Australine, a Pyrrolizidine Alkaloid That Inhibits Amyloglucosidase and Glycoprotein Processing†

Joseph E. Tropea,*,‡ Russell J. Molyneux,§ G. P. Kaushal,‡ Y. T. Pan,‡ Michael Mitchell,‡ and Alan D. Elbein‡

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284-7760, and Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

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ABSTRACT: Australine [(1*R*,2*R*,3*R*,7*S*,7a*R*)-3-(hydroxymethyl)-1,2,7-trihydroxypyrrolizidine] is a polyhydroxylated pyrrolizidine alkaloid that was isolated from the seeds of the Australian tree *Castanospermum australe* and characterized by NMR and X-ray diffraction analysis [Molyneux et al. (1988) *J. Nat. Prod.* (in press)]. Since swainsonine and catanospermine are polyhydroxylated indolizidine alkaloids that inhibit specific glycosidases, we tested australine against a variety of exoglycosidases to determine whether it would inhibit any of these enzymes. This alkaloid proved to be a good inhibitor of the α-glucosidase amyloglucosidase (50% inhibition at 5.8 μM), but it did not inhibit β-glucosidase, α- or β-mannosidase, or α- or β-galactosidase. The inhibition of amyloglucosidase was of a competitive nature. Australine also inhibited the glycoprotein processing enzyme glucosidase I, but had only slight activity toward glucosidase II. When incubated with cultured cells, this alkaloid inhibited glycoprotein processing at the glucosidase I step and caused the accumulation of glycoproteins with $Glc_3Man_{7-9}(GlcNAc)_2$-oligosaccharides.

Among the common types of glycoproteins that are found in eukaryotic organisms, both as cell-associated proteins and as secreted proteins, are those having N-linked or asparagine-linked oligosaccharides (Kornfeld & Kornfeld, 1976; Wagh & Bahl, 1981). The biosynthesis of the oligosaccharide portion of these molecules involves a complex sequence of events beginning with the synthesis of the $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryldolichol intermediate and the transfer of the carbohydrate portion of this intermediate to various asparagine residues on the newly synthesized polypeptide (Elbein, 1979; Struck & Lennarz, 1980; Kornfeld & Kornfeld, 1985). Following the transfer of this oligosaccharide to the protein, the newly formed glycoprotein undergoes a number of modification or "processing" reactions which begin in the endoplasmic reticulum and continue as the glycoprotein is transported through the Golgi to its final destination (Turco & Robbins, 1976; Grinna & Robbins, 1979; Hubbard & Ivatt, 1981; Elting et al., 1980).

The initial processing reactions, catalyzed by two endoplasmic reticulum membrane bound glucosidases, involve the removal of the three glucose residues. Glucosidase I removes the outermost α1,2-linked glucose residue, while glucosidase II releases the remaining two α1,3-linked glucoses (Chen & Lennarz, 1978; Grinna & Robbins, 1979, 1980; Ugalde et al., 1978; Kilker et al., 1981). These trimming reactions give rise to a $Man_9(GlcNAc)_2$-protein which may be acted upon by an endoplasmic reticulum bound α1,2-mannosidase to give a $Man_8(GlcNAc)_2$-oligosaccharide structure (Bischoff & Kornfeld, 1983; Bischoff et al., 1986). The $Man_{8-9}(GlcNAc)_2$-protein may be the direct precursor of the high-mannose glycoproteins; or it may be further processed, after translocation to the Golgi, to yield hybrid or complex types of glycoproteins.

In the Golgi, the $Man_{8-9}(GlcNAc)_2$-protein may be the substrate for mannosidase I which removes the remaining α1,2-linked mannose residues, generating a $Man_5(GlcNAc)_2$-protein (Kornfeld et al., 1978; Opheim & Touster, 1978; Tabas & Kornfeld, 1979; Forsee & Schutzbach, 1981; Tulsiani et al., 1982a). This glycoprotein can then serve as acceptor for GlcNAc transferase I which catalyzes the addition of a GlcNAc, from UDP-GlcNAc, to the mannose that is linked α1,3 to the β-linked mannose, resulting in the formation of a $GlcNAcMan_5(GlcNAc)_2$-protein (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980a; Narasimhan et al., 1977). Following this addition a second Golgi mannosidase, mannosidase II, catalyzes the removal of the α1,3- and α1,6-linked mannose residues, generating a $GlcNAcMan_3(GlcNAc)_2$-oligosaccharide (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980b). Failure to remove these two terminal mannose units can result in the formation of hybrid types of glycoproteins (Tulsiani & Touster, 1983; Kornfeld & Kornfeld, 1985). The $GlcNAcMan_3(GlcNAc)_2$-protein can then be the substrate for a series of glycosyltransferases forming the complex types of glycoproteins that contain galactose, sialic acid, fucose, etc. (Schachter & Roseman, 1980; Hubbard & Ivatt, 1981).

The study of the biosynthesis of the oligosaccharide portion of the N-linked glycoproteins has been greatly facilitated by the use of inhibitors that act at specific steps in the processing pathway (Elbein, 1987; Schwarz & Datema, 1982). Some of these inhibitors which have gained popularity over the past several years include swainsonine, a Golgi mannosidase II inhibitor originally isolated from the Australian plant *Swainsona canescens* (Colegate et al., 1979; Elbein et al., 1981; Tulsiani et al., 1982b), deoxymannojirimycin, a synthetic Golgi mannosidase I inhibitor (Legler & Julich, 1984; Fuhrmann et al., 1984; Elbein et al., 1984a), and castanospermine, a glucosidase I/glucosidase II inhibitor isolated from the seeds of the Australian tree *Castanospermum australe* (Hohenschutz et al., 1981; Pan et al., 1983; Szumilo et al., 1986).

During the purification of castanospermine from the seeds of *C. australe*, we found several alkaloid fractions, in addition to the castanospermine fraction, that had inhibitory activity against α-glucosidases. By a combination of ion-exchange chromatography and preparative centrifugal thin-layer chromatography, a new alkaloid, australine, was isolated and its structure (Figure 1) established as (1*R*,2*R*,3*R*,7*S*,7a*R*)-3-

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‡ The University of Texas Health Science Center.

§ Agricultural Research Service, USDA.

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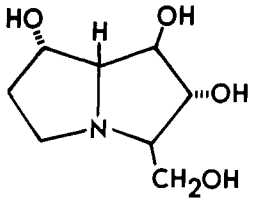

FIGURE 1: Stereochemical structure of australine.

(hydroxymethyl)-1,2,7-trihydroxypyrrolizidine (Molyneux et al., in press). This compound proved to be a good inhibitor of amyloglucosidase and the processing glucosidase I as reported in this paper. This is the first demonstration that a pyrrolizidine alkaloid may have glycosidase-inhibitory activity. In addition, australine inhibited the processing of glycoproteins in cultured cells. Thus, this pyrrolizidine structure adds to the repertoire of chemical structures that can have important inhibitory activity.

