GLUCURONYL GLYCINE, A NOVEL N-TERMINUS IN A GLYCOPROTEIN

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SUMMARY: Treatment of cutinase, an extracellular glycoprotein produced by *Fusarium solani* f. *pisi*, with NaB^3H_4 at pH 7.0 generated labeled enzyme. Acid hydrolysis showed that all of the label was in an acidic carbohydrate which was identified as gulonic acid. The N-terminal amino group of the enzyme is blocked; the precursor of gulonic acid has a free reducing group and it is attached via a linkage resistant to β-elimination. Furthermore, pronase digestion of NaB^3H_4-treated cutinase gave rise to a ninhydrin negative compound which contained the bulk of the ^3H and this compound was identified as N-gulonyl glycine. These results strongly suggest that the amino group of glycine, the N-terminal amino acid of this enzyme, is in amide linkage with glucuronic acid.

The N-terminal amino group of many proteins is blocked by an acetyl or a pyroglutamyl moiety. Our recent evidence showed that the N-terminal amino group of cutinase, an extracellular glycoprotein (1), does not react with the usual amino group reagents such as dansyl chloride, suggesting that the N-terminus of this protein is blocked. Evidence presented in this communication strongly suggests that the amino group of glycine, the N-terminal amino acid of this protein, is in amide linkage with glucuronic acid. Such an N-terminal blocking group has not been heretofore reported in any other protein.

EXPERIMENTAL

Materials. NaB^3H_4 (293 mCi/mmole) was purchased from Amersham/Searle Corp. γ-Gulonolactone and other sugar standards were gifts from Dr. Frank Loewus of this Department. Glycine methyl ester·HCl and protease (*Streptomyces griseus*) were purchased from Sigma Chemical Co. N-Methyl-N-nitroso-p-toluene sulfonamide was obtained from Aldrich Chemical Co. MN-Cellulose powder 300 was purchased from Macherey, Nagel & Co. Electrophoretically homogeneous cutinase I was prepared from the extracellular fluid of *F. solani pisi* as described before (2).

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NaB^3H_4 Treatment of Cutinase and Analysis of Products. This treatment was done essentially as described by Tanaka and Pigman (3) except for the pH. About 12 mg of electrophoretically homogeneous cutinase I was treated with 0.05 M Tris buffer, pH 7.0, containing 17 µmole NaB^3H_4 (5 mCi) for 216 hr at 4°C in the dark. Then 0.2 ml of 0.08 M $PdCl_2$ and one drop of n-octanal were added and the reaction mixture was incubated for 2 hr at 22°C. The final mixture was acidified and lyophilized repeatedly to remove the exchangeable tritium. The mixture was dissolved in 1 ml of H_2O and centrifuged. The clear supernatant was adjusted to pH 7.5 with 0.1 N NaOH, and was treated with 1.2 mg protease for 24 hr at 22°C. The mixture was centrifuged at 15,000*g* and the supernatant was loaded on a Bio-Gel P-2 column (1.2 × 110 cm) and eluted with H_2O. Since a substantial amount of ^3H was present in fairly large molecules, these radioactive fractions were pooled, lyophilized, and treated again with 1.2 mg protease as mentioned above. The reaction mixture was centrifuged and the supernatant was lyophilized.

The residue was dissolved in 1 ml of 0.1 N HCl and loaded on a column of Dowex 50W-X8 (50-100 mesh, 2.2 × 30 cm) and the column was eluted with 2 gel volumes of H_2O. The radioactive eluant contained about 80% of the ^3H applied to the column. A portion of this residue was treated with diazomethane essentially as described by Eisenbraum *et al.* (4). A cooled aqueous solution of the labeled material was acidified to pH 3.5 with HCl and an excess of an etherial solution of diazomethane was added. As the methyl ester remained in the aqueous layer, this phase was lyophilized. Another portion of the labeled material obtained from the above cation exchange column was purified by thin-layer chromatography on Silica Gel G with n-propanol:water (9:1 v/v) as the solvent system, and the isolated material (R_f, 0.23) was hydrolyzed by heating it with 6 N HCl in a sealed ampule at 110°C for 24 hr. The hydrolysate was lyophilized, dissolved in a minimal volume of water, and analyzed for amino acids by thin-layer chromatography on Silica Gel G with methyl ethyl ketone: acetic acid:methanol (4:2:1 v/v) as the developing solvent. Another portion of the hydrolysate was analyzed with a Beckman amino acid analyzer column (AA-15, cation exchanger, 9 mm i.d. × 55.5 cm).

