Reaction of glycolaldehyde with proteins: Latent crosslinking potential of α -hydroxyaldehydes*

(aldehyde/Amadori rearrangement/hydroxyethylation)

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ABSTRACT The Schiff base adducts of glycolaldehyde with hemoglobin undergo Amadori rearrangement to form stable ketoamine structures; this reaction is similar to the nonenzymic glucosylation of proteins. In the present studies the analogous rearrangement of the Schiff base adducts of glycolaldehyde with proteins has been demonstrated. However, the Amadori rearrangement of this Schiff base adduct produces a new aldehyde function, an aldamine, which is generated *in situ* and is capable of forming Schiff base linkages with another amino group, leading to covalent crosslinking of proteins. Sodium dodecyl sulfate gel electrophoresis of the glycolaldehyde-RNase A adduct showed the presence of dimers, trimers, and tetramers of RNase A, demonstrating the crosslinking potential of this α -hydroxyaldehyde. The crosslinked products exhibited an absorption band with a maximum around 325 nm and fluorescence around 400 nm when excited at 325 nm. The crosslinking reaction, the formation of a 325-nm absorption band, and the development of fluorescence were prevented when the incubation was carried out in the presence of sodium cyanoborohydride. This finding indicates that the Amadori rearrangement that generates a new carbonyl function is a crucial step in this covalent crosslinking. Glycolaldehyde could be a bifunctional reagent of unique utility because its crosslinking potential is latent, expressed only upon completion of the primary reaction.

We have been interested in the reaction of α -hydroxyaldehydes with proteins, especially in view of our observation that glycolaldehyde is an efficient antisickling agent *in vitro* that acts primarily at the stage of aggregation of sickle cell deoxyhemoglobin (1). Only five of the 24 amino groups per $\alpha\beta$ dimer of hemoglobin are capable of forming stable ketoamine linkages with glycolaldehyde (2, 3), in a reaction analogous to the Amadori rearrangement of glucosylated hemoglobin (4). Recently, we have found that even though valine-1 of the α -chain readily forms a Schiff base with glyceraldehyde, it is refractory to subsequent Amadori rearrangement (5). On the other hand, valine-1 of the β chain does form stable ketoamine adducts with glyceraldehyde or with glucose by rearrangement. The lysine ϵ -amino groups of hemoglobin also display selectivity in their pattern of reactivity with glyceraldehyde (2) or glucose (6). Therefore, it is clear that our knowledge of the rearrangement process as it applies to proteins is limited with respect to both the mechanistic and physiological consequences of the process. The present manuscript addresses the latter question by using glycolaldehyde, a simple α -hydroxyaldehyde. We have now investigated whether the Amadori rearrangement reaction also would occur with the Schiff base adducts of glycolaldehyde with proteins. The rearrangement of the glycolaldehyde-Schiff base adduct would generate a new aldehyde function, which should have the potential to react with another free amino group of the

protein and result in covalent crosslinking (Fig. 1). The present study demonstrates that such a reaction indeed takes place when proteins are incubated with glycolaldehyde in the *absence* of reducing agents. This approach differs from that for monofunctional modification of proteins by reductive alkylation (7). In that system, a reducing agent is present throughout the reaction; Amadori rearrangement and subsequent crosslinking of proteins is thereby precluded.

MATERIALS AND METHODS

Reaction of Glycolaldehyde with RNase A. RNase A (Sigma) (0.5 mM) in phosphate-buffered saline, pH 7.4, was incubated at 37°C for the indicated period at a given concentration of glycolaldehyde (Sigma). At the end of the reaction period, the sample was desalted by passage through a column (2.2 $\times$ 30 cm) of Sephadex G-25 (Pharmacia), equilibrated and eluted with 0.1 M acetic acid. The protein, isolated by lyophilization, is referred to as glycolaldehyde-RNase A adduct. Where indicated, the glycolaldehyde adducts were reduced with sodium borohydride as described for the reaction of glyceraldehyde with hemoglobin (1, 2).