Experimental Procedures

Materials. [2-^{3}H]Mannose (15 Ci/mmol) and [6-^{3}H]-galactose (15 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. [4,5-^{3}H]Leucine (50 Ci/mmol) was obtained from ICN, Pronase was from Calbiochem, and endo-β-*N*-acetylglucosaminidase H (Endo H)[1] was from Miles Scientific. Con A–Sepharose 4B, maltitol, amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), α-mannosidase (from jack bean), and all *p*-nitrophenyl glycoside substrates were purchased from the Sigma Chemical Co. β-Mannosidase was purified from *A. niger* as previously described (Elbein et al., 1977). Intestinal sucrase was purified to homogeneity from white rat (Sprague-Dawley) small intestine (Kolinska & Semenza, 1967). Crude homogenate (white rat small intestine) was used as the source of intestinal maltase. Glucosidase I and glucosidase II were partially purified from mung bean seedlings (Szumilo et al., 1986). [^{3}H]Glucose-labeled $Glc_3Man_9GlcNAc$-oligosaccharide was isolated from influenza virus infected MDCK cells labeled with [6-^{3}H]galactose in the presence of castanospermine (Szumilo & Elbein, 1985). Tissue culture materials were obtained from Flow Laboratories. Bio-Gel P-4 and Bio-Gel P-2 were purchased from Bio-Rad Laboratories, and Sephadex LH-20 was purchased from Pharmacia Fine Chemicals. Australine was isolated from *C. australe* mother liquors by ion-exchange chromatography and TLC (Molyneux et al., in press). Castanospermine was isolated from methanolic extracts of *C. australe* seeds by crystallization as reported elsewhere (Hohenschutz et al., 1981; Molyneux et al., 1986). All additional chemicals were analytical grade.

Enzyme Assays. (*A*) *Arylglycosidases.* The enzymatic activities of amyloglucosidase, β-glucosidase, α- and β-galactosidase, and α- and β-mannosidase were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (Rudick & Elbein, 1973). All reaction mixtures contained 20 μmol of sodium acetate buffer, pH 5, 2 μmol of *p*-nitrophenyl glycoside, and enzyme in a final volume of 0.4 mL. Incubations were at 37 °C for 15 min, and the reactions were stopped by the addition of 2.5 mL of 0.4 M glycine, pH 10.4. The *p*-nitrophenol liberated in the reaction was measured at 410 nm by using a Gilford spectrophotometer. Assays were done under conditions where the amount of *p*-nitrophenol released was linear with both time and protein concentration. For inhibition studies, australine (or castanospermine) was preincubated with enzyme (20 min) prior to the addition of substrate. For competition analysis, australine and substrate were added simultaneously.

(*B*) *Sucrase and Maltase.* Intestinal sucrase and maltase activities were determined by measuring the formation of reducing sugar from sucrose and maltitol, respectively. The reaction mixture contained 7.5 μmol of sodium citrate buffer, pH 6, 9 μmol of substrate (sucrose or maltitol), and enzyme, in a final volume of 0.3 mL. The mixtures were incubated at 37 °C for 10 min, and the formation of reducing sugar was determined by the Nelson method (Nelson, 1944). For both enzymes, activity was proportional with time and amount of protein. As described above for the arylglycosidases, inhibitors were preincubated with enzyme prior to the addition of substrate.

(*C*) *Glucosidase I and Glucosidase II.* Glucosidase I activity was determined by measuring the release of [^{3}H]glucose from [^{3}H]glucose-labeled $Glc_3Man_9GlcNAc$ as described previously (Szumilo & Elbein, 1985). Glucosidase II activity was measured by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl α-D-glucoside (Rudick & Elbein, 1973). The incubation mixtures for both enzymes contained 50 mM MES buffer, pH 6.5, 0.1% Triton X-100, enzyme, and substrate (25000 cpm of $Glc_3Man_9GlcNAc$ for glucosidase I; 1.25 μmol of *p*-nitrophenyl α-D-glucoside for glucosidase II), in a final volume of 0.25 mL. A typical incubation was for 1 h at 37 °C and was linear with both time and protein concentration for the duration of the assay. For inhibition studies, enzyme was preincubated with inhibitor prior to the addition of substrate.

Growth and Labeling of Influenza Virus. The NWS strain of influenza virus was grown in MDCK cells as previously described (Elbein et al., 1982; Pan et al., 1983). MDCK cells were maintained in 75-cm^2 tissue culture flasks in modified Eagle's medium containing 10% fetal bovine serum. At confluency, the cells were infected with influenza virus at a multiplicity of infection of approximately 75 PFU. One hour after infection, australine was added to a final concentration of 10–500 μg/mL. Following an incubation period of 2 h to allow the alkaloid to take effect, [6-^{3}H]galactose or [2-^{3}H]-mannose was added to each culture (both at 25 μCi/mL) and the cells were allowed to incubate for an additional 36–48 h to form mature virus. The presence of mature virus was determined by hemagglutination. When the titer reached a maximum, the medium, containing viral particles and cell debris, was removed and saved, and any cells still adhered to the flasks were removed with a rubber policeman and pooled with the medium. The pooled fraction was subjected to low-speed centrifugation to remove cell debris, and the supernatant was then centrifuged at 100000*g* for 18 h to pellet the virus.

Preparation and Analysis of Glycopeptides. The mature influenza virus, isolated by ultracentrifugation, was exhaustively digested with Pronase to obtain a glycopeptide fraction for analysis. Briefly, viral pellets were suspended in 2 mL of 50 mM Tris buffer, pH 7.5, containing 1 mM $CaCl_2$. An equal volume of a 5 mg/mL Pronase solution (in the same buffer) was added, and the mixtures were incubated at 37 °C for 18 h under a toluene atmosphere. The Pronase treatment was repeated a second time. The reaction mixture was then treated with TCA to a final concentration of 5%. After a

[1] Abbreviations: Con A, concanavalin A; Endo H, endo-β-*N*-acetylglucosaminidase H; MDCK cells, Madin–Darby canine kidney cells; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

30-min incubation in an ice–water bath, precipitated protein was removed by centrifugation, and the supernatant liquid was extracted repeatedly with ethyl ether to remove any remaining TCA. The neutralized solution, containing glycopeptides, was then concentrated to a small volume for analysis by gel filtration chromatography.

