One-proton catalysis by the α -L-arabinofuranosidase III of *Monilinia fructigena*

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1. Michaelis–Menten parameters for the hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside were measured as a function of pL (pH or pD) in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. 2. The variation of both V_{max} and V_{max}/K_m with pL is sigmoid, the pK governing V_{max} shifting from 6.34 ± 0.05 in $^1\text{H}_2\text{O}$ to 6.84 ± 0.07 in $^2\text{H}_2\text{O}$, and that governing V_{max}/K_m from 5.89 ± 0.03 in $^1\text{H}_2\text{O}$ to 6.38 ± 0.05 in $^2\text{H}_2\text{O}$. 3. In the plateau regions there is a small inverse solvent isotope effect on V_{max}/K_m (0.92), and one of 1.45 on V_{max} . 4. The variation of V_{max} with isotopic composition is strictly linear, indicating that the isotope effect arises from the transfer of a single proton.

INTRODUCTION

Partial proton transfer from a group on the enzyme protein to the departing aglycone during the action of glycosidases has been repeatedly suggested to be one factor in their efficiency as catalysts (e.g. see Thoma & Koshland, 1960; Vernon, 1967; Capon, 1971; Kirby, 1987). However, although hitherto there has been no direct evidence for the application of this form of catalysis by a glycosidase acting on a glycoside, it has been shown that several glycosidases will efficiently hydrolyse glycosyl derivatives without proton donation to the aglycone (e.g. see Jones *et al.*, 1977; Dale *et al.*, 1986). [Proton donation by an active-site group has, however, been implicated in the hydration of enol ethers catalysed by glycosidases (see Lehmann & Schlesselmann, 1983; Hehre *et al.*, 1986).]

Solvent deuterium kinetic isotope effects are the classic probe of proton transfer (e.g. see Bell, 1973), but measurement of a single deuterium solvent isotope effect gives little information about an enzyme-catalysed reaction, partly because exchange at a multiplicity of sites could alter the protein conformation, and partly because in general the relation between Michaelis–Menten parameters and rate constants for individual chemical steps is not simple. In the last decade, however, the ‘proton inventory’ technique, which in favourable cases enables the number of protons responsible for the observed solvent isotope effect to be determined, has given much useful information about proton transfer during enzyme catalysis (Venkatasubban & Schowen, 1984).

The technique is based upon rate measurements in mixtures of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. In the general case the variation of rate with isotopic composition is given by eqn. (1):

$$v_0/v_n = \frac{\pi(1-n+n\phi_T)}{\pi(1-n+n\phi_R)} \quad (1)$$

where ϕ_R are the fractionation factors for all the exchangeable protons in the ground state and ϕ_T the fractionation factors for the same protons in the transition state: therefore one only sees a kinetic effect from protons whose fractionation factors change. In principle, ground-state fractionation factors for these

protons should be taken into account, but the common protic groups in a protein, with the exception of the thiol group, have fractionation factors close to unity. It is likely that the α -L-arabinofuranosidase III of *Monilinia fructigena* has no free thiol groups, since it is extracellular. The denominator in eqn. (1) thus is unity, and the determination of the number of protons responsible for any solvent deuterium kinetic isotope effect reduces to determining whether the variation of rate with isotopic composition of the solvent is best described by a linear function (one-proton), a quadratic (two protons), a cubic (three protons) etc. In practice the precision required of rate and isotopic composition data to distinguish between various types of oligoprotic effects is not attainable with conventional techniques unless the solvent isotope effect is large, and for modest effects (less than about 2) a distinction can be made only between one-, two- and many-proton effects.

In addition to solvent isotope effects arising directly from transfer of protons in the rate-limiting step of an enzyme reaction, effects can also arise from alteration of the pK_a values of critical ionizations: for this reason where possible proton inventories are compiled at plateau regions of the pL (pH or pD) profiles.

We now report the variation of Michaelis–Menten parameters for the hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside by the title enzyme as a function of pL in $^1\text{H}_2\text{O}$ and in $^2\text{H}_2\text{O}$, and a proton inventory in equivalent buffers corresponding to a pH of 4.40. Aglycone–glycone cleavage completely determines k_{cat} for this substrate since an $^{18}(V_{\text{max}})$ effect of 1.03 was observed consequent upon isotopic substitution at O-1 (Kelly *et al.*, 1987).

MATERIALS AND METHODS

The isolation of enzyme (Kelly *et al.*, 1987) and the preparation of substrate (Kelly *et al.*, 1988) have been described elsewhere.

