EVIDENCE FOR A COVALENT INTERMEDIATE BETWEEN α-GLUCOSIDASE AND GLUCOSE*

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Summary - A stable enzyme-glucose intermediate has been obtained in the short-term reaction between α-methyl-D-glucosidase and α-methyl-D-^{14}C-glucopyranoside. A rapid-flow technique was employed in which phenol was used to terminate the reaction and to trap the product. It is believed that a covalent linkage is involved because (a) continued washing of the denatured protein failed to remove the radioactivity and (b) the radioactivity was retained by a tryptic peptide isolated by gel filtration. Treatment of the labeled protein with 2 N HCl at room temperature released over 80% of the radioactivity as a compound with the same chromatographic mobility as glucose. No radioactive product was formed when bovine serum albumin replaced the enzyme, nor when glucosylamine, a potent glucosidase inhibitor, was present with the enzyme.

Much speculation on the mechanism of action of glycosidases is based on studies of the acid catalysis of glycoside hydrolysis, on experiments with various glycosidase inhibitors such as glyconolactones (1), glycals (2), and glycosylamines (3), and on x-ray crystallography studies on lysozyme and lysozyme-substrate complexes (4). Such studies have supported the possibility of a carbonium-oxonium intermediate and have suggested a role for a carboxyl group in the active site (5). However, the important question as to whether or not a true enzyme-glycosyl intermediate is formed in the enzymatic reaction has not been answered.

Earlier attempts in our laboratory to isolate enzyme-glycosyl intermediates in the case of homogenous maltase and α-methyl-D-glucosidase from Saccharomyces oviformis by rapid denaturation of the reacting enzyme using trichloroacetic acid have been unsuccessful (6). The phenol denaturation method of Mitchell et al. (7) has been successfully employed to trap the phosphoryl enzyme intermediates of succinyl CoA synthetase (7), phosphoglycerate mutase (8), and of glucose-6-phosphatase (9). We wish to report here the successful application of this procedure to demonstrate the formation of a glycosyl intermediate of α-methyl-D-glucosidase.

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MATERIALS AND METHODS

α-Methyl-D-glucosidase from Sacchromyces oviformis was purified from freshly harvested cells by ammonium sulfate fractionation, chromatography on DEAE-Sephadex and hydroxyapatite C (10). This enzyme was homogeneous by disc gel electrophoresis at pH 8.3 (11). Methyl-^{14}C(U)-α-D-glucopyranoside was purchased from Amersham/Searle Corp. β-D-Glucosylamine was prepared by the method of Isbell, et al. (12). The Glucostat reagent and trypsin, free of chymotrypsin activity were purchased from Worthington Biochemical Corp.

The apparatus, similar to that described by Bieber et al. (13) consisted of two syringes arranged for synchronous discharge through an interconnecting T-tube was used for rapid mixing at room temperature. The incubation time was approximately 10 msec unless otherwise noted. The tip of the delivery tube was immersed in rapidly stirred liquid phenol (88%, 0.4 ml). One syringe contained 0.1 ml of α-methylglucosidase (0.88×10^{-5} mmoles, 0.35, mg based on 40,000 M.W.) in 0.067 M phosphate buffer, pH 6.8. The other syringe contained 0.1 ml of ^{14}C-methyl α-D-glucoside (2.0×10^{-4} mmoles) in phosphate buffer. The final concentrations after mixing but prior to inactivaction were α-methyl-glucosidase (4.4×10^{-5} M), phosphate buffer (0.067 M, pH 6.8) and ^{14}C-methyl α-D-glucoside (1×10^{-3} M) equivalent to 2.8×10^{5} cpm. The reaction mixture was carefully washed 5 or 6 times by thoroughly mixing with 5 ml of a phenol-saturated phosphate buffer (0.067 M, pH 6.8) containing 0.1 M methyl α-D-glucoside. The phenol layer was measured and transferred to scintillation fluid for ^{14}C counting. Four variations of the reaction mixture were used as controls: 1) α-Methylglucosidase was inactivated by phenol before the radioactive substrate was added (0 time). 2) α-Methylglucosidase was incubated with radioactive substrate for 10 min. to ensure that the reaction was complete, after which phenol was added. 3) Bovine serum albumin (5×10^{-5} M) replaced the α-methylglucosidase. 4) 0.025 M β-D-glucosylamine, a powerful competitive inhibitor of the enzyme (3), was included in the standard reaction mixture.

