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SEQUENCE-DEPENDENT REACTIVITY OF MODEL PEPTIDES WITH GLYCERALDEHYDE*

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ABSTRACT

Glyceraldehyde reacted faster with tripeptides than with dipeptides. The pH profiles of the reactions with tripeptides displayed optima in the range of 8.5–10.0, ~1–2 pH units higher than found with dipeptides. The second amino acid residue influences not only the rate of reaction but also the extent of formation of the product of the Amadori rearrangement, the ketoamine. The presence of histidine as the second amino acid residue of either di- or tri-peptides greatly accelerated the rate of reaction perhaps by facilitating the rearrangement. Conventional amino acid analysis and liquid chromatography procedures have been used to detect intermediates and the ketoamine product. ^{1}H-N.m.r. analysis of the reduced adducts was consistent with the assigned structures.

INTRODUCTION

In previous studies on the reaction of glyceraldehyde with hemoglobin, we found that only a few amino groups of the protein (5 of 24 per $\alpha\beta$ dimer) formed stable ketoamine adducts with the aldehyde[1]. Thus, the Schiff base adduct of glyceraldehyde with Val-1(β) is capable of undergoing the Amadori rearrangement to the ketoamine (Scheme 1), but the Schiff base adduct at Val-1(α) does not rearrange[2]. It seems likely that many amino groups of hemoglobin form Schiff base adducts with glyceraldehyde (and with D-glucose) but only a few of them undergo the Amadori rearrangement to form the stable ketoamine adduct. In an effort to decipher the details of this selective reaction on hemoglobin, we have chosen to study the reaction of glyceraldehyde in less complex systems with model peptides of various sequence. In an earlier study[3] using this approach, we were able to estimate the amount of Schiff base intermediate and to characterize in part the rearranged Amadori product of the reaction between glyceraldehyde and the dipeptide, Ala-His.

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The reaction of simple sugar aldehydes with amines has been studied extensively[4]. The substituted glycosylamine, which is an early intermediate in the reaction of hexoses and amines, can sometimes be isolated under the proper conditions. These isolated compounds, *i.e.*, in the crystalline state, usually do not rearrange further in the absence of solvent. In solution, however, glycosylamines can undergo the acid-catalyzed Amadori rearrangement to the ketoamine[4-6]. Under some conditions, this ketoamine can participate in further rearrangements and condensations in a process termed the Maillard reaction[4,5]. Thus, under some conditions, such as very low pH or high temperature, it is difficult to isolate in pure form the Amadori rearrangement product, the 1-amino-1-deoxy-2-ketose (ketoamine) derivative. However, as described in our previous communication[3], a product was isolated whose properties were consistent with those of a ketoamine adduct formed between glyceraldehyde and a dipeptide. This product was isolated chromatographically in reasonable yield by avoiding extremes of pH and temperature. Additional proof of structure for a related adduct is given in the present communication.

The purpose of the present investigation is to determine the effect of amino acid residues near the amino terminus in influencing the overall course of this reaction. Therefore, we have studied the reaction of a variety of di- and tri-peptides having different amino acid sequences in an effort to determine the factors on a peptide that contribute to the formation both of the intermediates and of the Amadori rearrangement product, the ketoamine. To this end, we have found conventional amino acid analysis and liquid chromatography (l.c.) procedures to be of considerable value.

EXPERIMENTAL

Materials. — The di- and tri-peptides (of the L-configurations), Ala-Asp, Ala-His, His-Ala, Leu-Arg, Gly-Ser, and Gly-His-Gly, as well as L-valine, ethanolamine, and DL-glyceraldehyde were obtained from Sigma Chemical Co., St. Louis, MO. Val-Leu amide hydrochloride and β-Ala-Gly were obtained from Chem. Dynamics Corp., South Plainfield, NJ. Val-Gly and Gly-Ser-Ala were purchased from Bachem Inc., Torrance, CA. The peptides were judged pure by elemental analysis (kindly performed by Mr. S. T. Bella of this institution), and each gave a single peak upon amino acid analysis. The separate D- and L-isomers of glyceraldehyde and D_2O were obtained from Aldrich Chemical Co., Milwaukee, WI.

Kinetics of reaction of glyceraldehyde with peptides. — The condensation of glyceraldehyde (50mM) with each amino group-containing compound (1mM) was carried out at various pH values and for different times of incubation at 30°. The pH values, measured before and after the incubation, did not change by more than 0.1 pH unit. Aliquots of each reaction mixture were removed at appropriate time intervals, and the amount of unreacted amine was measured by the Fluram assay[7,8]. The rate of disappearance of amine was plotted as a function of time of incubation.

These plots were linear when drawn as a pseudo-first-order reaction. The rate constant was calculated from the slope of the line.

Reduction of the reaction mixture of Ala-His and glyceraldehyde. — In those studies in which the reducing agent was present at initial times, 6mM Ala-His, 60mM glyceraldehyde, and 120mM $NaCNBH_3$ were incubated in 50mM potassium phosphate (pH 7.0) for 3 h at 50°. After lyophilization, the mixture was desalted at room temperature on a Sephadex G-10 column (90 × 1.5 cm), equilibrated with 60mM acetic acid. After lyophilization, the mixture was separated by l.c. on an Altex Ultrasphere-ODS column (25 × 1.0 cm), which was equilibrated with 0.1% trifluoroacetic acid.

