Mannostatin A, a New Glycoprotein-Processing Inhibitor†

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ABSTRACT: Mannostatin A is a metabolite produced by the microorganism *Streptoverticillium verticillus* and reported to be a potent competitive inhibitor of rat epididymal α-mannosidase. When tested against a number of other arylglycosidases, mannostatin A was inactive toward α- and β-glucosidase and galactosidase as well as β-mannosidase, but it was a potent inhibitor of jack bean, mung bean, and rat liver lysosomal α-mannosidases, with estimated IC_{50}'s of 70 nM, 450 nM, and 160 nM, respectively. The type of inhibition was competitive in nature. This compound also proved to be an effective competitive inhibitor of the glycoprotein-processing enzyme mannosidase II (IC_{50} of about 10–15 nM with *p*-nitrophenyl α-D-mannopyranoside as substrate, and about 90 nM with [^{3}H]mannose-labeled GlcNAc-Man_5GlcNAc as substrate). However, it was virtually inactive toward mannosidase I. The N-acetylated derivative of mannostatin A had no inhibitory activity. In cell culture studies, mannostatin A also proved to be a potent inhibitor of glycoprotein processing. Thus, in influenza virus infected Madin Darby canine kidney (MDCK) cells, mannostatin A blocked the normal formation of complex types of oligosaccharides on the viral glycoproteins and caused the accumulation of hybrid types of oligosaccharides. This observation is in keeping with other data which indicate that the site of action of mannostatin A is mannosidase II. Thus, mannostatin A represents the first nonalkaloidal processing inhibitor and adds to the growing list of chemical structures that can have important biological activity.

Glycoproteins are a most diverse group of biological polymers that are ubiquitous constituents of nearly all forms of life. They occur in cells, in both soluble and membrane-bound form, as well as in the intracellular matrix and extracellular fluids. Included in this class of compounds are enzymes, immunoglobulins, hormones, transport proteins, toxins, lectins, and structural proteins, to name a few (Wagh & Bahl, 1981). Over the years, it has become apparent that the carbohydrate portions of glycoproteins do not perform a single function but act in a variety of ways, probably depending on the glycoprotein in which they occur (Olden et al., 1982). In some cases, the sugar side chain may be directly involved in the biological activity of the glycoprotein (Dahms et al., 1989), whereas in other cases, it may perform any of a number of less obvious ancillary functions (Rademacher et al., 1988).

Among the common types of glycoproteins that are found in nature are those having N-linked or asparagine-linked oligosaccharides (Kornfeld & Kornfeld, 1985). The biosynthesis of the oligosaccharide portion of these N-linked glycoproteins involves a complex sequence of events that can be divided into three general steps: (1) synthesis of the common intermediate, $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol, (2) transfer of the oligosaccharide portion of this common precursor to specific asparagine residues on the protein, and (3) trimming of the carbohydrate unit and addition of peripheral sugars and other constituents to give a variety of different oligosaccharide side chains (Hubbard & Ivatt, 1981). Steps 1 and 2 appear to be common to the biosynthesis of all types of N-linked oligosaccharides, whereas the trimming and processing (step 3) may vary with different cell types (Snider, 1984). It is this variability in processing that gives rise to the different types of oligosaccharides (i.e., high-mannose, complex, and hybrid) that are found in nature.

The study of the biosynthesis and function of the oligosaccharide portion of the N-linked oligosaccharides has been greatly facilitated by the use of inhibitors that act at various steps in the biosynthetic pathway (Elbein, 1987). Several of the inhibitors that have been used extensively over the past several years include castanospermine (Pan et al., 1983) and deoxynojirimycin (Saunier et al., 1982) which inhibit glucosidase I and glucosidase II, swainsonine which inhibits the Golgi mannosidase II (Tulsiani et al., 1982; Elbein et al., 1981), and deoxymannojirimycin (Fuhrmann et al., 1984; Elbein et al., 1984) which inhibits the Golgi mannosidase I. A common feature of these, and all other processing inhibitors described thus far, is that they are polyhydroxylated alkaloids exhibiting a structural similarity to the sugar recognized by the particular glycosidase in question (Elbein & Molyneux, 1987).

Recently, a new compound called mannostatin A (Figure 1) was isolated from the culture filtrate of the microorganism *Streptoverticillium verticillus* var. quintum ME3-AG3 (Aoyagi et al., 1989) and was shown to be a potent inhibitor of the rat epididymal α-mannosidase. Since this compound represents the first reported example of a "nonalkaloid" inhibitor of aryl-α-mannosidase, it was tested as a potential inhibitor of glycoprotein processing. Mannostatin A was found to be a potent inhibitor of the Golgi processing mannosidase II but was inactive toward the processing mannosidase I. In cell culture, mannostatin A blocked glycoprotein processing and caused an increase in hybrid types of oligosaccharides,

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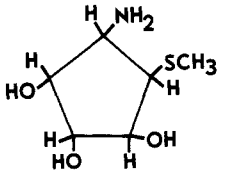

FIGURE 1: Structure of mannostatin A.

consistent with an inhibition in mannosidase II activity. The significance of these findings in relation to this new chemical structure is discussed.