Reaction of Glycolaldehyde-RNase A Adduct with 2,4-Dinitrophenylhydrazine. This reaction was carried out essentially as described by Fields and Dixon (8). The RNase adduct (0.25 μ mol) was treated with 1.5 ml of 25 mM 2,4-dinitrophenylhydrazine in 1 M HCl at room temperature for 15 min. The derivatized protein was isolated from the excess reagent by gel filtration on Sephadex G-25, equilibrated and eluted with 0.1 M acetic acid.

NaDodSO₄/Polyacrylamide Gel Electrophoresis. The extent of crosslinking of RNase A was analyzed by NaDodSO₄/polyacrylamide gel electrophoresis at pH 8.0 on gradient slab gels of 10-20% acrylamide. The stacking gel was made with 4% acrylamide in Tris-HCl buffer, pH 6.8, 10% in NaDodSO₄. Generally, the amount of protein was of the order of 20 μ g. The gels were routinely run at 7.5 mA for 1 hr to load the protein and the electrophoresis was carried out for another 1.5 hr at 15 mA in Tris/glycine, pH 8.0, 1% in NaDodSO₄. The protein bands were stained with Coomassie blue and destained by washing first with methanol/acetic acid/water, 40:10:50 (vol/vol), and then with 7% (wt/vol) acetic acid.

Amino Acid Analysis. To determine the extent of reaction of the lysine residues with glycolaldehyde, the adduct was reduced with sodium borohydride as described earlier for the reaction of glyceraldehyde with hemoglobin (2). The reduced adduct was dialyzed against water and lyophilized. The samples were hydrolyzed in 6 M HCl under reduced pressure for 22 hr and analyzed on an amino acid analyzer of the design of Spackman *et al.* (9).

Fluorescence Measurements. These determinations were

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* A preliminary report of these results has been published (25).

 FIG. 1. Crosslinking potential of glycolaldehyde. Dnp-NHNH₂, 2,4-dinitrophenylhydrazine.

made in a Perkin-Elmer MPF-4 spectrofluorometer. The solvent was 0.1 M acetic acid.

RESULTS

Amadori Rearrangement of Schiff Base Adducts of Glycolaldehyde with Proteins. The potential of glycolaldehyde to form Schiff base adducts with the amino groups of proteins has been demonstrated previously by the reductive hydroxyethylation studies of Geoghegan *et al.* (10). In an attempt to determine whether the Schiff base adducts of this aldodiol have the potential to undergo the Amadori rearrangement in the absence of a reducing agent, the presence of protein-bound carbonyl groups in a RNase A-glycolaldehyde adduct was measured by treatment of the adduct with 2,4-dinitrophenylhydrazine (Table 1). The amount of protein-bound dinitrophenyl chromophore increased with the concentration of glycolaldehyde used to prepare the adduct. If the Schiff base adducts had not rearranged, then the dinitrophenyl chromophore would not have remained with the protein. The covalent attachment of the dinitrophenyl chromophore to the glycolaldehyde-RNase A adduct clearly demonstrates that some of the Schiff base adducts of glycolaldehyde with the amino groups of RNase A underwent Amadori rearrangement to form the 2-oxoethylated protein (Fig. 1); the product of the Amadori rearrangement of the Schiff base adduct of glycolaldehyde with the amino groups of proteins is an aldamine rather than a ketoamine structure that is generated from the Schiff base adducts of glucose or glyceraldehyde.

Table 1. Dinitrophenylhydrazine assay of glycolaldehyde-RNase A adduct

Glycolaldehyde, mM	mol of dinitrophenyl per mol of protein
10	0.25
20	0.53
50	0.94
100	1.92

RNase A (0.5 mM), incubated with glycolaldehyde in phosphate-buffered saline for 3 hr, was desalted by passage through a Sephadex G-25 column that was equilibrated and eluted with 0.1 M acetic acid.

