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Glycation and Glycoxidation of Histones by ADP-ribose*

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The reaction of long lived proteins with reducing sugars has been implicated in the pathophysiology of aging and age-related diseases. A likely intranuclear source of reducing sugar is ADP-ribose, which is generated following DNA damage from the turnover of ADP-ribose polymers. In this study, ADP-ribose has been shown to be a potent histone glycation and glycoxidation agent *in vitro*. Incubation of ADP-ribose with histones H1, H2A, H2B, and H4 at pH 7.5 resulted in the formation of ketoamine glycation conjugates. Incubation of histone H1 with ADP-ribose also rapidly resulted in the formation of protein carboxymethyllysine residues, protein-protein cross-links, and highly fluorescent products with properties similar to the advanced glycosylation end product pentosidine. The formation of glycoxidation products was related to the degradation of ketoamine glycation conjugates by two different pathways. One pathway resulted in the formation of protein carboxymethyllysine residues and release of an ADP moiety containing a glyceric acid fragment. A second pathway resulted in the release of ADP, and it is postulated that this pathway is involved in the formation of histone-histone cross-links and fluorescent advanced glycosylation end products.

Both intracellular and extracellular proteins are subject to a variety of non-enzyme-catalyzed chemical modifications (reviewed in Ref. 1), which can adversely affect function. The accumulation of chemical modifications in long lived proteins has been implicated in the pathophysiology of aging (2, 3) and a number of specific diseases, including diabetes (4, 5) and Alzheimer's disease (6, 7). Two interrelated protein modifications that have received much recent attention are glycation and oxidation, which lead to the formation of protein glycoxidation products (2, 3, 8). Glycation is initiated by the reaction of a reducing sugar with a protein amino group to generate Schiff base adducts (9, 10) that can undergo the Amadori rearrangement to form ketoamine adducts (11–15). Many of the ketoamine adducts are unstable, and by a complex chemistry that involves oxidation, glycation often leads to protein glycoxidation products that have been termed AGE[1] (2, 3). The AGE include protein carboxymethyllysine (CML) residues (11) and a heterogeneous group of complex modifications characterized by their high fluorescence and ability to cause protein-protein cross-links (3, 16).

Glucose is assumed to be a major source of glycation and glycoxidation of extracellular proteins *in vivo* based on its abundance and association with diabetic complications (3–5). The sugar sources for the glycoxidation of intracellular proteins are less well understood, but pentoses have been implicated, because they are efficient precursors for the formation of fluorescent AGE (16). A likely intracellular source of a reducing pentose moiety is ADP-ribose, which is generated from NAD by multiple metabolic pathways (Fig. 1). The cell nucleus is a likely site for ADP-ribose modifications as oxidative stresses and other conditions that cause DNA strand breaks stimulate the synthesis of nuclear polymers of ADP-ribose, which are rapidly turned over generating ADP-ribose in close proximity to the long lived histones rich in lysine residues (17, 18). Additionally, ADP-ribose can be generated in other cell compartments by the turnover of cyclic ADP-ribose (19, 20) and by the removal of ADP-ribose from proteins modified by the action of protein-mono-ADP-ribosyltransferases (21).

The evaluation of a possible role of ADP-ribose in protein glycation is challenging, since the enzyme-catalyzed modification of proteins by ADP-ribose occurs at several different amino acid residues (21, 22). To distinguish between glycation and enzymatic modification of proteins by ADP-ribose, we have previously prepared model conjugates for ADP-ribose glycation and determined properties that allow the glycation adducts to be distinguished from enzymatic modifications (22). We report here that ADP-ribose is much more efficient than other possible pentose donors for glycation and glycoxidation of histones. Our results also suggest that previous reports of histone modifications *in vivo* may represent glycation and glycoxidation reactions initiated by ADP-ribose.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]NAD was from DuPont NEN. CHES, ADP-ribose, *n*-butylamine, histones H1, H2A, H2B, and H4, and bacterial alkaline phosphatase were from Sigma. For some experiments, ADP-ribose was purified by anion exchange chromatography prior to its use in studies of histone cross-linking and formation of fluorescent AGE. Snake venom phosphodiesterase was from Worthington. For all reversed-phase HPLC analyses except those shown in Fig. 11, μBondapack C_{18} reversed-phase HPLC columns (3.9 × 300 mm) from Waters were used. For the separation shown in Fig. 11, a Zorbax RX-C_{18} reversed-phase column (2.1 × 150 mm) from MAC-MOD Analytical was used. Hydroxylamine hydrochloride and ammonium formate were from Fisher. D_20 (99.8 and 100%) was from Cambridge Isotope Laboratories. D_2O (99.8%) containing 0.7% of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid was from Aldrich.

Preparation and Characterization of Model Glycation Conjugates of ADP-ribose—Model glycation conjugates for ADP-ribose, termed ketoamines 1 and 2, were synthesized from ADP-ribose and *n*-butylamine and purified as described previously (22). To study the products released from these conjugates at pH 9.0, 50–100 nmol of ketoamine was incubated in 200 mM CHES buffer, pH 9.0, at 37 °C, and aliquots were diluted in 100 mM potassium phosphate buffer, pH 5.0, and analyzed by

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[1] The abbreviations used are: AGE, advanced glycation end products; CML, N^{ϵ}-(carboxymethyl)lysine; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; G-ADP, 3″-ADP glyceric acid; ADP-ribose, adenosine diphosphoribose; HPLC, high performance liquid chromatography; Boc, *t*-butoxycarbonyl; PAGE, polyacrylamide gel electrophoresis.

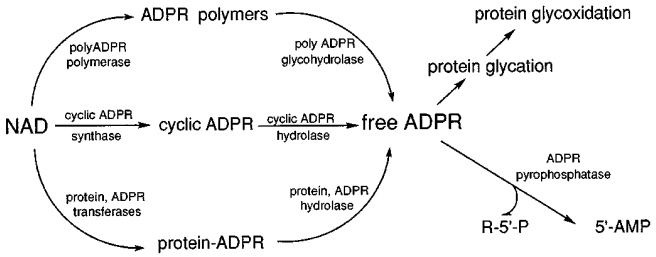

FIG. 1. **Pathways for the generation of free ADP-ribose and possible metabolic fates within cells.** ADP-ribose is abbreviated *ADPR*.

reversed-phase HPLC using 100 mM potassium phosphate buffer, pH 5.0, as the mobile phase at 1 ml/min. This chromatographic condition was designed to separate G-ADP from ADP-ribose, ADP, and AMP. Ketoamines 1 and 2 were not eluted from the column and their elution required the addition of 5–7% methanol to the mobile phase. For enzyme characterizations, the incubations at pH 9.0 contained 10 units/ml bacterial alkaline phosphatase and/or 10 units/ml snake venom phosphodiesterase.

