Protein Glycation by ADP-Ribose: Studies of Model Conjugates†

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ABSTRACT: Protein glycation by hexoses has been implicated in the pathophysiology of a number of diseases as well as the aging process. Studies of ADP-ribose polymer metabolism have shown that free ADP-ribose is generated at high rates in the cell nucleus following DNA damage. Protein glycation by ADP-ribose has been reported although the chemistry is not understood. Described here is the synthesis and characterization of model conjugates for protein glycation of lysine residues by ADP-ribose. Two stable conjugates derived from ADP-ribose and *n*-butylamine were isolated and characterized. Both conjugates were shown to be ketoamines derived from a Schiff base by an Amadori rearrangement. The chemical stability of the ketoamines allowed them to be differentiated from all classes of enzymic protein modification by ADP-ribose. Further, their chemical properties suggest that a previous report of histone H1 modification in carcinogen treated cells was due to glycation by ADP-ribose.

Besides its involvement in hydride transfer reactions central to cellular energy metabolism, NAD[1] is utilized in multiple classes of ADP-ribose transfer reactions in animal cells (Jacobson et al., 1990a). Enzymes that catalyze ADP-ribose transfer are widely distributed throughout the cell and include poly(ADP-ribose) polymerase (Althaus & Richter, 1987), protein mono-ADP-ribosyltransferases (Williamson & Moss, 1990), ADP-ribosyl cyclase (Lee & Aarhus, 1991), and NAD glycohydrolases (Yost & Anderson, 1982). These NAD-consuming reactions ultimately lead to the formation of free ADP-ribose as an intermediate in metabolism (Figure 1.) For example, during repair of DNA damage, the nucleus is the site of the synthesis of ADP-ribose polymers and their rapid turnover to free ADP-ribose (Jacobson et al., 1990a). The levels of free ADP-ribose in vivo and its metabolic fates are very poorly understood. An ADP-ribose pyrophosphatase activity with a low K_m for free ADP-ribose has been recently described (Miro et al., 1989), although its role in ADP-ribose metabolism has not been established.

Free ADP-ribose is a very reactive molecule that has the potential for protein glycation. The chemistry involved in protein glycation by hexoses has been well studied (Maillard, 1912; Lea & Hannan, 1950; Hodge & Rist, 1953; Reynolds, 1963; Bunn et al., 1975; Monnier & Cerami, 1982; Watala et al., 1985; Iberg & Fluckiger, 1986). It is known that hexoses such as glucose readily form Schiff bases with lysine residues, leading to labile aminoglycosides and more stable ketoamine products that result from an Amadori rearrangement. Previously, Kun et al. (1976) have reported glycation by ADP-ribose of histones or polylysine in vitro. More recently, Hilz et al. (1984) and Tanaka et al. (1989) have shown that glycation by ADP-ribose can occur with specific proteins in vitro. The occurrence of protein glycation by ADP-ribose has been postulated in vivo (Kreimeyer et al., 1984) although definitive evidence for its presence in vivo has not been reported.

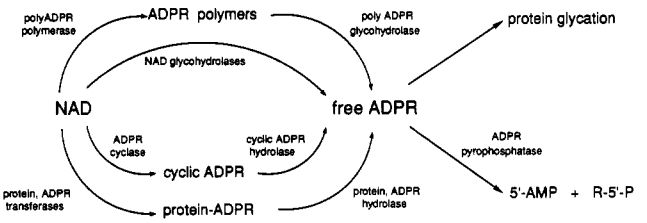

FIGURE 1: Metabolic pathways for the formation and utilization of free ADP-ribose.

The chemistry of protein glycation by ADP-ribose has not been well characterized. When proteins or polypeptides are used as acceptors, structural characterization of the products formed with high-resolution methods such as NMR is difficult. In this study, the formation and characterization of two stable model conjugates for protein glycation of lysine residues by ADP-ribose is described. These conjugates are derived from *n*-butylamine and ADP-ribose. To distinguish glycation by ADP-ribose from enzymatically catalyzed ADP-ribose modifications of proteins, the properties of these conjugates have been compared to ADP-ribosyl amino acids formed by protein mono-ADP-ribosyltransferases.

MATERIALS AND METHODS

Materials. ADP-ribose, *n*-butylamine, MOPS, and CHES were from Sigma Chemical Co. An analytical HPLC column (C_{18} μBondapak, 10 μm, 3.9 mm × 300 mm) was from Waters. A preparative HPLC column (C_{18} Dynamax-300A, 5 mm × 300 mm) was from Rainin. Hydroxylamine hydrochloride

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[1] Abbreviations: ADP-ribose, adenosine diphosphoribose; AMP, adenosine monophosphate; NAD, oxidized form of nicotinamide adenosine diphosphonucleotide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; DNA, deoxyribonucleic acid; D_2O, deuterium oxide; COSY, correlated spectroscopy.

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and ammonium formate were from Fisher Scientific. D_2O (99.8 and 99.9%), 1,2-*o*-dinitrobenzene, and premium 5-mm NMR tubes were from Aldrich Chemical Co. Potassium phosphate was from EM Science.