Crosslinking of RNase A with Glycolaldehyde. A consequence of the generation of an aldamine is the potential of producing another Schiff base adduct, involving this newly generated aldehyde and another amino group (Fig. 1); if the amino group were donated from another protein, intermolecular crosslinking would occur. To examine this possibility, RNase A that had been treated with glycolaldehyde in the absence of reducing agents was subjected to gel filtration on a Bio-Gel P-60 (Bio-Rad) column (Fig. 2). The glycolaldehyde RNase A adduct formed was yellow and most of it eluted in a high molecular weight region of the column ($M_r > 60,000$). The absorption spectra of this product and its dinitrophenyl derivative are shown in Fig. 3.

The presence of intermolecular covalent crosslinks in RNase A as a result of the reaction with glycolaldehyde was also established by NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 4). The gel electrophoresis clearly showed the formation of higher

FIG. 2. Gel filtration of RNase A after treatment with glycolaldehyde. RNase A (0.5 mM) was incubated with glycolaldehyde (50 mM) for 3 hr at 37°C. The sample, which had developed a yellow color during the incubation, was applied to a column (0.9 × 60 cm) of Bio-Gel P-60, which was eluted with 0.1 M acetic acid. The high molecular weight product retained the yellow color; the position of monomeric RNase A is marked.

FIG. 3. Absorption spectra of the glycolaldehyde-RNase A adduct and its derivatives. The spectra of RNase A (---), glycolaldehyde-RNase A (—), and the dinitrophenylhydrazone of the glycolaldehyde-RNase A (···) were recorded in an Aminco DW-2 spectrophotometer with a cell of 1-cm path length; the protein concentration was 1 mg/ml in 0.1 M acetic acid.

molecular weight species of RNase A—dimeric, trimeric, and tetrameric species (in decreasing amounts); some higher molecular weight species were also present. Because this Na-DodSO₄ electrophoretic analysis was carried out under denaturing conditions, it is clear that glycolaldehyde had introduced stable covalent crosslinks into RNase.

Effect of Sodium Cyanoborohydride on Crosslinking. In the scheme of the crosslinking reaction (Fig. 1), the formation of the Schiff base between glycolaldehyde and the free amino group of the protein is the initial step. These Schiff base adducts can be selectively reduced by sodium cyanoborohydride in the presence of excess free aldehyde (11–13). This reduction should thus inhibit the subsequent Amadori rearrangement of the initial Schiff base and thereby prevent the formation of the second aldehyde function. Indeed, when sodium cyanoborohydride is present during the reaction with RNase A and glycolaldehyde, the formation of higher molecular weight species is completely prevented (Fig. 4). In addition, the derivatized protein is not yellow as it is in the absence of NaCNBH₃. Thus, development of a 325-nm absorption band and the generation of the crosslinked adduct are interrelated because they are inhibited by the presence of NaCNBH₃. These observations indicate that the glycolaldehyde-RNase A adduct contains the newly generated 2-oxo group and explain the latent crosslinking potential of this simple hydroxyaldehyde.

FIG. 4. NaDodSO₄/polyacrylamide gel electrophoresis of glycolaldehyde-RNase A adducts. RNase A (0.5 mM) was treated with 20 mM glycolaldehyde in phosphate-buffered saline, pH 7.4, for 3 hr at 37°C and desalted on Sephadex G-25 (2.2 × 40 cm) equilibrated with 0.1 M acetic acid; the protein was isolated by lyophilization. Lane A, RNase A; lane B, glycolaldehyde-RNase A adduct; lane C, incubation of glycolaldehyde and RNase A in the presence of NaCNBH₃.

