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Cross-Linking of Proteins by Aldotriose: Reaction of the Carbonyl Function of the Keto Amines Generated in Situ with Amino Groups†

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ABSTRACT: Nonreductive modification of proteins with glyceraldehyde forming 2-oxo-3-hydroxypropylated protein is mechanistically analogous to nonenzymic glycation reactions. The latent cross-linking potential of glyceraldehyde as a consequence of the reactivity of the carbonyl function of 2-oxo-3-hydroxypropyl groups of nonreductively modified protein has been now investigated. Reaction of RNase A (0.5 mM) with glyceraldehyde (20 mM) at pH 7.4 and 37 °C for 4 h resulted in the intermolecular cross-linking of the protein, with the concomitant development of a yellow chromophore with two new absorption bands having maxima around 305 and 375 nm. The product exhibited a fluorescence that had excitation and emission maxima around 365 and 450 nm, respectively. The presence of $NaCNBH_3$ during the reaction, which selectively reduces the Schiff base adducts of aldotriose to form 2,3-dihydroxypropyl groups on proteins, inhibited both the cross-linking reaction and the development of the absorption and fluorescence characteristics. The hydroxymethyl group of the aldotriose is not an essential moiety since the cross-linking potential of glyceraldehyde is comparable to that of glyceraldehyde 3-phosphate. The formation of cross-links appears to involve the carbonyl function of the keto amines resulting in the formation of Schiff base adducts (ketimine linkages) as the initial event. Consistent with this, incubation of 2-oxo-3-hydroxypropylated RNase A with [^{14}C]glycine ethyl ester resulted in the incorporation of the reagent into the protein. The cross-linking reaction was inhibited when the reaction of RNase A with glyceraldehyde was carried out in the presence of amino compounds, such as glycine ethyl ester, ethanolamine, glucosamine, and aminoguanidine. An equimolar amount of aminoguanidine inhibited the nonreductive incorporation of [^{14}C]glyceraldehyde into RNase A by nearly 85%. The inhibition of the cross-linking reaction by the aminoguanidine and other amino compounds is predominantly a consequence of the inhibition of the nonenzymic glycation of RNase A. The results of the present study demonstrate that the protein cross-linking by aldotriose under physiological conditions is latent and is a consequence of the reactivity of the carbonyl function of keto amines generated in situ with the amino groups of protein.

The reaction of glyceraldehyde (2,3-dihydroxypropionaldehyde), an aldotriose, with hemoglobin (Hb) is mechanistically similar to nonenzymic glycation reaction (Acharya & Manning, 1980). Just as in the case of nonenzymic addition of glucose to Hb (Holmquest & Schroeder, 1966; Wold, 1981; Brownlee & Cerami, 1981; Brownlee et al., 1984), the reversible Schiff base adducts of glyceraldehyde with the amino groups of Hb undergo Amadori rearrangement to form stable keto amine adducts (Figure 1). Consistent with this similarity in the mechanism of the reaction of aldohexose and aldotriose, many of the sites on HbA that are reactive toward glyceraldehyde in vitro are the same as those that are nonenzymically glycated (Acharya & Manning, 1980; Shapiro et al., 1981).

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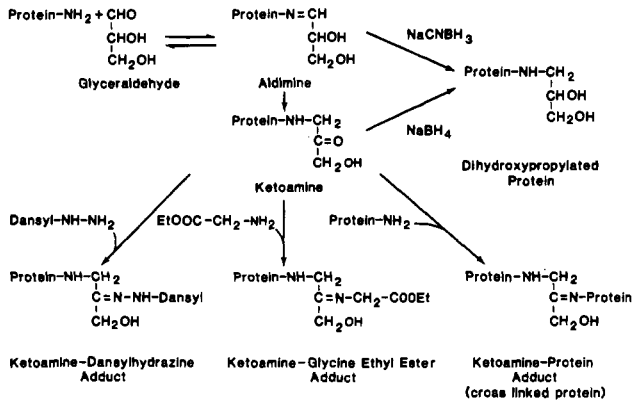


FIGURE 1: Schematic representation of the reaction of glyceraldehyde (aldotriose) with protein amino groups to introduce cross-links.

The study with aldotriose suggested that Amadori rearrangement may be a general property of Schiff base adducts of α-hydroxy aldehydes with the amino groups of proteins rather than being unique to aldohexoses. Indeed, the Schiff base adducts of glycolaldehyde, an aldodiose, with RNase A were also found to undergo Amadori rearrangement (Acharya & Manning, 1983). However, the reaction of aldodiose and aldotriose with proteins occurs at a significantly faster rate than that of aldohexose. The lower reactivity of aldohexose with proteins is, apparently, a reflection of the fact that only a small fraction of aldohexose is normally present in the aldehydic form, the reactive species for the formation of the initial Schiff base adducts (Angyl, 1979).

Though the Schiff base adducts of glycolaldehyde undergo the Amadori rearrangement just as the adducts of the higher homologues, one significant difference becomes apparent when the Amadori product of aldodiose is compared with that of aldotriose and aldohexose. The Amadori product of aldodiose is an aldo amine, whereas that of aldotriose and aldohexose is a keto amine. Since the Amadori rearrangement of aldodiose generates a new aldehydic function in situ, glycolaldehyde was expected to show a latent cross-linking potential. Our earlier studies have indeed demonstrated the introduction of covalent cross-links into proteins by glycolaldehyde (Acharya & Manning, 1983).