In those studies in which the reducing agent was added *after* the reaction, 5mM Ala-His and 50mM glyceraldehyde were incubated in 50mM potassium phosphate (pH 7.0) for 30 min at 30°. M $NaBH_4$ in 10mM NaOH was added dropwise to the mixture ([$NaBH_4$]:[glyceraldehyde] 1:2), maintaining the pH at 6.0 with acetic acid. After 10 min, the pH was adjusted to 2.0 with concentrated HCl to destroy excess $NaBH_4$. After lyophilization, the mixture was desalted at room temperature on Sephadex G-10 equilibrated with 60mM acetic acid. After another lyophilization, the components were separated by l.c. on a Bio-Rad TSK SP-5PW column (75 × 7.5 mm), which was equilibrated with 20mM sodium phosphate (pH 2.8) and 65mM NaCl.

In those studies in which the *isolated* component was reduced, 5mM Ala-His and 50mM glyceraldehyde were incubated in 50mM potassium phosphate, pH 7.0 for 30 min at 30°. After lyophilization, the mixture was desalted on Sephadex G-10 and subjected to l.c. as described above. The material that was eluted at 27–30 mL was reduced with $NaBH_4$ as described above.

Analyses. — The products of the reaction were analyzed by amino acid analysis on the system of Spackman, Stein, and Moore[9]. Aliquots of the reaction mixture, taken at various time intervals, were diluted with 0.2M sodium citrate buffer, pH 2.2, and applied to the 0.9 × 60 cm or the 0.6 × 20 cm column of the amino acid analyzer.

As reported previously[3], when the amounts of the dipeptide Val-His remaining after treatment with glyceraldehyde were compared by amino acid analysis and by the Fluram assay, the profiles of disappearance of the dipeptide were qualitatively very similar, although the recovery based on ninhydrin was always somewhat lower than the recovery of Fluram-positive material. (The amino acid analysis calculations of the dipeptide remaining were made after chromatographic separation of the Schiff base on the column of the analyzer[3], whereas the Fluram measurements were made on the unfractionated reaction mixture.) The difference in the two measurements was nearly equal to the amount of Schiff base found by amino acid analysis. Therefore, it is possible that the Schiff base reacts with the Fluram reagent at room temperature. In the present experiments, the amount of the glyceraldehyde-tripeptide Schiff base adducts constituted ~5% of the components in the reaction. Therefore, we assumed that the use of the Fluram assay is valid to ~95%

in generating the profile of disappearance of the tripeptide. The extent to which the Fluram reagent reacts with any of the other intermediates, *i.e.*, carbinolamines, is not known but these are present in only small amounts (see below). The assumption that the Fluram assay was a valid measure of the amount of peptide remaining was ascertained with the two tripeptides described below in this communication.

The determination of glyceraldehyde and the other carbonyl compounds present in the various fractions was carried out by the 2,4-dinitrophenylhydrazine assay as previously described[3,10].

^{1}H-N.m.r. studies. — The ^{1}H-n.m.r. spectra were recorded at 300 MHz with a Nicolet Spectrometer. Samples were prepared in D_2O after repeated replacement of the residual water with D_2O by lyophilization. Chemical shifts are given in δ values downfield from the signal of the external standard, tetramethylsilane (0.1%) in $CDCl_3$.

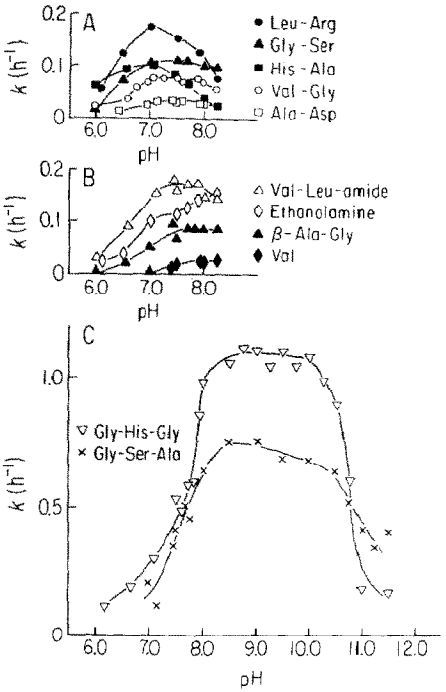


Fig. 1. Rate of disappearance of various peptides and related compounds in the presence of glyceraldehyde as a function of pH. Substrate (mM) was mixed with 50mM DL-glyceraldehyde at 30° in 50mM potassium phosphate (pH 6.0–11.5). Reaction times of 2–4 h were used for the peptides that react rapidly with glyceraldehyde but reaction times of up to 8 h were employed for the slowly reacting peptides. In general, incubation times were chosen in order to obtain sufficient data to plot the pseudo-first-order profile. The concentration of glyceraldehyde was found to be unchanged for reaction periods up to 8 h. For the reaction from pH 8.5 to 11.5, M potassium hydroxide was added to maintain the indicated pH by use of a pH stat apparatus (Radiometer). The amount of peptide remaining was measured with Fluram.