Reaction of n-Butylamine and ADP-Ribose. The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 25 mM ADP-ribose, and 3 M *n*-butylamine. Incubation was at 37 °C. Aliquots were taken at 0, 4, 8, and 12 h and diluted to 1 mL with 50 mM potassium phosphate buffer, pH 6.0, and subjected to analytical reversed-phase HPLC. The elution was performed isocratically with 100 mM potassium phosphate buffer, pH 6.0, 5% methanol, at a flow rate of 1 mL/min at room temperature. Detection was at 254 nm using an ISCO model 228 detector.

^1H and ^{13}C NMR Analysis of Products 1 and 2. Fifty milliliters of the reaction of *n*-butylamine and ADP-ribose was prepared. Products were purified using a preparative C_{18} reversed-phase column using 5 mM potassium phosphate buffer, pH 5.0, for product 1, and pH 6.0, for product 2, in 1% methanol with isocratic elution. Fractions containing the eluted peaks were pooled and lyophilized. The samples were adjusted to pH 5.0 and 6.0, respectively, for products 1 and 2, followed by two washes with 99.8% D_2O and one with 99.9% D_2O. The final samples were dissolved at a final concentration of approximately 7 mM in 99.9% D_2O containing 50 mM potassium phosphate buffer at same pH as mentioned above.

A Varian XL-300 NMR spectrometer operating at 299.9 MHz for ^1H and 75.4 MHz for ^{13}C was used to acquire spectral data. ^1H NMR spectral parameters were as follows: sweep width, 4400 Hz; data points, 32K; acquisition time, 3.6 s; acquisition delay, 1 s; 32 acquisitions in double precision mode. ^{13}C NMR spectral parameters were as follows: sweep width, 18 000 Hz; data points, 32K; acquisition time, 1 s; acquisition delay, 2 s; 20 000–25 000 acquisitions in double precision mode. ^1H spectra were referenced to HOD at 4.68 ppm, and ^{13}C spectra were referenced using the referencing software of the instrument.

The ^{13}C spectrum of ketoamine 1 exhibited the following absorptions: δ 207.6, 173.6, 157.4, 154.4, 151.5, 142.7, 134.4, 121.2, 117.9, 89.9, 86.3, 78.7, 76.9, 73.6, 72.9, 68.0, 67.4, 49.9, 41.9, 31.4, 30.1, 21.7, 21.6, and 15.4 ppm. The ^{13}C NMR spectrum of ketoamine 2 exhibited the following absorptions: δ 208.9, 157.4, 154.2, 152.0, 143.5, 122.3, 121.7, 90.4, 86.9, 78.5, 77.4, 74.5, 73.4, 68.5, 50.4, 30.4, 22.1, and 15.8 ppm.

Colorimetric Reaction of Products 1 and 2 with o-Dinitrobenzene. To a reaction mixture containing 5 mM potassium phosphate buffer, pH 6.0, and at least 60 μmol of products 1 or 2, was added 50 μL of 20% sodium hydroxide (w/v). This reaction mixture was stirred for 1 min, and 100 μL of an aqueous solution of 0.2% *o*-dinitrobenzene was added. The complete reaction mixture was kept on ice and stirred. After 1 min the appearance of a purple color indicated the presence of the ketoamine.

Reaction of Products 1 and 2 with Hydroxylamine. The reaction mixture contained 100 mM MOPS buffer, pH 7.0, 100 μM purified products 1 or 2, and 1 M hydroxylamine. At 0, 20, 40, 60, and 80 min, aliquots were diluted to 1 mL with 50 mM potassium phosphate buffer, pH 6.0, and subjected to analytical reversed-phase HPLC as described above.

^1H NMR Analysis of Oximes from Ketoamines 1 and 2. The purification procedure and preparation of the oximes for ^1H NMR analysis were the same as described for products 1 and 2.

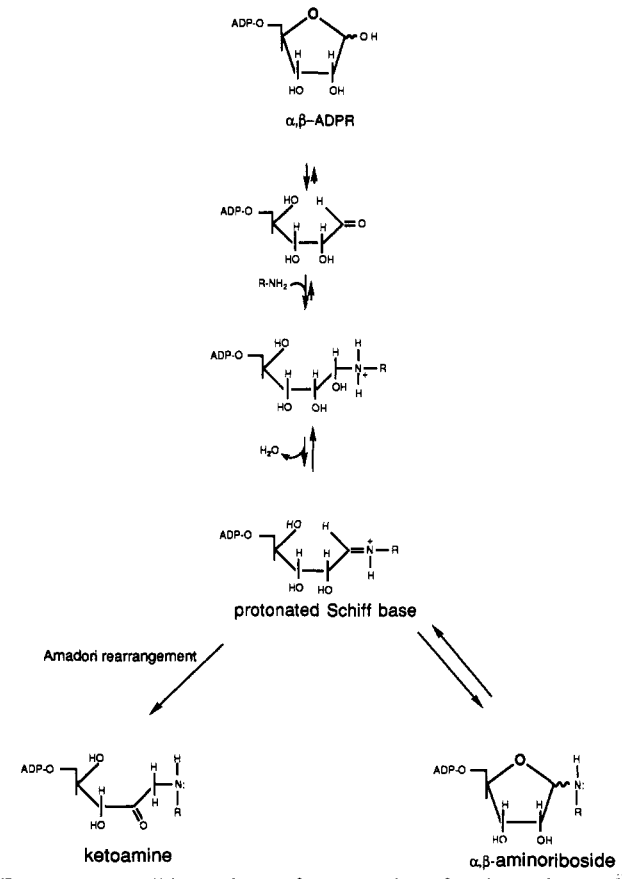

FIGURE 2: Possible products of the reaction of amines with ADP-ribose.

