Amino Acid-Specific ADP-ribosylation: Structural Characterization and Chemical Differentiation of ADP-ribose–Cysteine Adducts Formed Nonenzymatically and in a Pertussis Toxin-Catalyzed Reaction†

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ABSTRACT: ADP-ribosylation is a posttranslational modification of proteins by amino acid-specific ADP-ribosyltransferases. Both pertussis toxin and eukaryotic enzymes ADP-ribosylate cysteine residues in proteins and also, it has been suggested, free cysteine. Analysis of the reaction mechanisms of cysteine-specific ADP-ribosyltransferases revealed that free ADP-ribose combined nonenzymatically with cysteine. L- and D-cysteine, L-cysteine methyl ester, and cysteamine reacted with ADP-ribose, but alanine, serine, lysine, arginine, *N*-acetyl-L-cysteine, 2-mercaptoethanol, dithiothreitol, and glutathione did not. The ^{1}H NMR spectrum of the product, along with the requirement for both free sulfhydryl and amino groups of cysteine, suggested that the reaction produced a thiazolidine linkage. ADP-ribosylthiazolidine was labile to hydroxylamine and mercuric ion, unlike the ADP-ribosylcysteine formed by pertussis toxin and NAD in guanine nucleotide-binding (G-) proteins, which is labile to mercuric ion but stable in hydroxylamine. In the absence of G-proteins but in the presence of NAD and cysteine, pertussis toxin generated a hydroxylamine-sensitive product, suggesting that a free ADP-ribose intermediate, expected to be formed by the NADase activity of the toxin, reacted with cysteine. Chemical analysis, or the use of alternative thiol acceptors lacking a free amine, is necessary to distinguish the enzymatic formation of ADP-ribosylcysteine from nonenzymatic formation of ADP-ribosylthiazolidine, thereby differentiating putative NAD:cysteine ADP-ribosyltransferases from NAD glycohydrolases.

ADP-ribosylation is a posttranslational modification in which ADP-ribose is transferred from β-NAD to specific amino acid residues in proteins. Mono-ADP-ribosyltransferases are a diverse group of enzymes, including many bacterial toxins and eukaryotic enzymes (Moss & Vaughan, 1988; Aktories & Just, 1990; Collier, 1990; Ui, 1990; Wick & Iglewski, 1990; Williamson & Moss, 1990). The major acceptor proteins for mono-ADP-ribosylation by cholera toxin and pertussis toxin are the guanine nucleotide-binding (G-[1]) proteins involved in signal transduction (Moss & Vaughan, 1988; Ui, 1990). Cholera toxin-catalyzed ADP-ribosylation of the α-subunit of the G-protein G_s results in stimulation of adenylyl cyclase, whereas ADP-ribosylation by pertussis toxin of the α-subunit of G_t, G_i, and G_o disrupts the interaction of the G-protein with its receptor (Moss & Vaughan, 1988; Ui, 1990).

ADP-ribosyltransferases use specific amino acid residues as acceptors (Moss & Vaughan, 1977; Van Dop et al., 1984; West et al., 1985; Vandekerckhove et al., 1987, 1988; Moss & Vaughan, 1988; Tanuma et al., 1988; Sekine et al., 1989; Collier, 1990; Williamson & Moss, 1990). For example, cholera toxin ADP-ribosylates arginine residues in proteins (Moss & Vaughan, 1977; Van Dop et al., 1984). Pertussis toxin ADP-ribosylates a cysteine residue four amino acids from the carboxyl terminus of $G_t\alpha$ (Medynski et al., 1985; West et al., 1985), and presumably those in the α subunits of G_i and G_o (Van Meurs et al., 1987; Itoh et al., 1988). Another transferase, botulinum C3, modifies an asparagine residue in the ~20-kDa ras-like proteins of the *rho* and *rac* families (Didsbury et al., 1989; Sekine et al., 1989).

There is substantial interest in identifying ADP-ribosylation pathways with potential roles in regulating signal transduction processes. Endogenous ADP-ribosylation of both arginine and cysteine residues in proteins appears to be reversible, since NAD:arginine and NAD:cysteine ADP-ribosyltransferases and the enzymes that remove the modification, ADP-ribosylarginine and ADP-ribosylcysteine hydrolases have been identified in animal cells (Moss et al., 1980, 1985; 1986a; Yost & Moss, 1983; West & Moss, 1986; Tanuma et al., 1988; Tanuma & Endo, 1990). ADP-ribosylation of G_i by the animal cell NAD:cysteine ADP-ribosyltransferase attenuated epinephrine-induced inhibition of adenylyl cyclase in human platelet membranes, demonstrating a possible physiological function for this enzyme (Tanuma & Endo, 1989). In support of a regulatory role for this modification, proteins ADP-ribosylated on cysteine residues were identified in liver and were exclusively in the plasma membrane fraction (Jacobson et al., 1990).

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[1] Abbreviations: ADP-ribose, adenosine 5′-diphosphoribose; COSY, two-dimensional homonuclear-correlated spectroscopy; DTT, dithiothreitol; G-protein, guanine nucleotide-binding protein; G_i, "inhibitory" G-protein; G_o, G-protein found in high concentration in neural tissues; G_t, transducin; HPLC, high-performance liquid chromatography; MOPS, 3-morpholinopropanesulfonic acid; NADase, NAD$^+$ glycohydrolase (EC 3.2.2.5); NOE, nuclear Overhauser effect; ROESY, two-dimensional rotating-frame (or transverse) NOE spectroscopy; R_t, retention time; SAX, strong anion-exchange.