Glycopeptides were separated on a 1.5 × 100 cm column of Bio-Gel P-4 (100–200 mesh) equilibrated in 1% acetic acid. Aliquots of column fractions were analyzed for radioactivity to identify the positions of the glycopeptide and free sugar(s) peaks. Since this column did not completely resolve the complex types of glycopeptides from the high-mannose types, the entire glycopeptide peak was pooled and digested exhaustively with Endo H. For these incubations the entire glycopeptide peak was concentrated to dryness and then suspended in 2 mL of 50 mM citrate buffer, pH 6. Ten milliunits of Endo H (in the same buffer) was added, and the mixtures were incubated at 37 °C for 18 h under a toluene atmosphere. At the end of this time, a second addition of Endo H was made (10 milliunits) and the incubation was repeated. These digests were then rechromatographed on the same Bio-Gel P-4 column, and the Endo H sensitive peaks were identified by a shift of the radiolabeled glycopeptide peak to the right (i.e., eluting later from the column).

Characterization of Oligosaccharides. The structure of the Endo H sensitive oligosaccharide(s) produced in the presence of australine was determined by a combination of chromatographic, enzymatic, and chemical methods. Prior to analysis, each oligosaccharide fraction was subjected to affinity chromatography on Con A–Sepharose 4B to remove any contaminating complex type structures. One-milliliter columns of Con A–Sepharose 4B were equilibrated with 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The sample was applied to the column, and the column was washed with 20 mL of the equilibration buffer. Bound oligosaccharide was eluted from the column with 100 mM methyl α-mannoside in the above buffer, and the free methyl α-mannoside was removed from the oligosaccharide peak by desalting on a Bio-Gel P-2 column. The oligosaccharide eluting in the void peak of the Bio-Gel P-2 column was used for structural analysis.

(*A*) *Chromatographic Methods.* Endo H susceptible, Con A purified oligosaccharide was chromatographed on a calibrated 1 × 200 cm column of Bio-Gel P-4 (200–400 mesh), equilibrated in 1% acetic acid. The size of the oligosaccharide was determined after radioactive counting of the column eluant and comparison to migration of standard oligosaccharides.

(*B*) *Enzymatic Methods.* [^{3}H]Glucose-labeled oligosaccharides were treated with partially purified glucosidase I and glucosidase II to determine their enzymatic susceptibilities. Digestions were performed as described under Enzyme Assays (see above) and were analyzed by monitoring the release of free glucose and determining the size of the oligosaccharide product.

(*C*) *Methylation Analysis.* [^{3}H]Glucose-labeled oligosaccharides produced in the presence of australine were subjected to exhaustive methylation by a modification (Sanford & Conrad, 1966) of the method described by Hakomori (1964). Lyophilized oligosaccharide was dissolved in 2 mL of dimethyl sulfoxide under nitrogen and combined with 1 mL of dimethyl sulfoxide containing 100 mg of methanesulfinyl carbanion. The mixture was sonicated for 5 h at 40–50 °C and then chilled on ice to 4 °C. Two milliliters of CH_3I was added, and the sample was sonicated for 2 h at 4 °C with the addition of another 2 mL of CH_3I after 1 h. After standing at room temperature overnight, the mixture was passed through a column of Sephadex LH-20 equilibrated with 80% methanol (4 °C), in order to remove dimethyl sulfoxide, methanesulfinyl carbanion, and other salts. The column eluates, corresponding to methylated oligosaccharides, were pooled, concentrated to dryness, and hydrolyzed in 2 N TFA at 110 °C for 4 h under vacuum. Methylated sugars were then analyzed by thin-layer chromatography on 0.5-mm Kiesel Gel 60 F-254 plates (Merck) developed with benzene/acetone/water/ammonium hydroxide (50:200:3:1.5). Standard methylated glucose derivatives prepared from maltose, kojibiose, nigerose, and isomaltose, were run in parallel lanes. Methylated standards were visualized by charring the plates after spraying with 5% ethanolic H_2SO_4. Radioactive methylated sugars were detected by scraping the plates into 0.5-cm sections followed by liquid scintillation counting.

Synthesis of Lipid-Linked Saccharides and Protein. The effect of australine on the formation of lipid-linked saccharides and on protein synthesis was tested in uninfected MDCK cells. Confluent monolayers of MDCK cells, in six-well Linbro tissue culture dishes, were treated for 2 h with various concentrations of australine (50–500 μg/mL) in modified Eagle's containing 10% fetal bovine serum. The cells then were incubated for 15–20 min with either [2-^{3}H]mannose (20 μCi/mL) or [4,5-^{3}H]leucine (10 μCi/mL) to label lipid-linked saccharides and cellular proteins, respecitvely. At the end of the labeling period, the medium was removed by aspiration, and the monolayers were washed three times with PBS. One milliliter of PBS was added to each well, and the cells were dislodged by scraping and were then quantitatively transferred to reaction tubes. Each well was washed with another 1 mL of PBS, and this rinse was added to the initial tube. Radioactivity incorporated into lipid-linked monosacchrides and oligosaccharides was determined after extraction with chloroform/methanol/water (1:1:1) and chloroform/methanol/water (10:10:3), respectively, as previously described (Chambers & Elbein, 1975). For the incorporation of radiolabel into protein, cells were extracted with TCA at a final concentration of 20% in the presence of 500 μg of bovine serum albumin. Following an overnight incubation at 4 °C, precipitated protein was isolated by centrifugation. The precipitate was washed twice with 5% TCA, and once with absolute methanol, and then digested with Pronase as described above. Pronase-released radioactivity was measured by liquid scintillation counting.

Results

Effect of Australine on Various Arylglycosidases. Because of the structural similarity between australine and castanospermine (Molyneux et al., in press), we tested australine against a number of commercially available glycosidases to determine whether it had inhibitory activity. Like castanospermine, australine was found to be a potent inhibitor of amyloglucosidase, an α1→4, α1→6 exoglucosidase (Figure 2), showing 50% inhibition of enzymatic activity at a concentration of 5.8 μM. The type of inhibition (analyzed by the method of Lineweaver and Burk) was competitive in nature (data not shown), indicating a structural similarity to the substrate, similar to that found for castanospermine (Saul et al., 1984). In contrast to castanospermine, however, australine was found to be a poor inhibitor of β-glucosidase. For example, at levels of castanospermine that inhibited 50% of the β-glucosidase activity (66 μM), australine inhibited less than 5% (data not shown).

In addition to α- and β-glucosidase, australine was also tested against α- and β-mannosidase and α- and β-galactosidase for inhibitory activity. At concentrations up to 500 μM,

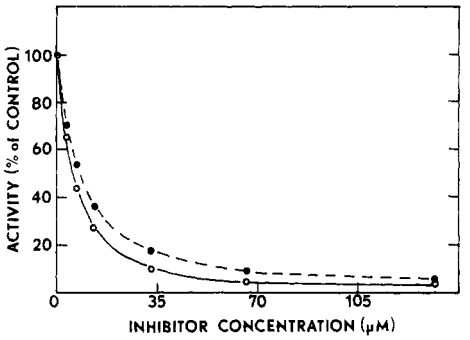

FIGURE 2: Inhibition of amyloglucosidase by australine and castanospermine. Incubations were as described under Experimental Procedures using *p*-nitrophenyl α-D-glucoside as substrate. Activity is expressed relative to untreated enzyme. (○) Australine; (●) castanospermine.