Experimental Procedures

Materials. [4,5-^{3}H]Leucine (50 Ci/mmol) and endo-β-*N*-acetylglucosaminidase H (Endo H[1] from *Streptomyces griseus*) were purchased from ICN Biochemicals. [2-^{3}H]-Mannose (27 Ci/mmol) was obtained from New England Nuclear Inc. Pronase (from *Streptomyces griseus*) was from Calbiochem, and 1-deoxymannojirimycin was from Genzyme. Concanavalin A–Sepharose, amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver or jack beans), α-mannosidase (from jack beans), β-*N*-acetylglucosaminidase (from jack beans), and all of the *p*-nitrophenyl glycoside substrates were purcahsed from Sigma Chemical Co. β-Mannosidase was purified from *A. niger* as previously described (Elbein et al., 1977). Mannosidase I, mannosidase II, and aryl-α-mannosidase were purified from mung bean seedlings (Szumilo et al., 1986; Kaushal et al., 1990). Rat liver fractions rich in aryl-α-mannosidase and mannosidase II were isolated from Percoll gradient fractionations (Tropea et al., 1988) of the 410000 g·min pellet as described (Ragab et al., 1967). [^{3}H]Mannose-labeled $Man_9GlcNAc$, for use as a substrate for mannosidase I, was prepared in influenza virus infected MDCK cells labeled with [2-^{3}H]mannose in the presence of the mannosidase I inhibitor deoxymannojirimycin (Szumilo et al., 1986). [^{3}H]Mannose-labeled GlcNAc-$Man_5GlcNAc$, for use as a substrate for mannosidase II, was produced from mannose-labeled $Man_9GlcNAc$ using the purified mung bean mannosidase I and a crude preparation of GlcNAc transferase I (Szumilo & Elbein, 1985; Kaushal et al., 1990). Tissue culture materials were from Flow Laboratories, Corning Glass Works, and/or Hyclone Labs. Biogel P-4, Biogel P-2, and Dowex 50-H^+ (100–200 mesh) were obtained from Bio-Rad Laboratories. Mannostatin A [4-amino-5-(methylthio)-1,2,3-cyclopentanetriol] was isolated from the culture filtrates of *S. verticillus* as previously described (Aoyagi et al., 1989). Swainsonine was isolated from *Astragalus lentiginosus* (Davis et al., 1984). All other chemicals were from reliable chemical sources and were of the best grade available.

Enzyme Assays. All enzyme assays were performed under conditions where hydrolysis of substrate was linear with time and protein concentration. For inhibition studies, mannostatin A and swainsonine were preincubated for 10 min with enzyme prior to the addition of substrate. For competition analysis, mannostatin A and substrate were added simultaneously.

(*A*) *Arylglycosidases.* The enzymatic activities of amyloglucosidase, β-glucosidase, α- and β-galactosidase, and α- and β-mannosidase, and the effects of mannostatin A on these enzymes, 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 4.5 for mung bean α-mannosidase, pH 4.0 for rat liver α-mannosidase, and pH 5.0 for all other enzymes), 2 μmol of *p*-nitrophenyl glycoside, various amounts of inhibitor, and enzyme, all in a final volume of 0.4 mL. Incubations were for 10–30 min at 37 °C, and the reactions were stopped by the addition of 2.5 mL of 0.4 M glycine buffer, pH 10.4. The *p*-nitrophenol liberated in the reaction was measured at 410 nm.

(*B*) *Mannosidase I and Mannosidase II.* Mannosidase I activity was measured by following the release of [^{3}H]mannose from [^{3}H]mannose-labeled $Man_9GlcNAc$ as previously described (Szumilo et al., 1986). Incubation mixtures contained 50 mM MES buffer, pH 6.0, 0.1% Triton X-100, 5 mM $CaCl_2$, 10000 cpm of $Man_9GlcNAc$, and enzyme, all in a final volume of 0.2 mL. The mixtures were incubated for 1 h at 37 °C, and the liberated radioactive mannose was measured with a concanavalin A binding assay (Szumilo & Elbein, 1985). Mannosidase II activity was determined by measuring the release of [^{3}H]mannose from [^{3}H]mannose-labeled GlcNAc-$Man_5GlcNAc$, or by measuring the release of *p*-nitrophenol from *p*-nitrophenyl α-mannoside. Incubation mixtures contained 50 mM MES buffer, pH 6.0, 0.1% Triton X-100, 10000 cpm of GlcNAc-$Man_5GlcNAc$ or 5 mM *p*-nitrophenyl α-mannoside, and enzyme, all in a final volume of 0.4 mL. Product formation was measured after a 30–60-min incubation at 37 °C, using the Concanavalin A binding assay for radioactive substrates or the colorimetric assay for the *p*-nitrophenyl glycoside. Rat liver membranes rich in mannosidase II activity were assayed by using the spectrophotometric assay described for mung bean mannosidase II.

Preparation of N-Acetylmannostatin A. Mannostatin A was N-acetylated according to the method of Distler (Distler et al., 1958) as modified by Carlson (1972). Briefly, 5 mg of mannostatin A (26.6 μmol) was reacted for 1 h at 0 °C with a 10-fold molar excess of acetic anhydride in 40% aqueous CH_3OH, containing 0.4 M $NaHCO_3$. The reaction mixture was then adjusted to pH 4.5 with HAc and applied to a Dowex-50-H^+ column. The wash of the column, containing the *N*-acetylmannostatin, was lyophilized. This recovered material was designated as *N*-acetylmannostatin on the basis of the following criteria: (1) its failure to bind to Dowex-50-H^+; (2) a negative ninhydrin reaction (relative to mannostatin A); (3) a positive Dragendorff's reaction; (4) an increase in mobility, relative to mannostatin A, on 0.2-mm silica gel G plates run in 1-butanol/acetic acid/water (3:1:1); (5) NMR and mass spectroscopic analysis consistent with the structure being *N*-acetylmannostatin (data not shown).

Growth and Labeling of Influenza Virus. The NWS strain of influenza virus was grown in Madin Darby canine kidney (MDCK) cells as previously described (Pan et al., 1983). Confluent monolayers grown in modified Eagle's medium with 10% fetal calf serum were infected with virus at a multiplicity of infection of about 75 plaque forming units/mL. The infected cells were placed in modified Eagle's medium containing 2% fetal calf serum, and various amounts of mannostatin A were added. Following an incubation of 1 h to allow the inhibitor to take effect, [2-^{3}H]mannose (10 μCi/mL) was added to each flask. Cells were then incubated at 35 °C for 40–48 h until visible lysis of cells had occurred and mature virus was formed. At this time, the media containing viral

[1] Abbreviations: Con A, concanavalin A; Endo H, endo-β-*N*-acetylglucosaminidase H; IC_{50}, concentration of inhibitor that gives 50% inhibition of enzymatic activity; MDCK cells, Madin Darby canine kidney cells; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

particles and lysed cells was removed and saved, and any cells still adhering to the plastic were removed with a plastic scraper. The pooled media and lysate were centrifuged at 10000*g* for 15 min to obtain the cell pellets, and the supernatant liquid was centrifuged at 100000*g* for 12 h to obtain the viral pellets.

