Peptide Research

Rearrangement, Racemization and Decomposition of Peptides in Aqueous Solution

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ABSTRACT

We reported earlier that peptides containing glycine as the third amino acid from the amino end undergo sequence rearrangement of the first two amino acid residues. In the course of this experimental verification of the suggested reaction mechanism, we found extensive racemization of the amino acid residue in position 1. Racemization is preferred over rearrangement in peptides containing amino acids different from glycine in position 3. We demonstrate that this reaction can be used for the selective labeling of peptides. Using model peptides, we suggest a mechanism that explains both the rearrangement and racemization of these peptides in aqueous solution. This mechanism is based on formation of a diketopiperazine-like (DKP-like) structure by attack of the N-terminal amino group on the amide carbonyl group of the second residue in the peptide chain. This tetrahedral intermediate, which contains a secondary amino group derived from the amide bond between the second and third amino acid residue, can (i) decompose with the formation of diketopiperazine and a shortened peptide sequence; (ii) form a bicyclic structure by transanular attack on the first amino acid carbonyl group in the DKP-like ring by the newly formed amino group, leading to the rearranged product; and (iii) form a bicyclic structure by transanular attack of the newly formed hydroxyl group on the carbonyl group in the DKP-like ring, leading to the racemized product.

INTRODUCTION

Peptides in aqueous solutions at neutral pH are rather stable compounds. It is a matter of general experience that in the absence of acids or bases most peptides can be stored in aqueous solutions without decomposition. However, there are two known mechanisms by which faster nonenzymatic transformations of peptides can occur. Both mechanisms are characterized by formation of cyclic intermediates. The first one applies to peptides which contain Asp/Asn or Glu/Gln and consists of decomposition, deamidation, isomerization, and racemization *via* succinimide intermediates (4). The other known mechanism of transformation of peptides through cyclic intermediates explains decomposition of peptides *via* the diketopiperazine-like (DKP-like) structure (Figure 1). This reaction has been studied comprehensively and there are many reports that describe cyclization of peptides or their derivatives to DKPs during storage of peptides in solution, deprotection by catalytic hydrogenation [see review (6)], or heating in aqueous solutions (7). But Steinberg and Bada (7), who studied decomposition of the tripeptide Leu-Gly-Gly *via* DKP formation in heating experiments, described the tripeptide sequence inversion as the most surprising observation. They suggested that the inversion reaction proceeds through formation of a cyclic tripeptide cyclo(Leu-Gly-Gly) with subsequent hydrolysis of the Gly-Gly peptide bond. Recently we reported on sequence change of peptides in aqueous solutions during heating experiments (6). We observed rearrangement of the first two amino acid residues in the peptide chain of Leu-enkephalin and related peptides. The same phenomenon described by Steinberg and Bada was observed, but it cannot be explained by the mechanism suggested by them. At first sight, it seems possible to rationalize formation of peptide with inverted sequence of first two residues by the following: At first, DKP and a shortened peptide are formed (in accordance with Figure 1); then the DKP ring is opened by the shortened peptide, either from one side or from the other side, and the initial peptide or the peptide with inverted sequence of the first two residues appears as the result of such an opening. This explanation is incorrect because if DKP can be opened by the shortened peptide, it can be opened by the initial peptide as well. This process should lead to formation of longer peptides, which were not detected. In our previous work (6) we suggested that the first step of rearrangement is the same as the first step of decomposition *via* DKP formation (Figure 1). However, the next step makes both mechanisms different. For DKP formation, the C-N bond of the tetrahedral intermediate must be cleaved. In the rearrangement mechanism, the exocyclic amino group of tetrahedral intermediate has

pronounced nucleophilic properties and effectively attacks transanular carbonyl carbon with formation of an intramolecular aza bridge (Figure 2). The bicyclic structure thus formed is "chemically symmetrical" with the exception of side chains of amino acid residues. Opening of the triazabicycloheptane intermediate can lead either to the original peptide or to the peptide with inverted sequence of the first two amino acid residues. Unfortunately, we could not detect or isolate any intermediates of the reaction studied, and hence, there is no direct proof for the suggested mechanism. Until now, all our conclusions about mechanism of rearrangement have been based only on logical arguments. In the present study, we report on new results that support the suggested rearrangement mechanism and demonstrate that the DKP-like intermediate plays an essential role not only for reactions leading to rearrangement and decomposition, but also for racemization of peptides in aqueous solution.