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The ADP-ribose–cysteine linkage can be differentiated from other ADP-ribose–amino acid bonds by its chemical stability. Pertussis toxin-catalyzed ADP-ribosylation of cysteine in proteins results in the formation of a thioglycoside bond, characterized by its lability to mercuric ion and its stability in neutral hydroxylamine (Horton & Hutson, 1963; Krantz & Lee, 1976; Hsia et al., 1985; Meyer et al., 1988). Low molecular weight model acceptors (e.g., amino acids) are ADP-ribosylated by some but not all ADP-ribosyltransferases. Pertussis toxin and the animal cell NAD:cysteine ADP-ribosyltransferase reportedly ADP-ribosylate free cysteine or other thiol compounds (Lobban & Van Heyningen, 1988; Tanuma et al., 1988).

In the current work, the mechanism of pertussis toxin-catalyzed ADP-ribosylation of G-proteins and of cysteine and other model acceptors was examined. ADP-ribosylation of free cysteine, under reaction conditions similar to those used in some previous studies of NAD:cysteine ADP-ribosyltransferases (Lobban & Van Heyningen, 1988; Tanuma et al., 1988), resulted in a product with chemical properties different from those of the ADP-ribosylcysteine produced in proteins by pertussis toxin in the presence of NAD. The conjugate, ADP-ribosylthiazolidine, was characterized by its ^{1}H NMR and mass spectra, incorporation of radiolabeled precursors, and analysis of its chemical degradation products.

MATERIALS AND METHODS

Materials. Nicotinamide [U-^{14}C]adenine dinucleotide (272 Ci mol^{-1}) and L-[U-^{14}C]cysteine (30–60 Ci mol^{-1}) were purchased from Amersham (Arlington Heights, IL); L-cysteine, alanine, serine, lysine, arginine, 2-mercaptoethanol, glutathione (reduced), ADP-ribose, and NAD from Sigma (St. Louis, MO); dithiothreitol, cysteamine, L-cysteine methyl ester, and *N*-acetyl-L-cysteine from ICN (Costa Mesa, CA); and MOPS, hydroxylamine hydrochloride, mercuric chloride, and D-cysteine from Fluka (Ronkonkoma, NY).

Preparation of [adenine-^{14}C]ADP-ribose. A NAD glycohydrolase (NADase) purified from rat brain was used to generate [*adenine*-^{14}C]ADP-ribose from [*adenine*-^{14}C]NAD. The enzyme was purified ~4000-fold, to 58 units mg^{-1}, from the particulate fraction of rat brain by partitioning into Triton X-114 aggregates at 37 °C, adsorption to concanavalin A–Sepharose, and chromatography on carboxymethyl cellulose, hydroxylapatite, and cellulose phosphate. A unit of NADase is defined as 1 μmol of NAD hydrolyzed min^{-1}. Products of NAD hydrolysis by the NADase were identified as ADP-ribose and nicotinamide by anion-exchange HPLC. [*adenine*-^{14}C]ADP-ribose was generated in 20 mM K-MOPS, pH 7.0, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1.25 μCi of [*adenine*-^{14}C]NAD (46 μM), and 5 milliunits of rat brain NADase activity (final volume 0.1 mL) incubated for 30 min at 30 °C. ADP-ribose was purified by SAX-HPLC and stored at −20 °C.

Formation of Radiolabeled ADP-ribosylthiazolidine. ADP-ribosylthiazolidine was typically formed in reactions containing 0.2 mM ADP-ribose, 50 mM L-cysteine, 20 mM Na-MOPS, pH 7.0, and 5 mM EDTA (final volume 0.1 mL). Where indicated, [*adenine*-^{14}C]ADP-ribose (~7000 cpm) or [^{14}C]-cysteine (~20 000 cpm) was included. Reactions were incubated at 30 °C for 60 min and then frozen on dry ice until analysis by HPLC. Specific reaction conditions are provided with the figures and tables.

Analysis by SAX-HPLC. Reaction products, substrates, or standard compounds were separated by anion-exchange HPLC on a Zorbax SAX column (0.46 × 25 cm; DuPont, Wilmington, DE) with a silica guard column, as described (Moss et al., 1983). To lower the ionic strength to approximately 0.05 M, certain samples were diluted with water. Samples were applied in 90 μL and eluted isocratically with 50 mM sodium phosphate, pH 4.5–4.6, at 1.0 mL min^{-1}. The column effluent was monitored for UV absorbance at 254 nm, and fractions were collected for scintillation counting. Standards were routinely run, as retention times (R_t) sometimes varied by up to 2–3 min in different buffer preparations. Within one experiment, compounds eluted reproducibly within 0.25 min R_t. A filter assay for ADP-ribosylcysteine (Tanuma et al., 1988) was tested but not used routinely because of its extreme sensitivity to ionic strength, which resulted in nonquantitative binding of the product to the filters, and also because of the poor selectivity of the filters in binding NAD, ADP-ribose, and ADP-ribose–cysteine adducts.

Preparation of ADP-ribosylthiazolidine for Physical Analyses. The synthesis of ADP-ribosylthiazolidine was insensitive to pH over the range from pH 4.5 to 6.2. In a typical preparation, ADP-ribose (160 mg, 0.27 mmol) and L-cysteine hydrochloride (30 mg, 0.17 mmol) were dissolved in 1 mL of water and the resultant mixture adjusted to pH 5 with triethylamine. After 4 h the reaction was complete, as determined by HPLC. The solution had turned yellow and a white precipitate formed that was not characterized. Following filtration to remove the precipitate, the ADP-ribosylthiazolidine adduct was purified on a C18 Lobar column (2.5 × 31 cm) with a 1-L, 0–9% methanol gradient in 20 mM triethylammonium bicarbonate, pH 7.0. The column effluent was monitored at 254 nm, and 10-mL fractions were collected. The ADP-ribosylthiazolidine eluted in fractions 55–72; fractions 58–72 were combined. Sodium bicarbonate (2 equiv) was added to the solution and water removed on a rotary evaporator. The bulk of the triethylammonium bicarbonate was removed by coevaporation with methanol (6 × 25 mL); the remainder was removed by passage of the sample over a column (10 mL) of Bio-Rex-70 (Na^+ form). Total yield of ADP-ribosylthiazolidine was 0.11 mmol as determined by UV absorption, using an extinction coefficient of 14 000 at 260 nm (assuming absorption at 260 nm is due solely to the adenine chromophore). Purity was established by HPLC, ^{1}H NMR spectroscopy, and mass spectrometry.