Preparation and Analysis of Glycopeptides. The virus, isolated by ultracentrifugation, was digested exhaustively with Pronase to generate glycopeptides for analysis (Tropea et al., 1989). The digests were treated with trichloroacetic acid to remove the protein, and the supernatant liquid was extracted with ethyl ether to remove the trichloroacetic acid. After concentrating to a small volume, the aqueous extract was fractionated on 1.5 × 80 cm column of Bio-Gel P-4 (100–200 mesh), equilibrated, and run in 0.35% HAc. Aliquots of every other tube were removed for the determination of radioactivity. Since this column did not completely resolve the complex types of glycopeptides from the hybrid or high-mannose types, the entire glycopeptide peak was pooled and digested exhaustively with endoglucosaminidase H. The digests were then rechromatographed on the same Bio-Gel P-4 column.

Characterization of Oligosaccharides Formed in the Presence of Inhibitor. The structures of the oligosaccharide(s) produced in the presence of mannostatin A were determined by a combination of chromatographic and enzymatic methods.

(*A*) *Chromatographic Methods.* The radiolabeled oligosaccharides released by Endo H were chromatographed on a long, calibrated column of Bio-Gel P-4 (1.5 × 150 cm, 200–400 mesh), equilibrated in 1% HOAc. A variety of standard oligosaccharides were run on this column for calibration. The various labeled oligosaccharides and glycopeptides were also chromatographed on small columns of concanavalin A–Sepharose (1.5 mL of gel in a 3-mL syringe) to compare the elution profiles to those of control cells. Samples of known radioactivity were applied to the concanavalin A columns, and the columns were washed well with buffer (20 mM Tris buffer, pH 7.2, containing 200 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$) to remove any unbound material. Fractions of 1.0 mL were collected and examined for their radioactive content. Material bound to the column was then eluted, first with 10 mM methyl α-glucoside to elute biantennary complex chains and hybrid structures, and then with 200 mM methyl α-mannoside to elute high-mannose structures. Fractions of 1.0 mL were collected and examined for their radioactive content.

(*B*) *Enzymatic Methods.* Mannostatin A induced oligosaccharides were digested with various enzymes (α-mannosidase, β-galactosidase from jack beans) to determine the sequence of sugars, as well as the structure of each oligosaccharide. Following each digestion, the products were identified by gel filtration on the calibrated Bio-Gel P-4 column. The assay mixtures for these digestions and the analysis of the products were as previously described (Tropea et al., 1989; Kaushal et al., 1988).

Effect of Mannostatin A on the Synthesis of Lipid-Linked Saccharides and Protein. The effect of this inhibitor on protein synthesis and on the formation of lipid-linked saccharide intermediates was examined in uninfected MDCK cells. Confluent monolayers were preincubated for 1 h in the presence of various amounts of mannostatin A in modified Eagle's medium containing 2% fetal calf serum. The cells were then incubated with [4,5-^{3}H]leucine (20 μCi/mL) for 15–120 min to label the cell proteins, or with [2-^{3}H]mannose to label the lipid-linked oligosaccharides and glycoproteins. After incubation with label, the monolayers were washed well with phosphate-buffered saline, and the cells were removed from the plates by scraping and placed in tubes. For the analysis of lipid-linked saccharides, the cell suspensions were sequentially extracted with $CHCl_3/CH_3OH/H_2O$ (1:1:1) and then with $CHCl_3/CH_3OH/H_2O$ (10:10:3) to isolate the lipid-linked monosaccharides and the lipid-linked oligosaccharides (Chambers & Elbein, 1975). For analysis of protein synthesis, cell suspensions were sonicated and then extracted with 20% trichloroacetic acid. Bovine serum albumin (5 mg) was added to the extracts to help precipitate the proteins. After standing overnight at 0 °C, the precipitated protein was isolated by centrifugation and washed twice with 5% TCA, once with absolute CH_3OH, twice with 50% CH_3OH, and once with absolute CH_3OH. The samples were then digested with Pronase, and the digests were counted to determine their radioactive content.

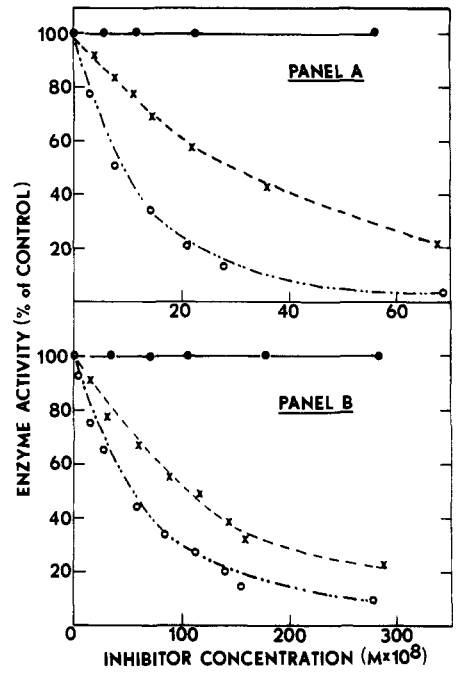

FIGURE 2: Comparison of the effect of concentration of mannostatin A, swainsonine, and *N*-acetylmannostatin A on the activity of jack bean α-mannosidase and mung bean α-mannosidase. Incubations were as described in the text, using *p*-nitrophenyl α-mannoside as substrate and the amounts of the various inhibitors as shown in the figure. The release of *p*-nitrophenol was measured at 410 nm. Activity is expressed relative to control incubations without inhibitor. (A) Jack bean α-mannosidase. (B) Mung bean α-mannosidase. (○) Mannostatin A; (×) swainsonine; (●) *N*-acetylmannostatin A.