Recovery of Lysine from the Glycolaldehyde-RNase Adduct. Because the suggested pathway for the formation of cross-linked RNase (see Fig. 1) involves the amino groups of the protein, the lysine content of the glycolaldehyde-RNase adduct has been determined (Table 2) under conditions in which there was a significant amount of crosslinking as estimated by Na-DodSO₄ gel electrophoresis. After the incubation but prior to the acid hydrolysis, the samples were reduced with NaBH₄ in order to convert any of the aldamine form to the stable hydroxyethyl derivative. Under these conditions about 30% of the theoretical lysine content could not be recovered either as free lysine or as the hydroxyethyl derivative, probably because of participation in crosslinks; no significant loss in any other amino acid was found. On the other hand, when a reducing agent was present during the incubation with glycolaldehyde (Table 2), under the conditions described by Geoghegan *et al.* (10), lysine and its derivatives were recovered quantitatively. These results support the direct involvement of lysine residues in the crosslinking reaction of glycolaldehyde with proteins.

In order to exclude the possibility that the guanidino side chain of arginine could participate in a reversible reaction with glycolaldehyde that would not be obvious from studies on acid hydrolysates, two types of experiments were performed. A sample of performic acid-oxidized RNase in which the amino groups had been completely modified by reductive alkylation with glyceraldehyde (dihydroxypropylation) was treated with glycolaldehyde and then with trypsin. Analysis of the tryptic peptides by HPLC indicated a pattern that was identical to that of dihydroxypropylated RNase. Therefore, significant reaction at arginine residues can be excluded. In another approach, the tripeptide Met-Arg-Phe was treated with glycolaldehyde under

Table 2. Recovery of lysine and its ϵ -hydroxyethylated derivatives from RNase A treated with glycolaldehyde in the presence or absence of NaCNBH₃

Glycolaldehyde, mM	NaCNBH ₃ , mM	Lysine and derivatives recovered, residues per molecule			
		Lysine	ϵ -Hydroxyethyl-lysine	ϵ -Di(hydroxyethyl)-lysine	Total
0	0	10.0	0	0	10.0
20	0	6.4	0.4	0.1	6.9
20	20	4.0	4.9	1.3	10.2

RNase (0.5 mM) was incubated at 37°C for 8 hr in the presence or absence of 20 mM glycolaldehyde. When NaCNBH₃ was present, its concentration was 20 mM. The sample that was incubated without NaCNBH₃ was treated with NaBH₄ after the incubation, prior to acid hydrolysis as described in the text. After incubations, the derivatized proteins were isolated by gel filtration on a Sephadex G-25 column (0.9 × 30 cm).

the same conditions used for proteins. Most of the phenylalanine and arginine (95%) was released by subsequent treatment with carboxypeptidase Y. This result also argues against extensive modification of arginine residues.

Role of Hydroxymethyl Groups of the Reagent in Crosslinking. As indicated above, the development of a 325-nm absorption band and the intermolecular crosslinking of RNase A upon reaction with glycolaldehyde are interrelated. In order to demonstrate that the type of covalent crosslinking observed with glycolaldehyde is a special rather than a general case of crosslinking found with simple aliphatic aldehydes such as formaldehyde, RNase A was incubated with glyoxylic acid (an aldehyde that cannot undergo Amadori rearrangement after Schiff base formation) in the absence of reducing agent. Such treatment of RNase did not result in the incorporation of adducts that were susceptible to derivatization with dinitrophenylhydrazine. Furthermore, there was neither development of a 325-nm absorption band nor covalent crosslinking of the protein. This lack of crosslinking of RNase A with glyoxylic acid cannot be ascribed to the failure of the carbonyl function of glyoxylic acid to form Schiff base adducts with the amino groups of RNase A because, when the incubation is carried out in the presence of sodium cyanoborohydride, carboxymethylation of the ϵ -amino groups of lysine residues took place as observed for allergen proteins (14); recent studies in this laboratory have shown similar reactivity for the α -amino groups of hemoglobin. Thus, it is clear that the generation of the 325-nm absorption band and covalent crosslinking on reaction of glycolaldehyde with RNase A (in the absence of reducing agent) is not simply a consequence of the formation of Schiff base adduct, rather it is a unique reaction for this aldodiolose containing the hydroxymethyl group adjacent to the free aldehyde function. It apparently reflects the consequence of the formation of a stable adduct containing the new carbonyl function.

