HPLC and electrochemical investigations of the salt-induced peptide formation from glycine, alanine, valine and aspartic acid under possible prebiotic conditions

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(Received March 30, 1992)

Abstract

Peptide formation by the recently discovered salt-induced mechanism, the simplest possible prebiotic peptide generation process so far, has been studied by the example of some amino acids known to form by Miller or Fischer-Tropsch mechanisms in simulated primitive atmosphere experiments. Valine and aspartic acid readily form peptides with glycine; self-condensation is observed for all acids except valine. Several findings give further indications towards the possible prebiotic relevance of the reaction: the observed preferential formation of peptide linkages, the shift from 'nature-irrelevant' to 'nature-relevant' peptide bonds in the presence of Cu(II) and the long conservation of optical purity in the reaction system. Additional data confirming the proposed reaction mechanism were obtained.

Introduction

Recently a new peptide formation reaction was discovered, which has, because of its simplicity, a strong probability of being a primordial way to this class of compounds, also with respect to the present view of the conditions on primitive earth [1–5].

The reagents needed for the reaction are sodium chloride, which can be assumed to have been ubiquitously present also on primitive earth, and copper(II), whose presence is indicated by precambrian 'green zones' containing malachite and azurite, and in accordance with the assumption that precambrian oceans acted as accumulators of divalent ions [6] (Cu(II) is still the most abundant transition metal ion found in seawater [7]). All previous proposals for prebiotic peptide formation in aqueous solution are by far not so simple and need larger amounts of condensation reagents [8] like ATP [9], linear [10] and cyclic [11, 12] inorganic polyphosphates, cynamides [13] or cyanates [14]; according to other proposals peptides could have been synthesized in the dry state within a well defined temperature range after evaporation of water in a melt [15] or on the surface of clays [16, 17].

In the recently found reaction, the removal of water is enabled by a high concentration of sodium chloride (>3 M), where the ions are unsaturated in water with respect to their first hydration shell [18–20]. The kinetic barrier of the reaction is lowered by the catalytic effect of Cu(II) ions, which seem to play an important role also in other aspects of this amino acid condensation [1, 2].

The yield found under optimal conditions, i.e. 5 M NaCl, an amino acid/copper ratio near two, high amino acid concentration and temperatures around 80 °C, is more than 5% dipeptide in the case of glycine. In systems containing both glycine and alanine more than 10% of glycine and 5% of alanine are incorporated into the four possible dipeptides [3]. Furthermore the formation of Gly_3, Gly–Ala_2 and all mixed peptides containing two glycine molecules has been proven [3]. In that context it was also found that the equilibrium ratio of mixed dipeptides can also be determined by a proton catalyzed sequence inversion and not only by the electrophilic/nucleophilic reactivity of the amino acids themselves.

Investigations including valine as the next simplest representative of biologically relevant amino acids, showed the formation of Gly_2, Gly–Val, Val–Gly, Gly–Ala, Ala–Gly and Ala_2 in good yields. The formation of Ala–Val, Val–Ala and Val_2 is hampered, apparently because of the low electrophilic reactivity of valine [5].

In the present study a reinvestigation of valine reactivity focused once more on the question of Val_2 formation. Furthermore, the experiments were extended to aspartic acid, because it is the next amino acid known to form in relatively good yields in simulated prebiotic amino acid synthesis reactions of Miller and

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Fischer-Tropsch types [21–27]. The existence of a polar sidechain in aspartic acid is another point of interest. The validity of the proposed correlation between decreasing electrophilicity (e.g. from glycine to valine) and the preference of mixed peptide formation can also be examined with aspartic acid, which is a stronger electrophile than glycine. Secondly, the formation of natural peptides (α-Asp–Asp and α-Asp–Gly) versus the condensation via the β-carboxylate of Asp (β-Asp–Asp and β-Asp–Gly), as it is observed in a melt [15], was another target of this work, as it could provide further indications about the relevance of the salt-induced amino acid condensation for the formation of simple peptides on the primitive earth.

In a further part of the present work, the electrochemical potentials of the solutions were recorded during reaction in order to obtain further knowledge about the underlying reaction mechanism(s). For this investigations, alanine was also included, whose peptide formation under the same experimental conditions has already been reported [3].

