The mechanism of action of β -glucosidase from *Botryodiplodia theobromae* Pat.

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1. The activity of the high-molecular-weight β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) obtained from culture filtrates of *Botryodiplodia theobromae* Pat. was affected by added NaCl in such a way that an initial phase of stimulation was followed by a phase of rapid non-linear decrease in velocity and finally by a phase of slow linear decrease in velocity as the concentration of NaCl was increased. 2. In the presence of 0.014 M-sodium acetate/acetic acid buffer (pH 5.0) there was a slight increase in enzymic activity in the presence of low concentrations of dioxan (up to about 10% dioxan) and a rapid decrease in enzymic activity at higher dioxan concentrations, but both effects were mitigated in the presence of 0.1 M buffer. 3. The order of efficiency of added glucosyl acceptors in β -glucosidase-catalysed reactions was found to be fructose > sucrose > glycerol > methanol. 4. The enzyme was inactivated by the active-site-directed compound conduritol-B-epoxide; but this inactivation was concentration-dependent, was prevented by 10 mM-glucose, and involved an acidic group with pK_a 4.3. 5. A rate equation has been derived on the assumption of a mechanism of action involving a solvent-separated and an intimate glucosyl cation-carboxylate ion-pair intermediate and an α -glucosyl enzyme intermediate [Umezurike, G. M. (1981) *Biochem. J.* **199**, 203–209]. 6. Calculations based on the application of the derived rate equation and the calculated kinetic parameters show that the rate equation explains the peculiar properties of β -glucosidase in the presence of added glucosyl acceptors or of NaCl.

INTRODUCTION

Like most glycosidases, the high-molecular-weight β -glucosidase from *Botryodiplodia theobromae* Pat. is believed to act by an initial general-acid catalysis by an acidic group on the enzyme to generate a glucosyl cation that is stabilized by an ionized carboxylate group (Umezurike, 1977, 1979). The increase in V_{max} observed in the presence of efficient alcoholic or polyhydric glucosyl acceptors has been explained by assuming that these glucosyl acceptors react more readily with a solvent-separated glucosyl cation-carboxylate ion-pair than with an intimate ion-pair, and water reacts more readily with the intimate ion-pair than with the solvent-separated ion-pair (Umezurike, 1981). This assumption is in consonance with the observation that alcohols are more reactive than water towards carbonyl-type carbon (a glucosyl cation being isoelectronic with a protonated aldehyde) whereas water is more nucleophilic than alcohol to saturated carbon in both S_N1 and S_N2 reactions (Streitweiser, 1962; Sinnott & Viratelle, 1973). The intimate ion-pair is postulated to be in equilibrium with a preponderance of a covalent α -D-glucosyl-enzyme (Umezurike, 1981).

Fructose, sucrose, glycerol and methanol have been found to be efficient glucosyl acceptors in reactions catalysed by β -glucosidase from *Botryodiplodia theobromae* Pat., confirming that the acceptor site of this enzyme has a broad specificity (Umezurike, 1975a). Double-reciprocal plots (Lineweaver & Burk, 1934) of results obtained when these compounds were present in the assay mixtures were linear and intersected to the right of the vertical axis, indicating that enzyme activity was inhibited in the presence of low concentrations but enhanced in the presence of high concentrations of the donor (*p*-nitrophenyl β -D-glucopyranoside). Both the

Michaelis constant (K_m) and the maximum velocity (V_{max}) increased in the presence of these compounds (Umezurike, 1978), although fructose and sucrose elicited these effects at much lower concentrations than glycerol and methanol (Umezurike, 1978, 1979).

Unlike the efficient glucosyl acceptors mentioned above, D-maltose has been found to be an intercept-hyperbolic non-competitive inhibitor (cf. Cleland, 1963, for definition) of β -glucosidase activity with respect to the donor when activity was measured by the release of *p*-nitrophenol (Umezurike, 1971b, 1975a, 1978). Thus the apparent K_m for donor is not affected by maltose in the absence or presence of glycerol but V_{max} is reduced (Umezurike, 1975a). A K_i value of 50 mM-maltose was calculated from the points of intersection of replots of slope and intercept values obtained from primary double-reciprocal plots as $1/(\text{slope}_x - \text{slope}_0)$ or $1/(\text{intercept}_x - \text{intercept}_0)$ against the reciprocal of maltose concentration where the subscripts x and 0 denote the presence and absence of inhibitor respectively (Umezurike, 1975a; cf. Cleland, 1963).

In the present paper, the properties of β -glucosidase are discussed in terms of the proposed mechanism of action involving two ion-pair intermediates (Umezurike, 1981).