Results

Effect of Mannostatin A on Various Arylglycosidases. Mannostatin A was originally reported to be a potent competitive inhibitor of rat epididymal α-mannosidase with an estimated IC_{50} of 0.1 μM (Aoyagi et al., 1989). In order to evaluate the specificity of this inhibitor, we further tested mannostatin A against a number of other arylglycosidases. This compound was found to be a very potent inhibitor of both jack bean and mung bean α-mannosidase, inhibiting the enzymatic activity by 50% at concentrations of 70 and 450 nM, respectively (Figure 2). These values are significantly lower than the IC_{50} values of swainsonine, an indolizidine alkaloid which until now was considered to be the most potent inhibitor of aryl-α-mannosidase. Kinetic analysis by the method of Lineweaver and Burk showed competitive inhibition (data not shown). In addition, mannostatin A also inhibited rat liver lysosomal α-mannosidase (data not shown) but with an IC_{50} equivalent to swainsonine (approximately 160 nM). Interestingly enough, when an acetyl group was chemically added

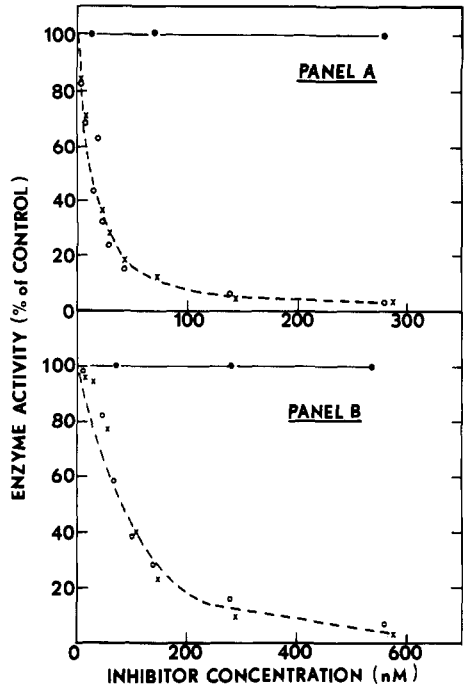

FIGURE 3: Effect of mannostatin A, *N*-acetylmannostatin A, and swainsonine concentrations on the activity of mung bean processing mannosidase II. Assay conditions were as described in the text. In panel A, purified mannosidase II was assayed with *p*-nitrophenyl α-mannoside as substrate, whereas in panel B, the enzyme was assayed with [^{3}H]GlcNAc-Man$_5$GlcNAc as substrate. (O) Mannostatin A; (×) swainsonine; (●) *N*-acetylmannostatin A.

to the amino group of mannostatin A, the corresponding *N*-acetylmannostatin A was completely inactive as an inhibitor (Figure 2), indicating that the integrity of the primary amine is required for inhibitory activity.

Mannostatin A was also tested against amyloglucosidase, β-glucosidase, α- and β-galactosidase, and β-mannosidase. At concentrations up to 100 μM, mannostatin A was completely inactive toward these other enzymes (data not shown).

Effect of Mannostatin A on the Processing Mannosidases. Mannostatin A was also tested as an inhibitor against partially purified mannosidase I and mannosidase II, isolated from mung bean seedlings. These glycosidases are involved in the oligosaccharide trimming reactions of N-linked oligosaccharides that occur in the early Golgi compartments (Kornfeld & Kornfeld, 1985). As shown in Figure 3, mannostatin A was found to be a very potent inhibitor of mannosidase II but was inactive against mannosidase I even at concentrations of 250 μM (data not shown). Thus, as shown in Figure 3, the inhibitory profile of mannostatin A was very similar to that of swainsonine toward mannosidase II when either the *p*-nitrophenyl α-mannoside was used as substrate (Figure 3A; IC_{50} about 10 nM) or the [^{3}H]mannose-labeled GlcNAc-Man$_5$-GlcNAc was the substrate (Figure 3B; IC_{50} about 90 nM). The type of inhibition (analyzed by the method of Lineweaver and Burk) was competitive when *p*-nitrophenyl α-mannoside was used as substrate. Similar results were observed with rat liver light membranes rich in mannosidase II activity (data not shown). Using the *p*-nitrophenyl glycoside substrate, an IC_{50} of approximately 15 nM was observed for both mannostatin A and swainsonine. In contrast, *N*-acetylmannostatin A was again completely inactive toward mannosidase II, regardless of which substrate was used.

Effect of Mannostatin A on Glycoprotein Processing in Culture. Since mannostatin A was found to be a potent α-mannosidase inhibitor and was strongly active against mannosidase II, we tested this compound to determine whether

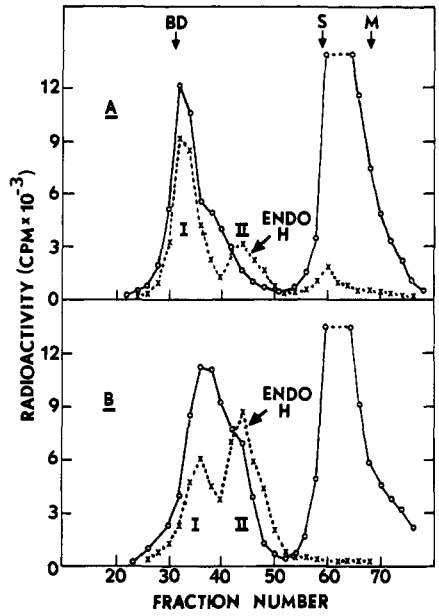

FIGURE 4: Effect of mannostatin A on the oligosaccharide composition of the influenza viral glycoproteins. Infected MDCK cells were incubated for 1 h in the absence (profile A) or presence (profile B) of 20 μg/mL mannostatin A (110 μM) and then labeled by the addition of [2-^{3}H]mannose to the culture medium. After an incubation of about 40 h, the mature virus was isolated by ultracentrifugation and the viral glycoproteins were digested with Pronase. The glycopeptides were separated on the Bio-Gel P-4 columns (shown by the solid lines and open circles). The entire glycopeptide peak (fractions 26–50 in each profile) was pooled, treated with Endo H, and rechromatographed on the column (dashed line, ×). Aliquots of every other fraction were removed for the determination of radioactivity. Arrows indicate the following standards: BD = blue dextran T-2000; S = stachyose; M = mannose.

it would alter normal glycoprotein processing in cell culture. For these studies, influenza virus infected MDCK cells were incubated in the absence or presence of 20 μg/mL mannostatin A (110 μM) and then labeled for 40–48 h in the presence of [2-^{3}H]mannose. The mature virus was isolated by centrifugation and disgested exhaustively with Pronase to obtain glycopeptides. The glycopeptides were then isolated by gel filtration on columns of Bio-Gel P-4, as shown in Figure 4. Since these columns did not give good resolution of the complex types of glycopeptides from the high-mannose types, the entire glycopeptide peak was pooled, digested with endoglucosaminidase H to cleave the high-mannose (and hybrid) glycopeptides, and rechromatographed on the column.