Experimental

Reaction system

NaCl and $CuCl_2 \cdot 2H_2O$ were obtained from Fluka Co., amino acids (D-Asp, L-Asp, L-Val and Gly) and reference peptides were purchased in analytical grade quality from Senn Chemical Co. OPA/MPA reagent was obtained from Hewlett Packard Co. and sodium n-hexylsulfonate from Sigma Chemical Co. All chemicals were used without further purification.

Solutions were prepared in distilled water and heated in glass flasks without stirring on a thermostatized sandbath with reflux cooler under air, the cooler ends closed with aluminum foil to prevent contamination, or under argon atmosphere.

Chromatographic methods

At regular intervals HPLC analysis of the 50-fold diluted reaction mixture was carried out. A Hewlett Packard 1090M LC system with diode array detection was used to monitor the amino acids and dipeptides, respectively after separation on a Shannon Hypersil column (ODS, 5 μm, 200×2.1 mm).

Valine

Val and Val_2 were separated within 7 min by a mobile phase composition of 96% 50 mM KH_2PO_4, pH = 2.5 (H_3PO_4), 4% MeCN at a flow of 0.35 ml/min, a column temperature of 40 °C and detection at 195 nm.

Aspartic acid

Sufficient resolution of Asp and Asp_2 was achieved with a mobile phase of 50 mM KH_2PO_4, 7.2 mM $C_6H_{13}SO_3Na$, pH = 2.0 (H_3PO_4), detection at 200 nm and a flow of 0.35 ml/min.

Aspartic acid plus glycine

After pre-column derivatization with *ortho*-phthaldialdehyde/3-mercaptopropionic acid (OPA/MPA), the isoindoles of amino acids and peptides were separated using a gradient from 100% 50 mM phosphate buffer, pH = 6.8 to 30% MeCN after 12 min, as illustrated in Fig. 1, at a column temperature of 35 °C, a flow of 0.45 ml/min and detection at 338 nm. The peak areas of peptides correspond to 15.0 pmol/μl each, except β-Asp_2 (30.4 pmol/μl) and Gly_2 (60.0 pmol/μl). The peak labeled 1 appears also in blank runs, and is reproduceable in retention time and area; the peak labeled 2 always accompanies the main glycine peak at higher concentrations of this amino acid in connection with this derivatization reagent.

All products were identified by comparison of retention times of authentic samples. Experiments with Asp were also analyzed by using the method for the mixed system described above, because precolumn derivatization with OPA/MPA has the advantage of just monitoring the products with a primary amino group, without oxidation products. Due to the high resolution and reproduceability in retention times of the chromatographic system used and the unambiguous data concerning the formation and identification of the peptides in previous studies [2, 3, 5], this method of identification by using two different chromatographic methods seemed sufficient.

Electrochemical measurements

The condensation reactions of systems containing a single amino acid (Gly, Ala, Val, Asp) were performed under identical conditions, with the only difference that

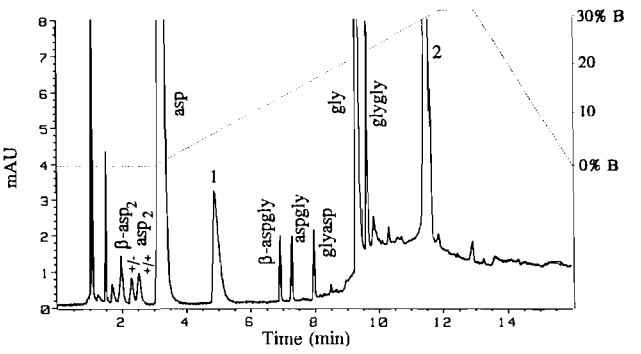

Fig. 1. Separation of a standard mixture containing Asp, Gly and all possible dipeptides, together with the gradient used. Other parameters of method and explanation for the numbers is given in the text.

oil bath termostatization at 70±3 °C was used. Besides a pH electrode, two electrodes for monitoring redox potentials were used: a platinum metal electrode and an AgS/CuS electrode. The latter was produced by precipitation of sulfides from a 0.01 M nitrate solution containing Cu and Ag in the ratio 3:10. After several steps of purification and drying, the electrode material was pressed and polished for use in the measurement. Calibration was performed with $CuCl_2$ solutions in 5 M NaCl, in order to reflect experimental conditions as closely as possible. Nernstian behaviour was found for the whole concentration range from 0.0 to 0.5 M $CuCl_2$.