An aspect of nonenzymic glycation of proteins that has not been addressed in full detail in the past is the possible reactivity of the carbonyl function of the keto amine adducts. If the carbonyl function of the keto amine exhibits reactivity toward amino groups of proteins (to form ketimines), this may provide one of the possible pathways, if not the only one, for the formation of cross-linked proteins as a chemical consequence of nonenzymic glycation (Figure 1). In principle, the anticipated reactivity of the keto amine of the aldotriose is similar to that of the carbonyl function of dihydroxyacetone and fructose. The 2-oxo-3-hydroxypropyl groups (OHP) on the ϵ-amino groups of lysine residues of a protein nonreductively modified with glyceraldehyde could be considered as substituted acetol. The carbonyl group of acetol has been demonstrated to form Schiff base adducts with the amino groups of proteins (Geoghegan et al., 1979). Thus, it is conceivable that the carbonyl function of OHP moieties on the protein is also reactive. This aspect of reactivity of the carbonyl function of OHP groups as well as the cross-linking of proteins as a chemical consequence of this reactivity has been investigated in the present study.

Materials and Methods

Reaction of Glyceraldehyde with RNase A. RNase A (Sigma) (0.5 mM) in phosphate-buffered saline (PBS), pH 7.4, was incubated at 37 °C for the indicated period at a given concentration of the aldotriose (Sigma). At the end of the reaction period, the sample was desalted by passage through a column (2.2 × 30 cm) of Sephadex G-25 (Pharmacia) in 0.1 M acetic acid. The protein isolated by lyophilization is referred to as glyceraldehyde RNase A adduct. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the adducts was carried out as described earlier (Acharya & Manning, 1983).

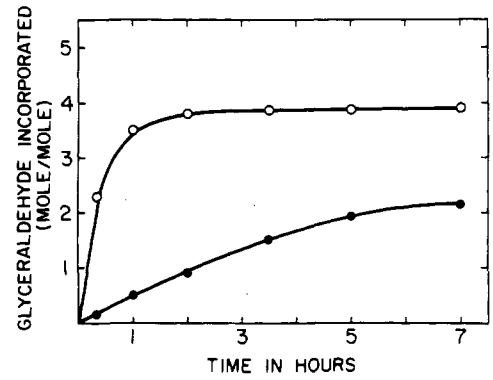


FIGURE 2: Kinetics of reductive and nonreductive modification of RNase A with glyceraldehyde: (●) nonreductive modification; (○) reductive modification in the presence of $NaCNBH_3$.

Reaction of Glyceraldehyde RNase A Adduct with 2,4-Dinitrophenylhydrazine. The reaction was carried out essentially as described earlier (Acharya & Manning, 1983). Excess reagent was removed by gel filtration on Sephadex G-25 in 0.1 M acetic acid, and the derivatized protein was lyophilized.

Fluorescence Measurements. These determinations were made in a protein MPF-4 spectrofluorometer. The solvent used was 0.1 M acetic acid.

Sodium Borohydride Reduction of Cross-Linked Material. After the indicated reaction period, the reaction mixture was diluted with an equal volume of 1 M phosphate buffer, pH 6.0, and 2 drops of 1-octanol was added. A 1 M $NaBH_4$ solution in 0.1 N NaOH is added to the sample in two to three aliquots over a period of 30 min at room temperature so that the final concentration of $NaBH_4$ becomes 0.5 M. After the addition of $NaBH_4$ was completed, the sample was kept at room temperature for another $^1/_2$ h and then dialyzed extensively against 10 mM potassium phosphate buffer, pH 6.0 at 4 °C.

Reaction of Glycine Ethyl Ester with Glyceraldehyde RNase A Adduct. A sample of glyceraldehyde RNase A adduct was prepared by incubating RNase A (0.5 mM) in PBS at pH 7.4 for 90 min with 10 mM glyceraldehyde and then desalted on a Sephadex G-25 (0.9 × 30 cm) column equilibrated and eluted with PBS, pH 7.4. The adduct was then reacted with 200 mM [^{14}C]glycine ethyl ester at pH 7.4 at room temperature for 16 h, the protein concentration being 1 mg/mL. After 16 h of incubation, the sample was desalted on a Sephadex G-25 (0.9 × 30 cm) column eluted and equilibrated with 0.1 M acetic acid, and the [^{14}C]glycine ethyl ester incorporated into the protein was estimated.

Results

Nonreductive Modification of RNase A by Glyceraldehyde. The incubation of RNase A (0.5 mM) with 10 mM [^{14}C]-glyceraldehyde at pH 7.4 and 37 °C resulted in a time-dependent incorporation of glyceraldehyde into the protein (Figure 2). Nearly 2 mol of glyceraldehyde was incorporated into the protein in about 8 h.

The glyceraldehyde RNase A adducts when incubated with dinitrophenylhydrazine incorporated the dinitrophenyl (DNP) chromophore. This demonstrates the presence of protein-bound carbonyl groups in the adduct, i.e., 2-oxo-3-hydroxypropylation of the protein has taken place. The 8-h reaction product incorporated nearly 1.8 mol of dinitrophenyl chromophore, indicating a nearly quantitative derivatization of the ketonic carbonyl group of 2-oxo-3-hydroxypropyl RNase A. In control experiments, dinitrophenylhydrazine did not react with native RNase A or with the glyceraldehyde RNase A adduct after the latter was subjected to reduction with $NaBH_4$.

In the presence of $NaCNBH_3$, the incorporation of glyceraldehyde into the protein took place at a significantly faster rate (Figure 2). Nearly 4 mol of glyceraldehyde was incorporated per mole of RNase A. Amino acid analysis showed the presence of nearly 3.5 mol of N^{ϵ}-2,3-DHP-lysine residues per mole of RNase A. The dihydroxypropylated protein did not show any reaction with dinitrophenylhydrazine. Thus, the presence of $NaCNBH_3$ completely inhibits the Amadori rearrangement. Mechanistically, the reductive dihydroxypropylation and the nonenzymic glycation (2-oxo-3-hydroxypropylation) proceed through the formation of an aldimine intermediate (Figure 1). Therefore, the results demonstrate that the Amadori rearrangement of the aldimine is the rate-limiting step in the nonreductive mode of modification of RNase A by glyceraldehyde.