The upper profile (A) of Figure 4 shows that the mannose-labeled glycopeptides from control virus emerged from the column as a rather broad peak with a shoulder, as shown by the solid line. This glycopeptide peak was pooled (fractions 26–50), treated with endoglucosaminidase H, and rechromatographed on the same column. The dashed line shows that this treatment resolved the pooled glycopeptides into two distinct peaks. The major peak (72% of the radioactivity), which is designated peak I, remained in the same position (fractions 28–38) as the original, while about 28% of the radioactivity was shifted to a slower migrating peak (fractions 40–50), designated peak II. Peak I has previously been characterized as being composed of complex types of N-linked glycopeptides, whereas peak II is composed of high-mannose structures (Pan et al., 1983). The elution profiles of these peaks from concanavalin A–Sepharose and Bio-Gel P-4, as described below, are consistent with these structures.

The lower profile of Figure 4 shows the glycopeptide profiles obtained from virus grown in the presence of 20 μg/mL

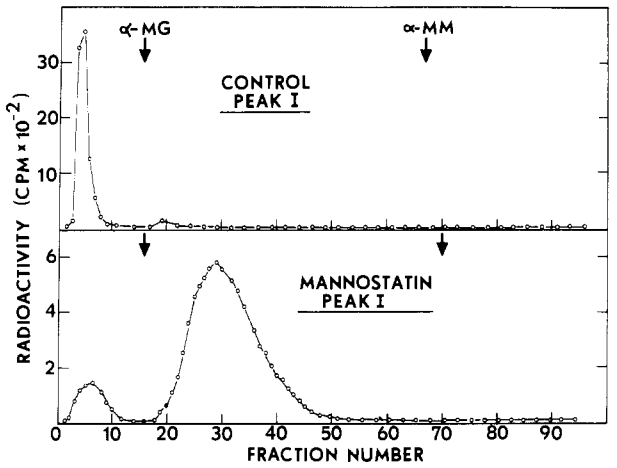

FIGURE 5: Identification of glycopeptides in peak I of control virus and mannostatin A treated virus by chromatography on columns of concanavalin A–Sepharose. Peak I of control virus (upper profile) was placed on the Con A column, and the column was washed well with equilibration buffer. Fractions were collected as shown. Then the column was eluted first with 10 mM methyl α-glucoside (α-MG) and then with 200 mM methyl α-mannoside (α-MM). The lower profile is that from peak I of mannostatin-treated virus.

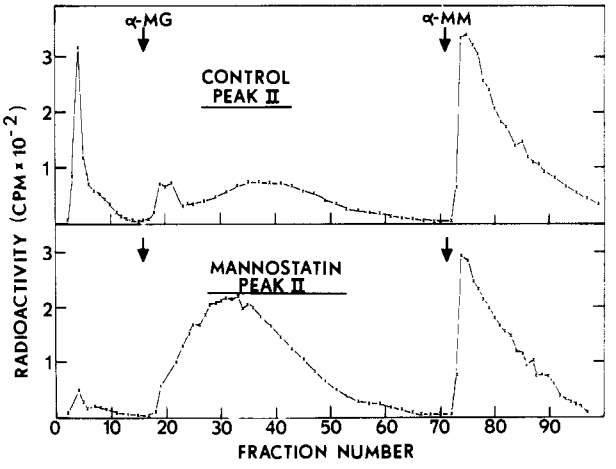

FIGURE 6: Identification of oligosaccharides in peak II of control virus and mannostatin-treated virus by chromatography on columns on concanavalin A–Sepharose. Columns were prepared and run as described under Experimental Procedures, and after sample application, the columns were washed with equilibration buffer and then eluted with 10 mM methyl α-glucose (α-MG) and then with 200 mM methyl α-mannoside (α-MM). Fractions were collected and analyzed for radioactivity. The upper profile is that of peak II of control virus, while the lower profile is peak II (see Figure 4) of the mannostatin-raised virus.

mannostatin A (110 μM). Again, the solid line shows the glycopeptide pattern before treatment with Endo H, while the dashed line shows the effects of Endo H treatment. It can be seen that mannostatin A caused a complete shift in the position of the initial glycopeptide peak so that it now emerged somewhat later from the column (i.e., peak fractions at tubes 36–38, rather than 32). When this glycopeptide was pooled and treated with Endo H, it gave rise to two peaks. One of these peaks (designated peak I) remained in the same position as the initial mannostatin A induced glycopeptide peak (dashed line; fractions 32–40), while the second peak (designated peak II) was shifted to later fractions. This second peak in the mannostatin A treated virus accounted for about 60% of the total radioactivity. Thus, mannostatin A caused marked alterations in glycoprotein processing and appeared to prevent the formation of complex types of N-linked oligosaccharides.