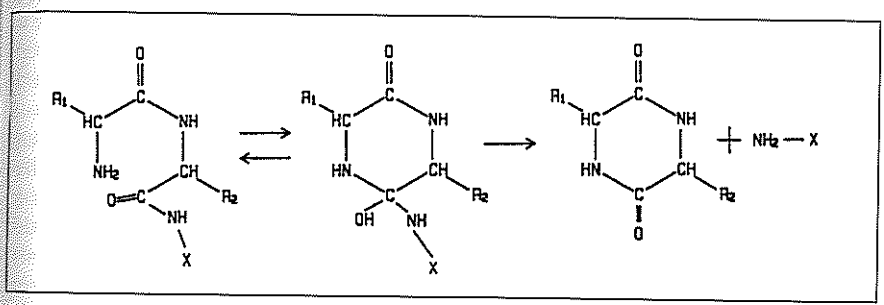

Figure 1. Decomposition of peptides *via* DKP formation.

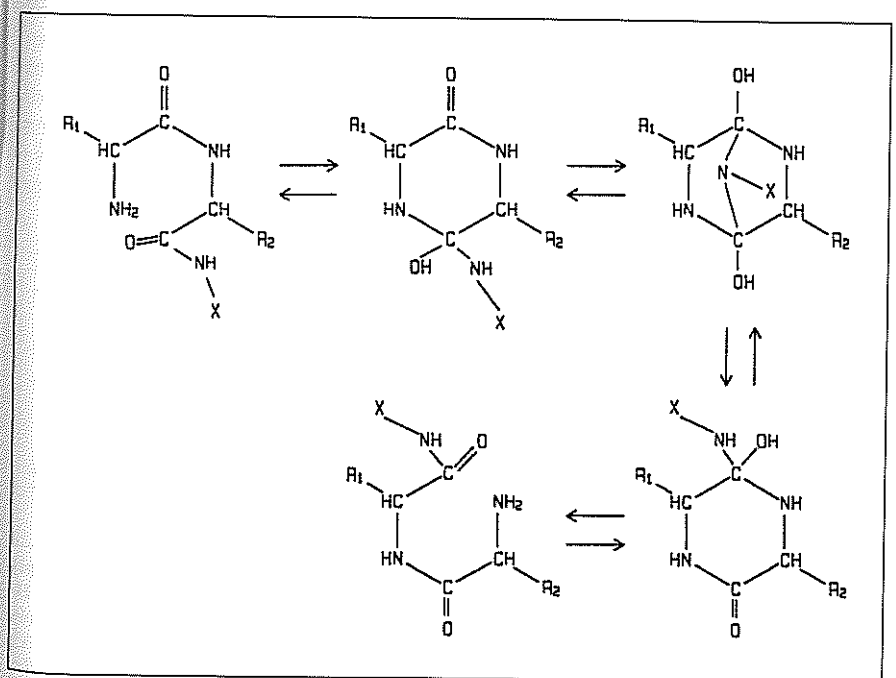

Figure 2. Suggested mechanism of rearrangement of the first two amino acid residues in peptides in aqueous solutions.

EXPERIMENTAL

The peptides used in this study were either described earlier, or synthesized for this purpose by the application of classical methods of peptide synthesis in solution (8). They were purified by reversed-phase HPLC and characterized by ^{1}H NMR spectroscopy (500 MHz). Synthesis of the peptide containing the reduced peptide bond is described below.

Boc-Alaninal

Boc-Alaninol (520 mg, 3.0 mmol) in dry dichloromethane (6.0 ml) was added to a stirred solution of 1,1,1-tris(acetoxy)-1,1-dihydro-1,2-benziodoxol-3(H)-one (periodinane; 1.60 g, 3.75 mmol) in dichloromethane (15 ml) at room temperature. The mixture was stirred for 10 min and unreacted periodinane was destroyed by addition of a solution of sodium thiosulfate (3.0 g) and sodium bicarbonate (3.0 g) in water (50 ml). The organic layer was separated and concentrated on a rotary evaporator at 25°C. The residue was dissolved in *tert*-butyl methyl ether (5 ml), and the solution was diluted by hexane (5 ml) and filtered through a short pad of silica gel. The eluant was evaporated at reduced pressure and the residue (498 mg, 95%) was shown to be homogeneous by TLC (benzene-hexane-acetone 2:2:1). The melting point was 145°–147°C (lit. 150°C [3]).