Preparation and Characterization of G-ADP Derived from Ketoamine 1—A reaction mixture (5.0 ml) containing 3 M *n*-butylamine, 10 mM ADP-ribose, 50 mM potassium phosphate buffer, pH 5.0, was incubated at 37 °C for 7 days. At that time, more than 90% of the ADP-ribose had been consumed as judged by HPLC. The reaction mixture was diluted with water to 100 ml, and 10 ml was applied to 10 separate 1.0-ml columns of Dowex 1-X2 equilibrated as described previously (23). After application, the columns were successively washed with 10 ml of ammonium formate, pH 5.0, of the following concentrations: 50 mM, 100 mM, 200 mM, 250 mM, and 500 mM. G-ADP was eluted with 5 × 1-ml washes of each column with 1.0 M ammonium formate. The material eluted from the column by 1.0 M ammonium formate was further purified by reversed-phase HPLC using 10 mM ammonium formate, pH 5.0, as the mobile phase.

A Varian VXR-400 NMR spectrometer operating at 399.95 MHz for ^{1}H and 100.58 MHz for ^{13}C was used to acquire NMR spectral data. Samples were lyophilized three times in D_2O prior to NMR analysis and dissolved in D_2O at a concentration of approximately 10 mM. ^{13}C NMR spectral parameters were as follows: sweep width, 22,371.36 Hz; acquisition time, 1 s; acquisition delay, 1 s; transients, 70,000 in double precision mode. ^{13}C was referenced using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt. The ^{13}C spectrum of G-ADP showed the following absorptions: δ 179.98, 155.93, 152.20, 151.80, 143.66, 121.31, 90.04, 86.75, 77.13, 73.03, 71.18, 67.88 ppm.

For analysis of G-ADP by mass spectroscopy, a Kratos CONCEPT 1H two-sector instrument was used. Glycerol was used as a matrix for positive and negative ion determinations, and the sample was spiked with cesium iodide.

Glycation of Histones by ADP-ribose—[^{32}P]ADP-ribose containing radiolabel in the adenosine proximal phosphate and [^{14}C]ADP-ribose containing radiolabel in the reducing ribose were obtained by incubation of the corresponding radiolabeled NAD with immobilized B. fasciatus NADase as described previously (22).

For glycation of histones by [^{32}P]ADP-ribose, incubation mixtures (100 μl) contained 50 μM [^{32}P]ADP-ribose (2.7×10^8 dpm), 1 mg/ml histone, 50 mM sodium pyrophosphate, 50 mM potassium phosphate buffer, pH 7.5, with incubation overnight at 37 °C. After incubation, trichloroacetic acid was added to 20% (w/v), the mixtures were placed on ice for 15 min and acid insoluble material was collected by centrifugation. The pellets were dissolved in 50 mM sodium acetate buffer, pH 5.0, and applied (Fig. 5) to 15% acid-urea acrylamide gels (24) or used for chemical stability studies (Fig. 6).

For glycation of histone H1 by ADP-ribose containing radiolabel in different positions (see Results), reaction mixtures (100 μl) contained 500 μM ADP-ribose with either ^{14}C in the reducing ribose (1.2×10^6 dpm) or ^{32}P in the adenosine proximal phosphate (8.2×10^7 dpm), 1 mg/ml histone H1, 50 mM sodium pyrophosphate, 50 mM potassium phosphate buffer, pH 7.5. The mixture was incubated for 5 h at 37 °C. Following incubation, trichloroacetic acid was added to 20% (w/v), the mixtures were placed on ice for 15 min and acid insoluble material was collected by centrifugation. The pellets were dissolved in 50 mM sodium acetate buffer, pH 5.0 and radiolabel was determined by liquid scintillation counting.

To examine for the presence of histone H1 cross-linking (Fig. 10), reaction mixtures (1.5 ml) contained 0.67 mg/ml histone H1, 500 μM ADP-ribose, 0.025% SDS, 50 mM sodium acetate, adjusted to pH 9.0 with NaOH, with incubation for 2 h at 37 °C. Following incubation, samples containing approximately 5 μg of protein were applied to 12% SDS-PAGE gels for electrophoresis. Following electrophoresis, gels were stained with Coomassie Blue.

Chemical Stability Studies of Glycated Histones—Aliquots of [^{32}P] glycated histones H1, H2A, H3, and H4 (approximately 1×10^5 dpm) were incubated under conditions described previously to determine the stability of the glycation conjugates (22). Incubations were terminated by adjusting to a final concentration of 20% (w/v) trichloroacetic acid, and the samples were placed on ice. After 15 min, samples were subjected to centrifugation and radioactivity in the supernatant fraction was quantified by liquid scintillation counting. Material released from histone H1 by treatment at pH 9.0 was analyzed by reversed-phase HPLC with 100 mM potassium phosphate buffer, pH 5.0, as the mobile phase at 1.0 ml/min (Fig. 6).

Formation of Fluorescent Products from Histone H1 and ADP-ribose—An incubation mixture (1.5 ml) contained 0.67 mg/ml of histone H1, 500 μM ADP-ribose, 0.025% SDS, 50 mM sodium acetate, adjusted to pH 9.0 with sodium hydroxide (2 M). After incubation at 37 °C for 2 h, the samples were adjusted to pH 5.0 by the addition of HCl, and fluorescence measurements were performed in a Hitachi fluorometer with excitation at 335 nm and emission at 385 nm.

To examine for the fluorescent products formed from histone H1 and ADP-ribose by HPLC (Fig. 11), a reaction mixture (10.0 ml) contained 500 μM ADP-ribose, 0.67 mg/ml histone H1, 0.025% SDS, 50 mM sodium acetate, adjusted to pH 9.0 by addition of NaOH, with incubation overnight at 37 °C. Following incubation, the sample was subjected to lyophilization, 11 M HCl was added, and the sample was purged with nitrogen gas and sealed. After incubation at 110 °C for 24 h, HCl was removed by evaporation, water was added, and the sample was neutralized by the addition of NaOH. An aliquot was subjected to reversed-phase HPLC using a Zorbax column with a gradient elution described elsewhere (25). Fluorescence was monitored using a Hewlett-Packard programmable detector using an excitation wavelength of 335 nm and an emission wavelength of 385 nm.

Amino Acid Analysis of Histone H1 Glycated by ADP-ribose—A reaction mixture (1.5 ml) of 0.67 mg/ml histone H1, 2.5 mM ADP-ribose, 100 mM sodium acetate was adjusted to pH 9.0 and incubated overnight at 37 °C. The sample was lyophilized and hydrolyzed with 11 M HCl for 24 h at 110 °C and subjected to amino acid analysis as described previously (11). A standard of CML was kindly provided by Dr. John Baynes, University of South Carolina.