Samples were analyzed by HPLC using a Beckman C-18 analytical column (0.46 × 25 cm) eluted at 1.0 mL min^{-1} with a mobile phase of 20 mM triethylammonium bicarbonate, pH 7.0/methanol (94.5/5.5, v/v). The column effluent was monitored at 260 nm. ADP-ribosylthiazolidine eluted as a broad, yet apparently homogeneous peak with a retention time of 16 min.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained on either a General Electric QE-300 or a GN-500 instrument operating at 300 or 500 MHz, respectively. Chemical shifts (25 °C) are reported in parts per million relative to internal 3-(trimethylsilyl)[2,2,3,3-d_4]propionate (TSP) for aqueous solutions. ^{1}H NMR data are tabulated in Table II. Samples, prepared in 50 mM sodium phosphate, pH 7.0, were lyophilized twice from D_2O before being dissolved in "100%" D_2O for spectral analysis. Typical sample concentration was 10 mM for 1D spectra and 40 mM for ROESY spectra.

Pertussis Toxin Reactions. ADP-ribosylcysteine was prepared using pertussis toxin by the method of Lobban and Van Heyningen (1988). Pertussis toxin, 4 μg (List Biological Laboratories, Campbell, CA), was incubated for 4 h at 30 °C with 0.05 mM [*adenine*-^{14}C]NAD (0.1 μCi), 250 mM L-cysteine or DTT, 1 mg mL^{-1} ovalbumin, and 50 mM sodium

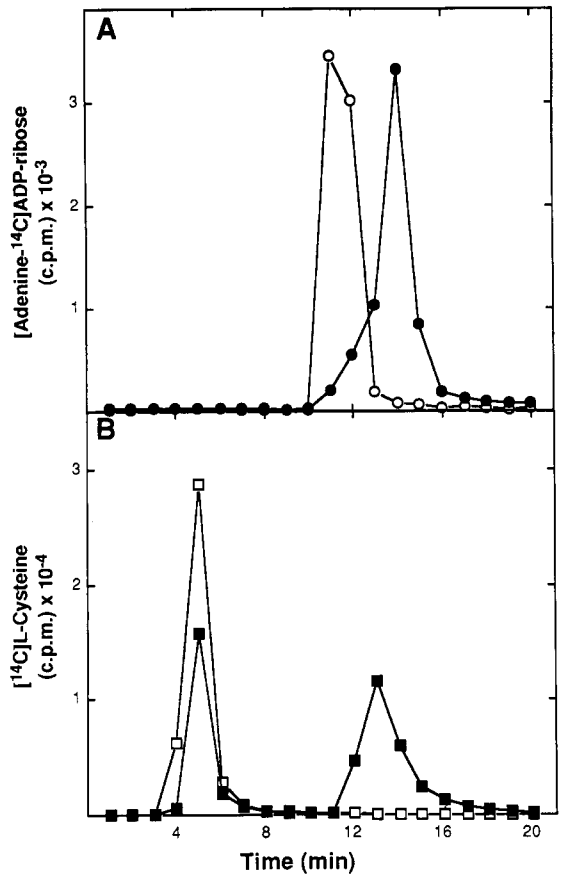

FIGURE 1: Products of the nonenzymatic reaction of cysteine and ADP-ribose. (A) Reactions containing 0.2 mM [*adenine*-^{14}C]ADP-ribose (~5200 cpm), 20 mM Na-MOPS, pH 7.0, and 5 mM EDTA, with or without 50 mM L-cysteine (final volume 0.1 mL), were incubated for 30 min at 30 °C before analysis of a sample (80 μL) by HPLC, with collection of 1-mL fractions: ADP-ribose alone (○); ADP-ribose plus cysteine (●). (B) Reactions containing 20 mM L-[^{14}C]cysteine (0.05 μCi), 20 mM Na-MOPS, pH 7.0, 5 mM EDTA, and 20 mM ADP-ribose (final volume 0.1 mL) were incubated at 30 °C for 0 (□) or 120 (■) min before analysis of a sample (80 μL) by HPLC. Data shown are from a single HPLC analysis (one of triplicates) from an experiment representative of four. R_t's: cysteine, 4–5 min; ADP-ribose, 11–12 min; ADP-ribosylthiazolidine, 13–14 min.

phosphate, pH 7.5 (final volume 0.1 mL). Samples (5 μL) were incubated at 30 °C for 120 min with or without 5 μL of 1 M NH_2OH, pH 7.0, then diluted with 90 μL of H_2O, and analyzed by HPLC.

RESULTS

During analysis of the reaction mechanisms for pertussis toxin-catalyzed ADP-ribosylation of the model acceptor cysteine, it was observed that incubation of cysteine with ADP-ribose produced a compound that incorporated radioactivity from both [*adenine*-^{14}C]ADP-ribose (Figure 1A) and L-[^{14}C]-cysteine (Figure 1B). The product was separated from cysteine and ADP-ribose by anion-exchange HPLC. The product formed readily in mixtures containing 50 mM L-cysteine and 0.2 mM ADP-ribose; the reaction was nearly complete within 2 h at 30 °C. During the reaction, the decrease in radiolabeled precursors, which occurred with one to one stochiometry, paralleled directly the formation of product.