All potentials were simultaneously recorded with an ABB-GOERZ SE 120 Metrawatt multi-channel recorder. As the system needs some time for equilibration of electrode potentials, solid amino acid was added to the hot aqueous $CuCl_2$/NaCl solution only after all electrodes showed constant potentials, and the reaction system was monitored after dissolution of the amino acid(s). In order to avoid complications by various oxidation processes, all measurements were performed only under inert gas atmosphere.

Results and discussion

Peptide formation

The copper(II)/Val/NaCl system

Although the sensitivity for Val_2 was increased by a factor of 3 compared to previous experiments with OPA/MPA precolumn derivatization, no Val_2 could be detected within 500 h of reaction time in a series of 15 experiments with Cu^{2+}/Val ratios varying from 2/1 to 1/1 and temperatures ranging from 50 to 95 °C, neither under air nor argon atmosphere.

This is the first example of an amino acid, whose condensation into dipeptide could not be observed under the conditions of the salt-induced peptide formation. The main reason for this may be the sterical hindrance of the voluminous and hydrophobic i-propyl sidechain of Val, which induces decreased mobility through the strongly polar solvent and low electrophilicity. On the other hand valine can act as a good nucleophile to form mixed peptides with other amino acids as shown in earlier studies [5]. Such a preference is still found in natural peptides: when we performed a sequence analysis of all known ribosomal proteins of procaryontic cells, it showed that valine is bonded 1.7 times more often to Gly, 1.4 times more often to Asp and 2.0 times more often to Ala than to itself.

The copper(II)/Asp/NaCl system

Figure 2 shows the typical proceeding of the reaction with time in solutions containing 0.66 M DL-Asp (or L-Asp) and 0.33 M $CuCl_2$ in 4.7 M NaCl. The two

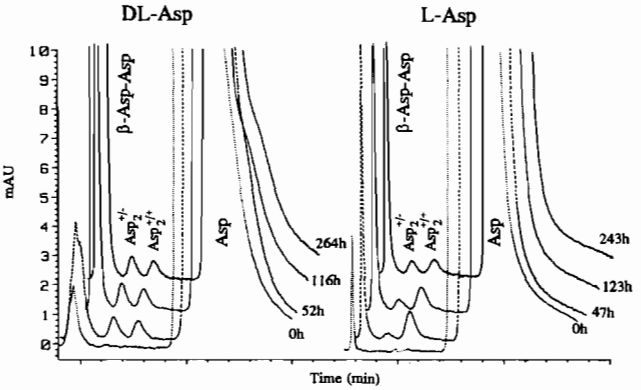

Fig. 2. Chromatograms of experiments starting from DL- and L-Asp at selected reaction times.

diastereomers of Asp–Asp are separated; DL,LD-Asp_2 elutes earlier than DD,LL-Asp_2, as was observed already for the diastereomers of Ala_2. The total yields of Asp_2, are the same for DL-Asp and L-Asp. This could help, together with the recorded UV spectra and the agreement of retention times by using two different chromatographic methods, in the doubtless identification of these compounds. It can also be observed that in the case of L-Asp full racemization of the product is only observed after about 11 days of reaction. This relatively long preservation of optical purity is not unimportant with respect to the prebiotic relevance of the reaction.

In Table 1 all experiments are summarized. In solutions containing Cu^{2+} the maximum yield of Asp_2 is obtained after around 100 h of reaction, except for 92 °C, where the maximum yield is reached already after 42 h.

A most remarkable finding was that solutions containing only NaCl at pH = 2 but no copper ions (always observed in parallel for all systems) also produce dipeptide in the case of aspartic acid. Such behaviour has been observed so far only for glycine [2]. The experiments without Cu^{2+} gave even higher peptide yields, but this difference should be due to the instability of Asp with respect to oxidation by Cu^{2+}, as illustrated in Fig. 3(a). Figure 3(b) and (c) show the corresponding curves of α-Asp_2 and β-Asp_2. As soon as the concentration of Asp has decreased to a level where it cannot complex all the Cu^{2+} in solution, the peptides are oxidized. This reaction has to be considered therefore, besides the acidic hydrolysis of peptides, as a second peptide-removing process.

