EVIDENCE FOR A GLUCOSYL-ENZYME INTERMEDIATE IN THE β-GLUCOSIDASE-CATALYZED HYDROLYSIS OF p-NITROPHENYL-β-D-GLUCOSIDE

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Summary

The reaction of almond β-glucosidase with p-nitrophenyl-β-D-glucoside has been investigated over the temperature range +25° to -45° using 50% aqueous dimethyl sulfoxide (DMSO) as solvent. At temperatures below those at which turnover occurs a "burst" of p-nitrophenol proportional to the enzyme concentration is observed. Such a "burst" suggests the existence of a glucosyl-enzyme intermediate whose breakdown is rate-limiting, and provides a method for measuring the active-site normality. At pH 5.9, 25°, the presence of 50% DMSO causes an increase in K_m from 1.7×10^{-3}M (0%) to 1.7×10^{-2}M, whereas V_{max} is unchanged. The DMSO thus apparently acts as a competitive inhibitor with K_i = 0.7M. The Arrhenius plot for turnover is linear over the accessible temperature range with E_a = 23.0 ± 2.0 kcal/mole.

Two major problems in studying the nature of intermediates in enzyme-catalyzed reactions are their low concentration and short life-time. One manner in which these obstacles may be overcome is by the use of very low temperatures to slow the reaction down sufficiently so that labile intermediates may be accumulated and studied. We have recently demonstrated the potential of this approach in the case of some proteases (1-3; A. L. Fink in preparation). Carbohydrate hydrolyses have several properties which make them well suited for study by this technique, and this report concerns some preliminary results with β-glucosidase.

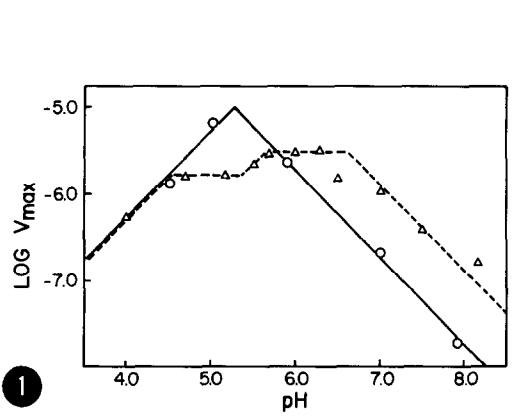
Relatively little is known about its mechanism of action. Raftery (4), on the basis of the α-deuterium isotope effect,

has suggested that a nucleophilic displacement reaction is involved. In an extensive series of papers, Legler (5-7), using the inhibitor conduritol B epoxide, has found evidence to implicate attack by a carboxyl group in the enzyme on the protonated epoxide to yield an inositol product with trans stereochemistry.

Aqueous-organic solvent systems are necessary in order to obtain fluid solutions at the low temperatures required for accumulating intermediates. Since the large molecular weight of β-glucosidase (approx. 10^5; (7)) suggests an oligomer we were particularly interested in the effect of the organic solvent on the quaternar structure.

Materials and Methods. Almond β-glucosidase was obtained from Worthington, Lot. No. EIID, and p-Nitrophenyl-β-D-glucoside (PNPG) from Cyclo Lot No. H3530. Aqueous dimethyl sulfoxide solutions were prepared as described previously (1). Exclusion chromatography wascarried out using a 100 x 0.9 cm column of CPG-10 120 Å porous glass beads, 120/200 mesh, from Electro-Nucleonics. The column eluant was monitored continuously at 280 nm with an Isco absorbance UA4 unit. A flow rate of 120 ml/h was used. Kinetic measurements were made using a Cary 118 spectrophotometer. Low temperature kinetic experiments were performed as described previously (1). Standard assay conditions for β-glucosidase activity were pH 5.4 (acetate), $25 \pm 0.1^\circ$, $\mu = 0.1$ M, and [S] = 1×10^{-2} M. p-Nitrophenol bursts were measured at -45° and -25°.

Results. Initial experiments were carried out to investigate the effect of DMSO on the β-glucosidase-catalyzed hydrolysis of PNPG. No activity was observed at 0° or 25° over a wide range of pH in 65% DMSO. In the 50% aqueous-organic solvent



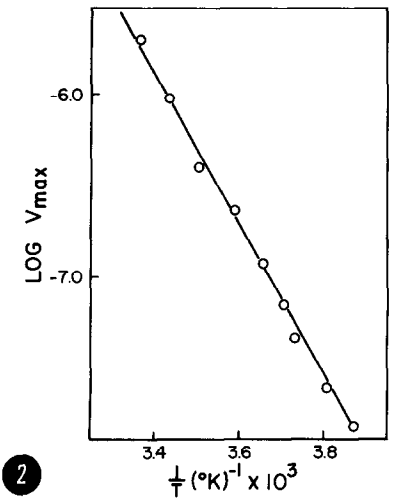


Figure 1. pH-Rate profiles for the β-glucosidase-catalyzed hydrolysis of PNPG in 0% (o) and 50%(Δ) DMSO, μ = 0.1M, 25±0.1°. E_o = 0.5 mg/ml, S_o = 1.2x10^{-2}M. The reaction was followed at 400 nm, V_{max} in M sec^{-1}.