Reactivity of the Carbonyl Function of 2-Oxo-3-hydroxypropylated RNase A with Carbonyl Reagents and Glycine Ethyl Ester. Glyceraldehyde RNase A adduct was treated with dansylhydrazine to establish the reactivity of carbonyl groups of keto amines toward substituted hydrazines (Fields & Dixon, 1972; Acharya & Manning, 1983; Anderson, 1986). Dansylhydrazine reacted with the OHP-RNase in 15% acetic acid as seen by the fluorescence excitation and emission spectra of the derivatized protein, which corresponded well with the fluorescence properties of the dansyl fluorophore. With an 8-h reaction product (prepared with 10 mM glyceraldehyde and 0.5 mM RNase A, pH 7.4 and 37 °C), the incorporation of dansyl fluorophores (~1.6 mol/mol) was comparable to that of the DNP chromophore (~1.8 mol/mol). In control experiments, neither RNase A nor DHP-RNase A reacted with dansylhydrazine, demonstrating that the incorporation of the fluorophore is a consequence of the modification of RNase A with glyceraldehyde in a nonreductive mode.

The reactivity of the carbonyl function of the keto amine linkages under physiological conditions was also investigated. The OHP-RNase A (prepared by incubating RNase A with 10 mM glyceraldehyde for 90 min; it contains nearly 1 mol of aldose/mol of protein) was incubated with [^{14}C]glycine ethyl ester (GEE) overnight in PBS at pH 7.4, 37 °C. Analysis of the products isolated by gel filtration showed the incorporation of nearly 0.6 mol of [^{14}C]GEE into the protein. In control experiments neither RNase A nor DHP-RNase A incorporated the GEE. This incorporation of GEE into OHP-RNase A clearly reflects the reactivity of the carbonyl function of the keto amine linkage toward the amino groups under the physiological conditions.

Cross-Linking Potential of Glyceraldehyde. The reactivity of the carbonyl function of the OHP groups toward the amino group of glycine ethyl ester suggests that glyceraldehyde should also exhibit a latent cross-linking potential in much the same way as glycolaldehyde (Acharya & Manning, 1983). RNase A (0.5 mM) was incubated with 20 mM glyceraldehyde at pH 7.4 and 37 °C for 4 h in the presence or absence of $NaCNBH_3$ (200 mM) and then desalted on Sephadex G-25 columns. The adducts generated were subjected to SDS–polyacrylamide gel electrophoresis (Figure 3). In the absence of $NaCNBH_3$, the reaction of RNase A with glyceraldehyde results in the formation of higher molecular weight bands, demonstrating the introduction of intermolecular cross-links into RNase A. If $NaCNBH_3$ is present during the incubation, the cross-linking reaction is completely inhibited. In the presence of $NaCNBH_3$, the Schiff base adducts of aldotriose with the amino groups of RNase A are reduced, resulting in the 2,3-dihydroxypropylation of the protein (Acharya et al., 1984, 1985, 1987). Thus, if the formation of keto amine is prevented, the cross-linking reaction is also inhibited. Thus, the Amadori rearrangement of the initial adducts to generate the keto amine adduct appears to be an obligatory intermediate step in the cross-linking reaction. The SDS–PAGE of the samples was run after the protein sample was reduced with β-mercaptoethanol; thus, the formation of oligomers of RNase A does not appear to be a consequence of the generation of