MATERIALS AND METHODS

The donor, *p*-nitrophenyl β -D-glucopyranoside, was purchased from Sigma Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). All other chemicals were obtained from BDH Chemicals (Poole, Dorset, U.K.).

The high-molecular-weight β -glucosidase from *B. theobromae* Pat. (I.M.I. 115626; A.T.C.C. 26123) used in the present work was obtained from culture filtrates and

Scheme 1. Reaction scheme for β -glucosidase-catalysed hydrolysis and transglucosylation in the presence of an added efficient acceptor (A)

E and ES are the free enzyme and the Michaelis enzyme-substrate complex respectively; $\text{EG}^+\parallel\text{C}^-$, EG^+C^- and EG-C are the enzyme-bound solvent-separated glucosyl cation-carboxylate ion-pair, the intimate ion-pair species, and the unreactive covalent glucosyl-enzyme respectively; P_1 , P_2 and P_3 are *p*-nitrophenol, a transglucosylation product and glucose respectively. It is assumed that the solvent-separated ion-pair intermediate reacts with the added glucosyl acceptor glycerol (A) whereas the intimate ion-pair reacts preferentially with water. The rate constants for the forward reactions are denoted as k_{+1} – k_{+6} and the reverse reactions as k_{-1} – k_{-4} . The rate constant $k'_{+6} = k_{+6}[\text{water}]$, but since the concentration of water is very high $k_{+6}[\text{water}]$ is regarded as a constant (k'_{+6}).

purified as described previously (Umezurike, 1971a, 1975b).

β -Glucosidase assay was carried out with *p*-nitrophenyl β -D-glucopyranoside as glucosyl donor in 0.05 M-sodium acetate/acetic acid buffer (pH 5.0) as described previously (Umezurike, 1971a). Maximum velocities (V_{max}) were calculated from plots of v against $v/[\text{donor}]$.

Conduritol-B-epoxide (m.p. 156–158 °C) was synthesized from *myo*-inositol (McCasland & Horswill, 1953; Legler, 1966; Quaroni *et al.*, 1974). Inactivation of β -glucosidase by conduritol-B-epoxide was carried out in the presence of 1.0 mM-epoxide in McIlvaine buffer (pH 2.6–7.4) at 27 °C. The reagent concentrations quoted were final concentrations in the reaction mixtures.

RESULTS AND DISCUSSION

Involvement of ion-pairs

β -Glucosidase-catalysed reactions in the presence of an efficient alcoholic or polyhydric glucosyl acceptor (A) have been considered in terms of the mechanism involving two ion-pair intermediates shown in Scheme 1, where $\text{EG}^+\parallel\text{C}^-$, EG^+C^- and EG-C are the solvent-separated ion-pair, the intimate ion-pair and the covalent α -D-glucosyl enzyme intermediate respectively. The steady-state rate equation for Scheme 1, derived by the method of King & Altman (1956), is of the Michaelis-Menten type (see eqn. 1 of Umezurike, 1981), and the equations for apparent K_m (apparent Michaelis constant), k_{cat} (the maximum rate of *p*-nitrophenol liberation), k_{trans} (the maximum rate of transglucosylation) and k_{hydr} (the maximum rate of hydrolysis) indicate that $k_{\text{cat}} = k_{\text{hydr}} + k_{\text{trans}}$ (Umezurike, 1981).

The assumption of the involvement of two ion-pair intermediates (i.e. a solvent-separated and an intimate ion-pair species of glucosyl cation and carboxylate anion) is predicated in the belief that in β -glucosidase-catalysed reactions the carboxylate anion would be solvated, as anions are capable of being solvated in protic solvents. Thus, the glucosyl cation-carboxylate ion-pair

Fig. 1. Effect of NaCl on β -glucosidase activity

(a) Plots of enzyme activity against the concentration of NaCl in the presence of 0.2 mM (Δ), 0.5 mM ($\blacktriangle$), 1.0 mM ($\circ$) and 2.0 mM donor (*p*-nitrophenyl β -D-glucopyranoside) ($\bullet$). (b) Replots of the ratio v_{ex}/v_o and v_s/v_o against the concentration of donor where v_{ex} , v_s and v_o represent the predicted velocity in the absence of inhibition (obtained by extrapolating the linear inhibition rate to zero concentration of NaCl), the velocity in the presence of 16.6 mM-NaCl when there was stimulation, and the velocity in the absence of added NaCl respectively.

first generated in β -glucosidase-catalysed reactions is believed to be solvent-separated.