RESULTS

pH Profile of the reaction of glyceraldehyde with various di- and tri-peptides. — Previously, we had demonstrated[3] that the pH profile of the reaction of glyceraldehyde with Val-His or Ala-His showed an optimum in the range of pH 6.5–6.8. The presence of a different basic amino acid, arginine, as the second residue showed nearly the same extent of reactivity of the dipeptide, except for a slight shift of the pH optimum to a higher value (Fig. 1A). Of particular note, the reaction of the peptide His-Ala is significantly different than that of Val-His. When histidine is the first amino acid residue and the second amino acid is neutral (His-Ala), the reaction rate of the NH_2-terminus was decreased by ~50%, compared to the reactivity[3] of Val-His. (We had previously established[3] that the reactivity of Ala-His is the same as that of Val-His.) The presence of two neutral amino acids, as in Val-Gly, also serves to reduce the rate of reaction to about the same extent as for His-Ala. The presence of an additional negative charge at the second residue, as in Ala-Asp, led to an extremely slow rate of reaction with glyceraldehyde. The pH profiles for the dipeptides that were studied showed optima in the range of pH 6.5–8.0.

The role of the carboxyl group in the rate of reaction was ascertained by studies illustrated in Fig. 1B. The reaction profile with ethanolamine was intermediate between those of dipeptides having a neutral substituent and those having a basic group at the second residue. However, the dipeptide amide, Val-Leu amide, displayed a reactivity similar to those of dipeptides having a basic group at the second position (Leu-Arg and Val-His, as reported previously[3]). In each case, the *net* charge of the second amino acid residue is zero. It appears, then, that the carboxyl group *per se* cannot alone account for the increased reactivity of dipeptides, especially since the free amino acid, L-Val, showed the lowest reactivity with glyceraldehyde. Furthermore, since β-Ala-Gly displayed about the same reactivity as Val-Gly (Fig. 1B), the pK_a value of the amino group does not seem to be the sole determinant in the reaction profile, especially since the pK_a values of the NH_2-terminal amino groups are not as different as are their reactivities with glyceraldehyde. Perhaps, the extent of interaction of these two functional groups at some finite secondary stage of their structure is a factor governing the reactivity (see below).

The reaction profiles of glyceraldehyde with tripeptides indicated distinct differences as compared with those for dipeptides. With tripeptides, the rates of the reaction were significantly increased, and the pH optima were shifted to higher pH values, in the range of pH 8–10 (Fig. 1C). The tripeptides were 3–5 times more reactive with glyceraldehyde than were the dipeptides or related compounds. The presence of a histidine residue at the second position, as in the tripeptide Gly-His-Gly, led to a very large increase in the reactivity of the amino terminal residue with glyceraldehyde. Thus, this generalization with respect to the basicity of the second amino acid residue is true not only for dipeptides but especially for tripeptides.

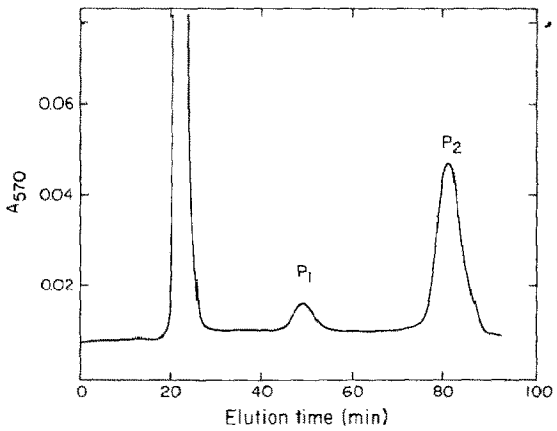


Fig. 2. Amino acid analysis of the components formed between Gly-His-Gly and glyceraldehyde. The experimental conditions were the same as those described in the legend to Fig. 1. The buffer used was 50mM potassium phosphate, pH 7.8, and the reaction time was 2.75 h. An aliquot was then removed, diluted to pH 2.2 with 0.2M sodium citrate, and applied to the 0.6 × 20-cm column of the amino acid analyzer. The column was eluted with 0.35M sodium citrate, pH 5.28.

Reaction products with Gly-His-Gly and glyceraldehyde. — Amino acid analysis of the reaction mixture of the tripeptide Gly-His-Gly with glyceraldehyde was performed after 3 h of incubation. The sample, which was colorless (indicating that a pronounced Maillard reaction had not occurred), showed three components which were eluted with pH 5.28 buffer (Fig. 2). This pattern closely resembled that found[3] for the reaction products of glyceraldehyde and the dipeptide Val-His. The ninhydrin-positive peak at 82 min of elution (P_2) is the unreacted tripeptide. These amounts of Gly-His-Gly remaining at various intervals, as determined by amino acid analysis, agreed very well with the values determined with the Fluram reagent.