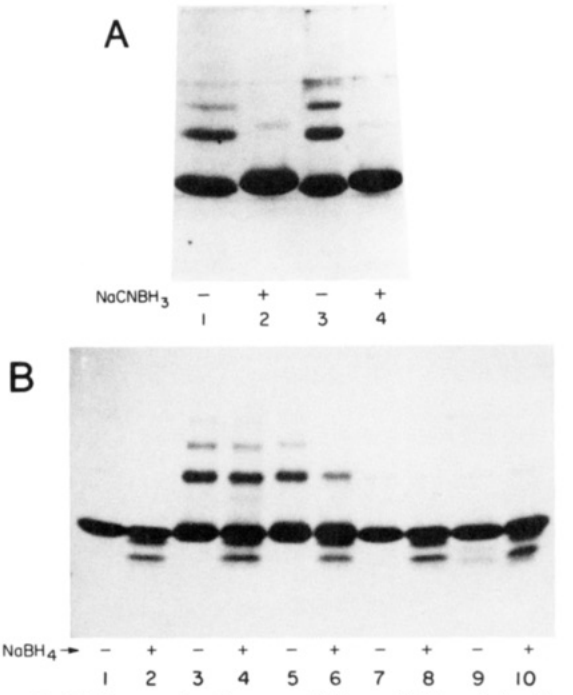


FIGURE 3: (A) Intermolecular cross-linking of RNase A by glyceraldehyde. SDS gel electrophoresis of glyceraldehyde RNase A adduct. (Lanes 1 and 2) RNase A reacted with glyceraldehyde in the absence and presence of $NaCNBH_3$; (lanes 3 and 4) RNase A reacted with glycolaldehyde in the absence and presence of $NaCNBH_3$. RNase A (0.5 mM) was treated with 20 mM aldotriose in phosphate-buffered saline for 4 h at 37 °C and then desalted on a Sephadex G-25 column in 0.1 M acetic acid, and the protein was isolated by lyophilization. (B) Influence of sodium borohydride on the intermolecular cross-linking of RNase A by α-hydroxy carbonyl compounds. RNase A (0.5 mM) was reacted with 20 mM α-hydroxy carbonyl compounds in phosphate-buffered saline for 8 h. The reacted samples divided into two equal parts. One part is directly desalted on a Sephadex G-25 column. The other part was diluted with an equal volume of 1 M phosphate buffer, pH 6.0, and reduced with 0.5 M $NaBH_4$ at room temperature for $^1/_2$ h. The sample was then desalted on a Sephadex G-25 column and the protein isolated by lyophilization. In both the control RNase A sample and the samples of RNase A reacted with α-hydroxy carbonyl compounds, sodium borohydride reduction appears to bring about some peptide bond cleavage. Such a reaction has been observed previously. (Lanes 1 and 2) RNase A with and without borohydride reduction, respectively; (lanes 3 and 4) RNase A reacted with glycolaldehyde without reduction and after reduction, respectively; (lanes 5 and 6) RNase A reacted with glyceraldehyde without reduction and after reduction, respectively; (lanes 7 and 8) RNase A reacted with dihydroxyacetone without reduction and after reduction, respectively; (lanes 9 and 10) samples of RNase A with acetol without reduction and after reduction, respectively.

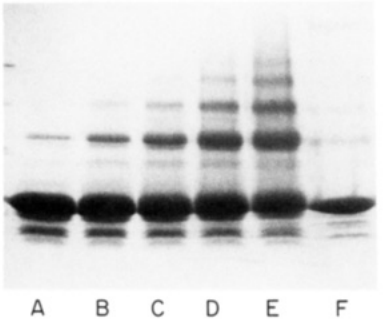


FIGURE 4: Influence of the concentration of glyceraldehyde on the cross-linking reaction. RNase A (0.5 mM) in phosphate-buffered saline, pH 7.4, was incubated with various amounts of glyceraldehyde at 37 °C for 6 h and then reduced with $NaBH_4$ as explained under Figure 3, and the protein was subjected to SDS gel electrophoresis (conditions same as in Figure 5). Glyceraldehyde concentration: (A) 5 mM; (B) 10 mM; (C) 20 mM; (D) 40 mM; (E) 80 mM; (F) 0 mM.

intermolecular disulfide bonds. Thus, the cross-linking mechanism appears to be distinct from the cross-linking through intermolecular disulfide bonds introduced on in vitro modification of crystallines by glucose (Monnier & Cerami, 1982).

The SDS gel pattern of RNase A treated with 20 mM glycolaldehyde is also shown in Figure 3A for comparison. The extent of cross-linking is somewhat higher than that seen with glyceraldehyde; trimers and small amounts of tetramers of RNase A were also present in glycolaldehyde-treated sample.

Influence of Sodium Borohydride Reduction on the Glyceraldehyde-Mediated Cross-Linking of RNase A. In an attempt to determine the influence of $NaBH_4$ reduction on glyceraldehyde-induced cross-linking of RNase A, the cross-linking pattern of the borohydride-reduced product is compared to that of the unreduced adduct (Figure 3B). SDS–PAGE of unreacted (control) RNase A shows that borohydride reduction of RNase A results in small amounts of fragmentation of the protein as well, as reported earlier (King et al., 1986). Samples of RNase A incubated with glyceraldehyde and subsequently reduced with $NaBH_4$ showed the presence of cross-linked products just as the unreduced product. However, the relative amounts of higher molecular weight products present in the sample of glyceraldehyde RNase A adducts are lowered if the reaction is terminated by $NaBH_4$ reduction prior to gel filtration (Figure 4). One of the steps in the borohydride reduction procedures is a 1:1 dilution of the reaction mixture with 1 M phosphate buffer, pH 6.0. Presumably, this dilution step results in the reversal of some of the Schiff base adducts (ketimines), thus leading to a relatively lower level of intermolecular cross-linking. The inhibitory influence of the reduction step on the glyceraldehyde-mediated cross-linking reaction is consistent with the lower propensity of the ketonic carbonyl function to form Schiff base adducts (i.e., higher propensity to reverse on dilution of the adducts).

The formation of intermolecular cross-linking on nonreductive modification of RNase A with glyceraldehyde should involve the participation of the carbonyl function of the protein-bound OHP group of one protein molecule and the amino group from another protein molecule. Therefore, one could speculate that at a given protein concentration increasing the amount of protein-bound OHP in the system would also increase the yield of the cross-linked products. Indeed, as shown in Figure 4, when the concentration of glyceraldehyde was increased from 5 to 80 mM, the yield of cross-linked products also increased. Only small amounts of dimers were seen with the 5 mM glyceraldehyde reaction product, whereas trimers and tetramers were generated when 80 mM glyceraldehyde

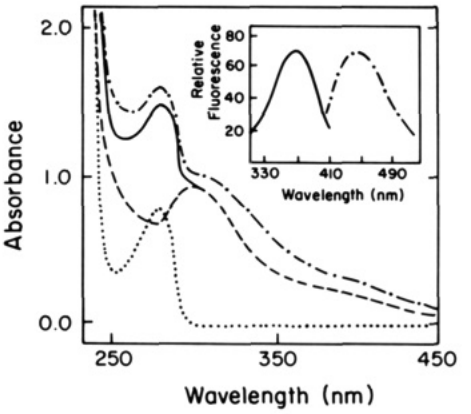


FIGURE 5: Absorption and fluorescence spectra of RNase A nonreductively modified with glyceraldehyde: (···) absorption spectra of RNase-A; (—) RNase A nonreductively modified with glyceraldehyde; (–•–) nonreductively modified with glyceraldehyde 3-phosphate in PBS, pH 7.4 and 37 °C for 16 h (aldehyde concentration 50 mM). Protein concentration 1 mg/mL. Inset shows the fluorescence excitation (—) and emission (–·–) spectra of the glyceraldehyde RNase A adduct in 0.1 N acetic acid at room temperature.

was used in the cross-linking reaction.