Studies on β -glucosidase from *B. theobromae* have shown that in the presence of a low concentration of NaCl (16.6 mM) the activity of the enzyme was stimulated and the apparent Michaelis constant (K_m) of the donor decreased, whereas in the presence of higher concentrations (up to 500 mM-NaCl) enzymic activity was inhibited and the apparent K_m for donor increased with increasing NaCl concentration (Umezurike, 1979). The data presented in Fig. 1 indicate that, after the phase of stimulation, there was a phase of rapid non-linear decrease in velocity followed by a phase of slow linear decrease in velocity as the concentration of NaCl was increased (Fig. 1a). These plots show three other striking features. First, the linear rate of decrease in velocity is roughly the same (0.001 nmol/min per mM-NaCl) in the presence of 0.2–2.0 mM donor (*p*-nitrophenyl β -D-glucopyranoside). Secondly, the predicted velocity in the absence of inhibition (obtained by extrapolating the linear inhibition rate to zero concentration of NaCl) is less than the observed velocity in the absence of NaCl when the donor concentration is low. The extrapolated velocity (v_{ex}) approaches the experimentally observed velocity (v_o) as the concentration of donor increases, so that a plot of v_{ex}/v_o against donor concentration is linear, and the value of v_{ex}/v_o increases from 0.82 in the presence of 0.2 mM donor to 1.0 in the presence of about 1.6 mM donor (Fig. 1b). Thirdly, the stimulation observed in the presence of a low concentration of NaCl

decreases non-linearly as the donor concentration increases, and the value of v_s/v_o (where v_s is the velocity on stimulation) decreases from 1.23 in the presence of 0.2 mM donor to 1.0 in the presence of 2.0 mM donor (Fig. 1b).

It is possible to explain the features of Fig. 1 by reference to Scheme 1. The stimulatory effect of a low concentration of NaCl is attributable to increased ionic strength which is known to favour the formation of carbonium ions (Alder *et al.*, 1971, p. 83). The rapid non-linear decrease in velocity observed when the concentration of NaCl was further increased can be attributed to 'medium effect' on donor binding, presumably arising from the effect of NaCl on the ionization of functional groups on the enzyme (Umezurike, 1979). Both effects (stimulation and rapid non-linear decrease in velocity) are abolished if the enzyme active site is saturated with the donor, and velocity is maximal, particularly as enzymic activity is determined by the rate of liberation of *p*-nitrophenol (P_1). The linear decrease in rate observed in the presence of high concentrations of NaCl, even when the concentration of donor is high (Fig. 1a), indicates that the generation of the intimate ion-pair from the solvent-separated ion-pair is reduced in the presence of increasing concentration of NaCl which interacts with the solvent-separated ion-pair.

NaCl would stabilize (i.e. decrease the activity coefficient of) the solvent-separated ion-pair more than it would the transition state for the addition of water to the intimate ion-pair (deglucosylation), and thus cause a decrease in rate. On the other hand, organic solvents would destabilize the solvent-separated ion-pair relative to the transition state for deglucosylation and tend to cause rate increases. Thus, in the presence of NaCl, the 'medium effect' on donor binding (Umezurike, 1979) can be represented as:

where E' is the modified and thus unreactive enzyme. The interaction of NaCl with the solvent-separated ion-pair can be represented as:

where the enzyme active-site carboxylate anion (C^-) involved in ion-pair formation with the glucosyl cation is displaced temporarily by the chloride ion (Cl^-) and is then stabilized by sodium cation (Na^+). If these two processes involving NaCl are included in Scheme 1, the rate equation will become:

$$\frac{v}{[E]_0} = \frac{k_{cat.}/(1 + [I]/K_I)}{1 + \frac{K_m}{[S]} \left\{ \frac{(1 + [I]/K_I)}{(1 + [I]/K_I)} \right\}} \quad (1)$$

where

$$[I] = [\text{NaCl}]$$

$$K_I = k_{-7}/k_{+7}$$

$$K_I = \frac{k_{-8}\{k_{-4}[k_{+5}[A](k_{-3} + k'_{+6}) + k_{+3}k'_{+6}\} + k_{+2}[k_{-4}(k_{-3} + k'_{+6}) + k_{+3}(k_{+4} + k_{-4})\}}{k_{+2}\{k_{-4}[k_{+5}[I](k_{-3} + k'_{+6})\}}}$$

Fig. 2. Plots of reciprocal of velocity against NaCl concentration in the presence of donor

Δ , 0.2 mM-, $\blacktriangle$, 0.5 mM-, $\circ$, 1.0 mM- and $\bullet$, 2.0 mM- *p*-Nitrophenyl β -D-glucopyranoside. The points were calculated from experimental data whereas the lines were calculated from eqn. (1), assuming that $k_{cat.} = 4.35$ mmol/min, $K_m = 0.3$ mM, $K_I = 0.5$ M and $K_I = 15$ M.