Figure 2. Arrhenius plot for the β-glucosidase-catalyzed hydrolysis of PNPG. pH 5.4, 50% DMSO, S_o = 7.5x10^{-2} M, E_o = 0.68 mg/ml, V_{max} in M sec^{-1}.

system at 25°, pH 5.9 the enzyme had lost 6% of its initial activity after 30 min and continued to lose activity at the rate of 30% per hour. The enzyme was quite stable at 0° in 50% DMSO and could be stored for several days without loss of activity. No deviations from linearity were observed when the initial velocity (under substrate-saturating conditions) was measured as a function of enzyme concentration from 0 to 1.5 mg/ml. The values calculated for K_m in 0% and 50% DMSO, pH 5.9, 25° were 1.7x10^{-3} M and 1.7x10^{-2}M respectively. The V_{max} values were the same, namely 5.0x10^{-6}M sec^{-1} per mg enzyme. The pH-rate profile is shown in Fig. 1 for 0% and 50% DMSO. Exclusion chromatography on porous glass beads showed a major component with approximate MW 71,000 and a minor component (MW ~ 25,000) in 0% pH 7.0 buffer. In 50% DMSO the ratio of these two components was changed from 2:1 to 1:4. Both components exhibited β-glucosidase activity.

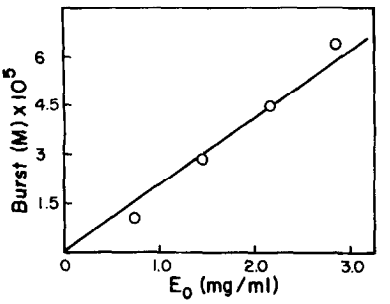


Figure 3. Magnitude of p-nitrophenol bursts as a function of enzyme concentration. T = 248°K, pH 5.4, 50% DMSO, S_o = 9x10^{-3} M, monitored at 380 nm. No subsequent reaction was observed.

The initial rate of p-nitrophenol production under substrate-saturating conditions in 50% DMSO, pH 5.4, was measured as a function of temperature (Fig. 2). The Arrhenius plot is linear and gives a value of E_a = 23.0 ± 2.0 kcal/mole. This strong temperature dependence results in the turnover reaction becoming essentially negligible at temperatures below -20° (pH 5.4). Rapid bursts of p-nitrophenol were observed at temperatures below -15°, the magnitude of the bursts being proportional to the enzyme concentration (Fig. 3). The active site normality was calculated to be 2.35x10^{-5}M per mg/ml of enzyme.

Discussion. The presence of bursts of p-nitrophenol under conditions in which the turnover reaction is negligible may be accounted for by a minimum scheme of the form

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{EG} \xrightarrow{k_3} E+P_2 \qquad \text{Scheme 1}$$

where ES = Michaelis complex, EG = glucosyl-enzyme, P_1 = p-nitrophenol and P_2 = glucose, and in which $k_3 \ll k_2$. This is the first report of the trapping of a glucosyl-enzyme intermediate in β-glucosidase-catalyzed hydrolysis of a substrate. Based on the magnitude of the α-deuterium isotope effect (4)

the glucosyl-enzyme would appear to involve a covalent rather than electrostatic bond between the glucosyl moiety and the enzyme. Bursts of p-nitrophenol such as those observed may be used to determine the active site normality (8). In the case of oligomeric enzymes such knowledge is useful in determining the MW of the active-site-containing unit. Since the enzyme preparation used in these experiments contained two different β-glucosidase components, as determined by polyacrylamide gel electrophoresis (pH 4) and chromatography on CM-Sephadex (A.L. Fink and T. Alber, unpublished results), as well as other minor proten contaminants, we cannot establish a precise MW. However, the burst experiments indicate a minimum MW of 43,000 per active site unit. The results of both the exclusion chromatography and pH-rate profile suggest that the DMSO causes a change in some fraction of the enzyme to a lower MW form with a higher pH optimum. Preliminary experiments with purified enzyme suggest that one of the β-glucosidase components undergoes dissociation from an oligomer to active monomers in the presence of 50% DMSO. We are currently making a highly homogeneous enzyme preparation for future studies of both the structural and catalytic properties. The effect of 50% DMSO on the kinetic parameters is consistent with the dimethyl sulfoxide acting as a competitive inhibitor with K_i = 0.7M. An alternate possibility is that the lower molecular weight form of the enzyme has a much higher K_m.

In the β-glucosidase-catalyzed hydrolysis of 6-purinyl-β-D-glucothiopyranoside, deuterium isotope effects of 1.1 and 0.6 have been reported for V_{max} and K_m respectively (9). Since $V_{max} = k_3E_o$ and $K_m = k_3(k_{-1} + k_2)/k_2k_{-1}$ from scheme 1, these isotope effects eliminate general base catalysis in the break-

down of the glucosyl-enzyme and indicate a substantial isotope effect on the rate of formation of glucosyl-enzyme (k_2), and/or substrate binding.

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