Hydrolysis of Ketoamines 1 and 2. The reaction mixture contained 100 mM CHES buffer, pH 9.0, and 100 μM purified products 1 and 2. Incubation was at 37 °C. At 0, 20, 40, and 60 min, aliquots were diluted to 1 mL with 100 mM potassium phosphate buffer, pH 6.0, and subjected to analytical reversed-phase HPLC as described above.

RESULTS

Reaction of n-Butylamine with ADP-Ribose. The reaction of hexoses such as glucose with amines has been studied previously in detail (Maillard, 1912; Lea & Hannan, 1950; Hodge & Rist, 1953; Reynolds, 1963; Bunn et al., 1975; Monnier & Cerami, 1982; Watala et al., 1985; Iberg & Fluckiger, 1986). Figure 2 shows analogous products that may be formed between ADP-ribose and an alkyl amine group such as that found in lysine. This series of reactions shows the formation of a Schiff base adduct that can undergo either a ring closure to form α- and β-aminoribosides or an Amadori rearrangement to form a ketoamine. To characterize the adducts formed between *n*-butylamine and ADP-ribose, the reactants were incubated at 37 °C, pH 8.0, and the products were monitored by reversed-phase HPLC. Figure 3 shows analysis by HPLC over a 6-h period. At 2 h, primarily a single product was formed, while at longer times a second product was observed. These compounds were termed products 1 and 2, respectively. When the pH of the reaction was between 4.0 and 6.0, only product 1 was formed. At pH 8.0 both products 1 and 2 were relatively unstable. When product 1 was isolated and incubated at pH 8.0, it was partially converted to product 2 (data not shown). Incubation of isolated product 2 at pH 8.0 did not result in detectable formation of product 1. These results suggested that product 2 was derived from product 1.

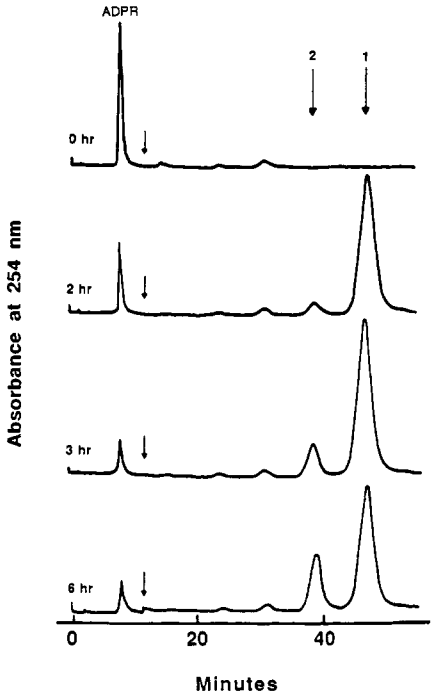

FIGURE 3: Time course of incubation of *n*-butylamine with ADP-ribose. The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 25 mM ADP-ribose, and 3 M *n*-butylamine. Incubation was at 37 °C. At the times indicated, aliquots were diluted to 1 mL with 50 mM potassium phosphate buffer, pH 6.0, and subjected to reversed-phase HPLC with UV detection at 254 nm. At 10 min of each run, the sensitivity was increased by a factor of 10 as indicated by the arrow. The position of ADP-ribose is shown. The positions of products 1 and 2 are shown by the numbered arrows.

Qualitative and ^{13}C NMR Analyses of Products 1 and 2. The formation of two products suggested several possibilities: (i) the two peaks could correspond to the formation of α and β anomers of an aminoriboside, (ii) the peaks could represent formation of an aminoriboside and a ketoamine, or (iii) the peaks could correspond to two ketoamines. In order to test these possibilities, products 1 and 2 were isolated by preparative reversed-phase HPLC. Product 1 was prepared at pH 5.0 since it was unstable at higher pH. The presence of a carbonyl group in these compounds was tested using the color test with *o*-dinitrobenzene described by Fearon et al. (1943). Ketoamines give a purple color in this test while aminoribosides do not. When products 1 and 2 were incubated with this reagent, an intense purple color was obtained for both, indicating that both products were ketoamines produced by an Amadori rearrangement.