At first sight, this result seems to favour a peptide formation mechanism without Cu^{2+} as demonstrated in Fig. 4, which also shows the decrease of peptides due to oxidation after reaching equilibrium. However, the ability of Cu^{2+} to accelerate the condensation of Asp (by lowering the kinetic barrier) is also well visible

TABLE 1. Results of experiments containing Asp, Cu^{2+} and NaCl

Reaction conditions	[Asp] (mol/l)	Atmosphere	Temp. (°C)	Max. yield of Asp_2 (%)
[L-Asp]/[Cu^{2+}] = 2:1, 4.7 M NaCl	0.66	air	80	0.20 (106 h)
	0.95	air	80	0.20 (107 h)
	0.66	Ar	82	0.24 (114 h)
	0.66	air	83	0.22 (99 h)
	0.66	air	92	0.28 (42 h)
[DL-Asp]/[Cu^{2+}] = 2:1, 4.7 M NaCl	0.24	air	75	0.14 (98 h)
	0.66	air	83	0.20 (95 h)
	0.66	Ar	80	0.29 (99 h)
	0.66	Ar	81	0.21 (116 h)
	0.75	air	78	0.24 (150 h)
pH 2, 4.7 NaCl without Cu^{2+}	0.56	air	99	0.34 (224 h)
	0.66	air	82	0.34 (242 h)
	0.66	air	95	0.34 (211 h)

Yields are given in percent of amino acid converted into peptide.

for the first day of reaction. Of much more relevance, especially in relation to prebiotic processes, is the fact that Cu^{2+} strongly enhances the formation of 'natural' peptide (α-Asp_2), which is obtained in double yield, and lowers the amount of β-Asp_2, which is the main product without Cu^{2+}. The ratio of α-Asp_2 to β-Asp_2 after 1 day is 0.2 in the presence of the metal ion, compared to 0.1 without it. Even after 5 days, this factor remains still higher (0.16).

In chromatograms without precolumn derivatization, besides the oxidation products of Asp, α-Asp_2 and β-Asp_2, a further condensation compound could be found: cyclo(Asp–Asp) is not derivatizable with OPA/MPA, because it lacks a primary amino group, and thus not visible at the detection wavelength of isoindoles. Such cyclic dipeptides are of considerable interest in prebiotic peptide chemistry. In the case of cyclo (Gly–Gly), identified already in earlier experiments, enlargement of the peptides via reaction of Gly or oligo-Gly with the disubstituted diketopiperazine and the possible relevance of this mechanism for prebiotic peptide formation has already been reported [28, 29].

Cyclo(Asp–Asp) was found in all experiments with a maximum yield of 0.42% after 12 h of reaction. In solutions without Cu^{2+} the maximum yield was 0.17%, which leads to the conclusion that Cu^{2+} favours the formation of both 'relevant' peptides (α-Asp_2 and cyclo(Asp–Asp)) with respect to the peptide β-Asp_2, which is irrelevant for peptides/proteins occurring in nature. This, together with the speed-up of the reaction, seems to be more important than the disadvantage of the metal ion through oxidation of Asp and peptides at longer reaction times.

The Copper(II)/Asp/Gly/NaCl system

In all experiments at 80 °C with a ratio of [Asp]/[Gly]/[Cu^{2+}] = 1:1:1 ([Asp] = 0.33 M) α-Asp_2, α-Asp–Gly, Gly–Asp, Gly_2, β-Asp_2 and β-Asp–Gly were identified. Also in these systems oxidation of amino acid strongly affects the peptide yields, besides acidic hydrolysis and oxidation of peptides. Figure 5(a) shows the change in the amount of free amino acid with time, under air and argon atmosphere, respectively. The corresponding peptide yields are illustrated in Fig. 5(b) and (c). Under air, Gly_2 is the dominating peptide, all peptides containing Asp continuously decrease after 50 h of reaction and after 10 days Asp_2 falls below the detection limit, whereas Gly_2 still gives a yield of 1.15% after 20 days. In comparison to this, the yield of Gly_2 under argon is much lower, but that of Asp peptides slightly better, because of their higher stability in the non-oxidizing atmosphere. (The estimations of O_2 content for the atmosphere of the primitive earth vary between 10^{-70} and 10^{-1}% of the present level; it seems likely that small amounts were present – enough to guarantee the presence of Cu^{2+}, but not critical for the 'survival' of amino acids and peptides [30–36].)