The parameters $k_{cat.}$ and K_m are as given by Umezurike (1981) in eqn. (1) of that paper. Eqn. (1) above can be rearranged to give eqn. (2), according to which plots of the reciprocal of velocity against $[\text{NaCl}]$ will give a series of straight lines in the presence of various fixed concentrations of donor (S).

$$\frac{v}{[E]_0} = \frac{k_{cat.}/(1 + K_m/[S])}{1 + [I]([S]K_I + K_m K_I)/K_I K_I([S] + K_m)} \quad (2)$$

When replotted as $1/v$ against $[\text{NaCl}]$, the experimental data of Fig. 1(a) are found to fit the data calculated from eqn. (1), as shown in Fig. 2.

In the presence of the efficient glucosyl acceptor glycerol and of a high donor concentration (> 0.5 mM), there was increase in velocity with increasing glycerol concentration. On the other hand, there was inhibition when the donor concentration was low (< 0.5 mM), and this inhibition increased with increasing glycerol concentration (Fig. 3). The concentration of donor (0.5 mM) at which this transition appears to take place is close to the K_m for the donor (about 0.3 mM-*p*-nitrophenyl β -D-glucopyranoside; Umezurike, 1971b, 1975a). By determining the levels of the various products released by β -glucosidase action in the presence of glycerol, it was found (Umezurike, 1981) that there was an increase in the rate of transglucosylation at the expense of hydrolysis, particularly when the overall rate of production of *p*-nitrophenol increased in the presence of high donor concentrations (> 0.5 mM).

Previous studies have indicated that dioxan affects

Fig. 3. Plots of the ratio of velocity in the presence of glycerol (v_{gly}) to that in its absence (v_0) against the concentration of glycerol in the presence of donor

The donor was *p*-nitrophenyl β -D-glucopyranoside: ○, 2.0 mM; ●, 0.05 mM.

donor binding and also encourages ion-pair collapse in β -glucosidase-catalysed reactions as a result of increased reactivity of the carboxylate anion (Umezurike, 1979). The results obtained when the activity of β -glucosidase was measured in the presence of increasing dioxan concentration in either 0.014 M- or 0.1 M-(final concentration) sodium acetate/acetic acid buffer (pH 5.0 in water) when $[S] = 35 K_m$ are shown in Fig. 4. The slight increase in rate observed in the presence of the less concentrated buffer when dioxan concentration was low (up to 10%) was abolished in the presence of the more concentrated buffer. On the other hand, the rapid decrease in rate observed in 0.014 M-buffer at higher dioxan concentrations was mitigated in the more concentrated buffer. These results strongly support the involvement of two ion-pair species in addition to a covalent α -glucosyl enzyme in β -glucosidase-catalysed reactions, the effects in the presence of up to 25% dioxan deriving from the formation of the intimate ion-pair from the solvent-separated ion-pair, and the effects at higher dioxan concentrations deriving largely from the conversion of the intimate ion-pair to the α -glucosyl enzyme (cf. Scheme 1). Both processes of conversion would be affected by dioxan and increased ionic strength of the solution in opposite ways.

It is not clear yet why the solvent-separated ion-pair does not collapse under the influence of the carboxylate anion to release an α -glucopyranose. Account must, however, be taken not only of water being a weaker base and nucleophile than the carboxylate anion or of the fraction of the potential nucleophile which is unprotonated at the pH (pH 5.0) of the medium, but also of the possibility of the polarity of the enzyme active site being somewhat low owing to the nearness of some hydro-

Fig. 4. Effect of dioxan on β -glucosidase activity in the presence of 0.014 M- (●) or 0.1 M- (○) sodium acetate/acetic acid buffer, pH 5.0, in water

Fig. 5. Plot of the reciprocal of the difference between the maximum velocities in the presence (V_x) and absence (V_0) of glycerol against the reciprocal of glycerol concentration in β -glucosidase-catalysed reactions

phobic centre. Direct ion-pairing between the enzyme-bound glucosyl cation and the carboxylate anion, without the intervening water molecule, would seem to be favoured under such conditions.