Synthesis of N-Gulonyl Glycine Methyl Ester. Three mmoles of gulonolactone, 3 mmoles of glycine methyl ester, and 1.7 mmoles of anhydrous sodium carbonate were dissolved in 40 ml of absolute methanol and the mixture was heated under reflux at 65°C under nitrogen for 4 hr. The mixture was evaporated to dryness and dissolved in a minimal volume of H_2O. This solution was loaded on a Dowex-50W (H^+) column, the product was eluted with water, and the eluant was lyophilized. The unreacted gulonolactone present in the reaction product was separated by thin-layer chromatography using a MN-cellulose 300 with ethyl acetate:pyridine:H_2O (1:1:1 v/v) as the developing solvent.

Chromatographic Analyses. Thin-layer chromatography of carbohydrates were carried out employing both activated Silica Gel G and MN-cellulose 300 plates with methyl ethyl ketone:acetic acid:methanol (4:2:1 v/v) and ethyl acetate:pyridine:H_2O (1:1:1 v/v) as the developing solvents, respectively. Benzidine-KIO_4 spray was used to locate the carbohydrates on the thin-layer chromatograms. Radio gas-liquid chromatographic analyses were conducted with a Perkin-Elmer 811 gas chromatograph attached to a Barber-Colman radioactivity monitor. A coiled stainless-steel column (78" × 1/4") was packed with 5% OV-1 on 80 to 100 mesh Gas Chrom Q with an argon flow rate of about 85 ml/min. For the high pressure liquid chromatography, a Waters Associates model ALC/GPC 244 instrument equipped with a µBondapak/carbohydrate column (4 mm i.d. × 30 cm) and differential refractometer was used.

Preparation of Derivatives. Trimethyl silyl derivatives of gulonolactone and N-gulonyl glycine methyl ester were prepared by treatment with a 2:1 mixture of bis-N,O-trimethyl silyl acetamide and pyridine at 90°C for 1 hr.

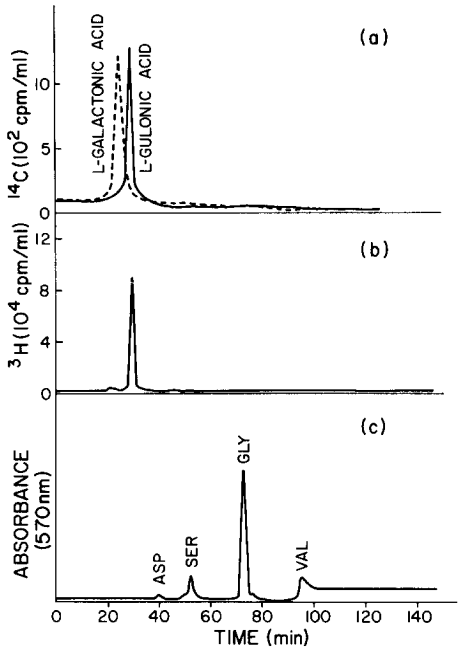

Figure 1. (a) Elution of authentic labeled gulonic acid and galactonic acid from the amino acid analyzer. (b and c) Analysis of the hydrolysate of the ninhydrin negative component isolated from the pronase digest of NaB^3H_4-treated cutinase; the eluant from the amino acid analyzer was analyzed for ^3H (b) and for the ninhydrin reaction products (c).

The excess reagent was evaporated off with a stream of N_2, and the product was dissolved in chloroform-methanol for gas chromatography. The gulonate collected from the amino acid analyzer was desalted with a Dowex 1X column (1.2 × 30 cm; formate). The lactone of gulonic acid was prepared by evaporating to dryness the acidic solution at 80°C, followed by heating of the dry residue at 80°C for 4 hr.

Determination of the Radioactivity. Radioactivity on thin-layer plates was determined by assaying the scraped gel directly in counting vials containing 15 ml of a 0.4% solution of Omnifluor in a 7:3 mixture of toluene and ethanol with a Packard liquid scintillation spectrometer. Radioactivity in liquid samples was determined similarly with Scinti Verse as counting fluid.