Boc-Ala[CH_2NH]Phe-Gly-OMe

Sodium cyanoborohydride (31 mg, 0.5 mmol) in methanol (2.0 ml) was added over a 1-h period at room temperature to a stirred solution of TFA.H-Phe-Gly-OMe (175 mg, 0.5 mmol) and Boc-Ala-al (104 mg, 0.6 mmol) in methanol (3.0 ml) containing 1% acetic acid. The reaction was complete in 90 min (TLC). Solvent was evaporated and the residue partitioned between ethyl acetate and 1 M citric acid. The aqueous layer was adjusted to pH 11 with cold 1 M sodium hydroxide and the base was extracted by *tert*-butyl methyl ether. Evaporation of the solvent produced an oily Boc-ester (155 mg, 79%), with a single peak on HPLC (Lichrosorb RP18, 250 × 4 mm, 5 µm, 70% methanol). FAB-MS: 394 (M+1, 100%).

Boc-Ala[CH_2NH]Phe-Gly-NH_2

The Boc-tripeptide ester (130 mg, 0.33 mmol) was dissolved in methanol saturated with ammonia at 0°C (5 ml) and the sealed flask was shaken for 2 days at room temperature. The reaction mixture was cooled and evaporated repeatedly *in vacuo* from methanol. The oily residue was purified by HPLC (Vydac C_{18}, 250 × 10 mm, gradient from 25%–50% methanol in 0.05% trifluoroacetic acid in 50 min). Yield: 86 mg (69%). FAB-MS: 379 (M+1).

Scheme of Heating Experiments

The peptide under investigation was dissolved in water or appropriate buffer, and the pH was adjusted by addition of HCl or KOH solution. The solution was incubated at various temperatures

(50°–140°C) for various lengths of time (from several minutes to several days). To compare directly the behavior of peptides that did not undergo rearrangement, they were incubated with the peptide prone to the rearrangement (Figures 3 and 4). After incubation, the solution was analyzed by HPLC (HP 1090, Hewlett Packard) using a Beckman Ultrasphere C18 reversed phase column (250 × 4.6 mm). In some experiments the products of incubation were isolated by preparative HPLC on a Gilson HPLC system (Model 303 pump, 802C manometric module, 116 UV detector, 201 fraction collector) using a Beckman Ultraprep C18 column (150 × 21.2 mm). ^{1}H NMR spectra were obtained on a Bruker WH-500 instrument (500 MHz).

pH-Velocity-Dependence Curve Fitting

The dependence of reaction velocity (expressed as the amount of a particular product formed in the first 30 min of reaction) on the pH of the reaction mixture was evaluated using the following equation:

$$-\lg[(v_{AH}-v)/(v-v_{A^-})] = n(pK-pH)$$

where v_{AH} is the velocity of the reaction when all functional groups participating in or influencing the observed reaction are in the fully protonated state; v_{A^-} is the same velocity with all deprotonated groups; v is the current velocity value; n is the Hill's coefficient expressing cooperation of participating groups. In our case, the value of n = 1 (with this value, the equation given above becomes the classical Henderson-Hasselbach equation) was not satisfactory for the experimental fitting. A much better fit was obtained with n = 0.6. This may mean that the velocity of the described transformation depends on the ionic state of several functional groups having pK values close to each other.

RESULTS AND DISCUSSION

In accordance with the suggested mechanism, several factors can prevent rearrangement or can change the rate of this reaction. One group of factors is connected with formation of a DKP-like intermediate, another with formation of a bicyclic intermediate. There are several possibilities for influencing the formation of the DKP-like structure: (a) blocking of the α-amino group of the peptide completely prevents cyclization of the N-terminal part of peptide; (b) nucleophilicity of the free α-amino group (and hence the tendency to form a DKP-like structure) can be affected considerably by variation in pH of the solution; (c) incorporation of proline or glycine into positions 1 and 2 of the peptide chain can facilitate cyclization; (d) presence of β-Ala in the first or second position of the peptide leads to formation of a seven-membered cycle which is less stable. There are also ways to influence formation of the bicyclic intermediate. It is possible to completely prevent the appearance of such an intermediate by incorporation of a reduced peptide bond between the first and the second residues or by alkylation of the amino group on the third residue. Surely, the probability of formation of the bicyclic intermediate depends on the nucleophility of the α-amino group of the third residue. It is well known that the glycine amino group is more reactive than amino groups of other residues; hence, the presence of glycine in the third position can promote the rearrangement reaction. All these approaches were used to test the suggested rearrangement mechanism.