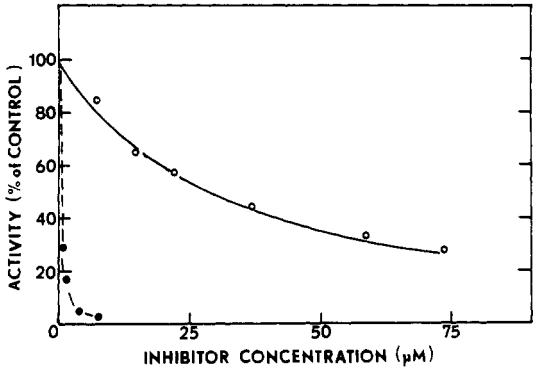

FIGURE 3: Comparison of the effect of australine and castanospermine on intestinal sucrase activity. Incubations were as described under Experimental Procedures using sucrose as substrate. Activity is expressed relative to untreated enzyme. (○) Australine; (●) castanospermine.

australine had no apparent effect on any of these hydrolases (data not shown).

Effect of Australine on Sucrase and Maltase. Since australine showed very little inhibitory activity toward β-glucosidase, an enzyme inhibited by micromolar amounts of castanospermine, we decided to examine the effects of australine toward other enzymes that are sensitive to castanospermine, namely, intestinal sucrase and maltase (Trugnan et al., 1986). The results of these studies are shown in Figures 3 and 4 for sucrase and maltase, respectively. Although australine did show significant levels of inhibition within the micromolar range for both enzymes (50% inhibition at approximately 28 μM for sucrase and 35 μM for maltase), relative to castanospermine the inhibition was poor (50% inhibition for both enzymes less than 1 μM).

Effect of Australine on Processing Glucosidases. Australine was also tested for inhibitory activity against partially purified glucosidase I and glucosidase II from mung bean seedlings. These hydrolases, which are inhibited by micromolar amounts of castanospermine (see Figure 5), are involved in the initial oligosaccharide trimming reactions in the endoplasmic reticulum during N-linked glycoprotein processing. The results of Figure 5 demonstrate quite clearly that australine selectively inhibits glucosidase I (Figure 5B), having very low activity against glucosidase II (Figure 5A) even at concentrations up to 500 μM. Although the inhibitory activity toward glucosidase I is less potent relative to castanospermine (50% inhibition at approximately 20 μM for australine vs 50% inhibition at approximately 1 μM for castanospermine), australine is the first example of a compound inhibiting glucosidase I without also inhibiting glucosidase II.

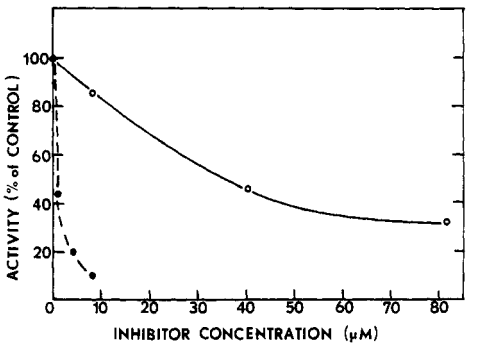

FIGURE 4: Comparison of the effect of australine and castanospermine on intestinal maltase activity. Incubations were as described under Experimental Procedures using maltitol as substrate. Activity is expressed relative to untreated enzyme. (○) Australine; (●) castanospermine.

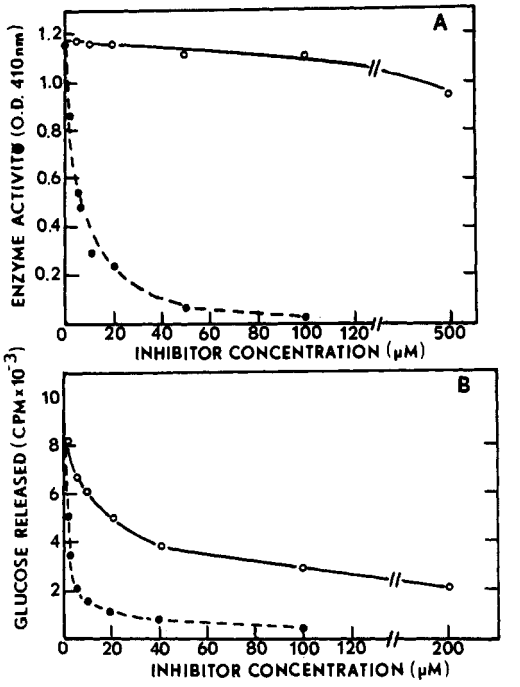

FIGURE 5: Effect of australine and castanospermine on the activity of the mung bean processing enzymes glucosidase I and glucosidase II. Conditions were as described under Experimental Procedures. (A) Glucosidase II activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl α-D-glucoside. (B) Glucosidase I activity was determined by measuring the release of [^{3}H]glucose from glucose-labeled $Glc_3Man_9GlcNAc$ oligosaccharide. (○) Australine; (●) castanospermine.

Effect of Australine on Glycoprotein Processing in Culture. Since australine was found to be a reasonable α-glucosidase inhibitor with activity against glucosidase I (see above), we tested this alkaloid to determine whether it would alter normal glycoprotein processing in cell culture. For these studies, influenza virus infected MDCK cells were incubated for 2 h in the absence or presence of various concentrations of australine, and then the cultures were labeled for 36–48 h with either [2-^{3}H]mannose or [6-^{3}H]galactose. The mature virus was isolated by differential centrifugation and digested exhaustively with Pronase to generate glycopeptides. The glycopeptides were then isolated by chromatography on columns of Bio-Gel P-4. Figure 6 shows representative radioactive profiles of those glycopeptides from virus raised in the absence (upper panels) or presence (lower panels) of 500 μg/mL australine (○). Since these columns did not give good resolution of the complex from the high-mannose structures, the entire glycopeptide peak was pooled for each sample, digested