Independent from the atmosphere, GlyAsp is formed preferentially under the conditions of the salt-induced peptide formation, although AspGly should be the favorite product, assuming that Asp should act as the electrophilic partner in the condensation and that the nucleophilic character of the amino group of Gly is higher than that of Asp because of the low electron donor ability of the β-carboxylate group. The only explanation for the obtained results is a very efficient and fast sequence inversion, as has already been found for Gly/Ala and Gly/Val peptides [3]. This inversion process could be proven by an experiment starting from the mixed peptides at 80 °C, as outlined in Table 2. AspGly rapidly gives β-AspGly and is decomposed into the amino acids under this conditions, which also leads

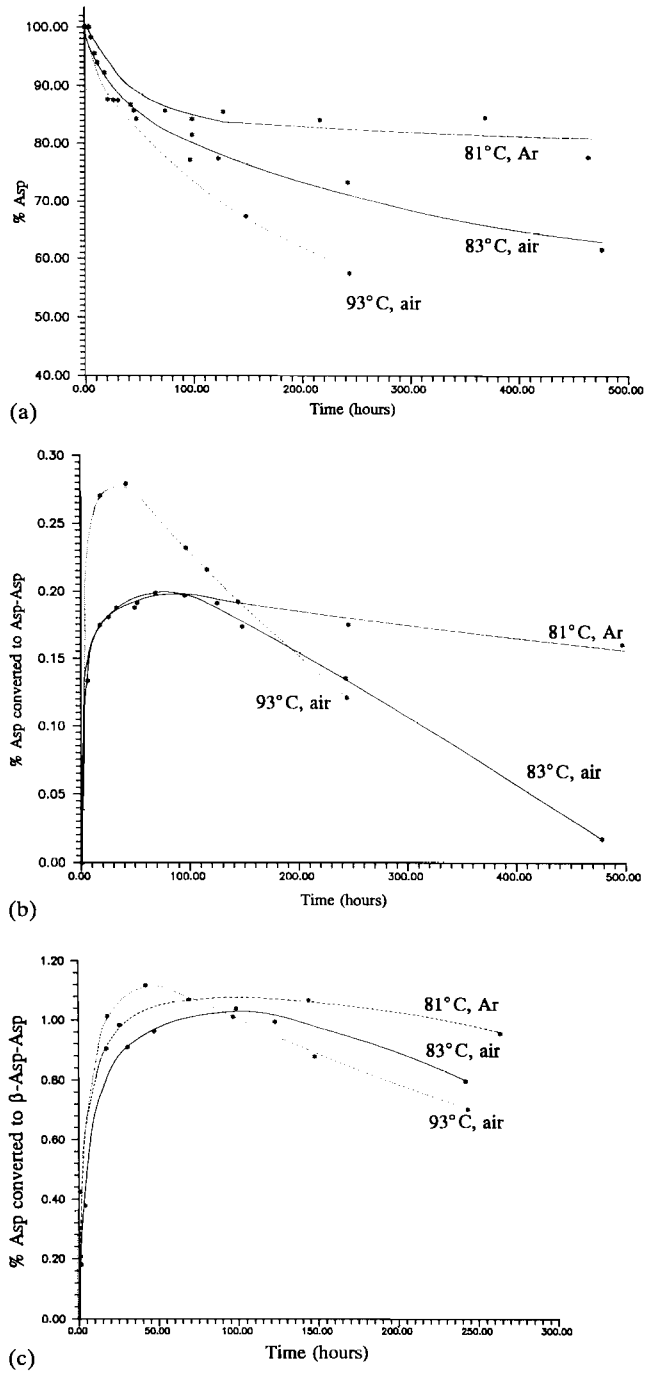

Fig. 3. Dependence of amino acid concentration (a), yield of α-Asp_2 (b) and yield of β-Asp_2 (c) on temperature.

to the conclusion that, in solutions containing Asp, Gly and Cu^{2+}, β-AspGly is formed from AspGly and not directly via condensation of the amino acids bound to Cu^{2+}. Also GlyAsp, which is more stable in such solutions is apparently formed from AspGly. Formation of β-AspGly and AspGly from GlyAsp is slower and occurs in smaller amounts.