Formation of Fluorescent Products from Arginine, Lysine, and ADP-ribose—A reaction mixture (1.5 ml) containing 10 mM ADP-ribose, 250 mM *N*-α-Boc-arginine, 50 mM *N*-α-Boc-lysine, in phosphate-buffered physiological saline, was adjusted to pH 9.0 and incubated for 7 days at 37 °C. The sample was lyophilized and hydrolyzed with 50 μl of trifluoroacetic acid as described previously (25). The trifluoroacetic acid was evaporated, and the sample was neutralized prior to analysis by reversed-phase HPLC using a gradient elution described elsewhere (25). Fluorescence was monitored using a Varian filter fluorometer using a deuterium lamp, a 220I excitation filter and a 370 nm cutoff emission filter. Material co-eluting with authentic pentosidine was isolated and its fluorescence properties were characterized using a Hitachi fluorometer. A standard of pentosidine was obtained from by Dr. Vincent Monnier, Case Western Reserve University.

RESULTS

Identification of Products Derived from ADP-ribose Glycation—Since the ϵ-amino groups of lysine residues contribute most of the amino groups in protein, we previously studied model conjugates representing lysine glycation by ADP-ribose (22). The Schiff base formed between ADP-ribose and *n*-butylamine undergoes an Amadori rearrangement that leads to the formation of two different ketoamine-containing adducts that differ in the position of the carbonyl group (22). Because proteins are also modified by ADP-ribose by the action of mono-ADP-ribosyltransferases, we compared the chemical properties of the ketoamine adducts, termed ketoamines 1 and 2, with enzymatic protein modifications by ADP-ribose. While all of the enzymatic modifications involving ADP-ribose were stable at pH 9.0, the ketoamines were very labile (22). Since the lability of the glycation conjugates at pH 9.0 readily distinguished them from the enzymatic modifications, the products released

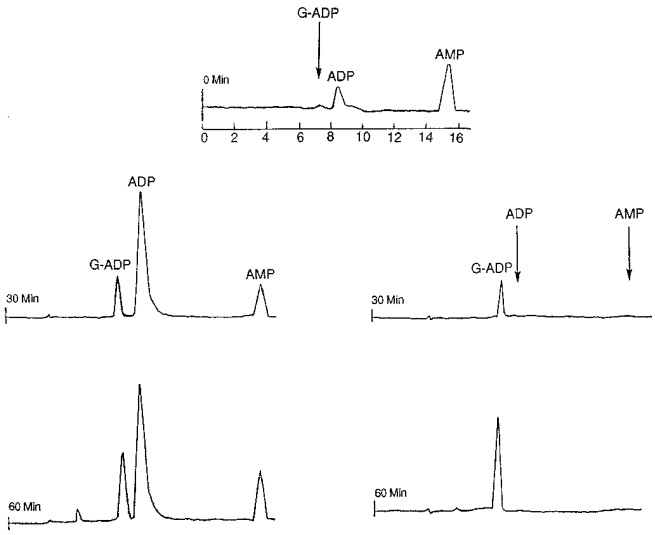

FIG. 2. **Analysis by reversed-phase HPLC of material released at pH 9.0 from a model glycation conjugate derived from ADP-ribose and *n*-butylamine.** Absorbance was monitored at 254 nm. Chromatography of a sample of ketoamine 1 (22) that was stored at pH 5.0, −20 °C and analyzed prior to incubation at pH 9.0 is shown at the *top* of the figure. The *left panels* show profiles from samples incubated at pH 9.0 and analyzed at the times indicated. The *right panels* show profiles from samples incubated at pH 9.0 in the presence of alkaline phosphatase. The elution positions of G-ADP, ADP, and AMP are shown.

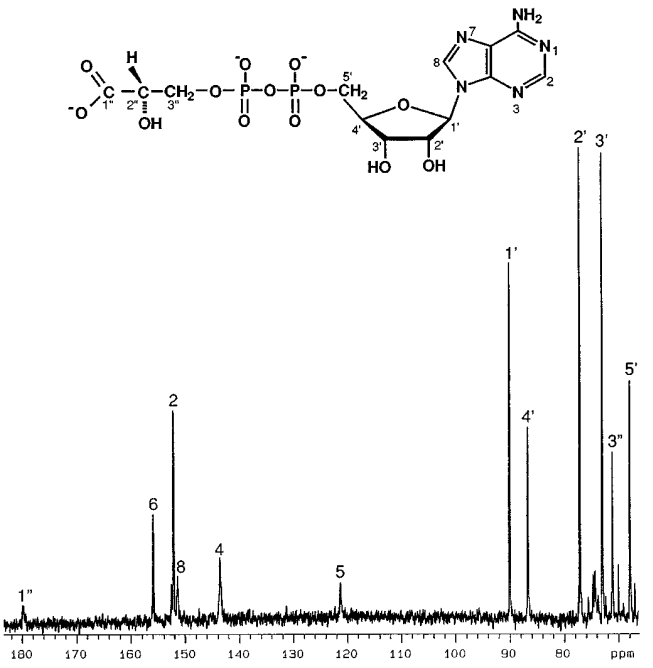

FIG. 3. **^{13}C NMR spectrum of a product (G-ADP) released from a model ADP-ribose glycation conjugate (ketoamine 1) by incubation at pH 9.0.** The proposed structure and numbering of G-ADP is shown at the *top* of the figure.

when the ketoamines were incubated at pH 9.0 were analyzed by HPLC. The results obtained for ketoamine 1 are shown in Fig. 2. The storage of ketoamine 1 at pH 5.0, −20 °C, resulted in a slow degradation to material that co-eluted with 5′-AMP, and to a lesser extent, to material that co-eluted with 5′-ADP (Fig. 2, *top*). In the HPLC running buffer, intact ketoamine 1 was not eluted from the column. At pH 9.0, ketoamine 1 is degraded by first order kinetics with a $t_{1/2}$ of approximately 15 min (22), thus the elution profiles at 30 and 60 min represent approximately 70 and 95% degradation of the ketoamine, respectively. With increasing time of incubation at pH 9.0, the major products formed co-eluted with 5′-ADP and with unidentified material which we termed G-ADP (Fig. 2, *left panels*). Also, a slight increase in material co-eluting with 5′-AMP was observed with prolonged incubation. The material co-eluting with 5′-AMP and 5′-ADP was not observed when alkaline phosphatase was included in the incubation (Fig. 2, *right panels*). However, G-ADP was unaffected by the presence of alkaline phosphatase, indicating that it lacked a phosphomonoester moiety. The same products were observed when ketoamine 2 was incubated at pH 9.0 (data not shown). Additionally, both ketoamines 1 and 2 yielded material that co-eluted with 5′-ADP and G-ADP when incubated at pH 7.4, although the rate of formation was approximately 20 times slower (data not shown).

The material co-eluting with 5′-ADP was converted to material that co-eluted with 5′-AMP following incubation with venom phosphodiesterase and to material that co-eluted with adenosine following incubation with venom phosphodiesterase and alkaline phosphatase (results not shown), indicating that it was 5′-ADP. The G-ADP was characterized further, since it represented a product potentially unique to protein glycation by ADP-ribose. Snake venom phosphodiesterase treatment of G-ADP resulted in material that co-eluted with 5′-AMP (data not shown), indicating the presence of a phosphodiester linkage.