Fluorescence Spectra of the Glycolaldehyde-RNase Adduct. The presence of a broad spectral band in the 300- to 380-nm region in the glycolaldehyde-RNase A adduct prompted us to investigate its fluorescent properties. The fluorescence excitation spectrum and the emission spectrum (Fig. 5) of this adduct have an excitation band with a maximum around 325 nm and an emission maximum around 400 nm. The appearance of fluorescence is prevented by the presence of NaCNBH₃, con-

FIG. 5. Fluorescence spectra of the glycolaldehyde-RNase A adduct. The excitation (---) and emission (—) spectra were recorded at a constant emission wavelength (400 nm) and a constant excitation wavelength (325 nm), respectively, at a protein concentration of 0.1 mg/ml in 0.1 M acetic acid.

comitant with the lack of development of crosslinking and a new absorption band.

Kinetics of Adduct Formation. The progress of the crosslinking reaction between glycolaldehyde and RNase A was followed by monitoring the development of the new absorption band characteristic of the crosslinked product. The kinetics of product formation are at least biphasic (Fig. 6). During the initial lag phase very little color is apparent and there is very little dimer formation seen by NaDodSO₄ gel electrophoresis. A yellow chromophore and crosslinked species are formed much more readily thereafter. As the concentration of glycolaldehyde is increased from 5 to 50 mM, at a constant protein concentration, the lag period for development of color decreased from about 2 hr to about 10 min. Thus it appears that, before the new absorption band is generated, a crucial concentration of the first Amadori rearrangement product, presumably 2-oxoethylated proteins, must be formed; increasing the glycolaldehyde concentration shifts the equilibrium in favor of more Schiff base adducts and therefore the Amadori rearrangement product. Because the second Schiff base involves the carbonyl function of the aldamine and another amino group of the protein, an increase in the concentration of the amino group (i.e., in protein concentration) at a constant concentration of glycolaldehyde should also significantly decrease the lag period for development of the chromophore. This prediction proved correct.

Preincubation of the glycolaldehyde solution before addition of RNase A has no influence on the kinetics of the color development. This result demonstrates that glycolaldehyde and not some condensation product of it is the actual crosslinking reagent. Because the generation of the absorption band and crosslinking of the protein appear to follow nearly the same kinetics, they probably reflect the same aspects of structural transformation in the protein.

FIG. 6. Kinetics of the development of the absorption band of the glycolaldehyde-RNase A adduct. RNase A (0.5 mM) was allowed to react with 5, 10, 30, and 50 mM glycolaldehyde in phosphate-buffered saline, pH 7.4, in quartz cuvettes in a Zeiss PMQ-6 spectrophotometer. The cell compartment was maintained at 37°C by using a circulating water bath. The absorbance of the samples at 310 nm was recorded at 5-min intervals. A blank containing 50 mM glycolaldehyde alone did not develop any absorbance at 310 nm. The absorption band was monitored at 310 nm within the limits of the UV lamp used in the Zeiss PMQ-6 spectrophotometer.

Spectral Properties of Adducts of Glycolaldehyde with Amino Acid Derivatives. As indicated above, the glycolaldehyde-RNase A adduct has an absorption band with a maximum around 325 nm, which coincides with the maximum of the fluorescence excitation spectrum. Because aldehydes would generate Schiff base adducts with both α - and ϵ -amino groups of lysine residues, free α -*t*-butoxycarbonyl (*t*-Boc)-lysine and glycine ethyl ester were treated with glycolaldehyde in order to determine whether both α - and ϵ -amino groups are capable of reaction with this simple α -hydroxyaldehyde to form a fluorescent compound. In each case a yellow product was formed. The reaction product of the ϵ -amino group of α -*t*-Boc-lysine with glycolaldehyde had a broad absorption band with a maximum around 325 nm—the same wavelength region where glycolaldehyde-RNase A adduct showed absorption and fluorescence excitation maxima. The products of reaction of glycolaldehyde either with α -*t*-Boc-lysine or glycine ethyl ester were also fluorescent. These results suggest that the details of the chemistry of the generation of the fluorescent adduct upon reaction of glycolaldehyde with proteins and the structure of the covalent crosslinks might be more easily identified by studies with these model compounds.