Characterization of the Glycopeptides and Oligosaccharides Produced in the Presence of Mannostatin A. The pooled glycopeptide peaks (i.e., peaks I in Figure 4) from control virus and from virus raised in the presence of mannostatin A were chromatographed on columns of concanavalin A–Sepharose as shown in Figure 5. The upper profile is that of the control virus (no inhibitor) and demonstrates that essentially all of the radioactivity in peak I emerges in the wash of the column, indicating that the radioactive mannose is present in complex glycopeptides of the triantennary and tetraantennary types. On the other hand, the lower profile shows that peak I from the mannostatin-grown virus (which migrated slower on Bio-Gel P-4 than peak I of controls) mostly bound to the concanavalin A–Sepharose columns and could be eluted with 10 mM methyl α-glucoside. These data indicate that this peak is mostly composed of biantennary and/or hybrid types of glycopeptides. When this peak was treated with jack bean α-mannosidase and the digestion mixture rechromatographed on the Bio-Gel column, there was a shift in the position of the oligosaccharide to a slower moving (i.e., smaller sized) peak with the release of free mannose (data not shown). These data suggest that peak I of the mannostatin A grown virus is composed mostly of hybrid types of N-linked oligosaccharides. More detailed characterization of these structures from peak II of the oligosaccharides produced in the presence of mannostatin A is discussed below and presented in Figure 7. Such hybrid structures would be consistent with the data presented earlier showing that mannostatin A is a potent inhibitor of mannosidase II and is inactive toward mannosidase I. The small peak of radioactivity that emerged in the wash of this column may represent small amounts of tri- or tetraantennary chains that are still produced in the presence of inhibitor. However, this peak represents less than 5% of the total radioactivity in the glycoproteins produced in the presence of mannostatin A.

Characterization of the Oligosaccharide Produced in the Presence of Mannostatin A. A combination of chromatographic and enzymatic techniques were employed to elucidate the structures of the oligosaccharides produced in the presence of mannostatin A and released by Endo H (i.e., peak II of Figure 4). This peak was first applied to concanavalin A–Sepharose columns to determine whether it would bind and to compare its elution pattern to that of peak II from control virus. Figure 6 presents the profiles obtained from these columns. The upper profile is that from control virus and demonstrates that most of the radioactivity in peak II binds firmly to the concanavalin A and requires 200 mM methyl α-mannoside for elution. This binding is consistent with high-mannose oligosaccharides. In this case, there was also some radioactivity that emerged in the wash and some that bound weakly and could be eluted with 10 mM methyl α-glucoside. It is likely that the material emerging in the wash is contamination from peak I since peaks I and II are not that well separated on the Bio-Gel P-4 column (see Figure 4). The radioactivity emerging in the methyl α-glucoside elution is probably due to small amounts of biantennary and hybrid structures that are produced by the normal virus.

The lower profile of Figure 6 demonstrates the results obtained with peak II of the virus grown in the presence of mannostatin A. In this case, two large peaks of radioactivity were eluted from the Con A column, one with methyl α-glucoside and the other with methyl α-mannoside. The peak eluting with methyl α-glucoside was further characterized by treatment with a number of exoglycosidases as shown in Figure 7. When this peak was initially chromatographed on the Bio-Gel P-4 column, it gave a series of peaks, but most of the radioactivity was in an area emerging with the he-

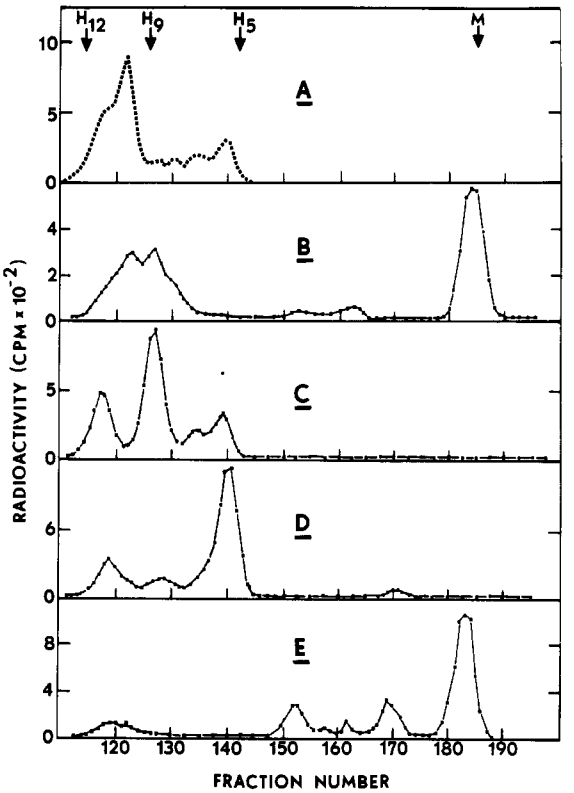

FIGURE 7: Identification of the structure of the peak II oligosaccharide produced in the presence of mannostatin A and eluted from Con A with methyl α-glucoside. Profile A shows the elution pattern of the methyl α-glucoside eluted radioactivity on a 1.5 × 150 cm column of Bio-Gel P-4. In other profiles shown in the figure, this oligosaccharide mixture was treated with various enzymes or mixtures of enzymes as follows: profile B, treatment with jack bean α-mannosidase; profile C, treatment with β-galactosidase; profile D, treatment with a mixture of β-galactosidase and β-*N*-acetylhexoasminidase; profile E, treatment with a mixture of α-mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase. In each case, the digestion mixture was rechromatographed on the column to identify the radioactive products. Standards shown by the arrows are as follows: H_{12}, hexose$_{12}$GlcNAc; H_9, hexose$_9$GlcNAc; H_5, hexose$_5$GlcNAc; M, mannose.

xose$_{10-11}$GlcNAc standards (profile A). This heterogeneity is not surprising since this material was synthesized in, and isolated from, virus-infected cultured cells. When this peak was treated with jack bean α-mannosidase, there was the release of free mannose and a shift in the size of the oligosaccharides to slower moving peaks (profile B). This α-mannosidase digestion released about 40% of the radioactive mannose, which is consistent with that expected for hybrid structures of the Man_5 type. There was also a shift in the size of some of the oligosaccharides to a smaller sized peak upon digestion with β-galactosidase (profile C). In this case there was no release of radioactive mannose. These data suggest that some of the hybrid structures contain a single terminal galactose residue, but the largest sized peak apparently does not have terminal galactose.