Generation of New Chromophores on Reaction of RNase A with Glyceraldehyde. The postulated pathway (Figure 1) for the formation of glyceraldehyde cross-linked RNase A is mechanistically similar to that of glycolaldehyde except that in the case of aldodiose the intermediate is an aldo amine. Accordingly, the spectral and the fluorescence properties of glyceraldehyde RNase A adduct have been investigated and compared with those of glycolaldehyde RNase A adduct (Figure 5). On reaction of RNase A (0.5 mM) with 50 mM glyceraldehyde for 16 h, a yellow chromophore is generated just as in the case of glycolaldehyde, resulting in a significant increase in the absorbance in the 250–400-nm region. The difference spectra revealed the presence of two new broad absorption peaks, one with a maximum around 305 nm and the other around 375 nm. This ultraviolet absorption spectrum of glyceraldehyde RNase A adduct is qualitatively similar to the spectrum of RNase A glycolaldehyde adduct (Acharya & Manning, 1983). Consistent with this similarity in the absorption spectrum, glyceraldehyde RNase adduct was also fluorescent (Figure 5, inset). The adduct exhibited fluorescence with an emission maximum around 450 nm and an excitation maximum around 370 nm. Thus, this fluorescence appears to correspond to the chromophore peaking around 375 nm. The presence of $NaCNBH_3$, which inhibits the cross-linking reaction, also inhibited the formation of the new chromophores. Thus, the cross-linking of RNase A by glyceraldehyde and the generation of new chromophores appear to be interrelated. Inhibition of Amadori rearrangement inhibits the cross-linking reaction as well as the development of new chromophores.

The progress of the cross-linking reaction between glyceraldehyde and RNase A was also followed by monitoring the generation of the new chromophore (Figure 6). As can be seen, the kinetics of the generation of this new chromophore is biphasic. There is an initial lag phase during which no new chromophore is developed and a second phase during which the new absorption developed rapidly. The duration of lag phase depends on the initial concentration of both the aldotriose and RNase A. With protein concentration remaining constant, the lag period could be decreased by increasing the aldotriose concentration. At a given concentration of glyceraldehyde, the duration of the initial lag phase also decreases with an increase in the protein concentration. Concomitant

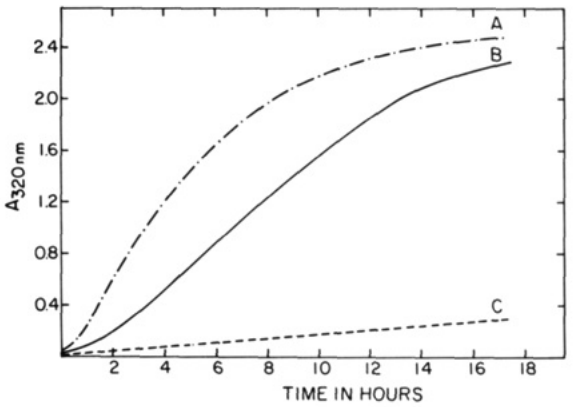


FIGURE 6: Kinetics of cross-linking reaction. The development of new absorption at 320 nm on incubation of RNase A (0.5 mM) in PBS at pH 7.4 and 37 °C with α-hydroxy carbonyl compounds is measured as a function of time: (A) glycolaldehyde; (B) glyceraldehyde; (C) dihydroxyacetone.

with the decrease in the lag period, the rate of development of color in the second phase increased, as is anticipated for the intermolecular cross-linking reaction. Preincubation of a solution of glyceraldehyde before the addition of RNase A has no influence on the kinetics of color development. This observation suggests that glyceraldehyde alone and not some condensation product of the aldotriose introduces the intermolecular covalent cross-links with RNase A.

Comparison of Propensity of α-Hydroxy Carbonyl Compounds To Introduce Covalent Cross-Links. The generation of the cross-linked products on reaction of the protein with glyceraldehyde involves the reaction of the carbonyl group of a ketonic function generated in situ. In the case of glycolaldehyde an aldehydic group generated in situ is involved in the cross-linking reaction. In view of the lower propensity of the carbonyl groups of ketones to form Schiff base adducts with the amino groups (Acharya et al., 1985), the kinetics of cross-linking of RNase A by glycolaldehyde and glyceraldehyde have been compared (Figure 7). Under identical conditions of reagent and protein concentration, the lag period for the development of color is shorter with glycolaldehyde (curve A) relative to that with glyceraldehyde (curve B). Besides, the exponential phase of the reaction also proceeds at a slower rate with glyceraldehyde. In contrast to the results with α-hydroxy aldehydes, generation of the new chromophore is only marginal with dihydroxy acetone (Figure 7, curve C). Similar results were obtained with hydroxyacetone as well.

Comparison of the SDS–PAGE pattern of RNase A incubated with 20 mM α-dihydroxyacetone and hydroxyacetone for 8 h with those of glyceraldehyde and glycolaldehyde revealed that only the α-hydroxy aldehydes are good cross-linking agents (Figure 3B). The α-hydroxy ketones do not introduce such high levels of intermolecular cross-links. Thus, the cross-linking by the trioses and the development of the new chromophore appear to be interrelated. When 100 mM dihydroxyacetone is used, the cross-linking potential of the ketone became more prominent (results not shown), suggesting that the lower levels of cross-linking are a consequence of the lower propensity of ketose to form Schiff base adducts.