Some additional experiments were performed with a ninefold excess of one of these amino acids ([aa] = 0.66 M, [aa]/[Cu^{2+}] = 2:1; 80 °C). If Asp is in excess, all peptides containing Asp can be observed, but no Gly_2, within the whole reaction time independent of the atmosphere. In the case of nine fold excess of Gly, no Asp_2 nor β-Asp_2 can be detected, but all possible mixed peptides and Gly_2. This shows that the actual presence of amino acids will surely have an influence on the products of the salt-induced peptide formation, but that mixed peptides are generally favoured over homo analogues.

Electrochemical results

After dissolution of the amino acids in the $CuCl_2$/NaCl solutions, the pH value remains virtually constant throughout the time of observation. The changes in platinum and copper electrode potentials with proceeding reaction are shown in Figs. 6(a)–(c) for glycine, alanine and aspartic acid, respectively. If valine is used, no significant changes in the potentials are observed, even over very long reaction times. This is in agreement

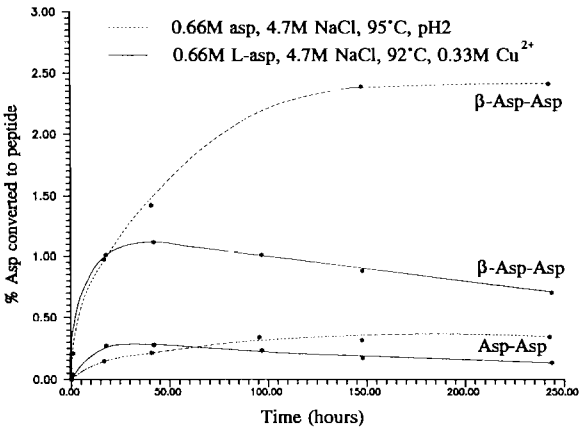

Fig. 4. Dependence of yields of dipeptides (in % amino acid converted into dipeptide) on time, with Cu(II) and at pH = 2, for Asp.

TABLE 2. Hydrolysis and sequence inversion of AspGly (a) and GlyAsp (b) at 80 °C

Time (h)	Amino acid (%)	AspGly (%)	GlyAsp (%)	β-AspGly (%)
(a)				
2.83	12.0	65.8	0	17.6
5.72	26.9	35.3	0.257	24.3
8.88	35.9	19.5	0.386	26.5
29.3	56.4	0.990	0.620	17.7
52.22	63.2	0.511	0.408	10.3
(b)				
2.8	1.84	0	74.9	0
5.55	5.25	0	63.0	0
8.85	9.93	0.285	54.2	0.0621
29.27	30.9	0.588	20.8	0.537
55.18	38.8	0.436	8.33	0.923