The conclusion that can be drawn from these observations is that glycerol reacts with the solvent-

separated ion-pair from the back-side in the transglucosylation process catalysed by β -glucosidase to produce 1-glyceryl β -D-glucoside (Umezurike, 1975a, 1978), whereas water reacts with the intimate ion-pair in hydrolysis to produce β -D-glucose. Any glucosyl acceptor which is more nucleophilic than water and which can thus react with the solvent-separated ion-pair would compete effectively with ion-pair collapse and thus favour transglucosylation at the expense of hydrolysis, irrespective of the high concentration of water (55 M). Indications that alcohols are more reactive than water towards the glucosyl cation (cf. Sinnott & Viratelle, 1973; Umezurike, 1979, 1981) and that methanol is less nucleophilic than water towards saturated carbon atoms in both S_N1 and S_N2 reactions (cf. Streitweiser, 1962; Sinnott & Viratelle, 1973) have been presented. The intimate ion-pair still has partial bonding between the carbon and the leaving group (carboxylate ion).

It has been observed that in the acetolysis of a number of alkyl arenesulphonates, at least two types of ion-pairs (a solvent-separated and an intimate ion-pair) are involved (cf. Alder *et al.*, 1971, p. 87). Furthermore, the involvement of reversibly formed ion-pairs in bimolecular nucleophilic substitution reactions with apparent S_N1 characteristics has been demonstrated (Sneen & Bradley, 1972; Sneen & Carter, 1972; Sneen & Kay, 1972). The process described in Scheme 1 therefore has some mechanistic precedents.

Acceptor efficiency

The reciprocal form of the equation for $k_{trans.}$ (Umezurike, 1981, eqn. 2) is shown here in eqn. (3), and indicates that a plot of $1/k_{trans.}$ against $1/[A]$ would give a straight line.

$$\frac{1}{k_{trans.}} = \frac{1}{(k_{cat.} - k_{hydr.})} = \frac{1}{k_{+2}} + \frac{1}{k_{+2}} \cdot \frac{K_{A1}}{[A]} \quad (3)$$

where

$$K_{A1} = \frac{k_{-4}[k_{+3}k'_{+6} + k_{+2}(k_{-3} + k'_{+6})] + k_{+2}k_{+3}(k_{+4} + k_{-4})}{k_{-4}k_{+5}(k_{-3} + k'_{+6})}$$

Thus, the values of k_{+2} and K_{A1} (see eqn. 3) can be calculated from a plot of $1/k_{trans.}$ against $1/[A]$. To the nearest approximation, the rates $k_{cat.}$ and $k_{hydr.}$ in eqn. (3) can be replaced with the maximum velocities in the presence (V_x) and absence (V_0) of an added glucosyl acceptor respectively, and these can be calculated from primary double-reciprocal plots or preferably from plots of v against $v/[donor]$. Fig. 5 shows a replot of experimental data as $1/(V_x - V_0)$ against $1/[glycerol]$. A K_{A1} value of 300 mM-glycerol was calculated by the least-squares method. The values of K_{A1} calculated from similar plots of data obtained when the added glucosyl acceptors were fructose, sucrose and methanol are 1.92 mM-fructose, 2.78 mM-sucrose and 11.1 M-methanol. In all these cases, the value of k_{+2} remained the same under the condition of the experiment. Thus, the order of efficiency of the added glucosyl acceptors is fructose > sucrose > glycerol > methanol. In other words, the effectiveness of fructose as acceptor is about 5.78×10^3 times that of methanol and about 156 times that of glycerol as acceptors.

It has been observed that very low concentrations (< 0.1 mM) of cellobiose activate but higher concentrations strongly inhibit β -glucosidase activity with the

Fig. 6. Plot of rate of inactivation (as percentage of maximum rate) by conduritol-B-epoxide of β -glucosidase from *B. theobromae* against pH

The concentration of conduritol-B-epoxide was 1.0 mM in the assay mixtures in McIlvaine buffer (pH 2.6–7.4) at 27 °C.

arylglucoside as glucosyl donor (Umezurike, 1971b), indicating that at low concentrations cellobiose can bind as a glucosyl acceptor to the acceptor site consisting of only the aglycone-binding sub-site, as the acceptor molecule would bind after the release of the leaving group, but at higher concentrations it can bind as a competitive inhibitor to the substrate-binding site consisting of both a glucose (glycone)-binding sub-site and the aglycone-binding sub-site.

