Ala-Gly-Gly and Ala-Gly-Ala. The percent yield of Ala_3 decreases slowly at glycine concentrations above 5 mM, whereas Ala-Gly-Gly and Ala-Gly-Ala seem

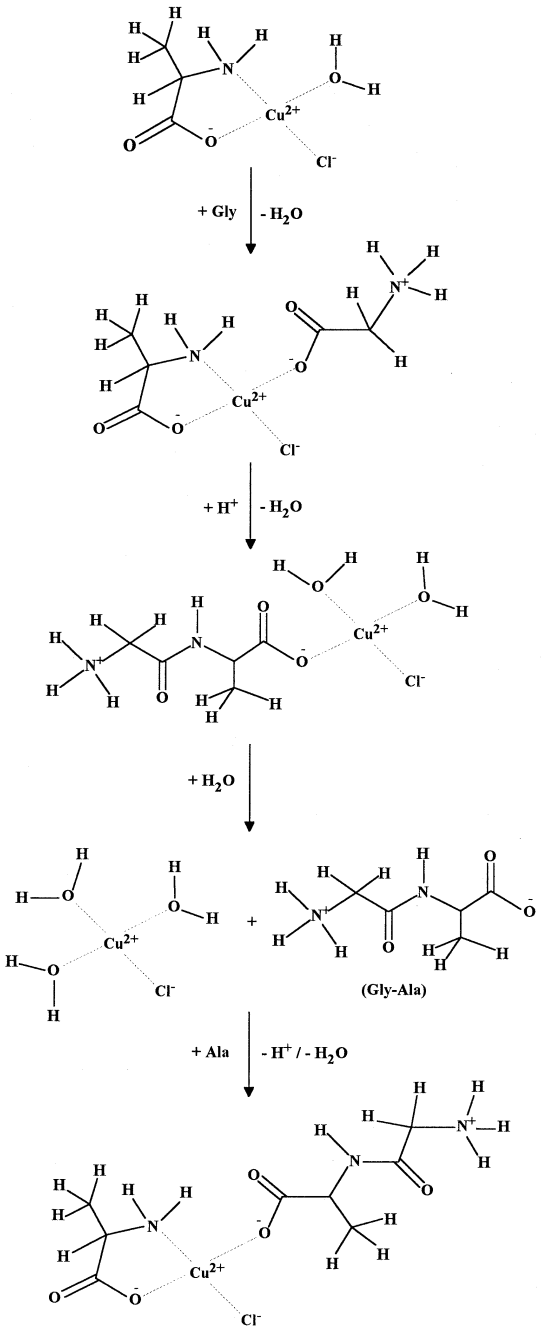

Figure 6. Reaction scheme for dialanine formation with glycine catalysis.