Potential acceptors were examined by HPLC for their ability to react with [^{14}C]ADP-ribose (Table I). Alanine, serine, lysine, and arginine were unreactive, suggesting that the cysteine sulfhydryl was involved in the reaction. L-Cysteine, D-cysteine, cysteamine, and L-cysteine methyl ester did react,

Table I: Reaction of ADP-ribose with Potential Acceptors[a]

acceptor	unreacted ADP-ribose (nmol)	reaction product (nmol)
amino acids		
none	18.0 ± 0.28	<0.1
L-cysteine	2.2 ± 0.22	15.2 ± 0.13
L-serine	18.0 ± 0.50	<0.1
L-alanine	17.4 ± 1.08	<0.1
L-lysine	17.3 ± 1.09	<0.1
L-arginine	18.2 ± 0.26	<0.1
sulfhydryl compounds		
D-cysteine	2.0 ± 0.04	16.2 ± 0.09
L-cysteamine	6.7 ± 0.19	11.8 ± 0.34
L-cysteine methyl ester	0.42 ± 0.04	16.7 ± 1.72
dithiothreitol	18.0 ± 0.30	<0.1
2-mercaptoethanol	17.4 ± 0.76	<0.1
glutathione	18.1 ± 0.14	<0.1
N-acetyl-L-cysteine	17.9 ± 0.20	<0.1

[a] [*adenine*-^{14}C]ADP-ribose (0.2 mM, ~7200 cpm) was incubated with acceptor (50 mM) in 20 mM K-MOPS, pH 7.0, 5 mM EDTA (final volume 0.1 mL) at 30 °C for 60 min and then placed on dry ice until a sample (0.09 mL) was analyzed by HPLC. Data are means ±SD of triplicate measurements from one experiment representative of two to four.

but dithiothreitol, 2-mercaptoethanol, glutathione, and *N*-acetyl-L-cysteine did not. Approximately 90% of the ADP-ribose was converted to product in a 60-min incubation with D- or L-cysteine. Cysteamine was less reactive, with 63% of the ADP-ribose converted in 60 min, and cysteine methyl ester was more reactive, with nearly 98% converted. With all the nonreactive compounds, no novel peaks of radioactivity or UV absorbance were detected in the HPLC analyses. Thus, reactivity with ADP-ribose required that the acceptor contain both a sulfhydryl group and a free primary amine. Absence of a free amino group, as in glutathione or *N*-acetylcysteine, resulted in no detected covalent adducts. These properties suggested that the product contained a linkage different from the thioglycoside bond formed by pertussis toxin-catalyzed ADP-ribosylation of cysteine in G-proteins in the presence of NAD. The reaction of ADP-ribose with cysteine described here is analogous to the reported reaction of cysteine with various aldehydes or aldoses to form thiazolidines, in which the amino and sulfhydryl groups of cysteine are linked to the aldose anomeric carbon (Schubert, 1936, 1939; Ratner & Clarke, 1947; Horton & Hutson, 1963).

To characterize further the nature of the reaction product, it was prepared on a larger scale and analyzed by ^{1}H NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectrum (Figure 2, Table II) showed the presence of an equimolar mixture of two diastereomeric forms. The corresponding resonances of the ribosyl and cysteine moieties of the two forms had readily observed differences in chemical shifts because of their proximity to the diastereomeric centers. The assignments were based on 2D-COSY and 2D-ROESY NMR spectra and ^{1}H–^{13}C correlated spectra (data not shown). Resonance doubling of the more remote adenosine A8 and A2′ resonances was also observed, analogous to that observed in the ^{1}H NMR spectrum of ADP-ribosylguanidine and ADP-ribosylarginine (Oppenheimer, 1978).

The values of the $^3J_{2'-3'}$ coupling constants of 7.8 and 7.5 Hz for the two forms, A and B, respectively, are considerably greater than those found in nucleotides, which typically range between 4 and 6 Hz as discussed by Altoona and Sundralingam (1972, 1973). Only ribofuranosyl conformations having the 2′ and 3′ groups eclipsed would yield values of $^3J_{2'-3'}$ comparable to those observed. Such conformations are

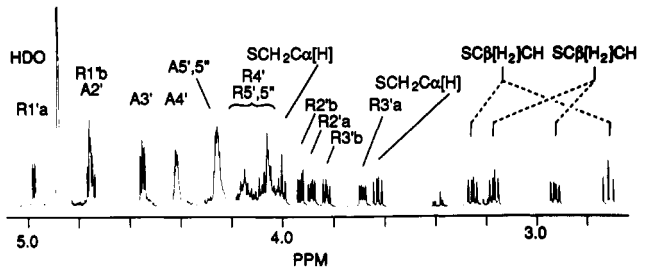

FIGURE 2: A portion of the 500-MHz ^{1}H NMR spectrum of ADP-ribosylthiazolidine obtained in D_2O at 25 °C. ADP-ribosylthiazolidine was prepared and analyzed as described under Materials and Methods. Resonances from the adenosine moiety are designated by "A" and from the ribosyl moiety by "R". The resonances from the two diastereomeric forms are designated either "a" or "b" without assignment of their absolute stereochemistry. Resonance peak assignments are also presented in Table II.