Earlier we had shown that rearrangement of the first two amino acid residues is impossible for peptides with blocked N-terminal α-amino groups. This is obvious from the suggested mechanism of the reaction, since a nucleophilic attack of the carbonyl group of the second residue by the N-terminal α-amino group marks the beginning of the rearrangement. In other words, blocking the N-terminal α-amino group abruptly decreases its ability for nucleophilic attack and thus prevents rearrangement. But the nucleophilicity of the N-terminal α-amino group can be affected considerably and smoothly by variation of the pH. At low pH, the N-terminal amino group of the peptide is in the protonated form and has low nucleophilicity. Increasing the pH makes amino groups more nucleophilic, promotes DKP-like structure formation, and thus should increase the rate of rearrangement. But at a pH higher than the pK value of the α-amino group, the rearrangement rate should increase more slowly, and the rate should become constant. This conclusion is in good agreement with the S-shape of pH-dependencies of the rearrangement rate that were obtained in the course of heating aqueous solutions of a few peptides. We studied pH-rate dependencies for Tyr-Gly-Gly-Phe-Leu, Tyr-D-Ala-Gly-Phe-Leu-Arg, Phe-Ala-Gly-Arg, Ala-Phe-Gly-Arg, and Pro-Leu-Gly-NH_2. Experimental data on the rate of rearrangement of the first two residues of Pro-Leu-Gly-NH_2 relative to pH, and the corresponding pH-rate profile plotted in accordance with the standard Hill equation (see Experimental) are

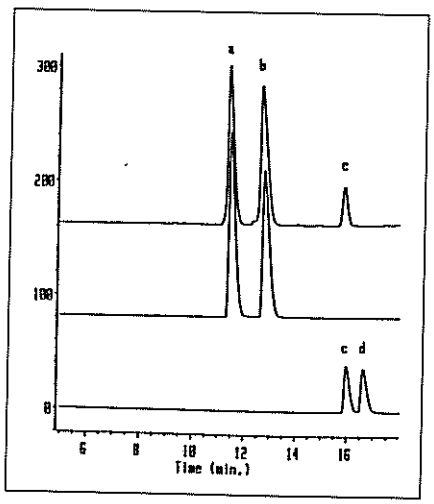

Figure 3. HPLC trace of aqueous solution (pH 8) of (a) H-Phe-Ala-Gly-Arg-OH and (b) H-Phe-βAla-Gly-Arg-OH. Middle trace - before heating; upper trace - after 180 min at 100°C; lower trace - mixture of synthetic model peptides with inverted sequence (c) H-Ala-Phe-Gly-Arg-OH and (d) H-βAla-Phe-Gly-Arg-OH.

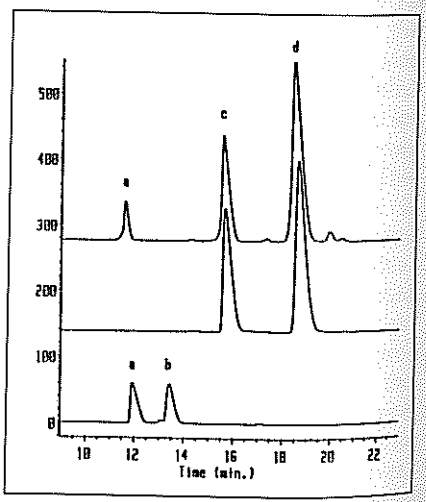

Figure 4. HPLC trace of aqueous solution (pH 8) of (c) H-Ala-Phe-Gly-Arg-OH and (d) H-Ala-Phe-MeGly-Arg-OH. Middle trace - before heating; upper trace - after 270 min at 100°C; lower trace - mixture of synthetic model peptides with inverted sequence (a) H-Phe-Ala-Gly-Arg-OH and (b) H-Phe-Ala-MeGly-Arg-OH.

displayed in Figure 5. The point at which there is a very sharp increase in reaction rate is quite near to the pK value of the imino group of the amino terminal Pro. It might be well to point out that this value is about 1.5 pH units higher than values obtained in our experiments for other peptides studied. It is in conformity with the fact that the pK of the Pro imino group is about 1.5 units higher than the pK values of the amino groups of other residues. (It must be remembered that the pK of the free amino acid NH_2 group is higher than the pK of the same group in peptides—Ala pK = 9.69, Ala-Ala pK = 8.30, Ala-Ala-Ala pK = 8.03, Ala-Ala-Ala-Ala pK = 7.94 [2].) Thus we can argue that the rate of rearrangement of the first two residues in the peptide is controlled in great part by the nucleophilicity of the N-terminal α-amino group.