The peak at about 50 min of elution (P_1) is most likely the Schiff base, which

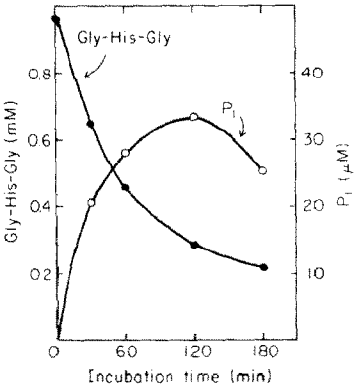


Fig. 3. Kinetics of utilization of Gly-His-Gly and appearance of intermediates. The reaction conditions and the analyses were the same as those described in the legend to Fig. 2. The analyses were performed at different times of incubation.

by analogy with previous studies[3], probably hydrolyzes to the free tripeptide (ninhydrin-positive) in the 100° heating coil of the amino acid analyzer *after* the chromatographic separation[9]. In the previous study[3], the compound designated as the Schiff base was reduced by sodium borohydride and, thus, rendered ninhydrin-negative. Since the chromatographic profiles found for these products of the di- and tri-peptide reaction with glyceraldehyde were so similar, it is assumed that P_1 is the Schiff base. Glyceraldehyde was eluted in the void volume of the column (20–25 mL) and reacted with ninhydrin to give an undefined chromophore. No other ninhydrin-positive materials were eluted from either column of the amino acid analyzer.

The kinetic profile of the reaction of Gly-His-Gly with glyceraldehyde is shown in Fig. 3. The putative Schiff base, P_1, reached a steady-state concentration that slowly decreased. This event happened concurrently with the disappearance of the tripeptide (P_2). However, it is evident from this profile that, after 3 h of reaction, a significant amount of material could not be accounted for by ninhydrin on either column of the amino acid analyzer. Thus, the recovery of unreacted tripeptide together with the Schiff base was only ~25%. Furthermore, it is apparent from an extrapolation of Fig. 3 that the yield would have been even lower for longer times of incubation.

In an effort to locate the ninhydrin-negative material, a large-scale incubation mixture of Gly-His-Gly with glyceraldehyde was applied to the 0.9 × 60-cm column of the amino acid analyzer. The eluent was collected in a fraction collector rather

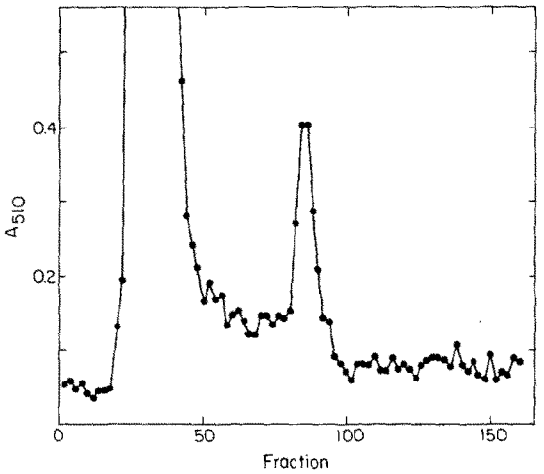


Fig. 4. Analysis for carbonyl compounds in the reaction mixture of Gly-His-Gly and glyceraldehyde. The tripeptide (5mM) was mixed with 50mM DL-glyceraldehyde in 50mM potassium phosphate buffer, pH 7.8. The reaction was carried out for 3.5 h at 30°. An aliquot (1.0 mL) was removed, and the pH adjusted to 2.2 with 0.2M sodium citrate (0.2 mL) and M HCl (60 μL). The sample was applied to the 0.9 × 60-cm column of the amino acid analyzer and eluted with 0.2M sodium citrate, pH 4.25. The collected fractions were assayed for the presence of carbonyl compounds with 2,4-dinitrophenylhydrazine as described in the text.

than passing it through the ninhydrin reaction coil[9]. Analysis[3] of these fractions with 2,4-dinitrophenylhydrazine[10] for 4 h indicated the presence of two components. A large amount of carbonyl-containing material was eluted in fractions 21–50 (void volume) in the position of glyceraldehyde (Fig. 4). Another compound, which was eluted with the pH 4.25 buffer at fractions 80–92, reacted with 2,4-dinitrophenylhydrazine but this material was ninhydrin-negative. Amino acid analysis of this component *after* acid hydrolysis indicated the presence of glycine and histidine in the ratio of 1.9:1. These results are consistent with the presence of a ninhydrin-negative derivative of Gly-His-Gly that contains a carbonyl moiety. The yield of this material was 41% and, therefore, it represents a significant portion of the remaining (*i.e.*, ninhydrin-negative) material. In an earlier study[3] with glyceraldehyde and the dipeptide Ala-His, a similar product was isolated by adsorption on Dowex 50 cation-exchange resin. Its chromatographic behavior was very similar to that of the ketoamine adduct formed between a hexose and valine, as reported by Walton *et al.*[18]. The compound that we isolated previously was ninhydrin-negative and positive to 2,4-dinitrophenylhydrazine. By elemental analysis, amino acid composition, analysis by the Pauly reagent, and composition of the isolated 2,4-dinitrophenylhydrazone derivative, the structure of that product was consistent with that of the ketoamine adduct, and it was isolated in 60% yield. Therefore, by analogy, the compound isolated in the reaction mixture of the tripeptide with glyceraldehyde was presumed to be the ketoamine adduct formed after Amadori rearrangement.