Table II: ^{1}H NMR Parameters from the 500-MHz Spectrum of ADP-ribosylthiazolidine Obtained in D_2O at 25 °C[a]

resonance	chem shift (ppm)	multip	integrtn	coupling const (Hz)
adenine A8	8.496	s	1/2	
adenine A8*	8.491	s	1/2	
adenine A2	8.192	s	1	
A1′ (ADP)	6.129	d	1	5.6 (1′–2′)
A2′ (ADP)	4.762	dd	1/2	5.0 (2′–3′)
A2′ (ADP)*	4.754	dd	1/2	5.0 (2′–3′)
A3′ (ADP)	4.556	dd	1	3.9 (3′–4′)
A4′ (ADP)	4.425	pent.	1	2–3 (4′–5′,5″,P)
A5′ (ADP)	4.262	m	2	nd[b]
R1′ (a)	4.984	d	1/2	4.5 (1′–2′)
R1′ (b)	4.767	d	1/2	3.2 (1′–2′)
R2′ (b)	3.936	dd	1/2	7.5 (2′–3′)
R2′ (a)	3.892	dd	1/2	7.8 (2′–3′)
R3′ (b)	3.833	dd	1/2	5.3 (3′–4′)
R3′ (a)	3.694	dd	1/2	4.3 (3′–4′)
R5′, 5″ (a, b)	(4.15, 4.07)	m	2	nd
R4′ (a, b)	(4.06–4.07)	m	1	nd
$SCH_2C[H\alpha]$	**3.634**	**dd**	1/2	**6.7** (α–β), **9.8** (α–β')
SCH[Hβ]CH	**3.256**	**dd**	1/2	**10.3** (β–β')
SC[Hβ′]HCH	**2.714**	**t**	1/2	
$SCH_2C[H\alpha]$	4.008	t	1/2	6.7 (α–β), 6.7 (α–β')
SCH[Hβ]CH	3.171	dd	1/2	10.5 (β–β')
SC[Hβ′]HCH	2.926	dd	1/2	

[a] ^{1}H NMR spectra of ADP-ribosylthiazolidine were obtained as described under Materials and Methods. Nomenclature of resonance assignments is described in the legend to Figure 2. [b] nd, not determined.

energetically most unfavorable and have not been observed in simple, unstrained nucleosides. The torsional freedom of an acyclic structure, on the other hand, does not present such limitations on vicinal coupling constants, and the observed values are consistent with a weighted average for the freely interconverting rotamer populations.

The ROESY spectrum of ADP-ribosylthiazolidine shows none of the long-range interactions among the ribosyl proton resonances (e.g., 1′–3′, 2′–4′, or 1′–4′) anticipated for a cyclic furanosyl structure. In contrast, these long-range interactions are readily observed among the resonances of the adenosine moiety. Although interpretation of the absence of NOEs should be approached with caution, in this case it is consistent with an extended structure such as for the proposed acyclic ribotide.

The mass spectrum of ADP-ribosylthiazolidine has a series of peaks at $M - H^+$ (663), $M - Na^+$ (685), $M - 2Na^+$ (707), $M - 3Na^+$ (729), and $M - 4Na^+$ (751). These masses correspond to the empirical formula $C_{18}H_{28}N_6O_{15}P_2S$. The mass of the molecular ion alone cannot distinguish between a cyclic or acyclic structure. The two structures can be distinguished by the presence of the diagnostic oxocarbenium ion derived from cleavage of glycofuranosyl bonds of simple nucleosides corresponding to the ADP-ribosyl oxocarbenium fragment.[2] The absence of this ion in the mass spectrum of ADP-ribosylthiazolidine is consistent with the noncyclized structure shown in Figure 6.

Table III: Chemical Reactivity of ADP-ribosylthiazolidine[a]

additions	ADP-ribosyl-thiazolidine (pmol)	% of control
none	940 ± 30	100 ± 3.1
HCl, 0.1 M	870 ± 50	92 ± 4.6
NaOH, 0.1 M	23 ± 3.7	2.4 ± 0.4
NH_2OH, 0.5 M	25 ± 6.6	2.6 ± 0.7
$HgCl_2$, 0.05 M	3.8 ± 2.8	0.4 ± 0.3

[a] [*adenine*-^{14}C]ADP-ribosylthiazolidine was prepared in a reaction mixture containing 10 mM K-MOPS, pH 7.0, 1.0% CHAPS, 0.1 M KCl, 0.2 mM [*adenine*-^{14}C]NAD (0.5 μCi) and 2 milliunits of rat brain NADase (final volume 0.1 mL). After incubation at 30 °C for 30 min, at which time NAD hydrolysis was complete, the reactants were filtered through a Centricon 10 (Amicon) to remove NADase. After addition of 5 μL of 1.0 M L-cysteine, the mixture was incubated at 30 °C for 120 min, resulting in essentially complete conversion of ADP-ribose to ADP-ribosylthiazolidine. Samples (5 μL) were transferred to 1.5-mL tubes, and chemcial agents were added in 5 μL to give the final concentration shown in the table. Following incubation at 30 °C for 120 min, reactions were mixed with 90 μL of water and frozen on dry ice until analysis by HPLC. Data are means ±SD of triplicate measurements, from one experiment representative of two.

Stability to chemical treatments has been used to distinguish different ADP-ribose–amino acid linkages. [*adenine*-^{14}C]-ADP-ribosylthiazolidine was stable in neutral solution and in 0.1 N HCl, with >90% remaining after a 2-h incubation (Table III). It was nearly completely degraded by incubation with 0.05 M $HgCl_2$ (<1% remaining), 0.1 M NaOH (3% remaining), or neutral 0.5 M hydroxylamine (3% remaining). Treatment of [*adenine*-^{14}C]ADP-ribosylthiazolidine with $HgCl_2$ generated free [*adenine*-^{14}C]ADP-ribose (Figure 3A), and treatment of ADP-ribosylthiazolidine (L-[^{14}C]cysteine) with $HgCl_2$ generated a ^{14}C-containing compound (Figure 3B) that eluted with the same R_t as a standard Hg^{2+}–cysteine adduct prepared by mixing L-[^{14}C]cysteine with excess $HgCl_2$ (Figure 3C). Treatment of [*adenine*-^{14}C]ADP-ribosylthiazolidine with hydroxylamine produced a ^{14}C-containing compound (Figure 4) that eluted with the same R_t as a standard produced by the reaction of free [*adenine*-^{14}C]ADP-ribose with hydroxylamine.