The most definitive data which strongly suggest that these are hybrid structures are shown in profiles D and E. In (D), it can be seen that when these oligosaccharides were treated with both β-galactosidase and β-*N*-acetylhexosaminidase, there was a considerable shift in the migration of most of the oligosaccharides to a single component that migrated with the $Man_5GlcNAc$ standard. This peak now accounted for 64% of the radioactivity. Most of the remaining radioactivity was still in two larger sized peaks which may also be hybrid structures but may have other terminal sugars that are resistant

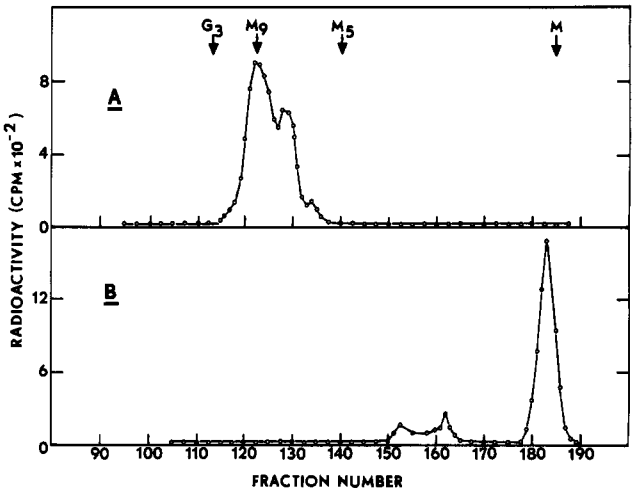

FIGURE 8: Identification of peak II oligosaccharide from virus grown in the presence of mannostatin A and eluted from Con A columns with methyl α-mannoside. The upper profile shows the elution pattern of the original oligosaccharides on a column of Bio-Gel P-4 (1.5 × 150 cm). In the lower profile, the oligosaccharide mixture was digested with jack bean α-mannosidase and rechromatographed on the same column. Standards shown by the arrows are as follows: G_3, $Glc_3Man_9GlcNAc$; M_9, $Man_9GlcNAc$; M_5, $Man_5GlcNAc$; M, mannose.

to these enzymes such as α-linked galactose or sialic acid. Finally, when the oligosaccharides were treated with all three glycosidases (i.e., α-mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase), much of the radioactive mannose was released as free mannose (>53%), and the remaining mannose was found in several smaller sized peaks. One of these peaks (fractions 167–172) is most likely Man-β-GlcNAc, whereas others may be hybrid structures that have other terminal sugars that are resistant to β-galactosidase or β-hexosaminidase, but which still have some susceptible mannose units. At any rate, although there are clearly several different oligosaccharide structures present in this peak II fraction, the data are consistent with them being mostly hybrid types of structures.

The other peak from the concanavalin A–Sepharose column that emerged with methyl α-mannoside (see Figure 6) was also characterized by enzymatic digestions to determine its structure, as shown in Figure 8. The upper profile shows that the initial peak was composed of several oligosaccharides that emerged from Bio-Gel P-4 in the same area as the $Man_{7-9}GlcNAc$ standards. As indicated by the lower profile, these oligosaccharides were almost completely susceptible to digestion by jack bean α-mannosidase, and this treatment resulted in the release of most of the radioactivity as free mannose (more than 80%). The remaining radioactivity was found in several small-sized peaks that migrated slower than the $Man_5GlcNAc$. One of these peaks is probably Man-β-GlcNAc, but the other is unidentified. These data indicate that most of the oligosaccharides that are found in the methyl α-mannoside elution of Con A columns of peak II from mannostatin-treated virus are of the high-mannose structure. Since the viral hemagglutinin normally contains about 25–30% of its oligosaccharides as high-mannose structures, these N-linked oligosaccharides would not be expected to be affected by mannostatin A.

Effect of Mannostatin on Protein Synthesis and on the Formation of Lipid-Linked Saccharides. Since mannostatin appeared to be an inhibitor of glycoprotein processing in cell culture, it was important to determine what effect it would have on the synthesis of protein in cells and also whether it would affect the synthesis of lipid-linked saccharides. For these

Table I: Effect of Mannostatin A on the Incorporation of [^{3}H]Leucine into Protein[a]

concn of mannostatin A (μM)	radioactivity (cpm) incorpd at time (min)			
	15	30	60	120
0	58 370	109 120	214 120	433 970
0.56	55 550	79 880	196 130	426 020
5.60	62 640	114 560	222 060	405 760
56.0	52 410	102 150	225 780	483 260
560.0	48 620	97 980	227 190	338 460

[a] Uninfected MDCK cells were grown in 25-cm^2 tissue culture flasks. At confluency, mannostatin A was added to some cultures to the final concentrations listed above. After a 1-h incubation to allow the inhibitor to take effect, [^{3}H]leucine 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 with PBS. The cells were released from the flasks by scraping, placed in tubes, and extracted with 20% TCA as described under Experimental Procedures. Radioactivity incorporated into total cellular protein was determined after Pronase digestion by liquid scintillation counting.

studies, uninfected MDCK cells were used. Thus, confluent monolayers of cells in MEM containing 2% fetal calf serum were incubated with various amounts of mannostatin A, and then either [2-^{3}H]mannose was added to label the lipid-linked saccharides and glycoproteins or [4,5-^{3}H]leucine was added to label the total proteins. Radioactivity incorporated into lipid-linked saccharides was determined after organic solvent extraction, and radioactivity incorporated into protein and glycoprotein was determined after Pronase digestion as described under Experimental Procedures.

Mannostatin A had no apparent effect on the overall rate of protein synthesis when added to cells at concentrations ranging from 0.1 to 100 μg/mL (0.56–560 μM), and for incubation times of up to 120 min. These data are shown in Table I. Similar results were observed for the incorporation of [^{3}H]mannose into lipid-linked saccharides as presented in Table II. In terms of the formation of dolichyl-P-mannose, the amount of radioactivity incorporated in two different experiments was somewhat low, but nevertheless there was no apparent inhibition seen even at 100 μg/mL (560 μM) mannostatin A. Likewise, in terms of mannose incorporation into lipid-linked oligosaccharides and into protein, there did not appear to be any significant inhibition, except perhaps of mannose incorporation into glycoprotein at the highest concentration used (i.e., 100 μg/mL, or 560 μM). However, this concentration of mannostatin A is at least 5-fold higher than the concentrations used in this study to inhibit glycoprotein processing.