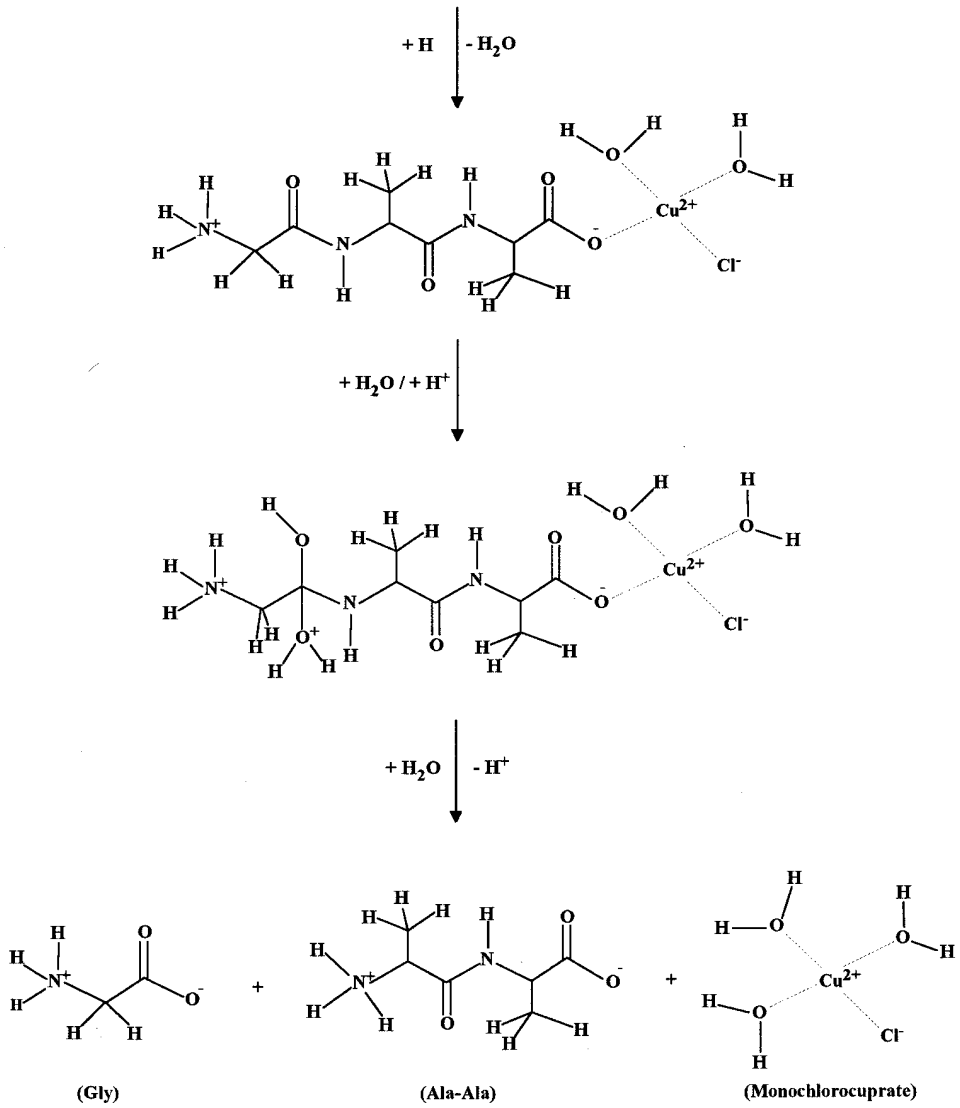

Figure 6. Continued

to remain rather constant. Ala-Gly-Ala is formed more easily than Ala-Gly-Gly but Ala-Gly-Gly dominates at high glycine concentrations. Gly-Ala-Gly and Gly-Ala-Ala do not appear at all, leading to the assumption that Gly-Ala-Ala could represent only an intermediate in the formation of Ala_2. Small amounts of Gly_3 and Gly-Gly-Ala are only produced in systems with high glycine concentrations.

Alanine concentrations under 40 mM (experiments 2, 3 and 4) produce some of the aforementioned tripeptides in trace amounts except for Ala_3 which is formed to

a considerable extent, and in traces even at the lowest concentration. Figure 3 also shows the percent yields of Ala_3 as a function of varying alanine concentration.

3.4. THE MECHANISM OF CATALYTIC ACTIVITY

The influence of glycine in catalysing Ala_2 production can be interpreted via the mechanism of the salt-induced peptide formation reaction (Eder and Rode, 1994): one amino acid (AA1) is chelate-bound in a monochlorocuprate complex, whereas another one (AA2) binds to Cu(II) only via its carbonyl function. The nitrogen atom of the chelating AA attacks the carbonyl carbon of the second AA, and a peptide bond is formed. In the case of glycine/alanine system, according to the order of nucleophilicity determined by the side-chain (Schwendinger and Rode, 1992), alanine is a better nucleophil and hence likely to act as the chelating molecule, i.e. as AA1. Glycine, therefore, acts as AA2, leading to the formation of Gly-Ala (Figure 4).

When a peptide and an amino acid are binding to Cu(II), the peptide always acts as AA2 (Rode *et al.*, 1993). Hence, Gly-Ala can form the tripeptide Gly-Ala-Ala (Figure 5), which, however, seems to be only an intermediate, as it is not found in the solutions. The most obvious explanation for this is a subsequent fast hydrolysis, producing Ala_2+ glycine. This reaction could occur, while the tripeptide is still connected to Cu(II) ion, before the newly formed tripeptide is released to the solution. Instead, Ala_2 and glycine are released, and glycine can re-act as partner for further alanine molecules. A schematic representation of this mechanism is given in Figure 6.

4. Conclusions

The formation of Ala_2 is clearly enhanced by the presence of glycine, compared to solutions containing alanine alone. Since glycine is released again to the solution after having played its helping role in Ala_2 synthesis, its effect can be clearly classified as catalytic. This catalytic effect does not only guarantee higher yields, but also enables the formation of Ala_2 starting from much lower alanine concentrations. The latter effect may have played a considerable role in prebiotic peptide evolution, where amino acid concentrations could have been quite low. The results reported here, therefore, encourage investigation of further possibilities of mutual catalysis of amino acids in the Salt-Induced Peptide Formation reaction.

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