According to this suggested mechanism, cyclization of the N-terminal portion of peptide with formation of a six-membered ring marks the beginning of a reaction leading to rearrangement of first two residues. Naturally, the size of the ring obtained is essential for the rearrangement reaction, since a six-membered ring is the most energetically favorable one. It is well known that a six-membered ring is considerably more stable than a seven-membered ring. We used this fact to support the suggested mechanism and studied the possibility of rearrangement in the peptides Phe-Ala-Gly-Arg and Phe-βAla-Gly-Arg. Cyclization of the N-terminal peptide with Ala should result in a six-membered ring, whereas cyclization with βAla should produce a seven-membered ring. Hence, if the cyclic intermediate plays an important role in rearrangement, this reaction should proceed for a peptide containing Ala, but it should not occur or proceed with negligible velocity for a peptide containing βAla. This assumption was confirmed by our experiments (Figure 3), showing that the peptide containing βAla was not influenced after 3 h at 100°C; at the same time a significant amount of rearrangement occurred in the Ala-containing peptide.

The next step after DKP-like structure formation is "attack" of the carbonyl of the first amino acid residue by the amino group of the third residue, resulting in a bicyclic structural appearance. Obviously this process can be disturbed by modification of any of the groups involved in cyclization (either the carbonyl of the first residue or the amino group of the third residue). Both possibilities were used in our studies. We have observed that replacement of the carbonyl of the first residue by the CH_2 group completely prevents the rearrangement of the first two residues in a peptide. Unfortunately, this is not strong evidence for our hypothesis, because the introduction of CH_2 instead of a carbonyl group not only eliminates the target of nucleophilic attack but also changes the character of the chemical bond between the first and second residues (the reduced peptide bond cannot be hydrolyzed). We also were not able to identify the DKP-like product of the cleavage of the first two residues (Ala[CH_2NH]Phe) in the reaction mixture. However, the explanation of the absence of this product is understandable based on our experience in preparing this cyclic pseudopeptide. All our attempts, starting either from H-Ala[CH_2NH]Phe-OMe or from the free peptide, failed, probably because of competition of the primary and secondary amino groups.

Most likely there is no way to modify the carbonyl group of the peptide bond without changing the character of the bond between residues. Meanwhile, the situation with other groups involved in formation of the supposed bicyclic intermediate (amino group of the third residue) is more favorable. There are at least two possible ways to affect (without essential changes of bonds in peptide molecule) the ability of the amino group of the third residue "to attack" the carbonyl of the first residue. These were already mentioned above: alkylation of an α-amino group of the third residue and replacement of glycine in the third position with a very reactive amino group by another residue. It might be well to point out that a characteristic feature of all peptides described above is the presence of glycine in the third position of the peptide chain. Presence of glycine in this position is of major importance, because we found that its replacement by another residue (for example, alanine) decreases considerably the rate of rearrangement and, in most cases, makes it negligible. Replacement of glycine in the third position by sarcosine (i.e., methylation of the α-amino group of glycine) completely prevents rearrangement (Figure 4).

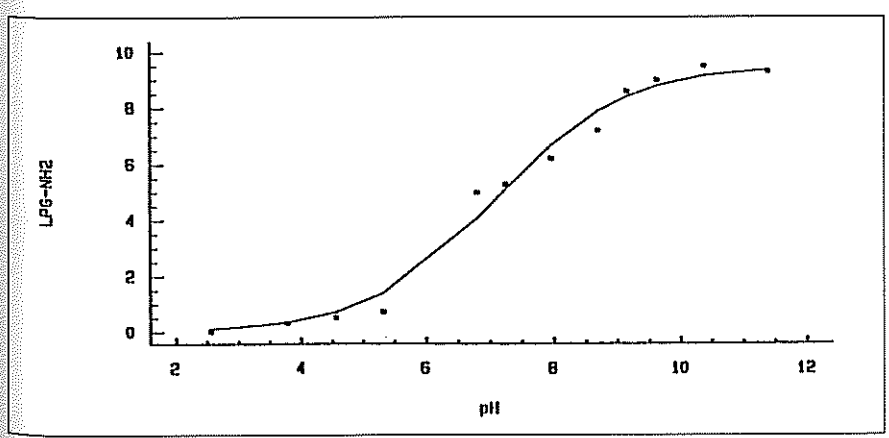

Figure 5. pH-rate profile for rearrangement of H-Pro-Leu-Gly-NH_2. The amount of H-Leu-Pro-Gly-NH_2 formed in 120 min at 100°C is given (%) on the y axis.