DISCUSSION

The studies presented in this communication demonstrate the potential of the two-carbon hydroxyaldehyde, glycolaldehyde, to generate covalent crosslinks in proteins in the absence of reducing agents (Fig. 1); when the reaction is carried out in the presence of reducing agents, monofunctional alkylation results, as shown by Feeney and his colleagues (7, 10). This potential for crosslinking is a result of the Amadori rearrangement of the initial Schiff base adduct of glycolaldehyde with the amino groups of proteins to form the aldamine—i.e., to generate a new aldehyde group; such rearrangement does not occur in the presence of reducing agents. This mechanism of crosslinking is different from that with formaldehyde in the absence of reducing agents (15, 16). On mechanistic grounds, crosslinking by formaldehyde cannot involve the intermediate generation of a second carbonyl function. The crosslinking by glycolaldehyde is also unique compared to that of bifunctional reagents such as dialdehydes, because the crosslinking potential of glycolaldehyde is latent, exposed only after the rearrangement of the initial Schiff base had occurred. This reaction mechanism thus distinguishes glycolaldehyde from reagents such as malonaldehyde and glutaraldehyde.

An interesting aspect of the reaction of glycolaldehyde with proteins is the development of fluorescence. Other aldehydes have also been shown to generate fluorescent adducts. Thus, *o*-phthalaldehyde reacts with the amino groups of amino acids to generate fluorescent adducts (17). The development of fluorescence as a result of reaction of malonaldehyde with proteins has been demonstrated, and its possible correlation to the browning reaction has been suggested (18, 19); glucose also reacts with proteins to form fluorescent adducts (20). Reaction of glutaraldehyde with proteins also generates a yellow chromophore (21), similar to that observed with glycolaldehyde. Under stringent conditions, glycolaldehyde and other sugar aldehydes have been shown to form substituted pyrazines (22). The presence of reducing agents such as sodium cyanoborohydride, which selectively reduces the Schiff base adducts and thus inhibits the potential for Amadori rearrangement, prevents the development of fluorescence on incubation of proteins with glycolaldehyde. It may be possible to correlate fluorescence with the crosslinking reaction as a marker to detect the secondary reaction of other hydroxyaldehydes through Amadori rearrangement in other systems.

One could conceive of a role for glycolaldehyde as a bi-

functional reagent of unique utility. Because it is a two-carbon crosslinking agent of a hydrophilic nature, it might have application in studies dealing with protein-protein interactions in which the proximity of two amino groups could be exploited. In addition, the stability of glycolaldehyde in aqueous solution should be greater than that of the commonly used imido esters and difluorodinitrobenzene, in which the second functional group of the derivative in which one group has reacted is susceptible to solvolysis. Because the second aldehyde group of glycolaldehyde is generated as a result of the first reaction of glycolaldehyde in a local protein environment, it might be expected to be more resistant to general solvolysis; the yield, as well as the specificity of the crosslinks, should be enhanced. One system that could be tested is deoxyhemoglobin S. Preliminary studies have shown extensive crosslinking of hemoglobin. The small size of the reagent should be an advantage in such a system because it should be able to penetrate deep into the intermolecular contact regions and generate crosslinks if two or more lysines of the same molecule or two different molecules are in the vicinity. The information thus generated should complement that being derived from x-ray diffraction (23) and electron microscopic studies (24) in defining the complete structure of the deoxyhemoglobin S aggregate.

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