Supporting evidence was also found in the ^{13}C NMR spectra of these compounds. Although limitations on sample quantity prevented a complete spectral analysis, each product exhibited the expected absorptions in four well-defined regions of the spectra shown in Figure 4. Peaks corresponding to the four *n*-butyl group carbons occurred between 10 and 55 ppm, and the signals at 207.6 ppm (product 1) and 208.9 (product 2) ppm were clearly from ketone carbonyl carbons. Additional peaks at 41.9, 31.4, and 21.7 ppm in the spectrum of product 1 were due to residual free *n*-butylamine which was not removed by HPLC. The adenine ring carbons appeared between 120 and 160 ppm in accord with previously reported data for ADP-ribose and related adenine-containing compounds (Miwa et al., 1977). Absorptions at 173.6, 134.4, and 117.9 ppm in the spectrum of product 1 and one of the two absorptions 122.3 or 121.7 in the spectrum of product 2 were unidentified minor impurities. In the region of ribose absorption between 65 and 90 ppm, one could not see all nine

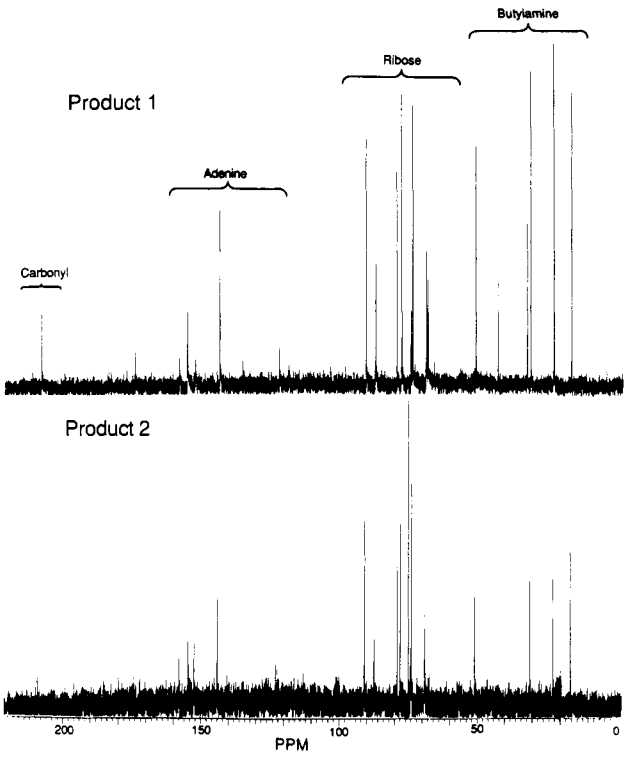

FIGURE 4: ^{13}C NMR analysis of product 2. Product 2 was prepared as described under Materials and Methods. NMR assignments for the ribose and adenine regions are based on published data of related adenosine-containing compounds (Miwa et al., 1977).

of the expected aliphatic carbon lines for one ribofuranose and four carbons from an opened ring. This might be due to overlaps or, more likely, to proton exchange with D_2O at the carbons α to a carbonyl (Silvertein et al., 1981; Oberfrank et al., 1984). This would lead to coupling, increased relaxation times, and line broadening, which could attenuate these signals into the baseline noise. The proton spectra for products 1 and 2 (data not shown) also exhibited attenuated signals for certain hydrogens.

Taken together, the qualitative data and the ^{13}C NMR spectra, by virtue of the signals at 207.6 and 208.9 ppm, provided evidence that both products 1 and 2 are ketoamines.

Reaction of Ketoamines 1 and 2 with Hydroxylamine. To characterize further the putative ketoamines, they were incubated with 1 M hydroxylamine at pH 7.0 and analyzed by reversed-phase HPLC, as shown in Figure 5. After 2 h of incubation at 37 °C, ketoamine 1 was quantitatively converted to a single product. Analysis of the product by ^{13}C NMR showed the disappearance of the peak at 207 ppm and the appearance of a peak at 160 ppm, which is characteristic of an oxime (data not shown). This compound has been termed oxime 1. When ketoamine 2 was incubated with hydroxylamine and analyzed by reversed-phase HPLC, two products (termed oximes 2 and 3) were formed.

^1H NMR Characterization of the Oximes Obtained from the Putative Ketoamines. To identify the positions of the carbonyl groups in ketoamines 1 and 2, the three oximes obtained were analyzed by ^1H NMR. Although the quality of these spectra was limited by the amount of available sample and also by the overlaps of proton signals, diagnostic features were evident in those portions of the ^1H NMR spectra of all three oximes shown in Figure 6. Since the samples contained varying amounts of free *n*-butylamine, the large triplets at 2.83 ppm in the bottom and middle panels and the weak signals slightly upfield of the triplet at 2.85 ppm in the top panel should be disregarded.