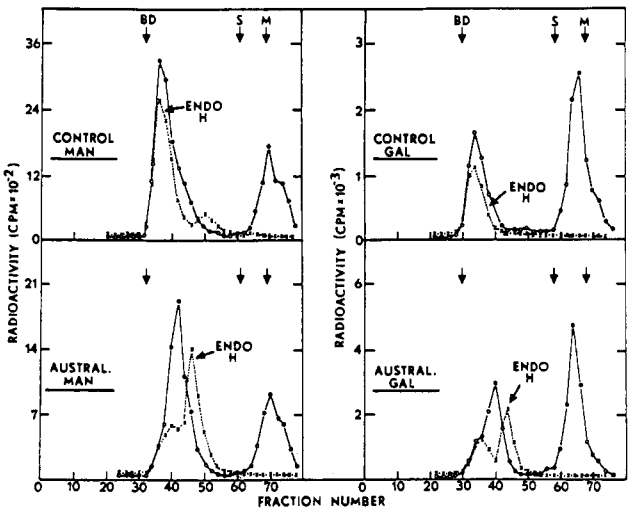

FIGURE 6: Effect of australine on the oligosaccharide composition of influenza virus glycoproteins. Infected MDCK cells were incubated for 2 h with 500 μg/mL australine (AUSTRAL., lower profiles) and then labeled with either [^{3}H]mannose (MAN, left) or [^{3}H]galactose (GAL, right). Control flasks (upper profiles) were done in the absence of alkaloid. After an incubation of 36–48 h, the virus particles were isolated from treated and untreated cultures by ultracentrifugation and digested exhaustively with Pronase as described under Experimental Procedures. The glycopeptides were separated on columns of Bio-Gel P-4 (1.5 × 100 cm) (O—O). The entire glycopeptide peak (fractions 32–52 for mannose-labeled material or fractions 30–48 for glucose-labeled material) was pooled, digested with Endo H, and rechromatographed on the same Bio-Gel column (×···×). Radioactivity was determined by liquid scintillation counting. The arrows indicate the positions of calibration markers: BD, blue dextran T-2000; S, stachyose; M, mannose.

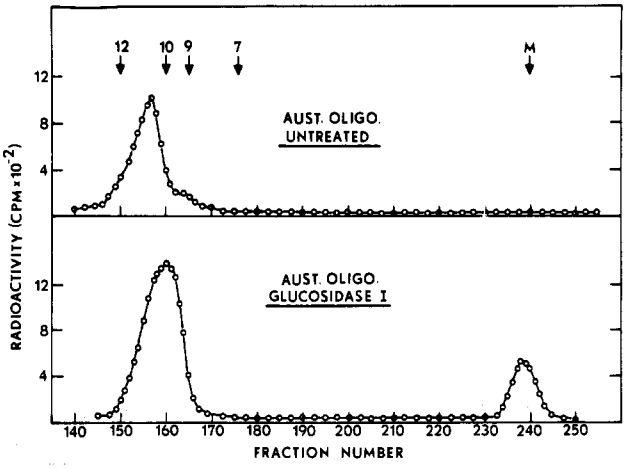

FIGURE 7: Partial characterization of the glucose-labeled oligosaccharide produced in the presence of australine. The Endo H released oligosaccharide (see Figure 6) from virus grown in the presence of 500 μg/mL australine (AUST. OLIGO.) was chromatographed on a 1 × 200 cm column of Bio-Gel P-4 (200–400 mesh). Radioactivity in various fractions was determined by liquid scintillation counting. The upper panel shows the molecular sizing of the untreated Endo H sensitive oligosaccharide while the lower panel shows the effect of glucosidase I treatment prior to chromatography. Standard oligosaccharides (arrows) are $Glc_3Man_9GlcNAc$ (12), $Glc_1Man_9GlcNAc$ (10), $Man_9GlcNAc$ (9), and $Man_7GlcNAc$ (7). M, mannose.

with Endo H, and rechromatographed on the same Bio-Gel P-4 (Figure 6; X).

It can be seen from the upper profiles of Figure 6 that the glycopeptides from control virus, labeled with either [^{3}H]-mannose or [^{3}H]galactose, emerged from the column as a single asymmetrical peak. Exhaustive digestion with Endo H of the glucose-labeled glycopeptide peak produced no change in the radioactive profile relative to the undigested material (fractions 30–42), while treatment of the mannose-labeled glycopeptide peak (fractions 32–46) with Endo H produced a smaller second peak (fractions 46–58) containing approximately 18% of the total incorporated radioactivity. The bulk of the radioactivity of the Endo H treated mannose-labeled glycopeptide, however, remained in a position identical with that of the untreated glycopeptide peak and, like the glucose-labeled material, represents glycopeptide with complex oligosaccharide structures. The smaller second peak most likely represents oligosaccharide of high-mannose type and is consistent with the observation that influenza viral hemagglutinin contains about 20–30% of high-mannose oligosaccharide side chains (Pan et al., 1983).

When virus was raised in the presence of 500 μg/mL australine, a much different glycopeptide profile was observed after Bio-Gel P-4 chromatography (Figure 6, lower profiles). For both mannose-labeled and glucose-labeled material, there was a shift of the glycopeptide peaks, with the bulk of the bound radioactivity eluting from the Bio-Gel P-4 columns at later positions relative to their controls. Furthermore, when these glycopeptide peaks were treated with Endo H, there was a massive release of labeled oligosaccharide comprising 71% of the bound radioactivity in the mannose-labeled glycopeptide and 65% of the bound radioactivity in the glucose-labeled oligosaccharide. These results indicate that australine altered the normal processing of the viral glycoproteins, generating a greatly increased proportion of high-mannose, Endo H sensitive oligosaccharides. Moreover, the observation that the Endo H released oligosaccharide from the glucose-labeled glycopeptide contained bound radioactivity suggested that australine blocked glucose removal, most likely by an inhibition of glucosidase I.

In addition to the studies shown in Figure 6, australine was also tested in virus-infected MDCK cell cultures at several intermediate concentrations and was found to affect glycoprotein processing in a dose-dependent manner. Thus, at a final concentration of 10 μg/mL of australine, Endo H susceptible oligosaccharide represented 43% of the total mannose-labeled glycopeptide, while at a dose of 50 μg/mL this proportion increased to 55%. Comparable values for glucose-labeled glycopeptide were 17% and 38% release of bound oligosaccharide at a final concentration of 10 and 50 μg/mL australine, respectively.