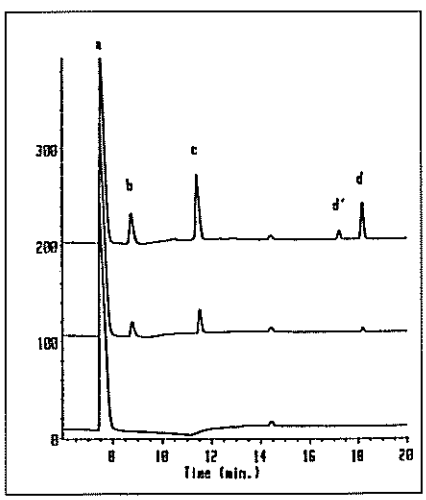

Figure 6. HPLC of aqueous solution of H-Pro-Leu-Gly-NH_2 (a) during heating experiment. Lower trace - before heating; middle trace - after 20 min at 100°C; upper trace - after 80 min at 100°C. Structure of products (based on ^{1}H NMR spectra): (b) H-Leu-Pro-Gly-NH_2, (c) H-D-Pro-Leu-Gly-NH_2, (d) c(Pro-Leu), (d') c(D-Pro-Leu).

Thus, reactivity of the third amino acid amino group plays a crucial role in rearrangement of the first two residues of the peptide, and this conclusion is in agreement with the suggested reaction mechanism.

Thus, it may be thought that the purpose of our studies (justification of the suggested rearrangement mechanism) is accomplished. But in the course of our investigations, we faced a phenomenon well known in peptide chemistry, yet its appearance in our experiments was unexpected. The case in point is racemization. It was surprising for us to find considerable racemization of amino acid residues at relatively mild conditions in the course of heating the peptides in aqueous solutions at neutral pH. We established that in all cases when racemization was observed only the first amino acid residue was found to be racemized. This fact was supported by heating experiments with deuterium oxide used as a solvent. The experiments revealed that deuterium is incorporated exclusively in the α-position of the first residue (Figure 8).

It seems that racemization of the first two residues and rearrangement of the first residues in peptides bear a relationship to each other. On the one hand, it may be said that racemization of the first residue in a peptide is competitive with the rearrangement of the first two residues. Once intensive rearrangement is observed (for example, for Tyr-Gly-Gly-Phe-Leu, Phe-Ala-Gly-Arg, etc.), there are no traces of epimeric products. But if there are some factors retarding rearrangement of the first two residues in peptide, racemization of the first residue occurs in parallel with (and sometimes instead of) rearrangement. As was mentioned above, the presence of an amino acid residue other than glycine in the third position is of primary importance. Racemization was detected in all our experiments with peptides containing residues other than glycine in the third position (for example, Arg-Tyr-D-Ala-Gly-Phe-Arg, Phe-Met-Arg-Phe, Ala-Gly-Phe-Met, etc.). Probably, the presence of proline in the first position also limits the rearrangement, because in heating experiments with Pro-Leu-Gly-NH_2, we observed simultaneous formation of a peptide with inverted sequence of the first two residues Leu-Pro-Gly-NH_2 and a peptide with D-Pro in the first position (Figure 6).

On the other hand, racemization of the first residue and rearrangement of the first two residues in the peptide have similar features. We found that racemization, as well as rearrangement, is impossible for peptides with blocked N-terminal α-amino groups. In cases when both racemization and rearrangement were observed (for example, for Pro-Leu-Gly-NH_2), these processes appeared and occurred simultaneously, that is, parallel to each other and have analogous S-shape dependencies of reaction rates on pH (Figures 5 and 7). In the cases when only racemization was observed (for example, Phe-Met-Arg-Phe), a similar S-shape pH-rate profile for racemization rate was also found. From our point of view, these facts indicate that racemization and rearrangement processes proceed through common intermediates.