RESULTS AND DISCUSSION

Cutinase, an extracellular enzyme which hydrolyzes the biopolyester cutin, was recently found to be a glycoprotein containing about 5 residues of carbohydrates per mole of the enzyme (22,000 mol. wt.). Elimination of the carbohydrates under basic conditions in the presence of NaB^3H_4 resulted in tri-

tiation of the protein. Hydrolysis of the protein, followed by analysis of the products, showed that, in addition to alanine, α-aminobutyric acid, phenylalanine, and tyrosine, an acidic carbohydrate was labeled (1). This labeled material eluted from the amino acid analyzer between cysteic acid and aspartic acid. The label coincided with L-gulonic acid, but not galactonic acid (Fig. 1a). Treatment of the protein with NaB^3H_4 under neutral conditions, followed by gel filtration with Bio-Gel P-2, showed that the protein was labeled, but hydrolysis of the protein followed by analysis of the products on an amino acid analyzer revealed that only the acidic carbohydrate component was labeled. No amino acid was labeled as might be expected, because labeling of the amino acids results from reduction of the dehydro-amino acids generated by β-elimination of the carbohydrate. These results showed that this protein contained a covalently attached acidic carbohydrate which does not undergo β-elimination. Furthermore, the hèmiacetal function of the acidic sugar was free to react with NaB^3H_4 and thus eliminating the possibility of any attachment involving a glycosidic linkage. Obviously, the linkage of this acidic carbohydrate involved either a hydroxyl or the carboxyl group. The N-terminal amino group of cutinase did not react with dansyl chloride strongly, suggesting that the N-terminal amino group is blocked. Therefore, it appeared possible that the amino group might be involved in an amide linkage with the carboxyl group of the acidic carbohydrate.

The tritiated acidic carbohydrate was retained by a Dowex 1 (formate) anion exchange resin, and the label was eluted with authentic gulonic acid when 0.1 N formic acid was used as the eluant. The labeled acidic component thus obtained was lactonized under conditions normally used for preparing gulonolactone. Radio gas-liquid chromatography of the trimethylsilyl derivative of the lactone showed that the radioactivity was coincident with the coinjected trimethylsilyl derivative of authentic gulonolactone (Fig. 2, left). High pressure liquid chromatographic analysis showed that virtually all of the radioactivity was eluted with coinjected gulonolactone (Fig. 2, right).

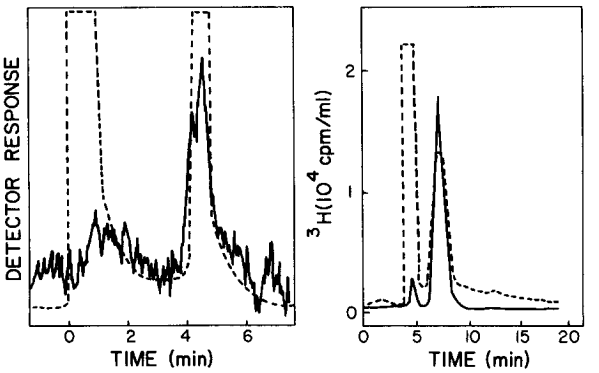

Figure 2. Radio gas-liquid chromatogram (left) and high-pressure liquid chromatogram (right) of the trimethylsilyl derivative of the lactonized acidic carbohydrate obtained by acid hydrolysis of NaB^3H_4-treated cutinase. The dotted lines indicate coinjected authentic standards; left, flame ionization detector response due to trimethylsilyl derivative of gulonolactone; right, differential refractometer response due to gulonolactone.

Therefore, we conclude that the labeled acidic carbohydrate is gulonic acid, which must have been derived by the NaB^3H_4 reduction of glucuronic acid attached to the protein.