Sensitivity to neutral hydroxylamine distinguishes ADP-ribosylthiazolidine from the thioglycoside-type ADP-ribosylcysteine produced by pertussis toxin in the presence of NAD and G-proteins. The known ADP-ribose–amino acid linkages differ greatly in their relative stabilities in neutral hydroxylamine. [*adenine*-^{14}C]ADP-ribosylthiazolidine was rapidly degraded in 0.5 M hydroxylamine at pH 7.0, with an exponential decay [half-life of 13.4 ± 0.3 min (n = 3)].

Pertussis toxin is a NAD:cysteine ADP-ribosyltransferase that also possesses NAD glycohydrolase activity (Moss et al., 1986b; Moss & Vaughan, 1988). It has been reported that pertussis toxin catalyzes ADP-ribosylation of free cysteine when incubated with NAD (Lobban & Van Heyningen, 1988). Results of incubation of [*adenine*-^{14}C]NAD and thiols (250 nM) with pertussis toxin, following previous procedures (Lobban & Van Heyningen, 1988), are shown in Figure 5. The predominant [^{14}C]adenine-containing product of the reaction with L-cysteine eluted from the SAX column with

[2] Handlon and Oppenheimer, unpublished results.

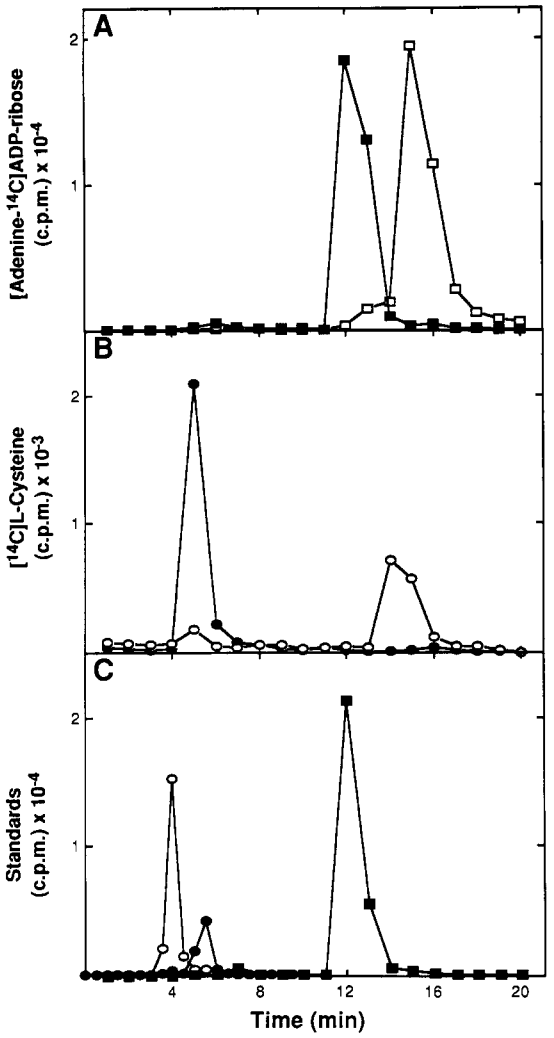

FIGURE 3: Products of incubation of ADP-ribosylthiazolidine with $HgCl_2$. (A) [*adenine*-^{14}C]ADP-ribosylthiazolidine was prepared and incubated with 5 mM EDTA (□) or with $HgCl_2$ (■) as described in Table III; samples (90 μL) were analyzed by HPLC. (B) ADP-ribosylthiazolidine ([^{14}C]cysteine) was prepared by incubation of 20 mM L-[^{14}C]cysteine (0.5 μCi), 4 mM ADP-ribose, and 20 mM K-MOPS, pH 7.0 (final volume 0.1 mL) at 30 °C for 4 h; the mixture was injected onto the HPLC column, and the peak of ADP-ribosylthiazolidine was collected. Samples (0.1 mL) of the purified compound were incubated for 30 min at 30 °C with 10 mM EDTA (○) or with 2 mM $HgCl_2$ (●) and then analyzed by HPLC. (C) Standard compounds analyzed by HPLC were (■) [*adenine*-^{14}C]-ADP-ribose, prepared as described under Materials and Methods, (○) [^{14}C]cysteine (0.01 μCi), and (●) [^{14}C]cysteine incubated with 2 mM $HgCl_2$. Data are from a single HPLC analysis (one of triplicates) in an experiment representative of three. R_t's: cysteine, 4 min; cysteine–mercuric ion adduct, 4.5–5 min; ADP-ribose, 12-13 min; ADP-ribosylthiazolidine, 15–16 min.

the same R_t as the nonenzymatically formed ADP-ribosylthiazolidine and was sensitive to hydroxylamine (Figure 5B). When dithiothreitol was tested as a possible ADP-ribose acceptor for pertussis toxin, the only [^{14}C]adenine-containing product of the reaction eluted from the column with the same R_t as ADP-ribose (Figure 5C). These results indicate that, under the stated conditions, neither cysteine nor dithiothreitol was directly ADP-ribosylated by pertussis toxin; i.e., the toxin did not produce the expected hydroxylamine-stable thioglycoside linkage between the thiol and ADP-ribose. Instead, the NAD glycohydrolase activity of pertussis toxin produced ADP-ribose, which reacted nonenzymatically with cysteine, yielding a distinct compound with a hydroxylamine-sensitive linkage.