G-ADP was further characterized by NMR and mass spectral methods. Fig. 3 shows a ^{13}C NMR spectrum of G-ADP. Signals corresponding to the five carbons of the adenine moiety and to the five carbons of ribose were readily identified by comparison with the spectra of other adenine-containing nucleotides (22, 26). Additionally, a signal at a position expected for the carbonyl carbon of a carboxylate group (179.98 ppm) and at a position expected for a carbon of a methylene group linked to a pyrophosphate bridge (71.18 ppm) could be identified. A weak signal at approximately 75 ppm suggested the possible presence of a 13th carbon atom present as a hydroxymethyl group, but this could not be established from the spectrum alone. The ^{1}H NMR spectrum allowed the identification of the adenine protons and the anomeric proton of the ribose bound to adenine (data not shown). To determine the molecular weight of G-ADP, the negative ion and positive ion fast atom bombardment mass spectra of G-ADP were obtained. These spectra (Fig. 4) showed a negative ion of m/z 514 and a positive ion of m/z 516, indicating a molecular weight of 515 for G-ADP.

The enzyme sensitivity and NMR data indicated that G-ADP contained ADP and an additional carbon fragment containing a methylene carbon and a carboxyl group derived from the second ribose originally present in the ADP-ribose. If the carbon fragment contained only the methylene and carboxyl groups, the predicted molecular weight for G-ADP would be 475. The molecular weight of 515 indicated that G-ADP contains an additional hydroxymethyl group. Taken together, the data indicate that the carbon fragment attached to ADP is glyceric acid. The proposed structure and numbering system is shown at the *top* of Fig. 3. We postulate that the proton of the 2″ carbon of G-ADP is acidic enough to partially exchange with D_20, resulting in a significantly reduced signal for this carbon in the ^{13}C NMR spectrum, such that it could not be unambiguously detected. In summary, our results demonstrate that model glycation conjugates of ADP-ribose degrade at pH 9.0 to form two primary products, 5′-ADP and G-ADP.

Histone Glycation by ADP-ribose: Formation of Ketoamine Adducts—To study the potential of ADP-ribose as a protein-glycating agent, histones were examined, since they are likely intracellular targets for ADP-ribose glycation due to their high

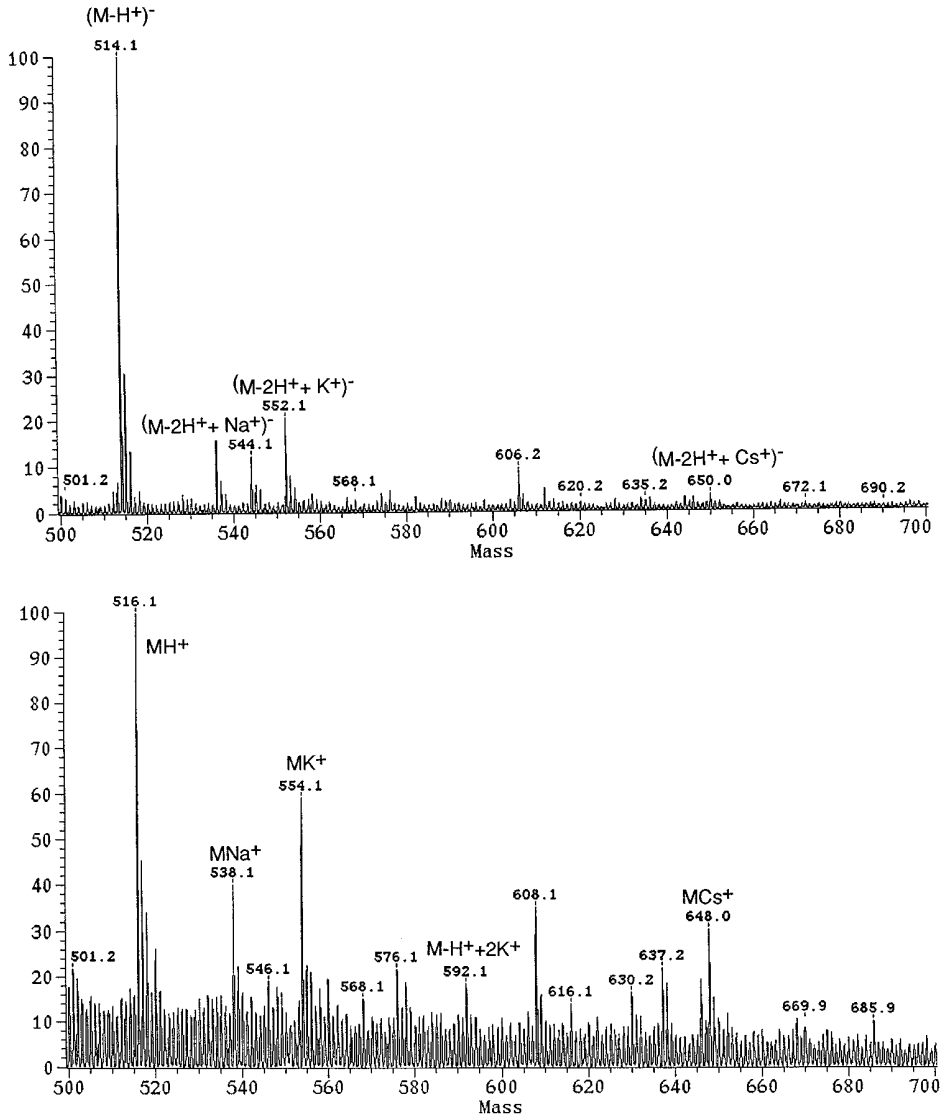

FIG. 4. **Negative ion (*top*) and positive ion (*bottom*) mass spectra of a product (G-ADP) released from a model ADP-ribose glycation conjugate (ketoamine 1) by incubation at pH 9.0.**

content of lysine and to their proximity to the turnover of ADP-ribose polymers (17, 18). We have previously observed that incubation of histone H1 with ADP-ribose results in stable modification of this protein (27). To examine glycation by ADP-ribose in more detail, histones H1, H2A, H2B, and H4 were incubated for 5 h at pH 7.5 and 37 °C with 50 μM [^{32}P]ADP-ribose containing the radiolabel in the adenosine proximal phosphate. Fig. 5 shows analysis of the histones by electrophoresis on acid-urea gels. Comparison of Coomassie Blue staining (*left panel*) and the autoradiogram of the gel (*right panel*) demonstrated that each of the histones was stably modified by ADP-ribose. The extent of modification for the individual histones under these conditions was (mmol of ADP-ribose/mol of histone): H1, 2.0; H2A, 4.7; H2B, 2.2; H4, 12.8. Additional studies demonstrated that the amount of modification was concentration-dependent for ADP-ribose (data not shown). To determine if the ADP-ribose modification of the histones involved formation of ketoamines, the chemical stability of radiolabeled histone adducts was compared with the model glycation conjugates of ADP-ribose studied previously (22). Like the model glycation conjugates, the histone conjugates were stable in formic acid and neutral hydroxylamine, but radiolabel was released at pH 9.0. When incubated at pH

