BIOSYNTHESIS OF GLUCOHYDROLASE I, A GLYCOENZYME FROM ASPERGILLUS NIGER*

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Summary: Enzymes which contain carbohydrate units as integral structural components are appropriately designated as glycoenzymes. Two aspects of the biosynthesis of glucohydrolase I, a glycoenzyme from Aspergillus niger have been investigated. First, the glyco-portion of the glucohydrolase is synthesized via nucleotide diphosphate hexose pathways and the synthesis does not seem to be under direct genetic control. Second, data on the properties of glucohydrolase isolated from inside and outside the cell establish that the carbohydrate moieties become attached to the enzyme inside the cell rather than during transport of the enzyme through the cell membrane and wall.

An increasing number of enzymes have been found to contain carbohydrate moieties in their structure and such enzymes are therefore glycoproteins. Enzymes of this type studied in our laboratory include glucose oxidase from Aspergillus niger (Pazur, et al., 1965), glucanohydrolase (alpha amylase) from Bacillus subtilis and glucohydrolase (glucoamylase) from Aspergillus niger (Pazur, et al., 1963) and Rhizopus delemar (Pazur, et al., 1967). These enzymes all contain unique combinations of mannose, glucose, galactose or glucosamine covalently linked to their polypeptide chains. Other enzymes which have been shown to be glycoproteins are glucanohydrolase from Aspergillus oryzae (Hanafusa, et al., 1955), ribonuclease B from bovine pancreas (Plummer and Hirs, 1964), chloroperoxidase from Caldariomyces fumago (Morris and Hager, 1966), beta glucuronidase from bovine liver (Plapp and Cole, 1967), malic dehydrogenase from bovine aorta (Dugan, et al., 1967),

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acid phosphatase from rat kidney (Dugan, et al., 1967), and deoxyribonuclease from bovine pancreas (Catley, et al., 1969). The term, glycoenzyme, seems appropriately descriptive for this unique group of enzymes. We have been investigating pathways for the biosynthesis of the glyco-portion of glycoenzymes with the view of assessing a possible function for the carbohydrate units in the transport system of the organism. In this communication, results of our studies on the biosynthesis of glucohydrolase I, a glycoenzyme from Aspergillus niger are being reported.

A. niger produces two isoenzymes of glucohydrolase (Pazur and Ando, 1959) but because of ease of purification most of our experiments have been performed with the more abundant isoenzyme, glucohydrolase I. This isoenzyme contains approximately 100 moles of monosaccharide residues per mole of enzyme representing about 15% of the total weight of the enzyme molecule. Mannose constitutes the major fraction of the carbohydrate residues, the remainder being glucose and galactose. Results from several types of experiments point to nucleoside diphosphate hexose pathways as the route of synthesis for the glyco-portion of the enzyme. First, specifically labeled carbohydrates are incorporated into the glyco-portion of the enzyme with little randomization of the label; second, pyrophosphorylase activities of several types are present in a cell-free extract from the organism; and third, a transfer of hexose units from nucleoside diphosphate hexoses to a modified enzyme molecule is effected by a cell-free enzyme extract. It is tempting to suggest that, since the addition of carbohydrate units appear to occur subsequent to protein synthesis, this aspect of glycoprotein synthesis is apparently not under genetic control.

While specific functions of carbohydrate moieties in glycoenzymes are at present unknown, the suggestion has been made that the carbohydrate units become attached to the glycoenzyme during passage through the cell membrane, thereby facilitating the transport of the enzyme. Data from our experiments show that glucohydrolase I from inside the cell possesses the same molecular

weight, electrophoretic mobility, carbohydrate content and carbohydrate composition as the enzyme isolated from the culture filtrate. The glucohydrolase is completely synthesized inside the cell prior to secretion through the cell membrane and wall. The possibility (Eylar, 1965) that the carbohydrate moieties, because of their neutral character, aid in the transport of the enzyme through the cell membrane has, of course, not been excluded by our findings.

EXPERIMENTAL

Incorporation of glucose-1-^{14}C and mannose-1-^{14}C into glucohydrolase — Aspergillus niger (NRRL 330) was grown in 500 ml cultures in media containing glucose-1-^{14}C or mannose-1-^{14}C, nonlabeled glucose or mannose, a nitrogen source (nutrient broth) and inorganic salts (Lineback, et al., 1966). The glucohydrolase production was followed by a new spectrophotometric assay method based on a coupled reaction with glucose oxidase; the details of the new method will be published elsewhere. Maximal enzyme synthesis occurred by 86 hours of incubation. At this point the glucohydrolases in the culture filtrate were precipitated with four volumes of ethyl alcohol and the glucohydrolase I was isolated by chromatography on DEAE-cellulose (Pazur and Ando, 1959), and further purified by centrifugation on density-gradient columns (Pazur, et al., 1963). Evidence for a high degree of purity for the glucohydrolase I was obtained from experiments on chromatography on ion-exchange materials, electrophoresis in gel-columns and ultracentrifugation on density-gradient columns. In all cases, the protein, the enzyme activity and radioactivity were located in the same fractions from the columns.