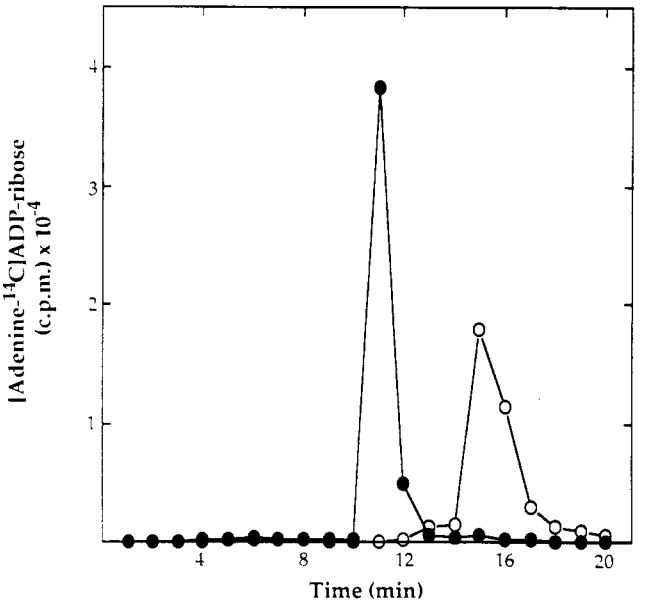

FIGURE 4: Products of incubation of ADP-ribosylthiazolidine with hydroxylamine. [*adenine*-^{14}C]ADP-ribosylthiazolidine was prepared and analyzed by HPLC as described in Table III, after incubation without (○) or with (●) 0.5 M hydroxylamine in 5 mM EDTA/0.1 M Tris–HCl, pH 7.0. Data are from a single HPLC analysis (one of triplicates) from an experiment representative of three. R_t's: ADP-ribosylthiazolidine, 15–16 min; ADP-ribose, 11–12 min; ADP-ribose–hydroxylamine adduct, 10–11 min.

DISCUSSION

The use of model acceptors for ADP-ribosylation reactions has allowed identification and purification of novel ADP-ribosyltransferases in cases where the physiological protein substrate is unknown. For example, model acceptor guanidino compounds were used to great advantage in the characterization and assay of cholera toxin and animal cell NAD:protein (arginine) ADP-ribosyltransferases (Moss & Vaughan, 1977, 1988; Moss et al., 1980; Yost & Moss, 1983; Soman et al., 1984; Tanigawa et al., 1984; West & Moss, 1986). Endogenous animal cell ADP-ribosyltransferases, including those catalyzing the ADP-ribosylation of cysteine and arginine residues in proteins may have a role in the regulation of signal transduction (Moss et al., 1980; Yost & Moss, 1983; Soman et al., 1984; Tanigawa et al., 1984; West & Moss, 1986; Tanuma et al., 1988; Tanuma & Endo, 1989, 1990). The model acceptors cysteine, cysteine methyl ester, and other thiol compounds have been utilized for known NAD:cysteine ADP-ribosyltransferases, pertussis toxin, and a human erythrocyte enzyme (Lobban & Van Heyningen, 1988; Tanuma et al., 1988).

In the present report, it is demonstrated that the major ADP-ribose–cysteine adduct formed by pertussis toxin results from the reaction of free ADP-ribose produced by the NAD glycohydrolase activity of the toxin with cysteine. These data do not exclude the formation of a small amount of thioglycoside-type ADP-ribosylcysteine by pertussis toxin-catalyzed reaction. This product, however, was not detected as a hydroxylamine-insensitive peak in the HPLC eluate. The present data also demonstrate that dithiothreitol is merely an activator of pertussis toxin, not an acceptor for the ADP-ribose, in contrast to a previous report (Lobban & Van Heyningen, 1988).

The thiazolidine linkage joining ADP-ribose and cysteine in the nonenzymatic product is chemically distinct from the bond formed by the pertussis toxin-catalyzed ADP-ribosylation of proteins on cysteine residues. Pertussis toxin forms a