If glucuronic acid is attached by an amide linkage to the N-terminus of the protein, NaB^3H_4 reduction followed by proteolytic digestion of the protein isolated after reduction might give us the gulonic acid amide of the N-terminal amino acid. Such an experimental approach showed that the bulk of the ^3H (80%) contained in the pronase digest was not retained by a cation exchange resin, which retained the amino acids present in the hydrolysate. When the labeled material was subjected to thin-layer chromatography all of the ^3H was found in a ninhydrin negative component. This material upon acid hydrolysis became reactive with ninhydrin. Therefore, it is obvious that the amino group which was not free in the labeled material was released by the hydrolysis. Thin-layer chromatography of the hydrolysate showed one ninhydrin positive component which had an R_f identical to that of glycine. The radioactivity was contained in a more polar material. When the acid hydrolysate was analyzed by an amino acid analyzer, glycine was found as the dominant

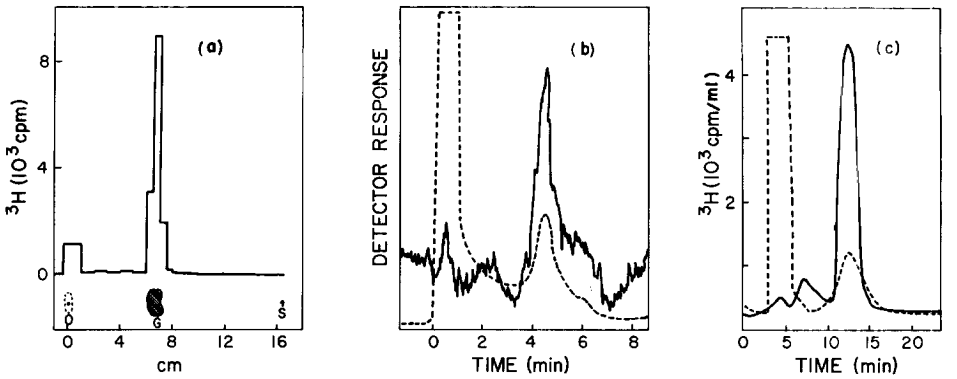

Figure 3. Thin-layer (a), gas-liquid (b), and high-pressure liquid (c) chromatograms of the ninhydrin negative material isolated from the pronase digest of NaB^3H_4-treated cutinase. G in a and the dashed lines in b and c represent synthetic N-gulonyl glycine methyl ester. S, solvent front.

amino acid (Fig. 1c). The radioactivity emerged from the amino acid analyzer in an acidic component coincident with gulonic acid (Fig. 1b). From the specific activity of the gulonic acid and from the amount of glycine as measured by the amino acid analyzer, it was calculated that at least 0.63 moles of labeled gulonic acid was present per mole of glycine. Since incomplete reduction of the glucuronic acid attached to the protein is a likely possibility under the present experimental conditions, which were designed to give high specific activity, it appears that the labeled ninhydrin negative material is a compound containing one mole of glycine and one mole of gulonic acid. The obvious conclusion is that it is a gulonic acid amide of glycine.

In order to further test the above conclusion, gulonic acid amide of glycine methyl ester was synthesized. The synthetic material was compared with methyl ester of the ninhydrin negative labeled compound isolated from the pronase digest of the NaB^3H_4-treated cutinase. In all chromatographic systems, the labeled material isolated from the protein was identical to synthetic gulonic acid amide of glycine methyl ester. For example, thin-layer chromatography on cellulose powder showed that virtually all of the ^3H was contained in a compound with an R_f identical to that of the synthetic amide

(Fig. 3a). Radio gas-liquid chromatography of trimethylsilyl ether of the labeled material showed that all of the radioactivity was coincident with the trimethylsilyl ether of the synthetic material (Fig. 3b). High pressure liquid chromatography showed that virtually all of the ^{3}H eluted from the column with the coinjected synthetic gulonic acid amide of glycine methyl ester (Fig. 3c). These results show that the labeled material isolated from the pronase digest of the $NaB^{3}H_{4}$-treated enzyme was gulonic acid amide of glycine.

The above results leave little alternative but the conclusion that $NaB^{3}H_{4}$ treatment of cutinase reduced glucuronic attached by an amide linkage to the amino group of glycine, the N-terminal amino acid of this enzyme. The present case constitutes the first report of the involvement of a uronic acid in blocking the N-terminal amino group of protein. A careful examination of some of the other extracellular glycoproteins might reveal that such a blockage is not restricted to the present enzyme.

Acknowledgments

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