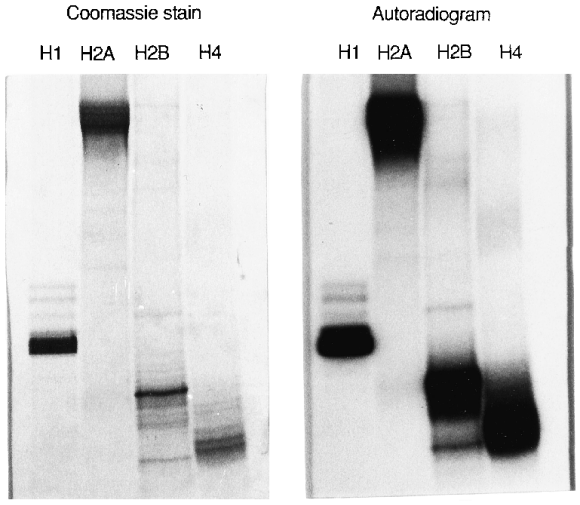

FIG. 5. **Electrophoretic analysis on acid-urea gels of histones incubated with [^{32}P]ADP-ribose.** Coomassie Blue staining is shown in the *left panel*, and the autoradiogram is shown in the *right panel*.

9.0, radiolabel was released from each of the histones by first order kinetics with $t_{1/2}$ values of approximately 35 min for H1, 30 min for H2A, 40 min for H2B, and 70 min for H4 (data not shown). Fig. 6 shows analysis by HPLC of the products released from histone H1 at pH 9.0 following a 1.0-h incubation. Material co-eluting with 5′-AMP, 5′-ADP, G-ADP, and a small amount of unidentified material were observed. Treatment of the released material with alkaline phosphatase prior to chromatography demonstrated that the material co-eluting with 5′-AMP and 5′-ADP was sensitive to phosphatase, but that the radiolabel co-eluting with G-ADP was unaffected by phosphatase treatment (data not shown). Material co-eluting with G-ADP also co-eluted with G-ADP on strong anion exchange HPLC, and it was converted to material that co-eluted with 5′-AMP following phosphodiesterase treatment (data not shown). These data indicate that histones can be stably glycated by ADP-ribose and that glycation involves the formation of ketoamine adducts.

Histone Glycation by ADP-ribose: Formation of CML—Fig. 7 shows a proposed mechanism for the formation of G-ADP from a protein glycated by ADP-ribose. This mechanism predicts that the release of G-ADP should result in the conversion of a protein lysine residue to a CML residue. To test this prediction, histone H1 was incubated overnight with 2.5 mM ADP-ribose and subjected to acid hydrolysis followed by amino acid analysis to examine for the presence of CML. Histone H1 was chosen due to its very low content of methionine, which is poorly separated from CML under the conditions of amino acid analysis (28). The region of the chromatogram corresponding to the elution position of CML for the control and glycated samples is shown in Fig. 8. The control sample showed a peak at an elution position of 30.46 min, the expected elution time of methionine. This peak corresponded to 85 pmol of methionine. In contrast, the glycated sample showed a peak corresponding to 730 pmol at an elution position of 30.77 min, which was shown to be the elution position of CML by analysis of a replicate sample to which authentic CML had been added (data not shown). Because of the similar elution times of methionine and CML, we made the assumption that the peak observed in the glycated sample was due to both methionine and CML. The analysis also showed that the content of most of the amino acids was unchanged in the glycated sample, except for lysine and arginine. The lysine content of the control sample was 2,210 pmol, which decreased to 1,510 pmol in the glycated sample. The decrease in lysine content of 700 pmol closely corresponded to the increase in the CML peak. The arginine content of the control sample was 148 pmol, which decreased to 34 pmol in the glycated sample. The decrease in arginine content as it relates to the formation of fluorescent AGE is described below. In summary, our results demonstrate that glycation of histone H1 by ADP-ribose can lead to the formation of CML.

Histone Glycation by ADP-ribose: Formation of Fluorescent AGE—Studies of protein glycation by glucose have shown that ketoamine glycation adducts degrade to release reactive dicarbonyl compounds such as 3-deoxyglucosone and undergo further reactions to generate a number of different fluorescent glycoxidation products (2, 3). Experiments were done with histone H1 to further characterize glycation by ADP-ribose to determine if the ketoamines formed from ADP-ribose were generating additional products. First, an experiment was done to determine which moieties of the ADP-ribose molecule were involved in histone modification. Histone H1 was incubated for

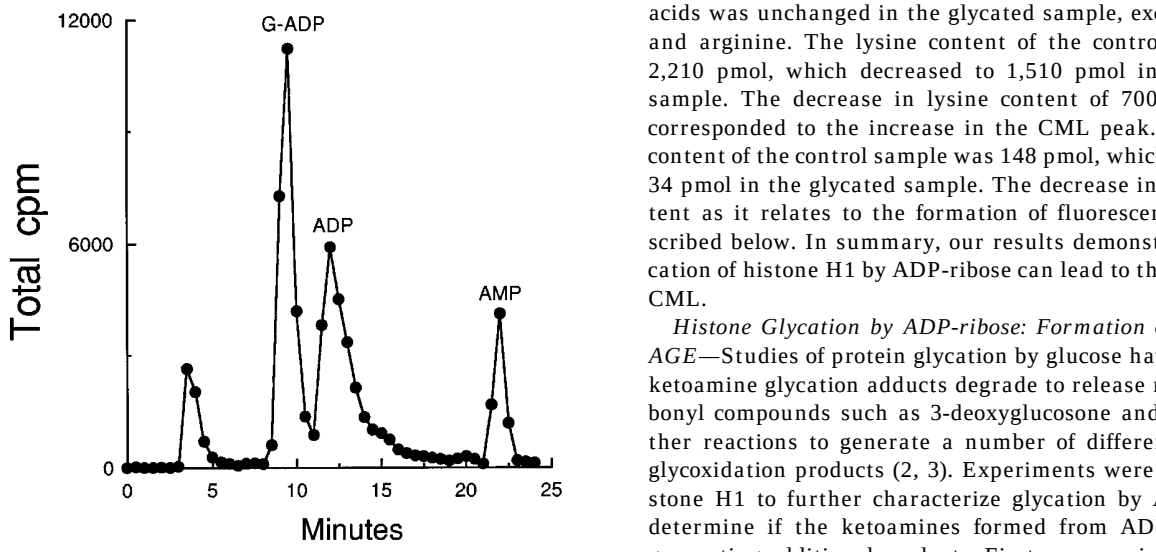

FIG. 6. **Analysis by reversed-phase HPLC of radiolabel released at pH 9.0 from histone H1 glycated by [^{32}P]ADP-ribose.** The elution positions of G-ADP, ADP, and AMP are shown.