^{1}H-N.m.r. spectra of adducts formed between alanylhistidine and glyceraldehyde. — A high-resolution ^{1}H-n.m.r. spectrum of the dipeptide, alanylhistidine, was obtained at 300 MHz for a solution in D_2O. Peak assignments were made based upon published low-resolution data (Aldrich map) and decoupling experiments. The chemical shifts at pD ~3.7 were assigned as follows: for CH_3-Ala, δ 1.51 (d, J 7.3 Hz); H_α-Ala, δ 4.03 (q, J 6.9 Hz); CH_2-His, δ 3.16 (q, d, J 15.2, 6.7 Hz); H_α-His, δ 4.46 (q, J 5.5 Hz); and imidazole-His, δ 7.13 and 8.19.

^{1}H-N.m.r. spectrum of reduced glyceraldehyde-alanylhistidine adduct. — The reduced form of ketoamine formed between alanylhistidine and glyceraldehyde was prepared as described in the Experimental section. The ^{1}H-n.m.r. spectrum of this compound confirmed the proposed structure. Resonances attributable to protons of amino acid residues were assigned by comparison with spectra of the corresponding dipeptide, alanylhistidine. The additional resonances observed were assigned to protons of the glyceryl residue which is attached to the amino terminus of the dipeptide.

Characteristic coupling pattern of H-1 protons centered at δ 3.05 suggested unequivalent environments of H-1a and H-1b. By analogy with the report of Walton *et al.*[18]. H-1a and H-1b are considered to represent the resonance of H-1 of the glyceraldehyde residue adjacent to the secondary amino group of the peptide. The chemical shifts of H-1a and H-1b were also shifted upfield and are clearly distinguished from typical methylene protons of sugar molecules which appear between δ 3.5 and 3.8. The complex resonance at δ 4 is due to H-2 which couples

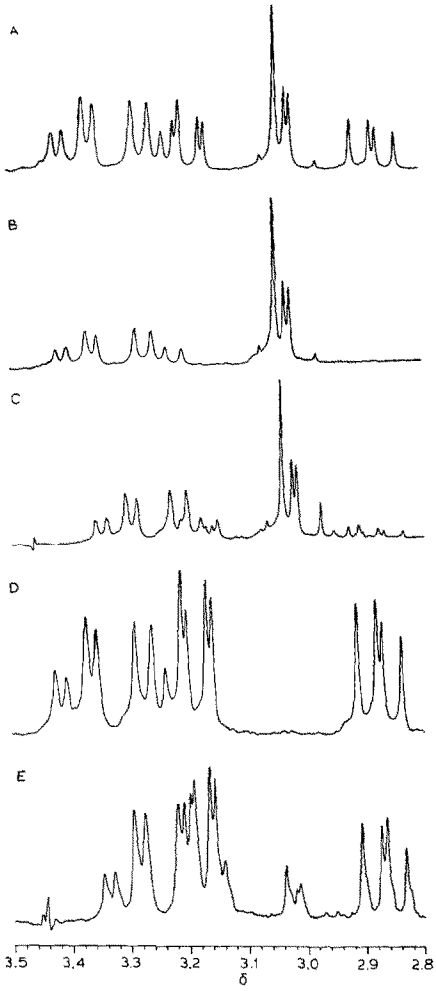


Fig. 5. ^{1}H-N.m.r. analysis after reduction of the reaction mixture of Ala-His and glyceraldehyde. Ala-His (6mM), 60mM glyceraldehyde, and 120mM $NaCNBH_3$ were incubated in 50mM potassium phosphate, pH 7.0, for 3 h at 50°. After lyophilization, the mixture was desalted on a column (90 × 1.5 cm) of Sephadex G-10, which was equilibrated with 0.06M acetic acid, at room temperature. After lyophilization, the mixture was separated by l.c. on an Altex Ultrasphere-ODS column (25 × 1.0 cm), which was equilibrated with 0.1% trifluoroacetic acid: (A) DL-Glyceraldehyde, (B) L-glyceraldehyde, and (D) D-glyceraldehyde. In (C) and (E), the reducing agent was added *after* the reaction and L-glyceraldehyde and D-glyceraldehyde were used, respectively. Ala-His (5mM) and 50mM glyceraldehyde were incubated in 50mM potassium phosphate (pH 7.0) for 30 min at 30°. After the pH was adjusted to 6.0 with acetic acid and maintained at that value by occasional addition of acetic acid on a pH stat, M $NaBH_4$ in 0.01M NaOH was added dropwise to the reaction mixture to achieve a final $NaBH_4$ concentration of 25mM. After 10 min, the pH was adjusted to 2.0 with concentrated HCl to destroy excess $NaBH_4$. After lyophilization, the mixture was desalted on Sephadex G-10, which was equilibrated with 0.06M acetic acid at room temperature, and then subjected to l.c. on a Bio-Rad TSK SP-5PW column (75 × 7.5 mm), which was equilibrated with 20mM sodium phosphate, pH 2.8, and 65mM NaCl. On this column, the compounds described in (A), (B), and (D) were eluted in the same position as the compounds described in (C) and (E).

with H-1a, H-1b, and the resonance at δ 3.62. The latter resonance indicated two protons by integration and represents H_2-3 of the glyceraldehyde residue.