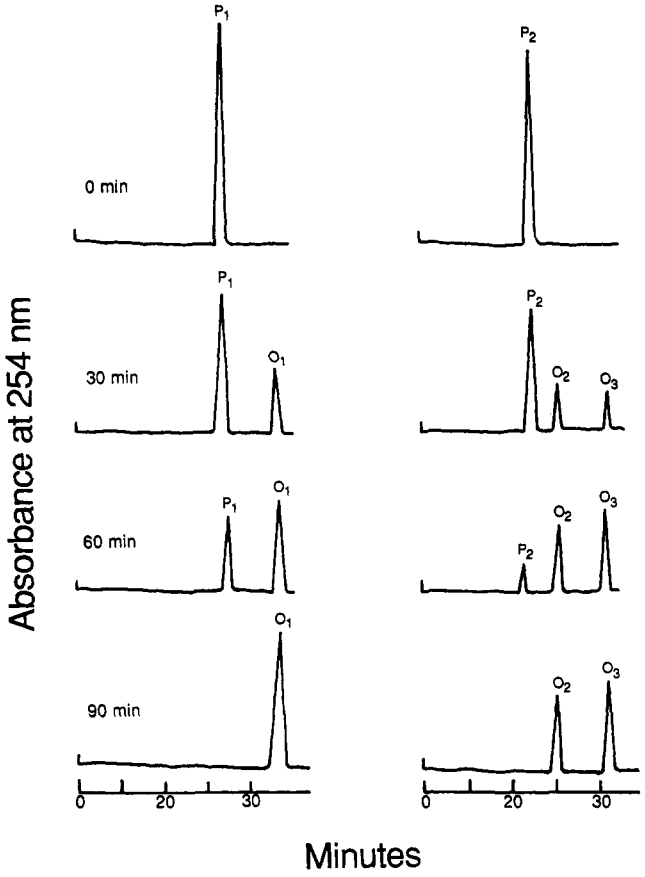

FIGURE 5: Time course of the reaction of products 1 and 2 with hydroxylamine. Purified products 1 and 2 were incubated in 100 mM MOPS, pH 7.0, and 1 M hydroxylamine at 37 °C. At the times indicated, aliquots were diluted to 1 mL with 100 mM potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with UV detection at 254 nm. The left panel shows the time course of the reaction of product 1 (P_1). The right panel shows the time course of incubation of product 2 (P_2).

For oxime 1 (bottom panel), the triplet at 2.95 ppm represents methylene protons on the nitrogen proximal carbon of the butylamino group. The diasteromeric methylene protons on carbon 1″ appear as an AB quartet with doublets (J = 13.0 Hz) at 3.75 and 3.87 ppm. The chemical shifts and multiplicities of these signals are consistent only with an isolated methylene group in the oxime having an imino carbon at position 2″. The assignment of the doublet (J = 7.0 Hz) at 4.26 ppm cannot be confirmed although the structure of oxime 1 contains only one proton at position 3″ that would be expected to appear as a doublet. The proton to which is coupled is obscured in the complex overlap between 3.8 and 4.0 ppm. Double irradiation of the sample at 3.85 ppm causes the doublet at 4.26 ppm to collapse to a singlet.

The middle and top panels show the ^{1}H NMR spectra of oximes derived from ketoamine 2. The key diagnostic information is the pair of doubled doublets (dd, J = 11.8, 4.3 Hz and dd, J = 11.8, 6.4 Hz) centered at 3.44 ppm in the middle spectrum and an identical absorption with the same coupling constants centered at 3.42 ppm in the top spectrum. These are the methylene protons and represent the AB portion of an ABC spin system such as that composed of protons at positions 1″ and 2″ of an oxime with an imino carbon at either C-3″ or C-4″. The limited sample quantity prevents a more thorough analysis to differentiate the isomers or to rule out the possibility that these oximes are syn and anti isomers derived from a single ketoamine. In turn, this supports the conclusion that ketoamines 1 and 2 differ in the position of the carbonyl group.