Water ($^1\text{H}_2\text{O}$) was distilled, deionized and degassed. $^2\text{H}_2\text{O}$ (99.8% ^2H) was obtained from Aldrich Chemical Co. and used without further purification. Buffer solutions were made from 0.1 M-sodium acetate/acetic acid (pH 3.50–5.58, pD 3.55–5.94) and 0.1 M- NaH_2PO_4 /

Na_2HPO_4 (pH 6.00–6.76, pD 6.32–7.11) with analytical-reagent-grade components.

Michaelis–Menten parameters were determined by continuous monitoring of absorbance at 347.3 nm, the isosbestic point between *p*-nitrophenol and *p*-nitrophenolate, in a Pye– Unicam SP.8800 u.v./visible-region spectrophotometer fitted with a thermostatically controlled cell block maintained at $30.0 \pm 0.05^\circ\text{C}$. Reactions were carried out in 1 mm-pathlength cells. Initial rates were obtained by least-squares analysis of the progress curve, using the manufacturer's software.

Because of the difficulty of weighing small quantities of substrate, its concentration was estimated by initial u.v. absorbance [$\epsilon = 3326 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ($^1\text{H}_2\text{O}$) and $\epsilon = 3166 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ($^2\text{H}_2\text{O}$)]. The absorption-coefficient difference between substrate and product was $2334 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in $^1\text{H}_2\text{O}$ and $2036 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in $^2\text{H}_2\text{O}$. These values were obtained by injecting an ethanolic solution of substrate or product (2.0, 6.0 and $10.0 \mu\text{l}$) into $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$ buffers (3.0 ml) from the same syringe, and measuring absorbance. No change in absorption coefficient was found with pH or with pD.

Michaelis–Menten parameters were calculated by using the program HYPER (Cleland, 1979). The program HBBELL (Cleland, 1979) was used to calculate the $\text{p}K_a$ values governing the pL-dependencies of V_{max} and V_{max}/K_m in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. Both programs were translated into BASIC from FORTRAN to run on a BBC Master microcomputer.

The pL values of the buffer solutions were measured with a Radiometer PM62 standard pH-meter equipped with a glass combination electrode: pD was taken as (pH-meter reading) + 0.40.

Measurements in mixtures of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ were carried out in 0.1 M-sodium acetate buffer at the constant buffer ratio that gave a pH of 4.40 in pure $^1\text{H}_2\text{O}$; isotopic composition was calculated gravimetrically. Initial-rate measurements were made at a substrate concentration of 5.78 or 7.80 mM. Accordingly, initial rates were corrected to complete saturation, assuming a linear dependence of K_m on isotopic composition. A linear dependence on isotopic composition was also assumed for the absorption-coefficient change.

Statistical analyses of proton inventory data were performed with the SAS (1985) statistical package (Sas Institute, Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Incubation of the enzyme for 12 h at 30°C in the presence of 1 mM-mercuric acetate (pH 4.40, 0.1 M-acetate buffer) produced no significant decrease in activity of the enzyme. This confirmed that the enzyme possessed no catalytically important thiol groups, and that therefore that the denominator of eqn. (1) can be taken as unity.

Fig. 1 shows the variation of k_{cat} for *p*-nitrophenyl α -L-arabinofuranoside as a function of pH and pD. The continuous lines represent the best fit to the data as calculated from HBBELL, and are described by eqns. (2) and (3). k_{cat} values are calculated from activities by using the maximum specific activity reported by Kelly *et al.* (1987):

$$\text{For } ^1\text{H}_2\text{O}: k_{\text{cat}} \text{ (s}^{-1}\text{)} = 10.49/(1 + 10^{-6.33}/[\text{H}^+]) \quad (2)$$

$$\text{For } ^2\text{H}_2\text{O}: k_{\text{cat}} \text{ (s}^{-1}\text{)} = 7.23/(1 + 10^{-6.84}/[\text{H}^+]) \quad (3)$$

Fig. 1. Dependence of k_{cat} for hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside by the α -L-arabinofuranosidase III of *Monilia fructigena* upon pL in $^1\text{H}_2\text{O}$ (Δ) and $^2\text{H}_2\text{O}$ ($\circ$) at 30°C

Fig. 2. Dependence of k_{cat}/K_m for hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside by the α -L-arabinofuranosidase III of *Monilia fructigena* upon pL in $^1\text{H}_2\text{O}$ (Δ) and $^2\text{H}_2\text{O}$ ($\circ$) at 30°C

Thus there is a significant deuterium solvent isotope effect on the pH-independent portion of the k_{cat} profile. Moreover, the difference in the $\text{p}K_a$ of the essential ionization between $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ is about what is expected for normal acids of about this strength (Venkatasubban & Schowen, 1984).