The profiles of the reaction intermediates and products were further examined by employing sodium cyanoborohydride or sodium borohydride as reducing agents, added either at the beginning of the reaction or after the reaction had taken place (Scheme 1). When the DL-glyceraldehyde was added to the dipeptide L-Ala-L-His, and sodium cyanoborohydride was present during the course of the reaction, a series of multiplets between δ 2.8 and 3.25 were observed by ^{1}H-n.m.r. spectroscopy (Fig. 5A). When the L-isomer of glyceraldehyde replaced the racemic mixture, and sodium cyanoborohydride was present during the course of the reaction, the ^{1}H-n.m.r. spectrum indicated that the multiplet at δ 3.0–3.1 was present but the other multiplets at δ 2.8–2.94 and 3.14–3.24 were absent (Fig. 5B). It is likely that the multiplet at δ 3.0–3.1 is due to the interaction of the methylene protons (H-1a,1b) with H-2 of the L-isomer of glyceraldehyde. The multiplets at δ 2.8–2.94 and 3.14–3.24 are likely due to the analogous coupling of H-1a and H-1b with H-2 of the D-isomer of glyceraldehyde. The latter conclusion was supported by the results shown in Fig. 5D in which D-glyceraldehyde was used.

Of particular interest is the finding that when sodium borohydride was added *after* the reaction with either L- or D-glyceraldehyde, then both isomers were found, although not in equal amounts (Fig. 5C and 5E). These results suggest that a finite amount of an intermediate without an asymmetric carbon atom, such as the ketoamine, was present and that its reduction with sodium borohydride produced both stereoisomers. A related explanation was that there was some labilization of H-1, perhaps through the enamine as suggested by Roper *et al.*[19]. Assuming that the most likely candidate for this species is the ketoamine through calculation of

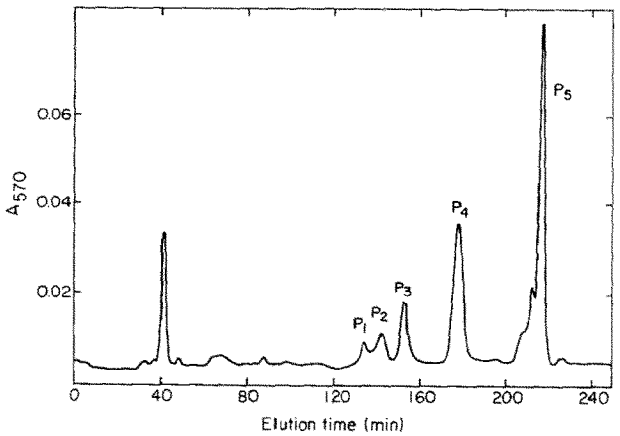


Fig. 6. Amino acid analysis of the components formed between Gly-Ser-Ala and glyceraldehyde. The experimental conditions were the same as those described in the legend to Fig. 1. The buffer used was 50mM potassium phosphate, pH 7.8, and the reaction time was 3.5 h. An aliquot was then removed, diluted to pH 2.2 with 0.2M sodium citrate, and applied to the 0.9 × 60-cm column of the amino acid analyzer. The column was eluted with 0.2M sodium citrate, pH 3.25, for 2.5 h, and then with 0.2M sodium citrate, pH 4.25, for 90 min.

the integration values for the H-1 protons (H-1a and H-1b), we have estimated the amount of ketoamine in the sample to be ~28% when either D- or L-glyceraldehyde were incubated for 0.5 h with L-Ala-L-His, and then reduced by $NaBH_4$. In the earlier study[3], a 4-h incubation time was used and a greater amount of ketoamine was found. This difference in the amounts of ketoamine as a function of time of incubation is consistent with the hypothesis that the Amadori rearrangement is a rate-limiting step in the reaction.

We also found that when $NaBH_4$ was added *after the isolation* on l.c. of the putative Schiff base between L- or D-glyceraldehyde and alanylhistidine, the H-1 protons showed complete racemization by ^{1}H-n.m.r. spectroscopy. This result suggests that during the isolation and purification of the Schiff base, H-2 becomes labile. On the other hand, if $NaCNBH_3$ was added at the beginning of the reaction, the configuration at C-2 was maintained.

Studies with the tripeptide Gly-Ser-Ala. — As shown in Fig. 6, amino acid analysis of the reaction mixture of Gly-Ser-Ala and glyceraldehyde showed more ninhydrin-positive components than were detectable from analysis of the reaction products formed with the tripeptide Gly-His-Gly (Fig. 2). Component P_5 is the unreacted tripeptide as determined by amino acid analysis. The profile of its disappearance was qualitatively similar to that found by Fluram analysis. However, the agreement between these assays was not as good as that found with Gly-His-Gly (described above). This finding is consistent with a larger pool of intermediates in the reaction of Gly-Ser-Ala with glyceraldehyde as described below. The peak at 40 min of elution time with the pH 3.25 buffer in the void volume was, most likely, unreacted glyceraldehyde. Four other peaks were eluted from the long column of the amino acid analyzer. The possible identity of these peaks is discussed below.