Discussion

Both plants and microorganisms have been rich sources of glycosidase inhibitors, many of which have been shown to alter glycoprotein processing by specifically inhibiting one of the processing glycosidases (Trusheit et al., 1981; Elbein, 1987).

Mannostatin A is a metabolite produced by the fungus *S. verticillus*, which was reported to be a potent, competitive inhibitor of rat epididymal α-mannosidase (Aoyagi et al., 1989). During our initial studies, we found this compound to be an effective competitive inhibitor of both jack bean and mung bean aryl-α-mannosidases (IC_{50} values of 70 nM and 450 nM, respectively), as well as partially purified lysosomal α-mannosidase from rat liver (IC_{50} of about 160 nM). In contrast, mannostatin A had no detectable inhibitory activity against α- or β-glucosidase, α- or β-mannosidase. Thus, this inhibitor shows some degree of selectivity.

Mannostatin A was also found to be a potent competitive inhibitor of the mung bean glycoprotein processing enzyme, mannosidase II, but was without effect on the other processing mannosidase, mannosidase I. Relative to swainsonine, a well-characterized mannosidase II inhibitor, mannostatin A was found to be equally potent when tested against the synthetic substrate (i.e., *p*-nitrophenyl α-mannoside, IC_{50} of about 10 nM) or the natural substrate (i.e., GlcNAc-Man_5-GlcNAc, IC_{50} of about 90 nM). Similar results were obtained when mannostatin A was tested against a Golgi-rich membrane fraction isolated from rat liver (IC_{50} of about 15 nM for mannosidase II), indicating that this inhibitor is active toward both plant and animal enzymes.

Table II: Effect of Mannostatin A on the Incorporation of [^{3}H]Mannose into Lipid-Linked Saccharides and Glycoproteins[a]

concn of mannostatin A (μM)	radioactivity (cpm)					
	lipid-linked monosaccharides		lipid-linked oligosaccharides		glycoprotein	
	set 1	set 2	set 1	set 2	set 1	set 2
0	280	260	8870	9680	18 870	21 180
0.56	270	290	11150	9710	21 075	17 550
5.60	250	280	7150	9480	17 490	19 590
56.0	270	370	9040	8270	17 520	21 220
560.0	230	230	7270	8180	13 740	13 750

[a] Uninfected MDCK cells were grown in 25-cm^2 tissue culture flasks. At confluency, mannostatin A was added to some cultures to the final concentrations listed above. After a 1-h incubation to allow the inhibitor to take effect, [^{3}H]mannose was added to a final concentration of 25 μCi/mL, and the incubations were continued. After 1 h the medium was removed by aspiration and the monolayers were washed with PBS. The cells were released from the flasks 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 residual protein pellet was digested with Pronase to solubilize labeled glycoprotein. The total radioactivity incorporated into the lipid-linked sugars and glycoprotein was determined by liquid scintillation counting.

Since mannostatin A inhibited mannosidase II 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 used. The results clearly indicate that mannostatin A did alter the normal processing of the viral N-linked glycoproteins, causing a complete change in the migration properties of the initial glycopeptide and a significant change in the profiles after digestion with endoglucosaminidase H. Partial characterization of these structures indicated that mannostatin A caused the accumulation of hybrid types of N-linked glycopeptides as well as an increase in the high-mannose chains. The effect of mannostatin A on glycoprotein processing was not due to any alteration in the formation of lipid-linked saccharides or the synthesis of protein, since neither of these reaction sequences was inhibited by mannostatin A, except perhaps at high concentrations. On the basis of the structures of the oligosaccharides produced in its presence, and the fact that mannostatin A inhibits mannosidase II in vitro, it seems most likely that this enzyme is also the site of inhibition in cell culture.

Until now, all reported glycoprotein-processing inhibitors have been alkaloids that are polyhydroxylated and are structurally related to the sugar that is recognized by the particular enzyme in question (Elbein & Molyneux, 1987; Elbein, 1989). For example, castanospermine, an indolizidine alkaloid that is structurally related to glucose, is a potent inhibitor of a number of glucohydrolases including the processing glucosidase I and glucosidase II (Pan et al., 1983; Szumilo et al., 1986). Others include the furanose derivative 1,4-dideoxy-1,4-imino-D-mannitol (Palamarczyk et al., 1985), the piperidine de-

rivative 1-deoxymannojirimycin (Fuhrmann et al., 1984; Elbein et al., 1984), and the previously mentioned indolizidine alkaloid swainsonine. These three alkaloids all show a structural similarity to mannose, inhibit α-mannosidase in vitro, and alter glycoprotein processing in cell culture due to their inhibitory activity on one of the two processing mannosidases.

Mannostatin A is structurally different from these inhibitors in that it has a unique cyclopentanol structure containing thiomethyl and amino functional groups (Morishima et al., 1989). It represents the first reported example of a nonalkaloid enzyme inhibitor capable of altering glycoprotein processing in cell culture. There are, however, certain critical features of this new compound which, like the alkaloids, may be essential for biological activity. These include a polyhydroxylated ring structure and a nitrogen functional group. In this latter respect, it is important to note that the *N*-acetyl derivative of mannostatin A is completely devoid of activity. Whether this is a result of steric interference of the acetylated amino group with the enzyme, an inability of the amino functional group to become charged, or simply a change in the overall conformation of the inhibitor, is not known. However, these results do suggest that the nitrogen may play a key role in the inhibitory activity of this new compound. Thus, mannostatin A represents the first member of a new class of glycoprotein-processing inhibitors and adds to the ever-expanding list of chemical structures that can have important biological activities.

Registry No. Mannostatin A, 102822-56-0; mannosidase II, 82047-77-6; α-mannosidase, 9025-42-7; *N*-acetylmannostatin A, 129467-31-8.

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