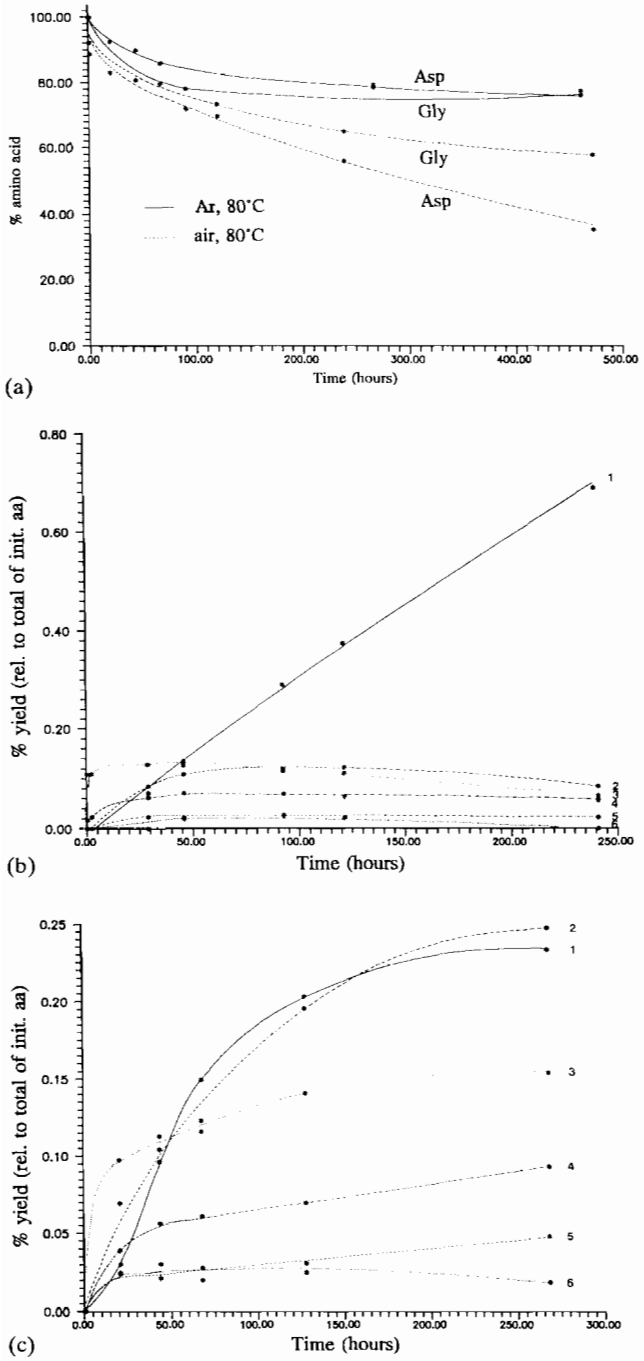

Fig. 5. Dependence of amino acid concentration (a) and yields of dipeptides (in % of total of initial amino acids) on time, under air atmosphere (b), and under argon (c), for the mixed Asp/Gly system. For (b) and (c): curve 1, GlyGly; curve 2, β-AspGly; curve 3, β-AspAsp; curve 4, GlyAsp, curve 5, AspGly: curve 6, AspAsp.