RESULTS AND DISCUSSION

α-Methylglucosidase, acting on ^{14}C-methyl-α-D-glucoside was found to incorporate ^{14}C into phenol-extractable protein. About 1.2% of the radioactivity associated with the substrate was incorporated into the enzyme after incubation for 10 msec (Table I). This corresponds to 0.23×10^{-5} mmoles of methylglucoside. Since the total amount of enzyme used in the experiment was 0.88×10^{-5} mmoles it would appear that the amount of enzyme in the intermediate form was 27% of the original assuming one molecule of glucose is

Table I

Incorporation of ^{14}C into α-Methylglucosidase

A mixture of protein (0.35 mg) and ^{14}C-methyl-α-D-glucoside (200 mμmoles, 280,000 c.p.m.) was allowed to react for the indicated time prior to inactivation with 88% aqueous phenol. The phenol layer containing the protein was washed repeatedly with phenol-saturated buffer containing non-radioactive 0.1 M methyl-α-D-glucopyranoside until the wash was free of counts. Radioactivity was measured in a Beckman scintillation counter.

Treatment	^{14}C Incorporated into protein	
	c.p.m.	mμmoles
A. Enzyme incubated for 10 msec prior to phenol addition	3300	2.3
B. Same as A but phenol added prior to substrate	400	0.28
C. Same as A but incubated for 10 min	350	0.25
D. Same as A but including 25 mM glucosylamine	350	0.25
E. Same as A but enzyme replaced by bovine serum albumin	370	0.26

bound per intermediate. The control value for the incorporation of ^{14}C by phenol inactivated enzyme was about 3% of that obtained in the test mixture.

Under the conditions described above, the enzyme can hydrolyze over 99.9% of the substrate in 3 min based on the assay of glucosidase by the Glucostat method. Thus after 10 minutes incubation, no glucosyl-enzyme intermediate should remain. The absence of protein-bound radioactivity after 10 minutes incubation was consistent with this prediction. When bovine serum albumin was substituted for the enzyme, only a negligible incorporation of ^{14}C into phenol-extractable protein was observed. In the presence of 25 mM β-D-glucosylamine, which should almost completely inhibit the enzyme, the incorporation of ^{14}C-glucose was also prevented. The results indicate that the inhibitor competes with substrate binding rather than with the decomposition of glucosyl-enzyme.

That the incorporated radioactivity resided in ^{14}C-glucose was shown as follows. The radioactive protein was precipitated by washing out the phenol with 0.067 M phosphate buffer, pH 6.8 and dissolved in 0.1 ml 0.05 M NH_4HCO_3

containing 0.1% sodium dodecyl sulfate. The solution was made 2 N with respect to HCl and incubated at 25°. An aliquot (0.05 ml) of the incubation mixture was chromatographed on Whatmann No. 1 paper. The labeled compound formed in the hydrolysis migrated with the same mobility as glucose when chromatographed on paper in butanol-pyridine-water, 6:4:3. After 1 and 18 hrs of incubation, 85 and 93%, respectively, of the counts were detected in the glucose region. Thus the radioactive protein appears to be a glucosyl-enzyme complex.

To confirm that the glucose was not merely mechanically trapped in the denatured enzyme, a preparation of the enzyme-glucosyl intermediate was subjected to tryptic digestion. Fig. 1 shows that most of the ^{14}C was bound to peptides and that only a small amount was eluted with the added cold glucose marker. The retention of the radioactivity by peptidyl fractions obtained from the Sephadex column suggested that the linkage between enzyme and the glucose moiety was covalent.