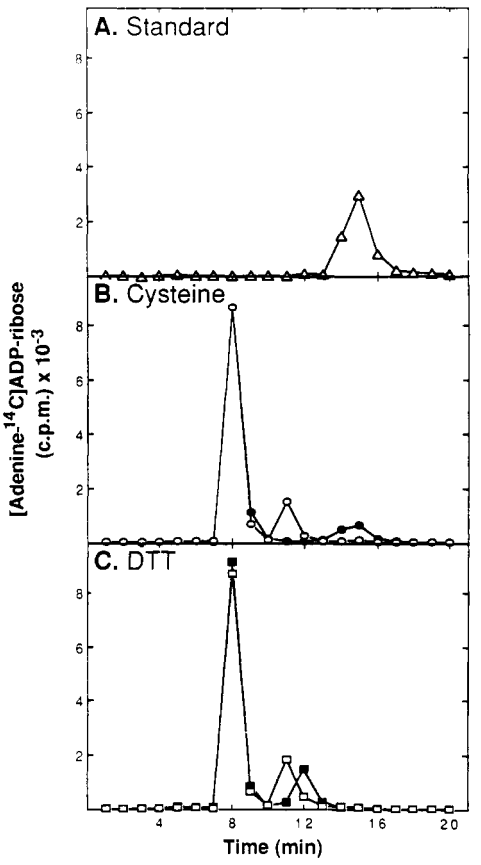

FIGURE 5: Products of incubation of pertussis toxin, [*adenine*-^{14}C]-NAD, and thiols incubated with or without hydroxylamine. (A) HPLC analysis of a standard preparation of [*adenine*-^{14}C]ADP-ribosylthiazolidine. (B) Pertussis toxin incubation with [*adenine*-^{14}C]NAD and cysteine. Samples of reactions were treated without (●) or with (○) hydroxylamine and analyzed by HPLC as described under Materials and Methods. (C) Pertussis toxin incubation with [*adenine*-^{14}C]NAD and DTT. Samples of reactions were treated without (■) or with (□) hydroxylamine and analyzed by HPLC. Data are from a single HPLC analysis (one of duplicates) in an experiment representative of two. R_t's: NAD, 8 min; ADP-ribose, 12 min; ADP-ribose–hydroxylamine adduct, 11 min; ADP-ribosylthiazolidine, 14–15 min.

thioglycoside bond between the cysteine sulfhydryl and the ribose anomeric carbon (Meyer et al., 1988). Thioglycoside linkages are characterized by their sensitivity to mercuric ion under mild conditions (Horton & Hutson, 1963; Krantz & Lee, 1976; Meyer et al., 1988), and their stability to hydroxylamine, relative to other ADP-ribosyl amino acid linkages (Hsia et al., 1985; Meyer et al., 1988). In contrast, the ADP-ribosylthiazolidine formed nonenzymatically was highly sensitive to both mercuric ion and hydroxylamine. Degradation of ADP-ribosylthiazolidine by either agent regenerated the precursors cysteine and ADP-ribose (or related chemical adducts). Because of the reversibility of thiazolidine formation, degradation by chemical agents could be either through direct attack on the thiazolidine ring or alternatively by trapping of the precursors when in the free form.

The proposed mechanism for the reaction of ADP-ribose and cysteine is shown in Figure 6. The first step involves a reversible attack by the free amino group of cysteine on the anomeric carbon of the ribosyl moiety to form a carbinolamine. Formation of carbinolamines is expected to be both rapid and reversible in aqueous solution (Cheng et al., 1987; Schendel et al., 1988). Their formation is especially favored at the high concentrations of the amine used in the synthesis. Subsequent dehydration of the carbinolamine generates a

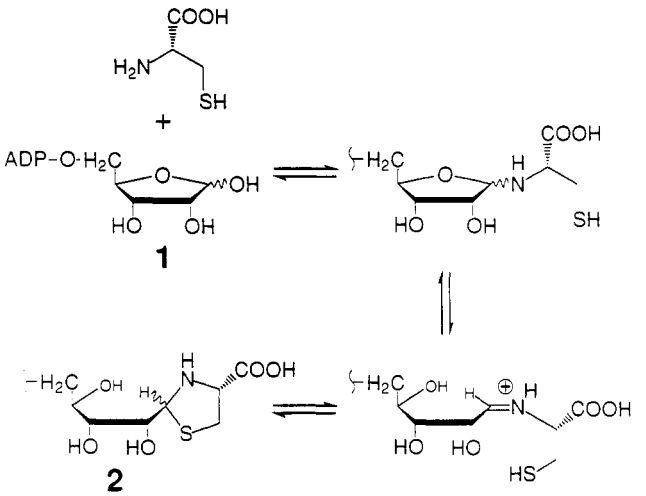

FIGURE 6: Proposed reaction scheme for the formation of ADP-ribosylthiazolidine from ADP-ribose and cysteine. Details of the reaction scheme are provided in the text; only some of the reaction intermediates are indicated, in order to highlight steps in the formation of ADP-ribosylthiazolidine: **1**, ADP-ribose; **2**, ADP-ribosylthiazolidine.

Schiff base which would be rapidly attacked by the thiolate to form the thiazolidine ring. The cyclization is driven entropically by proximity of the thiolate. In this scheme, thiol compounds not containing a primary amine should not be able to react with ADP-ribose, a result verified by the acceptor specificity shown in Table I. Two diastereomeric forms of ADP-ribosylthiazolidine were deduced from the ^{1}H NMR spectrum; they displayed selective differences in the relaxation times of the R1′-, R2′-, and α-H protons of ~30%. A speculative explanation is the existence of a hydrogen bond between the R2′-OH and the carboxylate in the cis isomer. Its presence would decrease the rotational averaging of the rotamer populations and also the average distance between the protons, hence enhancing the rate of dipolar relaxation. If correct, this would assign the A form as the cis isomer and the B form as the trans isomer.

The scheme outlined in Figure 6 is consistent with the observed properties of the synthesis. All the steps in the reaction are expected to be reversible, although at high cysteine concentrations cyclization would be favored thermodynamically. The overall reversibility of the reactions is borne out by the slow reversion to ADP-ribose and free cysteine observed in dilute solutions of ADP-ribosylthiazolidine. The reversibility of cyclization, which transiently generates the Schiff base, accounts for the susceptibility to attack by hydroxylamine with formation of ADP-ribosyl oxime.

The product ADP-ribosylthiazolidine was formed under conditions expected to be encountered in the analysis of putative cysteine-specific ADP-ribosyltransferases, i.e., in the presence of high concentrations of cysteine as a model acceptor, NAD, and a source of NADase activity. Stability to hydroxylamine can serve as a test for the enzyme-catalyzed formation of the product ADP-ribosylcysteine, which is expected to contain a hydroxylamine-stable thioglycoside bond. Alternatively, the use of thiol acceptors such as glutathione or *N*-acetylcysteine that are unable to form thiazolidines may obviate the complications arising from the chemically reactive aldose in ADP-ribose.

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