Characterization of the Oligosaccharide Produced in the Presence of Australine. The glucose-labeled Endo H released oligosaccharide produced in the presence of 500 μg/mL australine was isolated from the Bio-Gel P-4 column shown in Figure 6. After Con A-Sepharose 4B purification (see Experimental Procedures), the oligosaccharide was applied to a long calibrated column of Bio-Gel P-4 to obtain an accurate size determination. Figure 7 (upper panel) shows that the majority of the radioactivity eluted from this column in the position expected for a $hexose_{11}GlcNAc$ structure and was clearly distinct from the $hexose_{12}GlcNAc$ and $hexose_{10}GlcNAc$ standards. This oberservation was consistent with a $Glc_3Man_8GlcNAc$-oligosaccharide structure. Further evidence in support of this was obtained by enzymatic treatment of the Endo H released oligosaccharide with glucosidase I (Figure 7, lower panel). Since this enzyme will only act on structures containing a terminal α1,2-linked glucose unit, the appearance of free glucose after incubation is strong proof for the presence of a Glc_3-oligosaccharide structure. The results of Figure 7 (lower panel) demonstrate quite clearly that, after incubation of the glucose-labeled oligosaccharide with glucosidase I (free of glucosidase II), there was a release of free glucose concomitent with a change in the size of the resulting oligo-

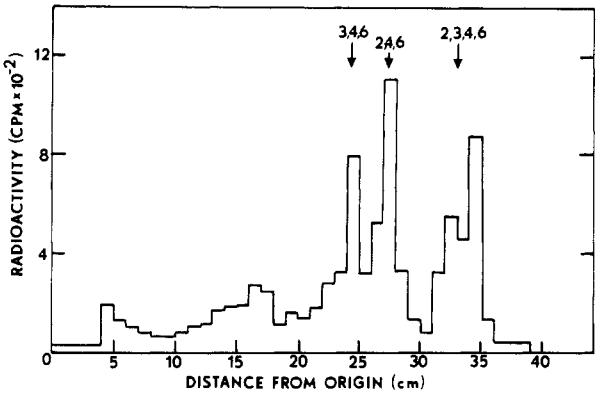

FIGURE 8: Methylation analysis of the [^{3}H]glucose-labeled oligosaccharide from australine-grown virus. The Endo H released hexose$_{11}$GlcNAc was isolated from the Bio-Gel P-4 column (see Figure 7) and subjected to complete methylation as described under Experimental Procedures. After acid hydrolysis the methylated sugars were analyzed by thin-layer chromatography. Radioactive sugar derivatives were located by scraping plates in 0.5-cm sections followed by liquid scintillation counting. Standard sugars are as follows: 3,4,6 = 3,4,6-trimethylglucose, 2,4,6 = 2,4,6-trimethylglucose, and 2,3,4,6 = 2,3,4,6-tetramethylglucose.

saccharide to a hexose$_{10}$GlcNAc structure, consistent with the loss of a single glucose unit. In addition to these observations, incubation of the Endo H released hexose$_{11}$GlcNAc with partially purified glucosidase II (free of glucosidase I) resulted in the release of only small amounts of radioactive glucose, suggesting that most of the australine-induced oligosaccharide contained three glucose residues.

Further evidence for a Glc$_3$Man$_8$GlcNAc-oligosaccharide structure was obtained by methylation analysis of the glucose-labeled, Endo H released oligosaccharide. For these studies, labeled oligosaccharide produced in the presence of australine was subjected to exhaustive methylation followed by acid hydrolysis, as described under Experimental Procedures. Methylated glucose derivatives were separated by thin-layer chromatography and identified by comparison with methylated glucose standards. The results are shown in Figure 8. Three major peaks of radioactivity, corresponding to 3,4,6-trimethylglucose, 2,4,6-trimethylglucose, and 2,3,4,6-tetramethylglucose, were observed on the thin-layer plates. Although the relative proportions of these sugar derivatives did not match the expected 1:1:1 ratio, the identification of three radiolabeled methylated glucose derivatives does indicate the presence of a Glc$_3$-oligosaccharide structure. The deviation from the expected ratio may be due to the fact that australine inhibition of glucosidase I is not complete and therefore small amounts of Glc$_2$Man$_9$GlcNAc or Glc$_1$Man$_9$GlcNAc could also be present in the oligosaccharide fraction used for methylation. Thus, the in vivo produced oligosaccharides probably have some inherent heterogeneity.

Effect of Australine on the Formation of Lipid-Linked Saccharides and Protein Synthesis. Since australine appeared to be an inhibitor of glycoprotein processing, it was important to determine whether it inhibited protein synthesis or lipid-linked saccharide formation. For these studies, uninfected MDCK cells were incubated with various amounts of australine and then labeled with either [^{3}H]leucine (for protein) or [^{3}H]mannose (for lipid-linked saccharide). Radioactivity incorporated into protein was determined after TCA precipitation, and radioactivity incorporated into lipid-linked saccharides was determined after organic extraction as detailed under Experimental Procedures.

Australine had no effect on the overall rate of protein synthesis at concentrations ranging from 50 to 500 μg/mL,

Table I: Effect of Australine on the Incorporation of [^{3}H]Mannose into Lipid-Linked Saccharides[a]

concn of australine (μg/mL)	radioactivity (cpm)					
	lipid-linked monosaccharides			lipid-linked oligosaccharides		
	15 min	30 min	60 min	15 min	30 min	60 min
0	90	140	160	1720	4720	8630
50	100	120	230	1670	5020	10770
100	100	150	240	2340	5340	11040
250	110	120	210	2070	5660	9950
500	100	180	240	2960	6170	11090

[a] Uninfected MDCK cells were grown in six-well limbro tissue culture dishes. At confluency, australine was added to some wells to the final concentrations listed above. After a 2-h incubation to allow the alkaloid to take effect, [^{3}H]mannose was added to a final concentration of 20 μCi/mL, and the incubations were continued. At the times shown, the medium was removed by aspiration, and the monolayers were washed three times with PBS. The cells were released from the wells by scraping, placed in tubes, and then sequentially extracted with chloroform/methanol/water (1:1:1) for lipid-linked monosaccharides, and chloroform/methanol/water (10:10:3) for lipid-linked oligosaccharides. The total radioactivity incorporated into the lipid-linked sugars was determined by liquid scintillation counting.

and for incubation times up to 120 min (data not shown). Similar results were observed for the incorporation of [^{3}H]-mannose into lipid-linked monosaccharides (Table I, left), although the results are somewhat tentative due to the low degree of incorporation of radiolabel. In contrast to these observations, however, was the finding that the incorporation of [^{3}H]mannose into lipid-linked oligosaccharide appeared to be stimulated by australine (Table I, right), although the magnitude of the increase was small. At present we have no explanation for this phenomenon, but it may be that australine is inhibiting competing reactions that utilize mannose, thus increasing the amount of mannose available for in vivo glycosylation.

Discussion

The studies reported here demonstrate that the pyrrolizidine alkaloid australine is a specific competitive inhibitor of amyloglucosidase, an α1→4, α1→6 exoglucosidase (see Figure 2 and text) but does not inhibit β-glucosidase or any other aryl α- or β-glycosidase tested. In addition, australine was a reasonably good inhibitor of glucosidase I, but was a very poor inhibitor of glucosidase II (Figure 5). Thus, this alkaloid appears to be the first glucosidase inhibitor that is active toward glucosidase I without also affecting glucosidase II.