Fig. 2 shows an analogous treatment of the data for k_{cat}/K_m . The continuous lines are described by eqns. (4) and (5) (the standard deviations on the numerators are 178 and 255 respectively):

$$\text{For } ^1\text{H}_2\text{O}: k_{\text{cat}}/K_m \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)} = 13235/(1 + 10^{-5.89}/[\text{H}^+]) \quad (4)$$

$$\text{For } ^2\text{H}_2\text{O}: k_{\text{cat}}/K_m \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)} = 14409/(1 + 10^{-6.38}/[\text{H}^+]) \quad (5)$$

Again the $\text{p}K_a$ shift is as expected, but now there is a small but statistically significant inverse solvent isotope effect on the pL-independent region [$^2\text{H}_2\text{O}(V_{\text{max}}/K_m) = 0.92 \pm 0.02$]. It is likely, therefore, that V_{max}/K_m is

Table 1. K_m values for hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside by the α -L-arabinofuranosidase of *Monilia fructigena* at 30 °C K_m values are given as means $\pm$ S.E.M.

pH	$10^4 \times K_m$ (M)	pD	$10^4 \times K_m$ (M)
3.50	8.5 ± 0.2	3.55	4.3 ± 0.3
3.78	7.1 ± 0.7	3.81	4.2 ± 1.0
4.06	7.6 ± 0.1	4.07	4.9 ± 0.8
4.30	9.3 ± 0.1	4.31	5.4 ± 0.2
4.77	9.0 ± 0.1	4.96	5.9 ± 0.7
5.00	9.4 ± 0.1	5.24	6.1 ± 0.1
5.25	8.6 ± 0.4	5.94	6.2 ± 0.3
5.58	9.8 ± 0.7	6.32	6.8 ± 0.7
6.12	10.7 ± 2.0	7.11	11.3 ± 0.3
6.55	16.3 ± 1.0		
6.76	21.9 ± 2.0

Fig. 3. Proton inventory for $V_{\max}$ for hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside by the α -L-arabinofuranosidase III of *Monilia fructigena* at 30 °C

governed by a different kinetic event than is $V_{\max}$, and this kinetic event is probably non-covalent. Unfortunately, with conventional techniques it was not possible to measure first-order rate constants with $[S] \ll K_m$ adequately precisely to demonstrate the absence of an $^{18}(V_{\max}/K_m)$ kinetic isotope effect.

K_m values are given in Table 1.

Fig. 3 illustrates a proton inventory for $V_{\max}$. Fitting the data to eqn. (6) was significant at the 99.9% confidence limit, and gave a value of ϕ of 0.75 ± 0.01 . The fit of the data to eqn. (7), corresponding to two-proton catalysis, showed no significant improvement in fit; it was not significant at the 80% confidence level. It gave

$\phi_1 = 0.71 \pm 0.02$ and $\phi_2 = 1.06 \pm 0.02$, i.e. the value for ϕ_1 was close to that obtained in the one-proton fit and that for ϕ_2 close to unity.

$$v_n/v_0 = (1 - n + n\phi) \quad (6)$$

$$v_n/v_0 = (1 - n + n\phi_1)(1 - n + n\phi_2) \quad (7)$$

We therefore conclude that the pH-independent solvent isotope effect on $V_{\max}$ is due to the transfer of a single proton. This is transferred to the leaving oxygen atom of the substrate. It is likely that the ionization of pK 5.90 in the free enzyme (6.18 in the ES complex) can be attributed to the ionization of the acid catalytic group. Therefore, if the acid catalytic group has a pK of around 6, proton transfer to a leaving group of pK around 7 fulfils Jencks' (1972) rule that general acid catalysis should be observed when the catalysing acid has a pK_a lower than that of the leaving group.

The fractionation factor for the transferred proton is sufficiently large that at the transition state it must be bound largely to the leaving group or to the acid. On the basis of the near-zero β_{1g} value obtained for a series of aryl α -L-arabinofuranosides (Kelly *et al.*, 1987) we have previously argued that it is largely transferred to the leaving group.

We thank the U.K. Science and Engineering Research Council for Grant GR/D/83002.

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Received 10 June 1988; accepted 6 July 1988