The carbohydrates in the glucohydrolase I from cells grown on glucose-1-^{14}C were liberated by acid hydrolysis, separated by paper chromatography and located on the chromatogram by radioautography. The labeled mannose, glucose and galactose were extracted from the chromatogram and obtained in pure form. An appropriate aliquot of the labeled mannose was degraded to carbon dioxide, ethyl alcohol and lactic acid (Gibbs, et al., 1963) with Leuconostoc mesenteroides (ATCC 10881) that had been adapted to grow on man-

nose. The distribution of the ^{14}C in the mannose from the labeled glycoenzyme was as follows: 86% in C_1, 2.5% in $C_2 + C_3$, 2.5% in $C_4 + C_5$, and 9% in C_6. Degradation of the labeled glucose and galactose have not yet been completed. Labeled glucohydrolase I was also isolated from Aspergillus niger grown on mannose-1-^{14}C. Since the yield of the enzyme in this experiment was low, only mannose was obtained in sufficient quantity for degradation studies. The labeling pattern of the mannose was similar to above.

Pyrophosphorylase activities and glycosyl transferase activities — The mycelium from a 4-day old culture of A. niger was collected on a filter and washed thoroughly with 0.1 M phosphate buffer of pH 7.2. The cells were suspended in a small amount of the phosphate buffer containing 0.01 M magnesium chloride, 0.005 M dithiothreitol and 0.1 M Triton X-100 and then broken in a Virtis homogenizer. The resulting mixture was centrifuged at 15,000 x g for 30 minutes and the residue was discarded. The supernatant was recentrifuged at 20,000 x g for 30 minutes. The clear supernatant from the second centrifugation was used as the enzyme preparation for detecting pyrophorylase and glycosyl transferase activities.

Samples of approximately 1 mg of nucleoside triphosphates and 1 mg hexose-1-phosphates were dissolved in 0.1 ml of the enzyme extract. The resulting digests were examined for the presence of new nucleotides after reactions times of 1, 3, 8 and 25 hours by a paper chromatographic method. UDP-glucose, TDP-glucose, UDP-galactose and GDP-mannose were detected in digests containing the appropriate triphosphate and hexose-1-phosphate. The organism elaborates the pyrophosphorylases capable of synthesizing these nucleoside diphosphate hexoses. The activation of the carbohydrate residues for synthesis of the glyco-portion of the enzyme probably occurs via these pyrophosphorylases.

A partially modified glucohydrolase I was prepared for use as the acceptor material for the glycosyl transferase experiments by treating pure glucohydrolase I with alpha mannosidase (Li, 1966). In the transferase ex-

periments, the digests contained 0.4 ml of the enzyme extract from the mycelium of A. niger, 1 mg of partially hydrolyzed glucohydrolase I, and 1 mg of guanosine diphosphate-mannose-^{14}C (total radioactivity approximately 2 x 10^6 cpm). After incubation for 6 hours at room temperature, the digest was fractionated into high and low molecular weight components on a Sephadex G-25 column with 0.05 M sodium acetate solution of pH 5.3. Fractions of 3 ml were collected from the Sephadex column and assayed for enzyme activity, protein content and radioactivity.

The glucohydrolase recovered from the digest was located in fractions 14, 15 and 16. The protein and enzyme activity were in comparable concentrations in these fractions. Total radioactivity in these fractions was 1,600 cpm. Unreacted guanosine diphosphate mannose and low molecular weight radioactive hydrolytic products were located in fractions 27 to 33. These fractions contained the major portion of the initial radioactivity. The intervening fractions (18 to 24) exhibited only traces of radioactivity. On the basis of results from several such experiments and adequate controls, it was concluded that radioactive glucohydrolase was synthesized from the guanosine diphosphate mannose-^{14}C and the modified glucohydrolase. The synthesis most likely occurred by a transfer of mannosyl units from the guanosine diphosphate mannose to the partially modified glucohydrolase. In mammalian systems, the transfer of galactosyl units (McGuire, et al., 1965), xylosyl units (Grebner, et al., 1966) and glucosyl units (Bosmann and Eylar, 1968) from the corresponding sugar nucleotides to glycoproteins has been demonstrated with enzyme preparations from different types of tissue.

Comparison of intra- and extracellular glucohydrolase — The mycelium from eight liters of a 46 hour culture of A. niger was collected by decantation and extracted with several aliquots of 0.05 M citrate-phosphate buffer of pH 4.0. Each extract was assayed for glucohydrolase activity. Enzyme activity in the sample from the seventh extraction was virtually nil. At this point the mycelium was broken in a Virtis homogenizer and the intracellular

glucohydrolase was liberated. The glucohydrolase I in this mixture was isolated and purified by the usual procedures. Data on enzyme yields and specific activities are recorded in Table I.

Suitable samples of the glucohydrolase I from inside the cell and from the culture filtrate were subjected to density gradient centrifugation, electrophoresis on paper and carbohydrate analysis. Identical values were obtained for the two preparations by all of these measurements. These findings rule out the possibility that the carbohydrate units become attached to the protein portion of the enzyme during passage through the cell wall. Rather, the glycoenzyme is synthesized completely inside the cell and then secreted through the cell wall.

TABLE I

Data on glucohydrolase isolated from a 46 hr. culture of A. niger

	Total activity units/l of culture	Specific activity units/mg protein
Extracellular glucohydrolase (I + II)*	15,000	--
1st extract (I + II)	1,400	--
7th extract	80	--
Buffer blank	70	--
Glucohydrolase (I)	--	2,300
Intracellular glucohydrolase (I + II)	5,500	15
Alcohol precipitation (I + II)	5,100	477
DEAE-cellulose chromatography (I)	4,000	2,290
Density-gradient centrifugation (I)	1,300	2,310

*Representing glucohydrolase I and glucohydrolase II, respectively.

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