Koshland (14) has suggested a double displacement mechanism for carbo-

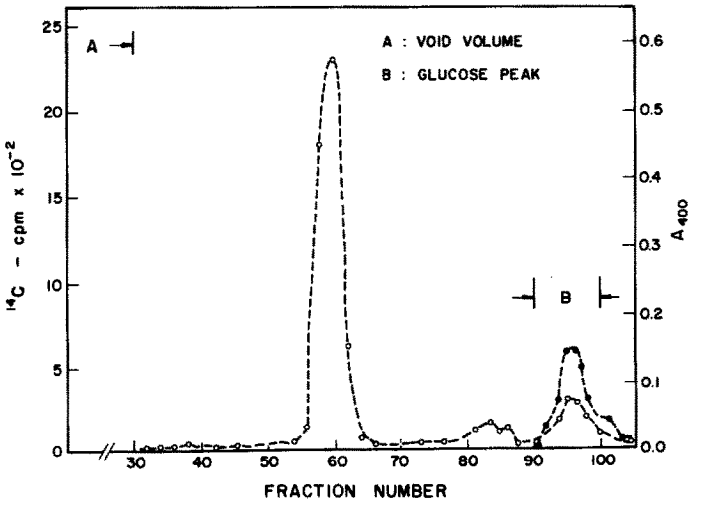

Figure 1. Chromatography of tryptic digest of radioactive enzyme-glucosyl intermediate. The intermediate was prepared as described in text but with 5 times as much α-methyl-D-^{14}C-glucopyranoside and 10 times as much enzyme. The washed product was precipitated from the phenol layer by the addition of 10 volumes of cold acetone. The precipitate was collected by centrifugation and suspended in 0.5 ml of 0.05 M NH_4HCO_3 containing 0.1% sodium dodecyl sulfate plus a drop of toluene and treated with trypsin (0.1 mg in 0.025 ml of 1 mM HCl). After 24 hours the digest was applied together with 0.1 ml of 1 M glucose as a reference to a G-25 Sephadex column (0.9 x 100 cm) equilibrated with 0.05 M NH_4HCO_3. The column was eluted with the same buffer at a flow rate of 30 ml/hr. One ml fractions were collected. Aliquots of 0.5 ml were counted for ^{14}C (0-0-0). Glucose was determined with the Glucostat reagent (●-●-●).

hydrase reactions proceeding with retention of configuration of the glycone. The enzyme used in these studies belongs to this class (15). He has proposed that an enzyme-substrate complex is formed with inversion of configuration at the C-1 position and then undergoes hydrolysis in the second displacement to yield the free sugar in its original configuration. The intermediate could be a covalent complex or simply a loose association held by ionic binding or other forces. Crystallographic studies of lysozyme substrate complexes (4) reveal a precisely defined spatial association between the two components. Various studies have supported the possibility of an ionic interaction between the γ-carboxylate of glutamic acid-35 of the enzyme and a carbonium-oxonium transient substrate (5). However these studies do not show whether or not a covalent bond exists.

Recently Fink and Good (16) have carried out low-temperature kinetic studies using 50% dimethyl sulfoxide as solvent. They have not only demonstrated a dramatic burst of the aglycone when β-glucosidase (a "retention" enzyme) acts on p-nitrophenyl-β-D-glucoside but have actually shown that below -20° no release of the glucosyl moiety occurs. These results provide convincing evidence for a glycosyl-enzyme intermediate in glycosidase-catalyzed reaction.

The intermediate trapped in our experiments now provides an opportunity to examine the active-site sequence of the enzyme. Moreover it should now be possible, to ascertain the configuration of the glucosyl-enzyme linkage in the glycosyl peptide with the aid of specific α- or β-glucosidases. In view of the indirect evidence that a carboxylate ion may be in the active center of glycosidases (see ref. 5), the potent and specific competitive inhibition of glycosidases by their corresponding glycosylamines, and the acid lability of the bound glucose it is tempting to speculate that a carboxylate group is involved in the active center.

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