Role of Hydroxymethyl Group of Glyceraldehyde in the Cross-Linking Reaction. It is conceivable that the enamine intermediate (of the initial Amadori rearrangement) with a double bond between C_1 and C_2 could isomerize to a structure wherein the double bond is between C_2 and C_3 and then rearrange to the aldo amine structure (3-oxo-2-hydroxypropyl group). The aldehydic carbonyl group of this Amadori product could then react with other protein amino groups to generate

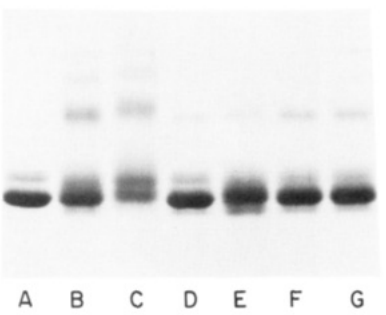


FIGURE 7: Inhibition of aldotriose-mediated protein cross-linking by amino compounds. SDS gel electrophoresis of RNase A reacted with aldotriose in the presence and absence of amino compounds: (A) RNase A control; (B) RNase A reacted with glyceraldehyde; (C) RNase A reacted with glyceraldehyde 3-phosphate; (D) RNase A reacted with 50 mM glyceraldehyde in the presence of 50 mM aminoguanidine; (E) RNase A reacted with 50 mM glyceraldehyde in the presence of 50 mM ethanolamine; (F) RNase A reacted with glyceraldehyde in the presence of 50 mM glycine ethyl ester; (G) RNase A reacted with 50 mM glyceraldehyde in the presence of 50 mM glucosamine. RNase A (0.5 mM) in PBS (pH 7.4) was incubated with 50 mM aldotriose for 16 h at 37 °C in the presence or absence of various amino compounds (50 mM). After the reaction, each sample is desalted on a Sephadex G-25 (0.9 × 30 cm) column equilibrated with 0.1 M acetic acid, and the protein was isolated by lyophilization.

protein cross-links. This reaction will be analogous to that of glycolaldehyde. In an attempt to determine whether the participation of the hydroxyl group at C_3 of glyceraldehyde is obligatory for efficient cross-linking, the reaction of glyceraldehyde 3-phosphate with RNase A has been studied. The intermolecular cross-linking of RNase A by glyceraldehyde 3-phosphate is compared with that obtained with glyceraldehyde (Figure 7). The triose phosphate did introduce the cross-linking. In addition, the overall cross-linking potentials of triose and triose phosphate appear to be nearly comparable. The reaction of RNase A with glyceraldehyde 3-phosphate also generates the absorbance (Figure 6) and fluorescence. Thus, the aldotriose-induced cross-linking of RNase A and the concomitant generation of chromophore and fluorophore could occur even without the participation of the hydroxyl group of the third carbon atom of the triose.

Inhibition of Cross-Linking of RNase A in the Presence of Aminoguanidine and Other Amino Compounds during Nonreductive Modification. As described above, the cross-linking of RNase A by glyceraldehyde is a consequence of 2-oxo-3-hydroxypropylation of the protein. Inhibition of this reaction resulted in a complete inhibition of the cross-linking reaction. In the absence of the Amadori product, cross-linking does not occur. Recently, Brownlee and his colleagues (Brownlee et al., 1986) have reported that aminoguanidine inhibits the glucose-mediated cross-linking of proteins by specifically trapping the Amadori products. Once the Amadori products are trapped by aminoguanidine, they are unavailable for the generation of advanced glycosylation end products (cross-linked products). Thus, this leads to the inhibition of cross-linking. It has also been suggested that aminoguanidine may be a reagent of therapeutic value to inhibit the sequela of the nonenzymic glycation.

In view of these studies, the propensity of aminoguanidine to trap the Amadori product of glyceraldehyde and hence its influence on the cross-linking potential of glyceraldehyde have been investigated (Figure 7). Incubation of RNase A with glyceraldehyde in the presence of an equimolar amount of aminoguanidine completely inhibited the cross-linking reaction. This inhibition of cross-linking has also been compared with that of other amino compounds such as glucosamine, ethanolamine, and glycine ethyl ester to determine the selectivity

Table I: Inhibition of 2-Oxo-3-hydroxypropylation of RNase A and the Concomitant Covalent Incorporation of Amino Compounds into the Protein[a]

amino compounds used	[^{14}C]glyceraldehyde incorporated (mol)	amino compounds incorporated (mol)
none	6.0	
glycine ethyl ester	4.8	0.5
ethanolamine	5.8	0.7
glucosamine	4.6	1.2
aminoguanidine	1.4	ND[b]

[a] RNase A (0.5 mM) in PBS, pH 7.4, was incubated with 50 mM glyceraldehyde for 16 h at 37 °C in the presence or absence of various amino compounds (50 mM). [b] ND, not determined.

and/or the generality of this inhibition reaction. All the amino compounds studied inhibited the cross-linking to some degree. The propensity of these amino compounds to inhibit the cross-linking reaction was decreased in the order aminoguanidine > glucosamine > ethanolamine > glycine ethyl ester. The inhibition of the aldotriose-mediated cross-linking of RNase A by aminoguanidine as well as other amino compounds could be considered as suggestive of the possible mechanistic similarity in the aldohexose- and aldotriose-mediated cross-linking reaction of proteins.