Inactivation by conduritol-B-epoxide

The β -glucosidase from *B. theobromae* is inactivated by the active site-directed inhibitor conduritol-B-epoxide. This inactivation is concentration-dependent and is prevented by the presence of 10 mM-glucose. The dependence of the inactivation rate on pH is shown in Fig. 6. This indicates the involvement of an acidic group with pK_a 4.3 in the activation reaction. A similar sigmoidal pH-dependence has been observed with the β -glucosidase from other sources, and the pK_a values obtained were 6.1, 5.6 and 7.3 for *Aspergillus wentii* β -glucosidase, almond β -glucosidase A and B respectively (cf. Legler, 1973). The results of studies on the inactivation of β -glucosidase from these sources indicate that the binding of conduritol-B-epoxide is followed by the protonation of the epoxide oxygen by the acidic group at the active site of the enzyme and by nucleophilic opening of the epoxide ring by a carboxylate ion of aspartate to form an ester of (+)-chiro-inositol, as treatment of the inactivated enzyme with hydroxylamine

Scheme 2. Proposed reaction scheme for β -glucosidase-catalysed hydrolysis and transglucosylation in the presence of both an efficient glucosyl acceptor (A) and an ineffective acceptor compound (B)

The forward reactions are denoted as k_{+1} – k_{+7} and the reverse reactions as k_{-1} – k_{-4} . P_4 is the transglucosylation product to B. Other details are as shown in the legend to Scheme 1.

leads to the release of (+)-*chiro*-inositol (Legler, 1973, 1975).

Since both the β -glucosidase from *B. theobromae* and that from *A. wentii* operate with retention of configuration of the anomeric carbon atom (Legler, 1973; Umezurike, 1975a, 1978), similar mechanisms would appear to be involved in their modes of action. Thus, the inactivation of these enzymes by conduritol-B-epoxide, in a process involving the formation of a covalent bond between the inactivating agent and the carboxylate functional group of the enzyme, is in consonance with

donor is high; Umezurike, 1981] are consistent with the postulated interconversion of a reactive C-1-trigonal and an unreactive C-1-tetrahedral intermediate (cf. Sinnott & Souchard, 1973; Umezurike, 1981). The observed α -deuterium kinetic isotope effect of 1.01 ± 0.01 and other results indicate that sweet almond β -glucosidase operates a similar process (Nath & Rydon, 1954; Dahlquist *et al.*, 1969).

β -Glucosidase A_3 from *A. wentii* catalyses the slow hydration of D-glucal (which has a half-chair conformation similar to the glucosyl cation) to 2-deoxy-D-glucose (Legler *et al.*, 1979). The hydrolysis of 4-methylumbelliferyl- β -D-glucopyranoside by this enzyme is inhibited by D-glucal (Legler *et al.*, 1979). By using ^{14}C -labelled D-glucal, the enzyme-inhibitor complex isolated after denaturation was found to contain ^{14}C -labelled D-glucal covalently bound to the same aspartate residue involved in covalent-bond formation with conduritol-B-epoxide and also in donor hydrolysis. Presumably, covalent intermediates which differ in reactivity are involved in these processes.

Kinetics

Consider the reaction scheme shown in Scheme 2, where glycerol (A) and maltose (B) react with the enzyme-bound solvent-separated ion-pair (glucosyl cation-carboxylate anion). The steady-state rate equation for Scheme 2, derived by the method of King & Altman (1956) where activity is measured by the release of P_1 (*p*-nitrophenol), is as shown in eqn. (4).

$$\frac{v}{[\text{E}]_0} = \frac{\left(k_{\text{H}} + \frac{k_{+2}[\text{A}]}{K_{\text{A1}}} + \frac{k_{+2}[\text{B}]}{K_{\text{B1}}} \right) / \left(1 + \frac{[\text{A}]}{K_{\text{A1}}} + \frac{[\text{B}]}{K_{\text{B1}}} \right)}{1 + \frac{K_{\text{m}} \{ (1 + [\text{A}]/K_{\text{A2}} + [\text{B}]/K_{\text{B2}}) \}}{[\text{S}] \{ (1 + [\text{A}]/K_{\text{A1}} + [\text{B}]/K_{\text{B1}}) \}}} \quad (4)$$

where

$$\begin{aligned}
 k_{\text{H}} &= \frac{k_{+2} k_{+3} k_{-4} k'_{+6}}{k_{-4} [k_{+3} k'_{+6} + k_{+2} (k_{-3} + k'_{+6})] + k_{+2} [k_{+3} (k_{-4} + k_{+4})]} \\
 K_{\text{A1}} &= \frac{k_{-4} [k_{+3} k'_{+6} + k_{+2} (k_{-3} + k'_{+6})] + k_{+2} [k_{+3} (k_{-4} + k_{+4})]}{k_{-4} [k_{+5} (k_{-3} + k'_{+6})]} \\
 K_{\text{A2}} &= \frac{k_{+3} k'_{+6}}{k_{+5} (k_{-3} + k'_{+6})} \\
 K_{\text{B1}} &= \frac{k_{-4} [k_{+3} k'_{+6} + k_{+2} (k_{-3} + k'_{+6})] + k_{+2} [k_{+3} (k_{-4} + k_{+4})]}{k_{-4} [k_{+7} (k_{-3} + k'_{+6})]} \\
 K_{\text{B2}} &= \frac{k_{+3} k'_{+6}}{k_{+7} (k_{-3} + k'_{+6})} \\
 K_{\text{m}} &= \frac{(k_{-1} + k_{+2})}{k_{+1}} \cdot \frac{k_{+3} k_{-4} [k'_{+6}]}{k_{-4} [k_{+3} k'_{+6} + k_{+2} (k_{-3} + k'_{+6})] + k_{+2} [k_{+3} (k_{-4} + k_{+4})]}
 \end{aligned}$$