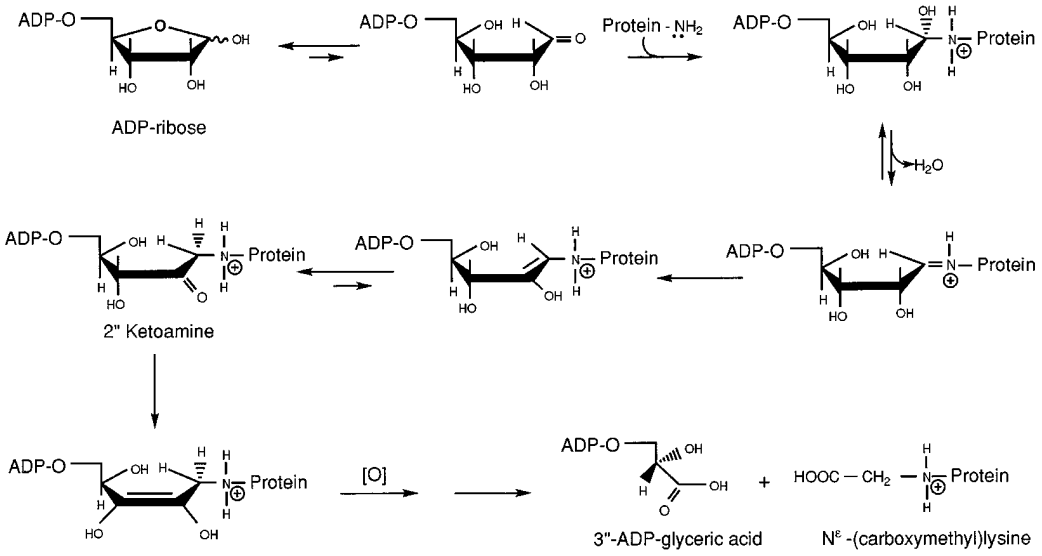

FIG. 7. **A mechanism proposed for the formation and degradation of a ketoamine adduct by reaction of ADP-ribose with a protein amino group.**

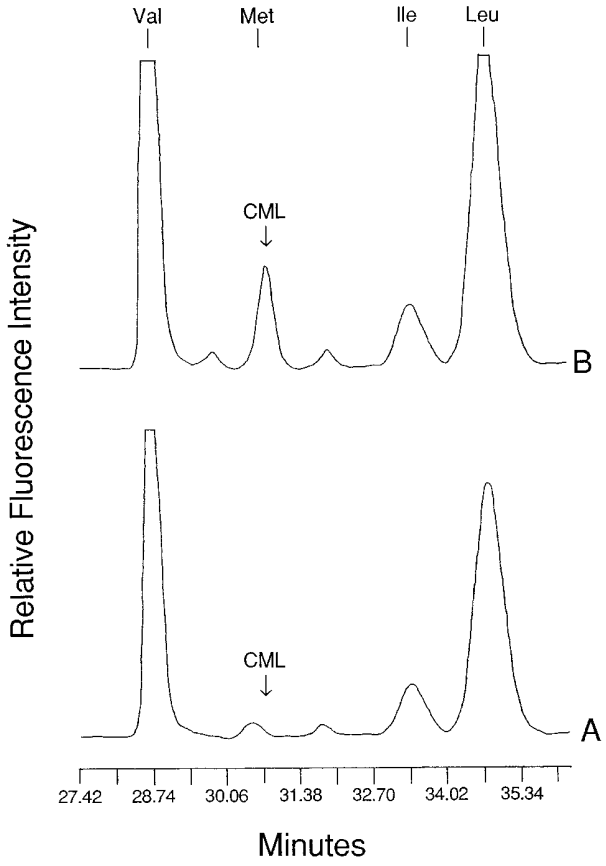

FIG. 8. **Amino acid analysis (11) of histone H1 (*A*) and histone H1 incubated with ADP-ribose (*B*).** The region of the chromatogram near the elution position of CML is shown.

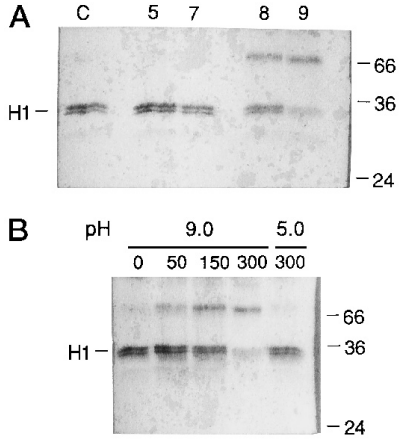

FIG. 9. **Analysis by SDS-PAGE of histone H1 incubated with ADP-ribose.** *A* shows Coomassie Blue staining of an unincubated sample (designated *C*) and samples incubated with 500 μM ADP-ribose where the numbers at the *top* of the gel indicate pH values. *B* shows Coomassie Blue staining of a sample incubated at pH 9.0 with the micromolar concentrations of ADP-ribose indicated at the *top* of the gel and a sample incubated at pH 5.0 with 300 μM ADP-ribose.

5 h at pH 7.5 with 500 μM ADP-ribose radiolabeled with either ^{32}P in the adenosine proximal phosphate or ^{14}C in the ribose moiety containing the free aldehyde. The H1 was then subjected to acid precipitation and the amount of radiolabel incorporated into histone was quantified. The amount of modification was 10.0 mmol of ^{32}P and 43.2 mmol of ^{14}C incorporated per mol of H1. These data indicate that much of the ADP-ribose glycation was accompanied by further reactions that involved the loss of the radiolabeled phosphate.

The study of Sell and Monnier (16) has shown that pentoses readily generate a number of different fluorescent AGE. One of these products, pentosidine, has been structurally characterized (16). The formation of these fluorescent AGE begins with the initial glycation of a lysine amino group but further reactions involve both oxidation and reaction with protein arginine residues. If the lysine and arginine residues are present on different polypeptide chains, the formation of pentosidine results in protein-protein cross-linking (2, 16). To determine if ADP-ribose glycation could lead to glycation products that result in protein cross-linking, histone H1 was incubated with ADP-ribose and subjected to analysis by SDS-PAGE. Fig. 9*A* shows that incubation of histone H1 with 500 μM ADP-ribose for 2 h at pH 5.0 and 7.0 did not affect its electrophoretic migration, but incubation at pH 8.0 and 9.0 resulted in the appearance of material at the expected position of a dimer of H1. At pH 9.0, the H1 was almost completely converted to the dimer position. No differences were observed when ADP-ribose purified by anion exchange chromatography was used, indicating that the activity was not due to reactive contaminants in the commercial preparation of ADP-ribose. With longer times of incubation, conversion of H1 to the dimer position also was readily observed at pH 7.0 (data not shown). Since the reaction was most rapid at pH 9.0, additional experiments were done this pH. Fig. 9*B* shows that conversion of H1 to a dimer was dependent upon the concentration of ADP-ribose. When the incubation contained ADP-ribose with ^{14}C in the ribose moiety containing the free aldehyde, radiolabel was incorporated into the dimer of H1 (data not shown). Aminoguanidine has been shown to serve as an effective inhibitor of protein glycoxidation (2). Addition of 500 μM aminoguanidine completely inhibited H1 dimer formation (data not shown). These results indicated that ADP-ribose was effective in causing protein cross-links by a mechanism involving glycation.