If the mechanism of inhibition of the cross-linking of RNase A with aminoguanidine and other amino compounds is indeed similar to that proposed for glucose (i.e., by trapping the Amadori products), it is anticipated that these amino compounds should react with the carbonyl functions of the Amadori products. Therefore, the influence of aminoguanidine on the incorporation of [^{14}C]glyceraldehyde has been investigated to determine whether it inhibits the nonreductive modification of the protein itself. The presence of 50 mM aminoguanidine during a 16-h reaction of 50 mM [^{14}C]-glyceraldehyde with RNase A inhibited the incorporation of glyceraldehyde into the protein nearly 80–85% (Table I). Thus, the inhibition of the glyceraldehyde-mediated cross-linking of RNase A by aminoguanidine appears to be a consequence of the inhibition of nonenzymic glycation rather than the trapping of the Amadori products.

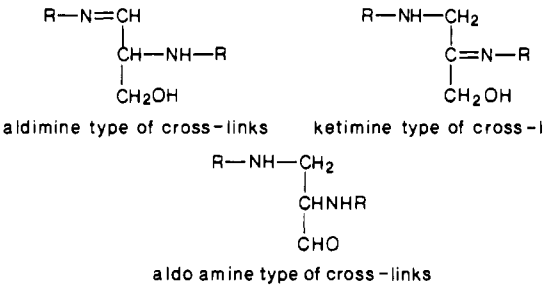
The propensity of other amino compounds, i.e., guanidine, glycine ethyl ester, glucosamine, and ethanolamine, to inhibit the nonenzymic modification of the protein and the extent of covalent attachment of these amines to proteins have been investigated (Table I). It may be seen that all the amines significantly inhibit the nonenzymic glycation, aminoguanidine being the most effective. All three other amines tested are also incorporated into the protein after 16 h of reaction. However, the amount of amine incorporated is only about 0.6–1 mol. These results suggest that inhibition of the nonenzymic glycation of RNase A by the amine studied is not as efficient as that by aminoguanidine.

Discussion

There has been considerable interest in recent years in elucidating the pathway(s) for the introduction of glucose-mediated protein cross-linking (Brownlee et al., 1984; Harding, 1985). Direct correlation appears to exist between the amount of cross-linking and the extent of nonenzymic glycation. Dicarbonyl compounds such as pyruvoylaldehyde generated in situ from the nonenzymically glycated proteins have been considered as the intermediate in the pathway(s) for the glucose-mediated cross-linking of proteins (Wolfe & Dean, 1987). The results of the present study demonstrate that the carbonyl function of keto amine linkage of the aldotriose bound to the protein (Amadori product) is reactive toward amines under physiological conditions. This reactivity appears to provide at least one of the pathways for cross-linking of proteins mediated through α-hydroxy aldehydes.

The reaction of the carbonyl function of the keto amine linkages of aldotriose with the amino functions of proteins results in the formation of keto amine–protein adducts (ketimines). Such a reaction introduces covalent cross-links into the proteins (Figure 1). There is a significant lag period before the protein cross-linking by aldotriose becomes apparent. It is conceivable that the earlier studies of Nigen and Manning (1978) presumably were in this lag period and hence should have failed to show any measurable cross-links. The cross-linking potential of glyceraldehyde is latent in that it becomes apparent only after the completion of the first phase of the reaction, i.e., Amadori rearrangement of the Schiff base adduct. In this respect, this cross-linking potential of aldotriose is comparable to that of glycolaldehyde (Acharya & Manning, 1983), except that in the glyceraldehyde system the reactive carbonyl species for the cross-linking reaction is ketonic rather than aldehydic.

The exact structure of the final cross-link introduced by glyceraldehyde is not clear at present. It is apparent that the initial step in this cross-linking scheme is the generation of a ketimine structure by the condensation of the ketonic carbonyl function of the keto amine with the amino function of the protein. Such a ketimine structure should be capable of equilibrating with the aldimine structure wherein the Schiff base is at C_1 of glyceraldehyde instead of at C_2 (in the ketimine structure). The results of the present study demonstrate the presence of at least some amount of ketimine type and/or aldimine type of structure in the samples of cross-linked RNase A.



The carbonyl function of the ketimine structure generated should also be capable of undergoing a second Amadori rearrangment to form the aldo amine-type structure. Such an aldo amine could react with the protein amino functions just as the aldo amines generated on nonreductive modification of proteins with glycolaldehyde (Acharya & Manning, 1983). Thus, glyceraldehyde could function as a trifunctional reagent. Though such a reaction appears to be feasible, the cross-linking through the aldo amines of glyceraldehyde does not appear to be an essential element of cross-linking of proteins by glyceraldehyde. An alternate aldo amine structure could also be generated by the isomerization of the enamine wherein the double bond between C_1 and C_2 of the molecule is rearranged to the 2,3-position. This enamine intermediate could then be converted to the aldo amine. For the formation of either of these aldo amine structures, the presence of the hydroxyl group on the third carbon is essential. Glyceraldehyde 3-phosphate, which does not contain the hydroxyl group on the third carbon atom (and hence could not generate aldo amine intermediates), introduces the cross-links with nearly the same efficiency as that of glyceraldehyde.