the formation of a covalent α -glucosyl enzyme in β -glucosidase-catalysed reactions.

Sinnott & Souchard (1973) have quoted reports that the glucosyl cation-carboxylate ion-pair of lysozyme does indeed collapse into an acylal. Many properties of β -glucosidase-catalysed reactions [e.g. the α -deuterium kinetic isotope effect ($k_{\text{H}}/k_{\text{D}}$) of 1.03 ± 0.01 for the hydrolysis of *p*-nitrophenyl β -D-glucopyranoside and the increase in rate in the presence of glycerol or other efficient glucosyl acceptors when the concentration of

The observed properties of β -glucosidase from *B. theobromae* (Umezurike, 1975a) agree with the predictions of eqn. (4) where for glycerol $K_{\text{A2}}/K_{\text{A1}} < k'_{+6}/k_{+5}$, $K_{\text{A2}} < K_{\text{A1}}$ and $k'_{+6} < k_{+5}$, and for maltose $K_{\text{B2}}/K_{\text{B1}} < k'_{+6}/k_{+7}$, $K_{\text{B2}} = K_{\text{B1}}$ and $k'_{+6} \gg k_{+7}$. When $k'_{+6} \gg k_{+7}$ (cf. eqn. 4) the contributions of the terms $k_{+2} (k_{-3} + k'_{+6})$ and $k_{+2} [k_{+3} (k_{-4} + k_{+4})]$ to K_{B1} are negligible and K_{B1} reduces to K_{B2} .

If only glycerol is present in the assay mixtures, all terms containing $[\text{B}]$, K_{B2} and K_{B1} in eqn. (4) drop out. Thus plots of $1/v$ against $1/[\text{S}]$ intersect to the right of

Fig. 7. Double-reciprocal plots of $1/v$ against the reciprocal of the concentration of donor (*p*-nitrophenyl β -D-glucopyranoside; PNPG)

The data were calculated from eqn. (4) assuming in (a) the absence (●) and presence of 50 mM- (○), 100 mM- (▲) or 200 mM-glycerol (Δ), and in (b) the absence (●) and presence of 25 mM- (○), 50 mM- (▲), 75 mM- (Δ) or 100 mM-maltose (□). Calculations were based on the assumption that in (a) $k_H = 33$ nmol/min, $k_{+2} = 65$ nmol/min, $K_m = 0.3$ mM-PNPG, $K_{A1} = 300$ mM-glycerol and $K_{A2} = 85$ mM-glycerol; and in (b) $k_H = 33$ nmol/min, $k_{+2} = 0.0625$ nmol/min, $K_m = 0.3$ mM-PNPG and $K_{B1} = K_{B2} = 50$ mM-maltose.

the vertical axis as observed experimentally (Umezurike, 1971b), since $k_{+5} > k'_{+6}$ and $K_{A1} > K_{A2}$. It can be shown that under conditions when the substrate is saturating and only glycerol is present, eqn. (4) reduces to a form which in reciprocal form gives eqn. (5).

$$\frac{1}{k_{cat.(A)}} = \frac{1}{(k_H + k_{+2}[A]/K_{A1})} + \frac{[A]}{K_{A1}(k_H + k_{+2}[A]/K_{A1})} \quad (5)$$

Since $k_{+2}[A]/K_{A1} > k_H$, plots of $1/k_{cat.(A)}$ against $[A]$ will be non-linear (concave down) and those of $k_{cat.(A)}$ against $[A]$ will also be non-linear (convex up). Replots of intercepts calculated from primary double-reciprocal

Fig. 8. Double-reciprocal plots of $1/v$ against the reciprocal of the concentration of donor (*p*-nitrophenyl β -D-glucopyranoside; PNPG)

The data were calculated from eqn. (4) assuming the absence (●) or presence of 100 mM-glycerol (▲) or of both 100 mM-glycerol and 25 mM-maltose (Δ). Calculations were based on the assumption that $k_H = 33$ nmol/min, $k_{+2} = 65$ nmol/min (with glycerol), $k_{+2} = 0.0625$ nmol/min (with maltose), $K_m = 0.3$ mM-PNPG, $K_{A1} = 300$ mM-glycerol, $K_{A2} = 85$ mM-glycerol and $K_{B1} = K_{B2} = 50$ mM-maltose.

plots or of V_{max} against glycerol concentration were found to be as so predicted (Umezurike, 1978).