It should be noted that racemization of the first amino acid residue in a peptide could be explained by the bicyclic intermediate described for rearrangement. Opening of this intermediate to the DKP-like structure can promote enolization of the carbonyl of the first residue, leading to racemization. But this is not the case, since there is a contradiction to this hypothesis. Heating an aqueous solution of Ala-Phe-(N-Me)Gly-Arg revealed small but detectable racemization of alanine (peak at 19.8 min on Figure 4). As already men-

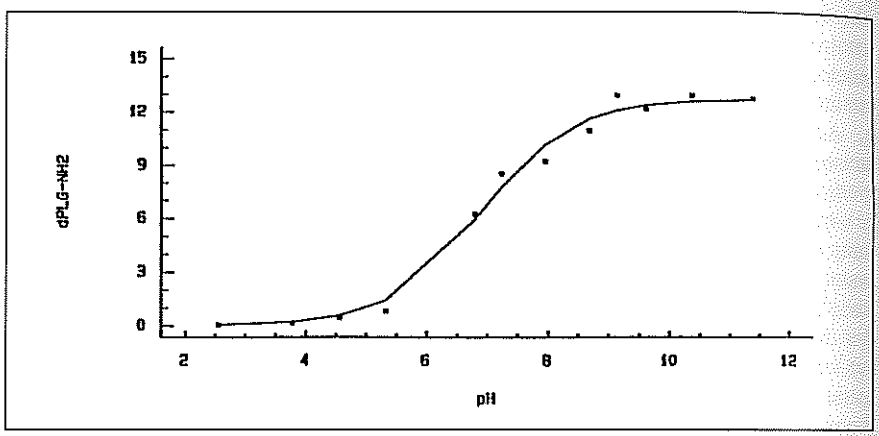

Figure 7. pH-rate profile for racemization of H-Pro-Leu-Gly-NH_2. On the y-axis the amount of H-D-Pro-Leu-Gly-NH_2 formed in 120 min at 100°C is given (%).

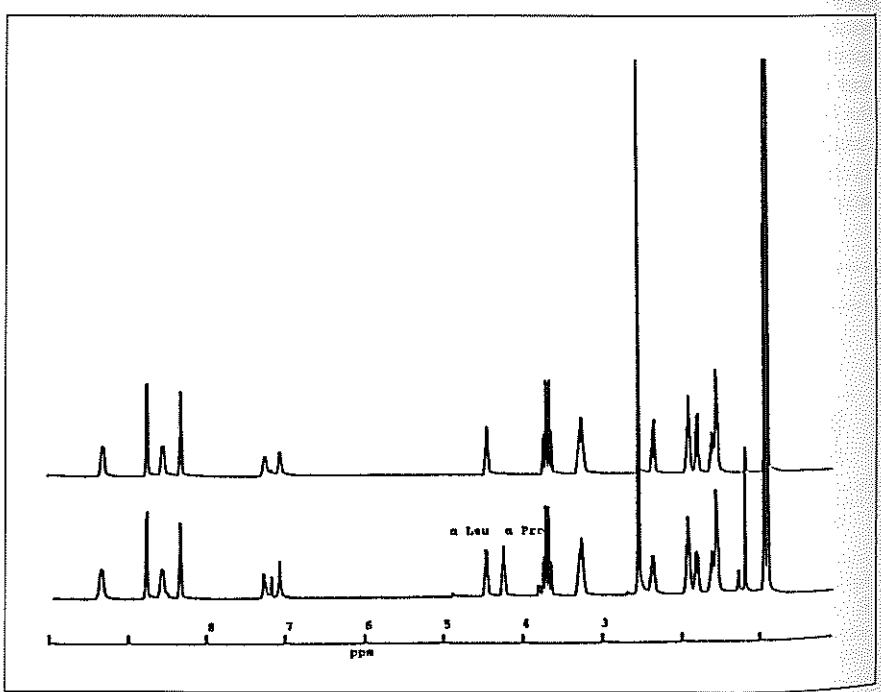

Figure 8. ^1H NMR spectra (500 MHz, in DMSO-d_6) of synthetic H-D-Pro-Leu-Gly-NH_2 (lower trace), and product isolated after heating H-Pro-Leu-Gly-NH_2 in deuterium oxide (upper trace).

tioned above, the bicyclic structure bridged, though nitrogen cannot be formed in peptides containing an N-methylated third residue. However, if we consider the possibility that the DKP-like intermediate can be bridged in the bicyclic structure by nucleophilic attack not only of the third residue NH group but also of the hydroxyl that results from DKP-like intermediate formation, then the contradiction just discussed can be overcome. We suppose that the bicyclic structure bridged through oxygen (Figure 9, right part) is responsible for racemization of the first residue. This hypothesis explains easily the peculiarities of racemization discussed above: racemization of only the first peptide residue, the S-shaped pH-rate profile for racemization, and the fact that racemization of the first residue is competitive with rearrangement of the first two residues.