Since australine inhibited glucosidase I activity in vitro, it was tested in cell culture as a potential inhibitor of glycoprotein processing. For these studies the influenza virus infected MDCK cell culture system was employed. The results presented clearly demonstrate that australine did alter the normal glycoprotein processing of viral glycoproteins (Figure 6) but did not do so by an inhibition of protein synthesis (see text) or lipid-linked oligosaccharide (or monosaccharide) formation (Table I). The major oligosaccharide produced in the presence of australine and released by Endo H appeared to be a Glc$_3$Man$_8$GlcNAc structure, on the basis of chromatographic (Figure 7), enzymatic (Figure 7 and text), and chemical (Figure 8) analyses. Thus, these data were consistent with the observed inhibition of glucosidase I by australine, in vitro. However, relative to other processing inhibitors, a fairly high concentration of australine was required to produce marked effects on glycoprotein processing. For example, castanospermine, a glucosidase I and glucosidase II inhibitor, at a concentration of 10 μg/mL, produced the same degree of inhibition (e.g., an increased proportion of Endo H susceptible

glycopeptides) that was observed for 500 μg/mL australine, in vivo (Pan et al., 1983).

Australine is, however, the first pyrrolizidine alkaloid that has been shown to have biological activity and to be a glycosidase inhibitor. From a structure–activity point of view, it would appear that the six-membered ring structure, which is characteristic of the indolizidine alkaloids (castanospermine, 6-epicastanospermine, and swinsonine; Elbein, 1987; Molyneux et al., 1986) as well as other related inhibitors (deoxynorjirimycin and deoxymannojirimycin; Saunier et al., 1982; Elbein et al., 1984a), is not necessary for a compound to be an inhibitor of glycosidases. Thus, the ring nitrogen and the configuration of the hydroxyl groups relative to this nitrogen may be the only factors required for such an inhibitor. In this regard, it is interesting to point out that several previous studies showed that the furanose derivatives, 1,4-dideoxy-1,4-imino-D-mannitol and 2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine, were inhibitors of mannosidase I and glucosidase I, respectively (Palamarczyk et al., 1985; Elbein et al., 1984b). Nevertheless, australine with its unique tetrahydroxylated pyrrolizidine structure does add to the growing list of chemical structures that can have important biological activity.

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Registry No. Australine, 118396-02-4; amyloglucosidase, 9032-08-0; glucosidase I, 73699-12-4.

REFERENCES

Bischoff, J., & Kornfeld, R. (1983) *J. Biol. Chem. 288*, 7907–7910.

Bischoff, J., Liscum, L., & Kornfeld, R. (1986) *J. Biol. Chem. 261*, 4766–4774.

Burns, D. M., & Touster, O. (1982) *J. Biol. Chem. 257*, 9991–10000.

Chambers, J., & Elbein, A. D. (1975) *J. Biol. Chem. 250*, 6904–6913.

Chen, W. W., & Lennarz, W. J. (1978) *J. Biol. Chem. 253*, 5780–5785.

Colegate, S. M., Dorling, P. R., & Huxtable, C. R. (1979) *Aust. J. Chem. 32*, 2257–2264.

Elbein, A. D. (1979) *Annu. Rev. Plant Physiol. 30*, 239–272.

Elbein, A. D. (1987) *Annu. Rev. Biochem. 56*, 497–534.

Elbein, A. D., Ayda, S., & Lee, Y. C. (1977) *J. Biol. Chem. 252*, 2206–2211.

Elbein, A. D., Solf, R., Dorling, P. R., & Vosbeck, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7393–7397.

Elbein, A. D., Vosbeck, K., Dorling, P. R., & Horisberger, M. (1982) *J. Biol. Chem. 257*, 1573–1576.

Elbein, A. D., Legler, G., Tlusty, A., McDowell, W., & Schwarz, R. T. (1984a) *Arch. Biochem. Biophys. 235*, 579–588.

Elbein, A. D., Mitchell, M., Sanford, B. A., Fellows, L. E., & Evans, S. V. (1984b) *J. Biol. Chem. 259*, 12409–12413.

Elting, J. J., Chen, W. W., & Lennarz, W. J. (1980) *J. Biol. Chem. 255*, 2325–2331.

Forsee, W. T., & Schutzbach, J. (1981) *J. Biol. Chem. 256*, 6577–6583.

Fuhrmann, U., Bause, E., Legler, G., & Ploegh, H. (1984) *Nature 307*, 755–758.

Grinna, L. S., & Robbins, P. W. (1979) *J. Biol. Chem. 254*, 8814–8818.

Grinna, L. S., & Robbins, P. W. (1980) *J. Biol. Chem. 255*, 2255–2258.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Harpaz, N., & Schachter, H. (1980a) *J. Biol. Chem. 255*, 4885–4893.

Harpaz, N., & Schachter, H. (1980b) *J. Biol. Chem. 255*, 4894–4902.

Hohenschutz, L. D., Bell, E. A., Jewess, P. J., Lewothy, D. P., Pryce, R. J., Arnold, E., & Clardy, J. (1981) *Phytochemistry 20*, 811–814.

Hubbard, S. C., & Ivatt, R. J. (1981) *Annu. Rev. Biochem. 50*, 553–583.

Kilker, R. D., Jr., Saunier, B., Tkacz, J. S., & Herscovics, A. (1981) *J. Biol. Chem. 256*, 5299–5303.

Kolinska, J., & Semenza, G. (1967) *Biochim. Biophys. Acta 146*, 181–195.

Kornfeld, R., & Kornfeld, S. (1976) *Annu. Rev. Biochem. 45*, 217–237.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Kornfeld, S., Li, E., & Tabas, I. (1978) *J. Biol. Chem. 253*, 7770–7778.

Legler, G., & Julich, E. (1984) *Carbohydr. Res. 128*, 61–72.

Michael, J. M., & Kornfeld, S. (1980) *Arch. Biochem. Biophys. 199*, 249–258.

Molyneux, R. J., Roitman, J. N., Dunnheim, G., Szumilo, T., & Elbein, A. D. (1986) *Arch. Biochem. Biophys. 251*, 450–457.

Molyneux, R. J., Benson, M., Wong, R. Y., Tropea, J. E., & Elbein, A. D. (1988) *J. Nat. Prod.* (in press).

Narasimhan, S., Stanley, P., & Schachter, H. (1977) *J. Biol. Chem. 252*, 3926–3933.

Nelson, N. (1944) *J. Biol. Chem. 153*, 375–380.

Opheim, D. J., & Touster, O. (1978) *J. Biol. Chem. 253*, 1017–1023.

Palamarczyk, G., Mitchell, M., Smith, P. W., Fleet, G. W. J., & Elbein, A. D. (1985) *Arch. Biochem. Biophys. 243*, 35–45.