To determine if the protein cross-linking by ADP-ribose involved the formation of fluorescent AGE, 500 μM ADP-ribose was incubated with H1, and fluorescence was monitored using the conditions of excitation and emission typical for pentosidine (16). Incubation of histone H1 at pH 9.0 with 500 μM ADP-ribose resulted in the rapid formation of putative fluorescent AGE (data not shown). Fluorescent products could be readily detected after a lag of approximately 8 min and increased in a linear manner as a function of incubation time. The presence of the lag phase is consistent with a mechanism by which the formation of ketoamine glycation products precedes the formation of AGE. Fluorescent products were also observed when incubations were done at pH 7.4, although the rate of formation was much slower. Fluorescent products also were observed when histone H1 was incubated with an ADP-ribose preparation that had been purified by anion exchange HPLC, but incubation of either histone H1 or ADP-ribose alone did not result in any detectable fluorescence. When analyzed after a 20-min incubation, formation of fluorescence was dependent upon the concentration of ADP-ribose in the incubation mixtures from 50 to 500 μM. Furthermore, the addition of 100 and 500 μM aminoguanidine inhibited the formation of fluorescent products by 65 and 98%, respectively. No reaction between ADP-ribose and aminoguanidine was detected by subjecting reaction mixtures to anion exchange HPLC, indicating that aminoguanidine was inhibiting oxidative steps rather than scavenging ADP-ribose and inhibiting glycation. Reactive oxygen species, whose formation can be catalyzed by metal ions, have been implicated in the oxidative phase of glycoxidation (2). Evidence that the fluorescent products observed here involved a similar mechanism was provided by the observation

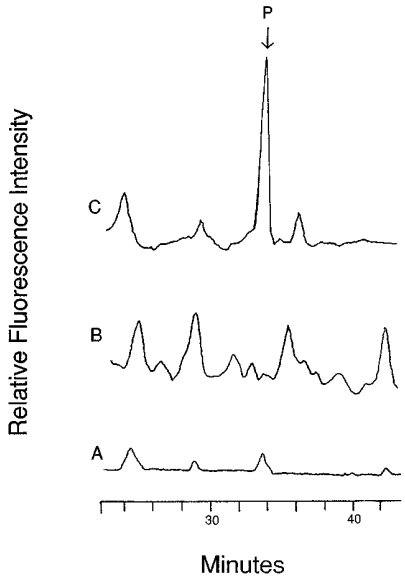

FIG. 10. **Analysis by reversed-phase HPLC of fluorescent products formed by incubation of ADP-ribose with *N*-α-Boc arginine (Chromatogram A), *N*-α-Boc lysine (Chromatogram B), or both *N*-α-Boc arginine and *N*-α-Boc lysine (Chromatogram).** The *N*-α-Boc protecting groups were removed prior to HPLC analysis. The elution position of a pentosidine standard (*P*) is shown by the *arrow*.

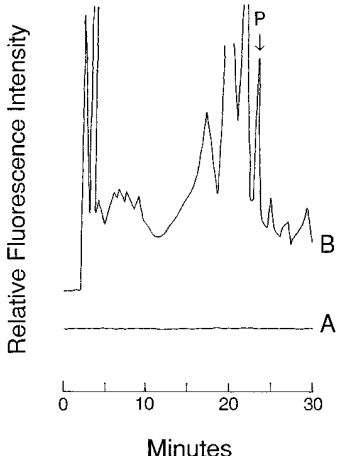

FIG. 11. **Analysis by reversed-phase HPLC of fluorescent products derived from incubation of ADP-ribose with histone H1.** The glycated histone H1 was subjected to acid hydrolysis prior to analysis. The elution position of a pentosidine standard (*P*) is shown by the *arrow*. The differences in elution times for pentosidine in Figs. 10 and 11 are due to the use of different reversed-phase columns (see "Experimental Procedures").

that their formation was inhibited by reduced ascorbate.[2] Also, the rate of formation of both fluorescent products and histone cross-links was increased at either pH 7.0 or 9.0 with either phosphate or acetate buffer when the buffer concentration was increased from 10 to 100 mM, consistent with traces of metal ion-catalyzing aerobic oxidation.[3]

It is noteworthy that ADP-ribose was much more effective than either ribose or ribose 5-phosphate in the formation of fluorescent AGE. While incubation with ADP-ribose with histone H1 resulted in intense fluorescence in minutes, neither ribose or ribose 5-phosphate resulted in detectable fluorescence when incubated under the same conditions for several days.

Formation of Fluorescent AGE from ADP-ribose and Amino Acids—Experiments were done to determine if fluorescent AGE could be formed from ADP-ribose, lysine, and arginine. *N*-α-Boc groups were used to block the α-amino groups of arginine and lysine to preclude their involvement in the formation of products. ADP-ribose was incubated with the amino acids alone and in combination. Following incubation, the Boc groups were removed and the products were analyzed by HPLC (Fig. 10). Incubation of ADP-ribose with *N*-α-Boc arginine alone resulted in a number of fluorescent compounds (Chromatogram A) and incubation with *N*-α-Boc lysine alone yielded a number of additional peaks (Chromatogram B). Incubation of ADP-ribose with both *N*-α-Boc arginine and *N*-α-Boc lysine resulted in the formation of a fluorescent compound that co-migrated with the pentosidine standard. Additionally, the material co-migrating with pentosidine had excitation and emission spectra indistinguishable from the pentosidine standard (data not shown).

Histone Glycation by ADP-ribose: Isolation of AGE—To determine if pentosidine and/or related glycation products are formed in proteins glycated by ADP-ribose, histone H1 was incubated with ADP-ribose, subjected to acid hydrolysis, and analyzed by reversed-phase HPLC. The glycated sample showed several fluorescent peaks with chromatographic migration similar to pentosidine including one peak that co-eluted with pentosidine (Fig. 11). Analysis of a replicate sample to which pentosidine had been added resulted in enhancement of the peak co-migrating with pentosidine, while the sample incubated in the absence of ADP-ribose did not contain any detectable fluorescent peaks.

DISCUSSION

Many lines of evidence indicate that the non-enzymatic modification of long lived biomolecules plays an important role in the aging process and the pathophysiology of diseases whose incidence increases as a function of age (1–7). Protein amino groups are sites of reaction with reducing sugars that result in protein modification by ketoamine adducts (11–15). Since ketoamine adducts are not very stable, many, and possibly most, ketoamine adducts are precursors to much more complex protein modifications (11, 16). Although less reactive, amino groups in DNA bases are also potential sites of modification by reducing sugars (29, 30).

