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β-Glucosidase: Substrate, Solvent, and Viscosity Variation as Probes of the Rate-Limiting Steps†

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Received August 6, 1985; Revised Manuscript Received November 20, 1985

ABSTRACT: The second-order rate constants (k_{cat}/K_m) for the β-glucosidase-catalyzed hydrolysis of aryl β-D-glucopyranosides show a bell-shaped dependence on pH. The pK_as that characterize this dependence are 4.4 ($\Delta H_{ion} \approx 0$) and 6.7 ($\Delta H_{ion} \approx 0$). In D_2O these pK_as are increased by 0.5 (±0.1) unit, but there is no solvent isotope effect on the pH-independent second-order rate constant. Nath and Rydon [Nath, R. L., & Rydon, H. N. (1954) *Biochem. J. 57*, 1-10] examined the kinetics of the β-glucosidase-catalyzed hydrolysis of a series of substituted phenyl glucosides. We have extended this study to include glucosides with phenol leaving groups of pK_a < 7. Brønsted plots for this extended series were nonlinear for both k_{cat}/K_m and k_{cat}. Brønsted coefficients for those compounds with leaving groups of pK_a > 7 (for k_{cat}/K_m) or pK_a > 8.5 (for k_{cat}) were nearly equal to −1.0, indicating substantial negative charge buildup on the leaving group in the transition state. The nonlinearity indicates an intermediate in the reaction. This was confirmed by partitioning experiments in the presence of methanol as a competing glucose acceptor. A constant product ratio, [methyl glucoside]/[glucose], was found with aryl glucoside substrates varying over 16 000-fold in reactivity (V/K), indicative of a common intermediate. Viscosity variation (in sucrose-containing buffers) was used to probe the extent to which the β-glucosidase reactions are diffusion-controlled. The results suggest that while k_{cat}/K_m may be limited by the association of the enzyme with the more reactive substrates (leaving group pK_a < 7), k_{cat} is not limited by product dissociation.

Glucosidases are widespread in nature, efficiently catalyzing the hydrolysis of various glycosides and oligosaccharides. Although β-glucosidase (EC 3.2.1.21) from sweet almond was one of the earliest enzymes investigated (Wohler & Liebig, 1837; Fischer, 1898), its mechanism of action is still far from clearly understood. Some indirect evidence suggests that β-glucosidase undergoes a double-displacement mechanism. Eveleigh and Perlin (1969) have shown that the reaction proceeds with retention of configuration at the anomeric carbon (C-1), and it is known that it is the glucosyl C–O bond that is cleaved (Bunton et al., 1954; Rosenberg & Kirsch, 1981). The β-glucosidase-catalyzed hyrolysis of phenyl β-glucopyranoside shows essentially no secondary deuterium kinetic isotope effect on V/K (Dahlquist et al., 1969). The simplest, but not only (Knier & Jencks, 1980), explanation of this is that there is no hybridization change at C-1 in the rate-limiting step (i.e., an S_N2 type mechanism). More recently, Weber and Fink (1980) have demonstrated "burst" kinetics with PNPGlc[1] at subzero temperatures. This is consistent with an intermediate in the β-glucosidase reaction. This work was undertaken in order to further probe the nature of this putitive intermediate.

Because of the rather broad specificity of β-glucosidase [see Dale et al. (1985)], it is ideally suited for structure–reactivity studies. Indeed, Nath and Rydon (1954), using a series of 21 substituted phenyl glucosides (leaving group pK_a > 7), persuasively demonstrated a structural sensitivity of the β-glucosidase reaction similar to that of the alkaline hydrolysis reaction. We report here an extension of this study to include phenyl glucosides with more acidic leaving groups. We also report the applications of solvent kinetic isotope effects, pH

† This research was supported in part by a grant from the Herman Frasch Foundation.

[1] Abbreviations: PNPGal, *p*-nitrophenyl β-D-galactopyranoside; PNPGlc, *p*-nitrophenyl β-D-glucopyranoside; DNPGal, dinitrophenyl β-D-galactopyranoside; DNPGlc, dinitrophenyl β-D-glucopyranoside; EDTA, (ethylenedinitrilo)tetraacetic acid; MES, 4-morpholineethanesulfonate; PIPES, 1,4-piperazinediethanesulfonic acid; SKIE, solvent kinetic isotope effect.

0006-2960/86/0425-2522$01.50/0

variation, product partitioning, and viscosity variation to probe the mechanism of action of β-glucosidase.

Materials and Methods

Materials

Sweet almond β-glucosidase was obtained from Sigma Chemical Co. (type I, sp. act. usually ~30 units/mg with salicin as substrate at pH 5.0, 37 °C; most of the work reported here was carried out with enzyme from lot 54F-4020). The enzyme yielded a single band on gel filtration (Fractogel HW55 or Sephadex G-200, pH 4.0, 5.5, and 8.5) corresponding to a M_r of ~100 000 both in the presence and in the absence of 8 M urea. Electrophoresis on polyacrylamide slab gels, carried out in the presence of sodium dodecyl sulfate (Laemmli, 1970), resulted in a major band (≥90%) corresponding to a M_r of 90 000. This suggests that the major component of the commercial enzyme is the monomeric isozyme B (Helferich & Kleinschmidt, 1968; Legler & Hasnain, 1970). Enzyme concentration was determined by absorbance at 278 nm with $E^{1\%} = 18.2$ (Legler & Hasnain, 1970), which corresponds to $\epsilon_{278} = 1.64 \times 10^5$ M^{-1} cm^{-1} on the basis of a M_r of 90 000.