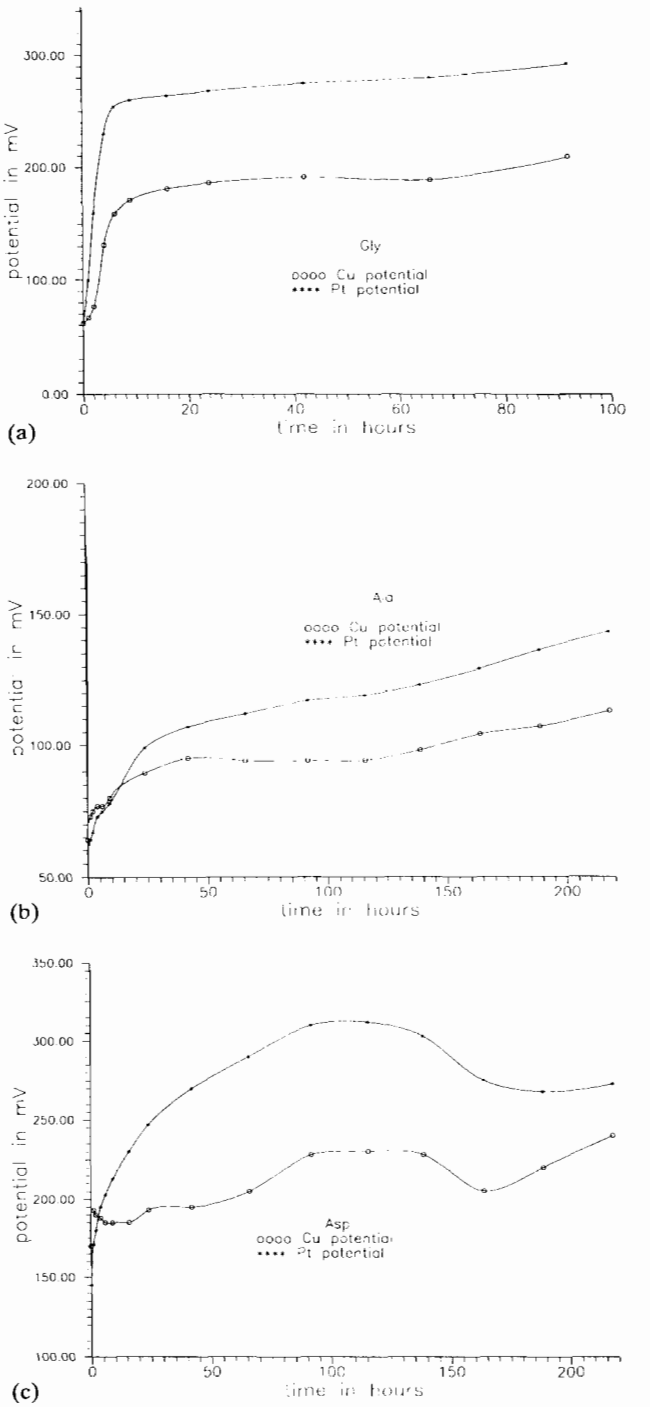

Fig. 6. Dependence of platinum and copper electrode potential on reaction time for glycine (a), alanine (b) and aspartic acid (c).

with the observation that valine alone does not react to form Val_2 under these conditions.

The Pt electrode potential – sensitive to all redox processes in the solution – develops almost exactly parallel to the copper electrode potential, indicating that all such processes occurring in the system involve copper ions. The initial potential value for the solution containing Asp is considerable higher than that for Gly and Ala solutions. This agrees with the observed faster decomposition of Asp by oxidation processes. The time dependence of the potentials in general indicates slow

processes, in accordance with the time needed to form the peptides from the amino acids. The relative change of the potentials within the observed reaction time is comparable with the peptide yields of the respective amino acids (Gly > Ala > Asp), but some additional features have to be considered within this context. In the case of glycine, the potential reaches the state of a steady slow increase after only 20 h of reaction. For alanine, this takes over 100 h, whereas in the case of aspartic acid, such a constancy is not seen within 200 h. With regard to the results discussed earlier this can be understood as a consequence of the relative occurrence of side reactions as amino acid/peptide oxidation and peptide hydrolysis. In a further experiment, Gly and Ala were added in identical amounts: in this case, a more or less constant potential behaviour is only observed after more than 150 h, more than for either of the two amino acids alone, indicating that the sequence inversion process also contributes to the overall chemical potential in this system.

Conclusions

The formation of Val_2 in solutions of Val, Cu(II) and sodium chloride could be definitely excluded, whereas the formation of Asp_2 by the salt-induced peptide formation reaction occurs readily. Dipeptide formation from Asp with similar yields is even observed without Cu(II) at pH = 2 and high concentrations of sodium chloride. Cu(II) enhances, however, the formation of cyclo(Asp–Asp), which presumably is important for a further condensation with amino acids and thus for the formation of peptides with higher prebiotic relevance. It also shifts the ratio α-Asp_2/β-Asp_2 from 0.1 to 0.2, a further aspect of the ability of Cu(II) to enable and favour formation of nature-like peptide bonds. The fact that racemization of Asp also occurs only after more than 10 days of reaction time – as found in the case of alanine too – is another important factor for the possible prebiotic relevance of this new peptide-generating reaction.

The formation of four 'nature relevant' (Gly_2, GlyAsp, AspGly and α-Asp_2) and two 'nature irrelevant' peptides (β-Asp_2 and β-AspGly) of Gly and Asp has been observed in solutions containing Asp, Gly, Cu(II) and high concentrations of sodium chloride. The instability of Asp at higher temperatures, especially under oxidizing atmosphere, is the main factor determining the yield of dipeptides. Acid catalyzed sequence inversion from AspGly to GlyAsp via disubstituted cyclic dipeptide was found to be the reason for the ratio of mixed peptides in the solutions, as in the case of the mixed peptides of Gly/Ala and Gly/Val.

The observed electrochemical potentials during the salt-induced condensation reaction can be well interpreted on the basis of reaction progress and yields, and thus confirm the assumption that the reaction mechanism depends on amino acid complexation by Cu^{2+}, as proposed in previous studies [1–5].

Acknowledgements

This work was supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (Project 8475-MOB), which also supplied the HPLC equipment. This generous support is gratefully acknowledged. Thanks are due to R. T. Kroemer who synthesized the reference peptides DL-Asp_2, LD-Asp_2 and β-Asp_2 for us.

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