Obviously, the formation of the bicyclic intermediate (N- or O-bridge) and, consequently, the pathway of peptide transformation (rearrangement or racemization) are determined by the relative reactivities of the NH and OH groups that in turn depend on the nature of the amino acid residues, steric restrictions and so on. Not all details of racemization of the first residue upon heating neutral aqueous solution of peptides are clearly understood, and these studies are still in progress. The possibility of using this phenomenon for isotopic labeling of peptides (5) is of specific interest. It looks promising, since the observed racemization is selective (only the first residue is racemized) and proceeds at relatively mild conditions.

In conclusion, we can say that there is a good reason to believe that all reactions observed in the course of heating neutral aqueous solutions of peptides (rearrangement, racemization, and decomposition) have a DKP-like structure as the first intermediate. The last can be cleaved, with DKP formation, or can be bridged to a bicyclic intermediate by the nucleophilic attack of either an OH or NH group, resulting in racemization or rearrangement. The reaction scheme is summarized in Figure 9. From a practical point of view, it is important to note that these transformations proceed not only at elevated temperatures but also at temperatures close to 0°C (6); therefore special care should be taken in storage and solution handling of peptides containing glycine in position 3.

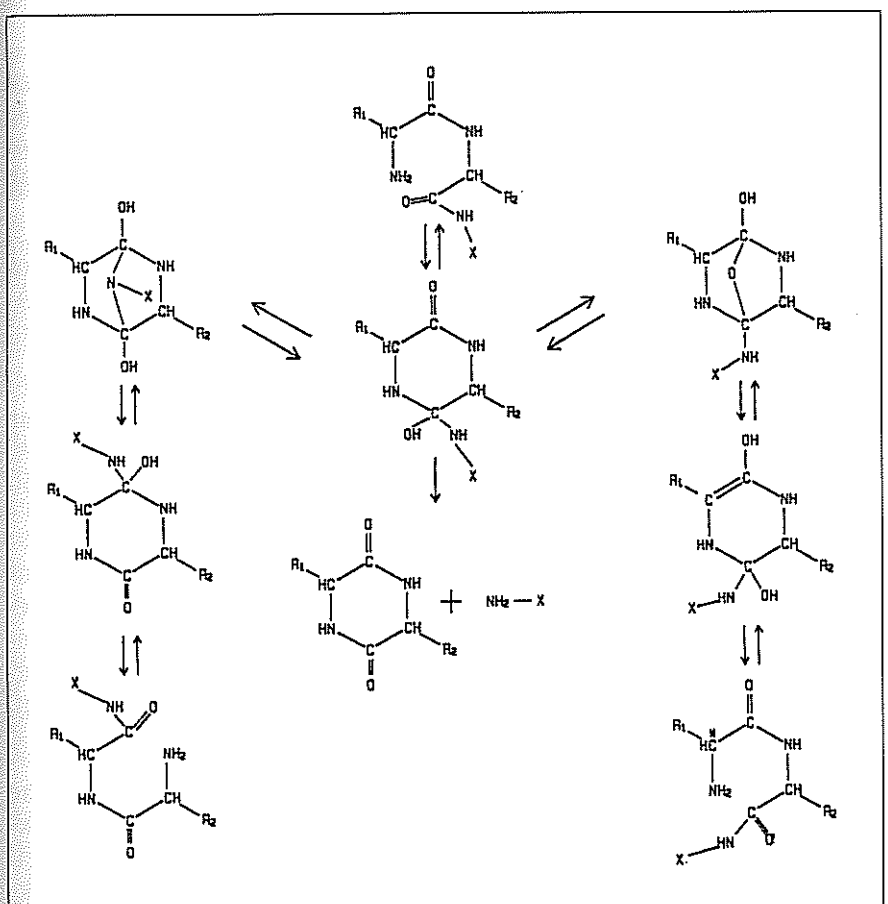

Figure 9. Possible pathways in the transformation of peptides in aqueous solutions. R_1 and R_2 - side chains of the first and the second residues, X = CH_2-CO-α-NH-CHR_n-COOH.

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