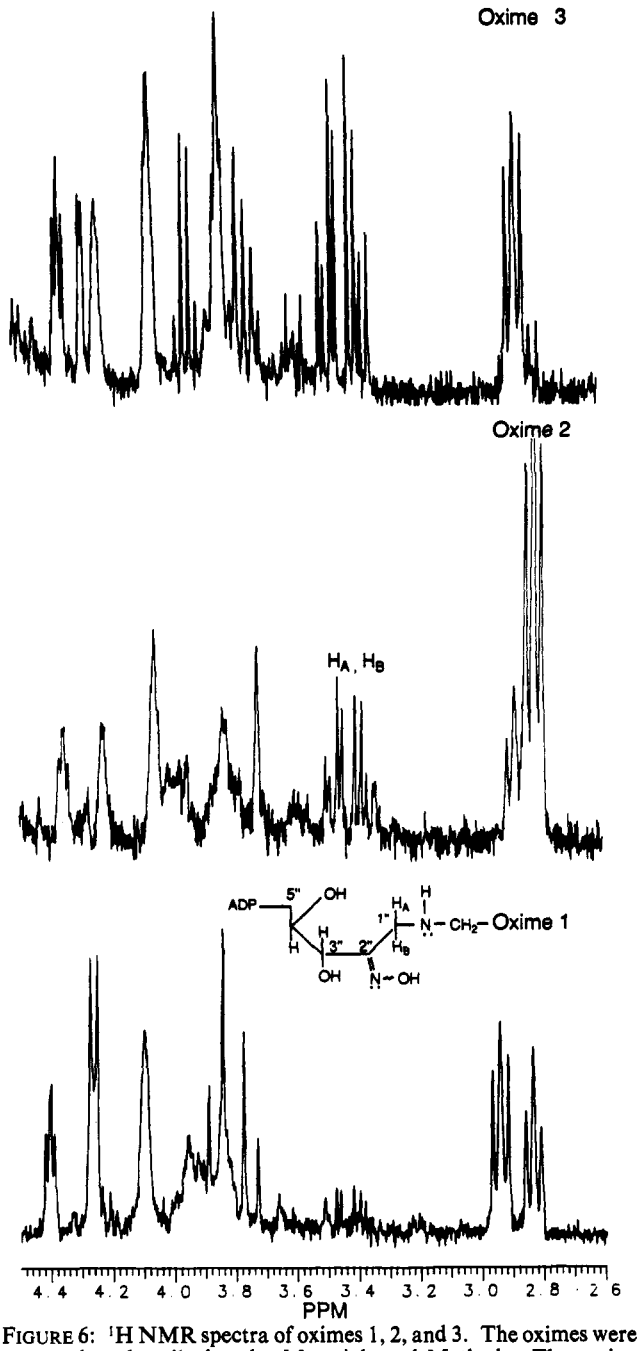

FIGURE 6: ^{1}H NMR spectra of oximes 1, 2, and 3. The oximes were prepared as described under Materials and Methods. The region corresponding to ribose and the nitrogen proximal methylene group from the butylamine is shown.

Stability Studies of Products 1 and 2. The chemical stability of ketoamines 1 and 2 has been studied with the aim of identifying conditions that would allow the differentiation of ADP-ribose lysine glycation products from the other ADP-ribosyl amino acids that can occur in proteins by the action of protein mono-ADP-ribosyltransferases. Previous studies from this laboratory have characterized the chemical stability of linkages between ADP-ribose and amino acid side chains known to be modified by protein mono-ADP-ribosyltransferases (Jacobson et al., 1990a,b; Payne et al., 1985). Table I summarizes chemical stability properties for ketoamines 1 and 2 and compares them to linkages between other amino acid side chains and ADP-ribose. An ADP-ribose conjugate was considered stable when its $t_{1/2}$ was at least 10 h. Both

Table I: Stability of the Linkage between Amino Acid Side Chain and ADP-Ribose

ADP-ribosyl linkage to	formic acid (44%)	hydroxylamine (1 M, pH 7.0)	Hg^{2+} (10 mM)	CHES (pH 9.0)	NaOH (1 M)
arginine	stable	released	stable	stable	released
cysteine	stable	stable	released	stable	released
histidine (diphtamide)	stable	stable	stable	stable	stable
asparagine	stable	stable	stable	stable	stable
serine, threonine	released	stable	stable	stable	stable
lysine (ketoamines)	stable	stable	stable	released	released

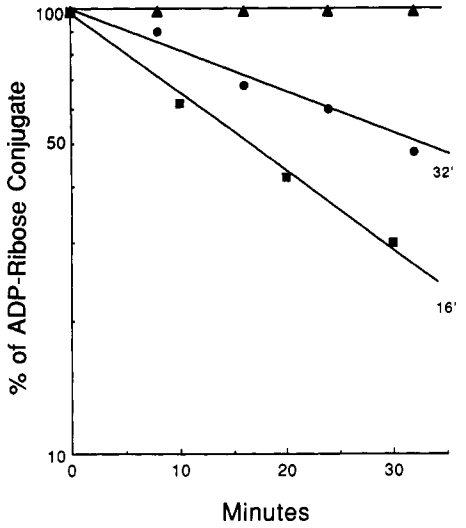

FIGURE 7: Kinetics of hydrolysis at pH 9.0 for products 1 and 2 and ADP-ribosyl arginine. Purified ketoamines 1 and 2 and ADP-ribosyl arginine were incubated in 100 mM CHES buffer, pH 9.0, at 37 °C. Aliquots were taken and diluted to 1 mL with potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. (■) Ketoamine 1; (●) ketoamine 2; (▲) ADP-ribosylarginine.

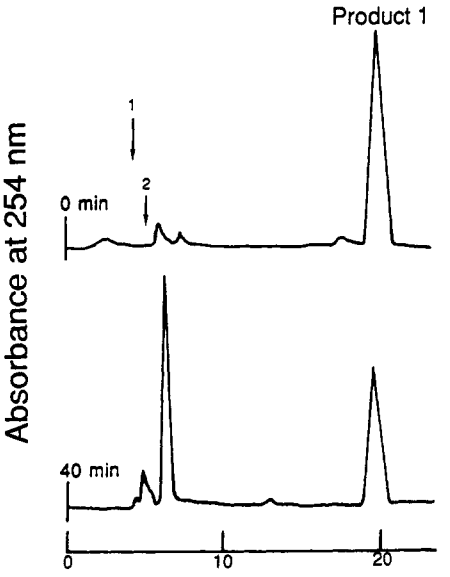

FIGURE 8: Analysis by HPLC of the hydrolysis of product 1 at pH 9.0. Purified product 1 was incubated at pH 9.0 as described in the legend to Figure 7. The positions of ADP-ribose and AMP are shown by the numbered arrows 1 and 2, respectively.

ketoamines 1 and 2 were quite stable in 44% formic acid with $t_{1/2}$ values of 13 and 16 h, respectively (data not shown). Likewise they were stable to Hg^{2+} treatment (data not shown) and stable in hydroxylamine as described above. Both ketoamines were extremely unstable in 1 M NaOH. The key feature differentiating the ketoamines from ADP-ribosyl amino acid conjugates formed enzymatically was the lability of both ketoamines at pH 9.0. Figure 7 shows that the ketoamines 1 and 2 were cleaved with $t_{1/2}$ of 16 and 32 min, respectively, at pH 9.0. For comparison, ADP-ribosylarginine is also shown.