The results presented in this study have potentially important implications for the non-enzymatic modification of long lived nuclear biomolecules involved in the maintenance of genomic integrity. Age-associated changes in chromatin structure have been described previously (3, 31), and both individual histones (32) and histones in nucleosomes (33) have been reported to be glycated *in vitro*. Genomic integrity requires efficient repair of molecular damage to DNA and repair of DNA damage is accompanied by the synthesis of ADP-ribose polymers, which are present only transiently, being rapidly degraded to ADP-ribose (17, 18). Although the intranuclear levels of ADP-ribose under specific physiological conditions are unknown, intracellular levels of NAD are estimated to be in the range of 100–300 μM and a large fraction of the NAD pool can flow through ADP-ribose polymers to ADP-ribose following DNA damage, indicating that appreciable concentrations of ADP-ribose should occur in the nucleus of DNA-damaged cells (17). The possibility that ADP-ribose might be a source of a ribose moiety *in vivo* for protein glycoxidation has been suggested by Sell and Monnier (16). As described here, ADP-ribose is an efficient source for glycation and glycoxidation of histones *in vitro*. Many previous studies of protein modification *in vitro* by reducing sugars have used sugar concentrations in the range of 20–500 mM (12, 32–34). In this study, ADP-ribose

[2] D. Schramm and D. Cervantes-Laurean, unpublished observations.
[3] D. Cervantes-Laurean, unpublished observations.

concentrations of 50–500 μM resulted in readily detectable histone glycation (Figs. 5 and 6) and glycoxidation (Figs. 9 and 11). While the reaction rates were much greater at pH 9.0, the lability of the ketoamine glycation conjugates, the cross-linking of histones and the formation of fluorescent AGE also were readily detectable at pH values in the physiological range, indicating that these reactions are likely to occur in intact cells.

Our studies with model ADP-ribose glycation conjugates (Figs. 2–4) and histone ADP-ribose glycation conjugates (Figs. 5 and 6) have demonstrated that ketoamines derived from ADP-ribose degrade by two primary pathways, one that generates ADP and a second that generates G-ADP. The efficacy of formation of glycoxidation products, specifically histone cross-links and other fluorescent AGE, likely relates to the degradation pathway that releases ADP (Figs. 2 and 6). The mechanism for the formation of AGE such as pentosidine postulates the condensation of a ketoamine derived neutral five-carbon fragment with a protein arginine residue (16). Pathways for the degradation of ADP-ribose derived ketoamines that result in the presence of a residual phosphate would interfere with this condensation, but the release of the ADP moiety results in an uncharged 5 carbon fragment that should readily react with arginine and thus promote histone cross-linking. This pathway may also explain the much greater efficacy of ADP-ribose in the formation of fluorescent products as compared with ribose 5-phosphate as ketoamines formed from ribose 5-phosphate may not degrade as readily to uncharged products. Another factor that may be related to the efficacy of ADP-ribose is the presence of a high affinity binding site for pyrophosphate in histone H1 (35), which may facilitate binding of ADP-ribose to the protein.

Our results suggest that the fluorescent products formed in histone H1 are derived from ADP-ribose, lysine, and arginine, since it was possible to generate a product indistinguishable from pentosidine by incubation of ADP-ribose only with lysine and arginine (Fig. 10). Further evidence that the fluorescent products resulted from glycoxidation was provided by the observation that aminoguanidine, an inhibitor of AGE formation, inhibited the formation of fluorescence. While the results described here have demonstrated histone cross-linking by ADP-ribose, the possibility that ADP-ribose may promote histone-DNA cross-links should also be considered.

The second pathway of degradation of ADP-ribose-derived ketoamines, which leads to the generation of G-ADP, has potential utility for the detection of ADP-ribose-specific glycation of proteins *in vivo*, since it likely represents a unique glycation product for this nucleotide. Additionally, the release of G-ADP predicted that protein glycation by ADP-ribose can result in the modification of protein lysine residues with carboxymethyl groups (Fig. 7). This prediction was confirmed in the experiments described here (Fig. 8). The occurrence of CML residues in proteins *in vivo* has been documented (11, 34, 36). The modification of histone lysine residues by carboxymethyl groups has the net result of converting a positively charged side chain to a negatively charged side chain, which would be expected to alter histone interaction with DNA. A search for the presence of CML in histones *in vivo* may be useful in assessing the possible role of nuclear generated ADP-ribose in histone glycation *in vivo*.

While additional studies will be needed to determine if ADP-ribose causes histone glycation *in vivo*, the results presented here are of interest with regard to a number of previous studies. Hilz and co-workers (37) have described ADP-ribose conjugates of histone H1 in hepatoma cells following DNA damage with chemical stability very similar to the histone glycation conjugates described here, raising the possibility that these conjugates may represent histone glycation. Smulson and co-workers (38) reported a stable complex containing a dimer of histone H1 and ADP-ribose polymers in HeLa cells, although the mechanism by which the histones were covalently cross-linked was not elucidated. Our results raise the possibility that the covalent linkage of the histone molecules is the result of histone glycoxidation initiated by ADP-ribose generated by polymer turnover. Gugliucci and Bendayan (39) have recently detected fluorescent AGE in histones isolated from rats and increased fluorescent AGE in streptozotocin-induced diabetic rats. Streptozotocin is a potent DNA damaging agent known to cause liver DNA damage and to stimulate poly(ADP-ribose) polymerase (17, 18), raising the possibility that the increases in histone AGE may have resulted from ADP-ribose polymer turnover.

The potentially deleterious effects of protein glycation and glycoxidation suggests that cellular mechanisms to repair or minimize these protein modifications are likely to exist. Several enzyme systems are possible candidates for reversing or limiting protein modifications of this type. Both bacteria (40) and fungi (41) contain glycation removal enzymes. An enzyme that reduces 3-deoxyglucosone, a reactive product released from glucose derived ketoamines, has been described in rat liver (42). An ADP-ribose pyrophosphatase catalyzes conversion of ADP-ribose to 5′-AMP and ribose 5-phosphate (43). Our observation that ribose 5-phosphate is much less effective than ADP-ribose in causing protein glycoxidation would indicate that the ADP-ribose pyrophosphatase could function to minimize histone glycoxidation. Protein glycation by glucose leads to the release of 3-deoxyglucosone, and the analogous product released following protein glycation by ADP-ribose would be 3″-ADP-deoxypentosone. It is interesting that 3″-ADP-deoxypentosone has been reported to be the product of the enzyme ADP-ribosylprotein lyase (44), an enzyme postulated to function in the turnover of ADP-ribose polymers by catalyzing the removal of the protein proximal ADP-ribose residue. In view of the results presented here, the possibility that ADP-ribosyl protein lyase may function in glycation removal should also be considered.

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