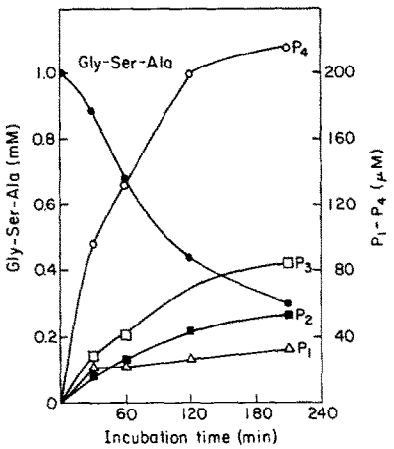
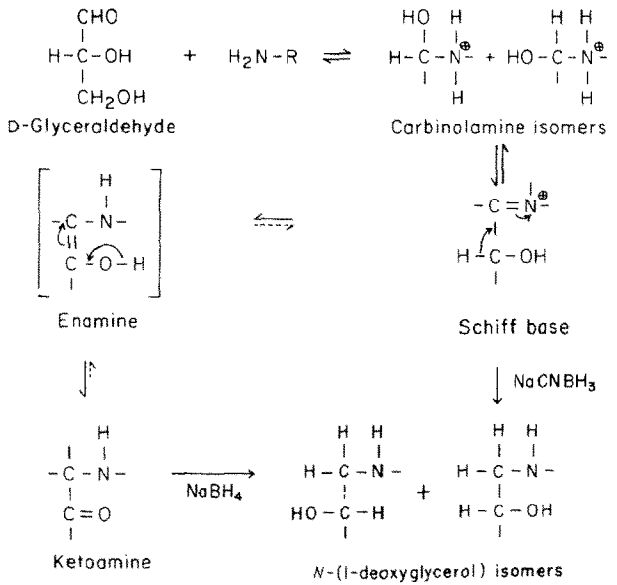


Fig. 7. Kinetics of disappearance of the tripeptide Gly-Ser-Ala and the appearance of intermediates. The reaction conditions and the analysis were the same as those described in the legend to Fig. 4.



Scheme 1. Reaction of peptides with glyceraldehyde.

As shown in Fig. 7, the kinetic profile of these components after different times of incubation is significantly different from that found with the tripeptide Gly-His-Gly (Fig. 3), which constitutes a less complex mixture of components. The disappearance of Gly-Ser-Ala occurred with the simultaneous appearance of four other ninhydrin-positive peaks. Component P_4, which is formed in highest yield, may represent the Schiff base. The other three peaks, which are formed in lesser amounts (P_1, P_2, and P_3), could represent the protonated carbinolamine diastereoisomers as discussed below (see Scheme 1). However, the absolute identification of these four components must await further study on larger amounts.

The significant difference between the reaction profiles of the tripeptides Gly-Ser-Ala and Gly-His-Gly is that the amount of recoverable ninhydrin-positive material in the former case (Gly-Ser-Ala) is 72% and in the latter case it is only 25%. Some of this material was located in the following way: A large amount of incubation mixture of Gly-Ser-Ala and glyceraldehyde was applied to the amino acid analyzer, and fractions were collected as described above for the tripeptide Gly-His-Gly. Analysis of the collected fractions with 2,4-dinitrophenylhydrazine and ninhydrin showed the presence of a carbonyl-containing, but ninhydrin-negative, component which was eluted just after the elution position of glyceraldehyde in the void volume of the column. This material was found to contain the amino acids glycine, serine, and alanine in the ratios of 1.0:1.0:1.1 *after* acid hydrolysis. The presence of the intact tripeptide in a ninhydrin-negative structure that reacts with 2,4-dinitrophenylhydrazine is consistent with a ketoamine structure of the adduct of glyceraldehyde and Gly-Ser-Ala formed after Amadori rearrangement. However, the amount of this material is much less than that found for the

corresponding adduct formed with Gly-His-Gly and glyceraldehyde, and its absolute identification will require the isolation of more material.