Pan, Y. T., Hori, H., Saul, R., Sanford, B. A., Molyneux, R. J. & Elbein, A. D. (1983) *Biochemistry 22*, 3975–3984.

Rudick, M., & Elbein, A. D. (1973) *J. Biol. Chem. 248*, 6506–6512.

Sanford, P. A., & Conrad, H. (1966) *Biochemistry 5*, 1508–1517.

Saul, R., Molyneux, R. J., & Elbein, A. D. (1984) *Arch. Biochem. Biophys. 230*, 668–675.

Saunier, B., Kilker, R. P., Tkacz, J. S., Quaroni, A., & Herscovics, A. (1982) *J. Biol. Chem. 257*, 14155–14162.

Schachter, H., & Roseman, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W., Ed.) pp 85–160, Plenum Press, New York.

Schwarz, R. T., & Datema, R. (1982) *Adv. Carbohydr. Chem. Biochem. 40*, 287–379.

Struck, D. K., & Lennarz, W. J. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W., Ed.) pp 35–83, Plenum Press, New York.

Szumilo, T., & Elbein, A. D. (1985) *Anal. Biochem. 151*, 32–40.

Szumilo, T., Kaushal, G. P., & Elbein, A. D. (1986) *Arch. Biochem. Biophys. 247*, 261–271.

Tabas, I., & Kornfeld, S. (1978) *J. Biol. Chem. 253*, 7779–7786.

Tabas, I., & Kornfeld, S. (1979) *J. Biol. Chem. 254*, 11655–11663.

Trugnan, G., Rousett, M., & Zweibaum, A. (1986) *FEBS Lett. 195*, 28–32.

Tulsiani, D. R. P., & Touster, O. (1983) *J. Biol. Chem. 258*, 7578–7585.

Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W., & Touster, O. (1982a) *J. Biol. Chem. 257*, 3660-3668.
Tulsiani, D. R. P., Harris, T. M., & Touster, O. (1982b) *J. Biol. Chem. 257*, 7936-7939.
Turco, S. J., & Robbins, P. W. (1976) *J. Biol. Chem. 254*, 4560-4567.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1978) *FEBS Lett. 91*, 209-212.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1980) *Eur. J. Biochem. 113*, 97-103.
Wagh, P. V., & Bahl, O. P. (1981) *CRC Crit. Rev. Biochem 10*, 307-377.

ADP-Ribosylation of ADPR-Transferase and Topoisomerase I in Intact Mouse Epidermal Cells JB6†

Georg Krupitza and Peter Cerutti*
Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland
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ABSTRACT: Poly(ADP-ribosylation) [poly(ADPR)] is a posttranslational modification of chromosomal proteins that affects the structural and functional properties of chromatin. We have studied poly(ADPR) of ADPR-transferase and topoisomerase I in intact mouse epidermal cells JB6 (clone 41) by a combination of affinity chromatography on phenylboronate and immunoblotting with monoclonal antibodies against poly(ADPR) chains and polyclonal antibodies against ADPR-transferase and topoisomerase I, respectively. Constitutive, steady-state poly(ADPR) substitution of ADPR-transferase was estimated at 4% and that of topoisomerase I at 0.1%. Active oxygen produced extracellularly by xanthine–xanthine oxidase and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine transiently increased the level of poly(ADPR) substitution of these enzymes by a factor of 6–10. While the poly(ADPR) substitution of ADPR-transferase remained elevated after 60 min of incubation, the poly(ADPR) substitution of topoisomerase I had returned to control values within this time. Benzamide (100 μM) partially prevented the stimulation of poly(ADPR) synthesis by these agents. We speculate that self-inactivation of ADPR-transferase by poly(ADPR) represents a feedback mechanism that has the function to avoid excessive poly(ADPR) synthesis and concomitant NAD and ATP depletion. Inactivation of topoisomerase I in the neighborhood of DNA breakage may temporarily shut down DNA replication and allow DNA repair to occur.

Constitutive poly(ADP-ribosylation) appears to play a role in several facets of chromatin and DNA metabolism (Althaus & Richter, 1987; Althaus et al., 1985). The relatively rapid turnover of poly(ADPR) chains renders this posttranslational protein modification particularly suitable for regulatory purposes. Poly(ADPR) can have both structural and functional effects. For example, the poly(ADPR) of histones may alter nucleosomal conformation (Niedergang et al., 1985; Poirier et al., 1982; Adamietz & Rudolph, 1984) and higher order chromatin structure in relationship to replication, repair, recombination, and transcription (Althaus & Richter, 1987; Althaus et al., 1985). Support for a structural role of poly(ADPR) derives from the observation that a majority of the poly(ADP-ribosylated) proteins are associated with the nuclear matrix (Adolph & Song, 1985a,b; Wesierska-Gadek & Sauerman, 1985; Cardenas-Corona et al., 1987). Poly(ADPR) of enzymes involved in DNA metabolism such as topoisomerases (Ferro & Olivera, 1982, 1984; Ferro et al., 1983), ligases (Ohashi et al., 1983; Teraoka et al., 1986), DNA-dependent RNA polymerase (Muller & Zahn, 1976), transcription factors, and ADPR-transferase itself (Adamietz & Rudolph, 1984; Kawaichi et al., 1981; Jump & Smulson, 1980; Singh et al., 1985) may regulate their activities. Poly(ADPR) is unique because it is stimulated as a consequence of DNA strand breakage caused by a variety of mutagens and carcinogens. Therefore, besides its physiological functions, poly(ADPR) is bound to participate in the processsing and expression of DNA damage.

Active oxygen (AO) and other oxidants act as tumor promoters (Cerutti, 1985, 1986). The mechanism of action of oxidant promoters appears to involve the modulation of the expression of growth- and differentiation-related genes and cytotoxic effects (Muehlematter et al., 1988; Crawford & Cerutti, 1988). AO and poly(ADPR) affect the redox state and energy metabolism of the cell, and AO induces DNA strand breaks that activate ADPR-transferase. Therefore, AO-induced poly(ADPR) of chromosomal proteins is likely to play a role in oxidant promotion (Singh et al., 1985).

In the present work we have focused on poly(ADPR) of ADPR-transferase, the biosynthetic enzyme that polymerizes NAD residues, and topoisomerase I in intact mouse epidermal cells JB6 (clone 41). Our results give insights into the steady-state levels of constitutive poly(ADPR) of these enzymes and of poly(ADPR) in response to AO and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) that was used as a "positive" control (Cerutti et al., 1987).

Materials and Methods

Materials

Xanthine and benzamide were purchased from Sigma, and xanthine oxidase was purchased from Böhringer Mannheim. Phenylboronate agarose matrix gel PBA30 and ultrafiltration

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