The generation of yellow chromophore as a consequence of the carbonyl–amine reaction has been the subject of consid-

erable interest. Malondialdehyde, a naturally occurring three-carbon dialdehyde produced by the oxidation of polyunsaturated lipids, reacts with amino acids/amino acid esters and proteins to yield N,N′-disubstituted 1-amino-3-aminopropene. The conjugated Schiff base of the derivatives of 1-amino-3-aminopropene possesses the characteristic ultraviolet and visible absorption properties as well as fluorescence (Chio & Tappel, 1969a,b). The enaminal intermediates also showed the characteristic ultraviolet absorption characteristics (Nair et al., 1980). The spectral properties of the adducts of methylglyoxal (α-keto aldehyde) with proteins have been investigated by Szent-Gyorgi and his colleagues (McLaughlin et al., 1980). The primary step in the reaction has been suggested to involve the formation of Schiff base adduct between the ϵ-amino groups of lysine and the aldehydic carbonyl of methylglyoxal. It is suggested that the yellow color (absorption peak at 328 nm and a shoulder around 350 nm) is consistent with a resonance-stabilized $n \rightarrow \pi$ transition for a Schiff base that is linked to a ketonic carbonyl group. The condensation of succinylacetone, a metabolic product present in the urine of patients with tyrosinemia, with amino acids and proteins to generate a yellow chromophore has been described by Manabe et al. (1985). Again the Schiff base appears to be stabilized by ketonic carbonyl group.

The formation of dicarbonyl compounds (keto aldehydes) as a consequence of the autoxidation of the hexose has been suggested to play a potential role in the generation of new yellow to brown chromophores as advanced glycosylation products (Wolfe & Dean, 1987). However, inhibition of this autoxidative glycosylation using metal chelating agent diethylenetriamine pentaacetic acid did not completely inhibit the generation of new chromophores. This observation suggests that more than one mode is available for the formation of advanced glycosylation products. The formation of new yellow to brown chromophores on reaction of aldoses with proteins, mostly the products generated after 7 days of incubation of protein with aldotriose and aldohexoses, was addressed by Candiano et al. (1985) and Gheggeri et al. (1985). The difference spectra of glyceraldehyde RNase A adducts studied here are distinct from that of α-*t*-Boc-Lys-glyceraldehyde adduct reported by Candiano et al. (1985), suggesting that the structures present in our sample apparently represent an earlier phase of the cross-linking reaction. Candiano et al. (1985) have ascribed the formation of yellow color to the formation of pyrrole structures. However, the procedure for the detection of the pyrrole structures subjects the derivatized protein to strong acidic conditions as well as to alkaline conditions. Possible structural transitions and stability of the yellow chromophore generated under the physiological conditions are not available at present.

Pangor et al. (1984) have isolated a fluorescent chromophore, 2-(2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole, from the acid hydrolysates of albumin that has been heavily glycosylated. However, the concentration of this fluorophore in the glycosylated albumin itself is significantly lower than in its acid hydrolysate (Chang et al., 1985). Thus, some of the fluorophore appears to have been generated by an acid-catalyzed rearrangement and/or transformation of a precursor chromophore. All these studies clearly demonstrate that a number of pathways are available for the formation of new chromophore(s) as a consequence of nonenzymic glycation. The mechanism that operates in the generation of yellow chromophore on reaction of proteins with glyceraldehyde appears to be more related to that suggested for malonaldehyde, methylglyoxal, and succinylacetone except that glyceraldehyde is not a true dicarbonyl compound. The second carbonyl in this system is latent and is generated only on completion of the Amadori rearrangement in situ.

Aminoguanidine has been suggested to function as specific inhibitor of cross-linking reaction of the nonenzymically glycosylated proteins by trapping the Amadori products (Brownlee et al., 1986). Though aminoguanidine inhibited the protein cross-linking by glyceraldehyde as well, the inhibition seen here appears to be mostly a consequence of the inhibition of the nonenzymic glycation reaction (initial aldimine formation) itself rather than by trapping the Amadori products (the formation of ketimine adduct, the second step). Nonenzymic glycation of RNase A by glyceraldehyde was inhibited nearly 80% in the presence of an equimolar amount of aminoguanidine. In the studies of Brownlee et al. (1986) aminoguanidine does not inhibit the nonreductive modification of bovine serum albumin (nonenzymic glycation) but specifically traps the Amadori products. This could be suggestive of the possible differences in the protein cross-linking pathways. However, it should be added here that the inhibition of the nonreductive incorporation of glyceraldehyde by aminoguanidine into RNase A is consistent with the fact that aminoguanidine is a highly reactive nucleophile, a substituted hydrazine, that readily reacts with aldoses (Szilagyi et al., 1986). The observation that aminoguanidine does not inhibit the nonenzymic incorporation of glucose into bovine serum albumin (Brownlee et al., 1986) is probably suggestive of the very high reactivity of amino groups of serum albumin to form adducts with glucose as compared with the amino group of aminoguanidine.

The cross-linking potential of glyceraldehyde 3-phosphate demonstrated here may be of physiological significance. This triose phosphate is an intermediate in the degradation of glucose in the erythrocytes. It is tempting to speculate that the triose phosphate reacts with the intracellular hemoglobin to form keto amine adduct, and derivatized Hb could then react with the membrane proteins through the carbonyl function of the keto amine generated in situ. It may be added here that the concentration of Hb near the membrane surface has been shown to be higher than that inside the red blood cell (Wang & Richards, 1975), and some amount of this appears to be irreversibly bound to membrane proteins.

In conclusion, the studies described here establish that the cross-linking potential of α-hydroxy aldehydes is a consequence of the reactivity of the carbonyl function of the keto amine (or aldo amine) generated in situ. The generation of this reactive carbonyl group is latent and is the result of the Amadori rearrangement of the initial Schiff base adducts of these aldehydes. However, it is also becoming clear that protein-bound carbonyls are also generated by other mechanisms (Ahn et al., 1987; Recsei & Snell, 1984; Ginsberg et al., 1973). It is conceivable that these carbonyl functions may also exhibit reactivities comparable to that of protein-bound 2-oxo-3-hydroxypropyl groups. Thus, the reactivity of the protein-bound carbonyl groups appears to be a subject of more general interest and is worthy of much more intensive investigation.

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