DISCUSSION

Scheme 1 outlines the details of a pathway for the reaction of peptides with glyceraldehyde. Parts of this Scheme have been suggested previously[4,5]. Some of the intermediates are more stable than others and therefore could conceivably be isolated. For example, whereas there would be little expectation of isolating the highly reactive enamine, other intermediates such as the carbinolamine and the aldimine (Schiff base) adducts could be detected under the relatively mild conditions of amino acid analysis used in the present study. Furthermore, the ketoamine adduct formed irreversibly after the Amadori rearrangement is sufficiently stable under such mild conditions that it can be isolated without the undesired browning (Maillard) reaction. Indeed, the reaction mixtures analyzed in the present study were colorless. In an earlier study[3], evidence for the presence of the Schiff base (aldimine) intermediate was based upon the observation that the preponderant ninhydrin-positive derivative formed in the reaction of glyceraldehyde with the dipeptides Val-His or Ala-His was rendered ninhydrin-negative upon reduction with $NaBH_4$. None of the other stable intermediates in this Scheme would have such a property. For example, the carbinolamine intermediates, which would probably also react with ninhydrin owing to dehydration in the 100° reaction coil of the amino acid analyzer, would not be expected to be reducible by $NaBH_4$. Perhaps the compounds P_1–P_3, detected by amino acid analysis in the reaction of the tripeptide Gly-Ser-Ala with glyceraldehyde (Fig. 5), are the diastereoisomers of the carbinolamine.

The results presented herein clearly show that different sequences in di- and tri-peptides can lead to significant variations in their rates of reaction with glyceraldehyde. This could be due to the primary structure alone or to interactions of other parts of the peptide with the intermediate adducts formed with glyceraldehyde. The results suggest an important role for positively-charged basic amino acid residues in increasing the rate of reaction. The pK_a values of the NH_2-terminal residues of the various peptides and their analogs studied are in the range of pH 8–10.5. However, the observed differences in their extent of reaction with glyceraldehyde does not appear to correlate with this property. Thus, the α-NH_2 group *per se* probably does not dictate either the reaction profile or the extent of formation of the products of reaction. These considerations make it seem likely that these differences in reactivity are due to positively-charged amino acid residues present at the second and perhaps other positions of the peptides with respect to the manner in which they interact with the glyceraldehyde adduct at the NH_2-terminus. This suggestion, which we have made previously[3], is reinforced by the results presented herein. Serum albumin also undergoes glycation by glucose[11], and a recent study[12] has noted the proximity of positively-charged amino acid residues to the sites of glycation by glucose.

The role of the carboxyl group of the di- and tri-peptides in facilitating the Amadori rearrangement seems marginal for two reasons. First, ethanolamine and Val-Leu amide are as reactive as any of the dipeptides that have free carboxyl groups. Second, the carboxyl group would be fully charged at all of the pH values under which these studies were conducted, so it is unlikely that such significant differences in the reactivity profiles could be ascribed to the presence of this functional group alone. However, it is conceivable that the stereochemical interaction of the carboxyl group with the α-NH_2 group carrying the glyceraldehyde moiety might be more favored with tripeptides, thus leading to their enhanced reaction rates compared with dipeptides. This possibility will be assessed in studies with longer peptides of varying composition. Whether the carboxyl group favors rearrangement in polypeptides may be a different question because of the greater distance between the NH_2-terminus and the internal carboxyl groups. Further studies with an extended series of amides or esters (or both) are needed to establish this point.

The difference in the pH optimum between di- and tri-peptides is also notable since the reaction profiles of tripeptides display pH optima that are generally shifted about 1–2 pH units higher than those for dipeptides. The amplitude of the reactivity with the tripeptides is also significantly increased, as compared to that for dipeptides. This could be due to the appearance of the early stages of secondary structure in the tripeptides that might not be present in the dipeptides. The increased rate of reaction of the tripeptide Gly-His-Gly could be due to the presence of an imidazole nitrogen atom that could lead to an enhanced rate of ketoamine formation due to proton abstraction from the Schiff base to form the enamine (see Scheme 1). High-resolution ^{1}H-n.m.r. spectroscopy provides independent evidence of the stereochemical course of the reaction. The studies described herein suggest that ^{1}H-n.m.r. spectroscopy could be used to estimate the amount of ketoamine adduct formed during the reaction.

Differences in the rate of reaction of various amino acids with glyceraldehyde were also noted recently by other investigators[13]. In those studies, a product of the Maillard reaction was assayed. However, in our studies, the reaction between glyceraldehyde and the peptide has been monitored at an earlier stage of the reaction, since the overall recovery of material in the present study is high and there is little detectable color in our reaction mixtures. The shorter times of incubation used in the present study most likely prevented the formation of yellow pigments observed by Candiano *et al.*[13].

The observation that both D-glucose[13–15] and glyceraldehyde[17] react with the terminal valine residue of the β-chain of hemoglobin to form a ketoamine adduct suggests that there is an environment around this particular residue which facilitates the Amadori rearrangement. The analogous ketoamine does not form readily at the terminus of the α-chain, although the Schiff base with glyceraldehyde is formed with ease[17]. The environment that favors ketoamine formation is apparently absent from this region of the molecule. It is tempting to speculate, from the results of the

present study, that the histidine residue at the second position of the β-chain of hemoglobin is involved in facilitating the Amadori rearrangement either with glyceraldehyde or glucose at the amino terminal valine residue[21]. Indeed, the other amino acid residues that undergo the Amadori rearrangement with glyceraldehyde[1] are also very near to histidine residues in the three-dimensional structure of hemoglobin.

It is clear from the studies presented herein that continued studies on such model peptide systems should provide information regarding those amino acid residues of a protein that facilitate the Amadori rearrangement and whether proximity factors are involved at the primary or secondary structure (or both) of the protein.

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