Figure 8 shows the HPLC analyses of the products formed when ketoamine 1 was incubated at pH 9.0. The released material did not comigrate with either ADP-ribose or AMP, suggesting that a unique product is released. The structure of the product has not yet been established.

DISCUSSION

In protein glycation by hexoses, the Schiff base formed between the hexose and amine can be stabilized by ring closure to form either a furanose or pyranose ring (Armbruster, 1987). Similarly, the ketoamine product of the Amadori rearrangement also can be stabilized by ring closure to either a furanose or pyranose ring. Under the conditions used here, a stable aminoriboside adduct of ADP-ribose and *n*-butylamine derived from a Schiff base was not detected. Although the ADP-ribose adduct could conceivably form an aminoriboside, it may be less stable than the hexose counterpart. It is also likely that the ketoamine adduct derived from ADP-ribose is less stable than the corresponding hexose ketoamine, since ring formation is not possible with ADP-ribose. This may have implications with respect to the rate of formation of post Amadori products that are known to occur with ketoamines. These products are formed through further reaction of the carbonyl group with other amino groups. It would be expected that for ketoamines derived from ADP-ribose, the formation of post-Amadori products would be more rapid than with hexoses due to the higher relative concentration of carbonyl groups. The inability of ADP-ribose derived ketoamines to undergo ring closure also may explain the formation of multiple ketoamines (Figure 3). A possible mechanism is shown in Figure 9. This involves migration, through an enol intermediate, of the carbonyl from position 2 to position 3. By an analogous mechanism, the carbonyl could migrate to position 4. This mechanism has been suggested in the reaction of reducing monosaccharides with phenylhydrazine and also in the oxidation of aldoses and ketoses with Tollen's reagent and Fehling's and Benedict's solutions (Morrison & Boyd, 1976).

Information concerning the structures of ketoamines 1 and 2 was obtained by treatment of these compounds with hydroxylamine, which resulted in the formation of stable oximes (Figure 5). This approach was useful because the NMR analyses of the oximes provided information that was not obtainable from the ketoamines. In the ketoamines, the acidic hydrogens α to the carbonyl group are exchangeable with D_2O and thus eliminated from the ^1H NMR spectrum. However, the presence of these hydrogens in the oximes aided in the interpretation of the data as presented under Results.

The formation of two oximes from ketoamine 2 (Figure 5) suggested that it was either a mixture of two compounds or that a further rearrangement occurred in the presence of hydroxylamine. The analysis of ketoamine 2 by ^{13}C NMR (Figure 4) clearly showed that it was a single compound. Thus,

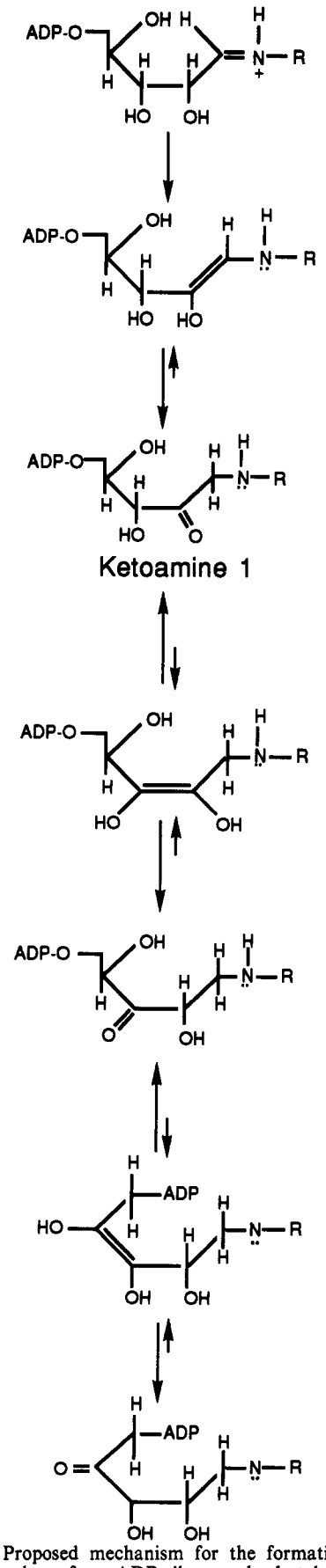

FIGURE 9: Proposed mechanism for the formation of multiple ketoamine products from ADP-ribose and *n*-butylamine.

we postulate that hydroxylamine is serving as a base that promotes migration of the carbonyl group. The carbonyl group in ketoamine 2 cannot be definitively assigned to position 3 or position 4 from the ^{1}H NMR of the oximes (Figure 6). The conclusion that ketoamine 1 is a true Amadori product with a carbonyl group at position 2″ is supported by the observations that ketoamine 1 was the first stable product formed, that under slightly acidic conditions it was the only product formed, and that the proton NMR spectrum of the derived oxime contains the signals expected for the isomer with an imino carbon at position 2″ and an isolated methylene group at position 1″. Even though the exact structures of oximes 2 and 3 remain unknown, the spectra confirm a rearrangement of the original Amadori product to one or two new ketones with displaced carbonyl groups.