Similarly, if only maltose is present in the assay mixtures, all terms containing $[A]$, K_{A1} and K_{A2} in eqn. (4) drop out. As $K_{B1} = K_{B2}$ and $k'_{+6} \gg k_{+7}$, maltose will behave like a non-competitive inhibitor as observed and double-reciprocal plots will intersect on the horizontal axis as observed experimentally (Umezurike, 1971a). Thus at substrate saturation eqn. (6) is obtained.

$$\frac{1}{k_{cat.(B)}} = \frac{1}{(k_H + k_{+2}[B]/K_{B1})} + \frac{[B]}{K_{B1}(k_H + (k_{+2}[B]/K_{B1}))} \quad (6)$$

Since $k_{+2}[B]/K_{B1} \ll k_H$, plots of $1/k_{cat.(B)}$ against $[B]$ will be hyperbolic. Plots of V_{max} or of $1/V_{max}$ against maltose concentration were found to be non-linear as predicted (Umezurike, 1975a, 1978).

In the absence of both maltose and glycerol, eqn. (4) reduces to eqn. (7).

$$\frac{v}{[E]_0} = k_H / (1 + K_m/[S]) \quad (7)$$

Thus the parameter k_H is the experimentally determined maximum velocity in the absence of added acceptors. The parameters K_{A1} and K_{B1} in eqn. (4) can be determined as already described above, and K_m for donor can be determined from the double-reciprocal plot

(or by other plots) of data obtained when no added glucosyl acceptor is present in the assay mixture as is obvious in eqn. (7). An examination of eqn. (4) shows that when only glycerol is added to the assay system, apparent Michaelis constant [K_m (app.)] will be as shown in eqn. (8).

$$K_m \text{ (app.)} = K_m[(1 + [A]/K_{A2})/(1 + [A]/K_{A1})] \quad (8)$$

To determine the value of K_{A2} , therefore, all that is necessary is to determine the value of K_m (app.) in the presence of a known concentration of glycerol and to substitute this value and those of the other parameters in eqn. (8). A value of 85 mM for K_{A2} for glycerol has been calculated. The value of k_{+2} when only glycerol or maltose was present in the assay mixture was calculated from eqn. (5) or eqn. (6) respectively. Figs. 7 and 8 show double-reciprocal plots of data calculated from eqn. (4) by assuming the values of these parameters calculated as described above. Fig. 7(a) shows that the plots intersect to the right of the vertical axis when only glycerol is assumed to be present. Fig. 7(b) shows that the plots intersect at a point on the horizontal axis when only maltose is assumed to be present in the system. It is observed in Fig. 8 that, when both maltose and glycerol are assumed to be present in the system, the apparent K_m is the same as when only the same concentration of glycerol is present, but the apparent K_m is higher than is observed when no added acceptor is present. The plots in Figs. 7 and 8 are similar to plots of experimental data (Umezurike, 1971b, 1975a, 1978). Inspection of Fig. 7 shows that the intercept values respond to the concentration of added acceptor, as was found experimentally (Umezurike, 1971b, 1975a, 1978).

Thus, the kinetics of β -glucosidase-catalysed reactions in the presence of an efficient glucosyl acceptor (e.g. glycerol) or of a very ineffective acceptor (e.g. maltose) or both are consistent with Scheme 2 and eqn. (4). Eqn. (4) is similar to an equation previously derived on the assumption that both donor (substrate) and acceptors bind to the enzyme in a random fashion to form ternary complexes (Umezurike, 1975a). This assumption was based on the increase in K_m (donor) observed in the presence of all the efficient glucosyl acceptors tested. The random mechanism is unlikely because the acceptor molecule is expected to bind to the acceptor site after the release of the leaving group, as shown in Schemes 1 and 2. The effects of cellobiose discussed earlier support this view. The increase in K_m (donor) thus appears to be due

to K_{A1} being higher than K_{A2} in a system apparently involving the formation of a covalent glucosyl-enzyme intermediate and where $k_{+5} > k'_{+6}$ (cf. Schemes 1 and 2, and eqn. 4).

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