In earlier studies, Hilz and co-workers have reported glycation by ADP-ribose of polyhistidine and polyarginine in addition to polylysine (Hilz et al., 1984). In this study, when ADP-ribose was incubated with imidazoyl or guanidino compounds under a wide variety of conditions similar to those used by Hilz and co-workers, no products were detected (data not shown). It is possible that this difference in reactivity is due to some chemistry unique to the polymers of histidine or arginine used as acceptors. Alternatively, it is possible that the glycation observed with polyhistidine and polyarginine was occurring only at the terminal α amino groups.

The stability of the ketoamines reported here is very similar to the stability of the mitochondrial protein conjugates reported by Hilz and co-workers (Hilz et al., 1985) and the cytosolic protein conjugates reported by Tanaka and co-workers (Tanaka et al., 1989). This suggests that the amino acid modified in these proteins was lysine and that the adduct(s) represented one or more ketoamine(s) resulting from an Amadori rearrangement. Recent evidence has shown that glycation by glucose can occur at specific lysine residues in albumin in vivo (Iberg & Fluckiger, 1986). The preferential sites observed for glycation such as Lys-Lys and Lys-His-Lys led these investigators to propose that a basic amino acid adjacent to the site of glycation promotes the Amadori rearrangement via combined acid–base catalysis. This proposal is also supported by the observation that the site of glycation of hemoglobin A_{IC} contains an adjacent histidine residue (Koenig et al., 1977). Thus, it is possible that a similar mechanism leads to the specific glycation of mitochondrial and cytosolic proteins by ADP-ribose (Hilz et al., 1984; Tanaka et al., 1989).

Free ADP-ribose is a product of metabolism generated at many locations within the cells (Figure 1). At present, its metabolic fates are poorly understood. The definitive detection of free ADP-ribose levels in vivo presents difficult technical challenges. Extractions normally used to obtain nucleotide pools are not suitable since acid extraction converts cellular NADH to free ADP-ribose and alkaline extraction converts NAD to free ADP-ribose. Extractions at neutral pH do not inactivate NADase activity, which generates free ADP-ribose. In view of these technical obstacles to the definitive detection of free ADP-ribose in vivo, its level may have to be monitored indirectly via protein glycation. The potential for protein glycation by free ADP-ribose at a particular cellular location will be related to the relative rates of formation and catabolism. At our current state of knowledge, the nucleus is a likely site of protein glycation by ADP-ribose. Cellular recovery from DNA damage involves the activation by DNA strand breaks of poly(ADP-ribose) polymerase. This results in high rates of formation of ADP-ribose polymers in chromatin and their rapid turnover to free ADP-ribose (Jacobson et al., 1990a). The generation of high local concentrations of free ADP-ribose in close proximity to histones rich in lysine residues

raises the likelihood of histone glycation. Indeed, Kreimeyer et al. (1984) have reported the occurrence of monomeric ADP-ribose conjugates of histone H1 in large excess over poly(ADP-ribose) conjugates following DNA damage in hepatoma cells. While proteins carrying monomeric ADP-ribose residues will occur as a consequence of ADP-ribose polymer turnover, these residues are linked to the acceptor proteins by carboxylate ester linkages that are very sensitive to hydroxylamine (Nishisuka et al., 1969). In contrast, the histone H1 conjugates observed by Kreimeyer et al. were stable in hydroxylamine. Taken together with the stability of the glycation model conjugates reported here, these histone conjugates are very likely to represent in vivo glycation by ADP-ribose.

The chemical stability studies of the ketoamines (Figure 8 and Table I) show that they can be differentiated from ADP-ribosyl amino acids that occur in proteins by the action of protein mono-ADP-ribosyltransferases (Table I). The selective release of glycation products from proteins at pH 9.0 should be possible. Further, the results described here (Figure 8) suggest these products are very likely unique to ADP-ribose glycation. Thus, they should prove very useful to monitor glycation of proteins by ADP-ribose both in vitro and in vivo. The structure of these hydrolysis products is currently being investigated.

It is likely that cells possess mechanisms to minimize protein glycation by ADP-ribose. Most likely a metabolic system that degrades ADP-ribose exists. This is supported by the finding of a highly specific ADP-ribose pyrophosphatase, which has a low K_m for ADP-ribose (Miro et al., 1989). Also, polyamines may be protective against glycation by serving as acceptors of ADP-ribose (Williams-Ashman, 1972). Nevertheless, a variety of nonenzymic modifications of proteins occurs as a function of age, and glycation has been implicated in this process (Stadman, 1990). The results reported here should be useful in examining the possible role of protein glycation by ADP-ribose in biological systems.

The use of *n*-butylamine ADP-ribose conjugates as models for ADP-ribose modified lysine residues in protein provides an approach for identifying this posttranslational modification in protein in situ. It is possible that in the environment of the protein the stability may be different than that of the model conjugates. However, in preliminary studies incubation of histone H1 with free ADP-ribose yielded conjugates with very similar chemical stabilities to the model conjugates described here. The products released at pH 9.0 from the protein conjugate were indistinguishable from the butylamine conjugates (data not shown). Thus these model conjugates should prove useful for understanding the chemistry of this post-translational modification of protein.

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