Enzymes used for analysis of products (e.g., glucose-6-phosphate dehyrogenase, galactose dehydrogenase, and hexokinase) or of substrates (*Escherichia coli* β-galactosidase) were also obtained from Sigma, as were most of the substrates; *m*-nitrophenyl β-D-glucopyranoside was obtained from Koch-Light Laboratories.

The dinitrophenyl glycosides were prepared by reaction of the 2,3,4,6-tetra-*O*-acetyl-α-D-glycopyranosyl bromide (Sigma) with 2,4-dinitrophenol (Sigma) or 3,4-dinitrophenol (Fluka) by the methods of Ballardie et al. (1973) and Sinnott and Viratelle (1973). Pentafluorophenyl β-D-glucopyranoside was prepared by the method of Jermyn (1966). *p*-Chlorophenyl β-D-glucopyranoside was prepared essentially as described by Nath and Rydon (1954). The recyrstallized glucosides were judged pure by thin-layer chromatography on silica gel (1:1:1 ethyl acetate–methanol–benzene) and by alkaline and enzymic hydrolysis end points. Melting points were found to be in reasonable agreement with literature values. 3,4-DNPGal was found to have a decomposition temperature of 138–141 °C.

Methods

Kinetics. The β-glucosidase-catalyzed hydrolysis of the nitrophenyl and dinitrophenyl glucosides was followed spectrophotometrically on a Beckman Model 3600 spectrophotometer as described previously (Dale et al., 1985). The hydrolysis of the phenyl glucosides was followed by removing aliquots of the reaction mixture at various times, diluting them into 1 mM NaOH, and reading the absorbance at 287 nm due to phenoxide ($\epsilon = 2600$ M^{-1} cm^{-1}).

The kinetic parameters (K_M and V_{max}) for the enzymic reaction under initial velocity (zero-order) conditions were estimated by the median method of Cornish-Bowden and Eisenthal (1974). The nonparametric 95% confidence limits for the parameters were calculated by the method of Cornish-Bowden et al. (1978). Under first-order conditions ([S] $\ll K_M$), the kinetic parameter V/K could be estimated graphically from a plot of log $(A_\infty - A_t)$ vs. time where A is the absorbance of the aglycon product. These plots were linear for at least three half-lives. Rate constants were determined at least in triplicate for each reaction. Plots of the pseudo-first-order rate constant vs. enzyme concentration were linear over a 400-fold concentration range (M. DeLaPaz and L. Byers, unpublished observations). The second-order rate constant k_a ($=k_{cat}/K_M$) was obtained by dividing the V/K value by the enzyme concentration, assuming a M_r of 90 000 for the active species.

Buffers containing sucrose (Sigma, grade I) or other viscosogenic agents were prepared by dissolving the required amount of the viscosogen in a previously prepared solution containing 0.01 M PIPES, 0.02 M sodium acetate, and 0.1 mM EDTA, pH 5.6). The relative viscosities η_{rel} ($=\eta/\eta^0$) of the buffer solutions were calculated from the solution densities and measurements with an Ostwald viscometer at 25 °C by using the buffer solution containing no added viscosogen as reference, η^0 (Brouwer & Kirsch, 1982).

Partitioning Experiments. The transfer of the glucose moiety from the aglycon to water or to an alternate acceptor (usually methanol) was investigated under first-order conditions. the buffer solutions were prepared by adding the alternate acceptor to the standard buffer solution (0.01 M PIPES, 0.02 M sodium acetate, and 0.1 mM EDTA, pH 5.6). In the case of methanol, the final concentration was usually ~3.5 M. The concentration of the methyl glucoside produced in the presence of methanol was calculated as the difference between the amount of aglycon liberated and the amount of glucose produced. The amount of glucose released was determined by use of the coupled hexokinase/glucose-6-phosphate dehydrogenase enzyme system as described previously (Dale et al., 1985; Schachter, 1975). Under most of the reaction conditions employed, the hydrolysis of methyl β-D-glucopyranoside was found to be negligible. The methanol concentrations used were found to have no adverse effect on β-glucosidase. When the enzyme (1.7 mg/mL) was incubated in 15% v/v CH_3OH for 3 h, there was no evidence of irreversible activity loss (when assayed with PNPGlc). Partitioning experiments with PNPGal were carried out under conditions similar to those of the transglucosylation reactions. The amount of galactose released was determined with galactose dehydrogenase.

Solvent Isotope Effects. Solutions were prepared in deuterium oxide (99.8%, Norsk Hydro, Oslo, Norway). The pD values were estimated from the formula pD = pH (meter reading) + 0.41 (Covington et al., 1968) and adjusted with either NaOD (48% in D_2O) or DCl (38% in D_2O). pD(H) fluxuations during any kinetic run did not exceed 0.05 unit. EDTA (0.1 mM) was added to both H_2O and D_2O buffer solutions to avoid interference by any divalent cations that may be present in one solvent but not the other. The enzyme is stable in D_2O. When aliquots from stock solutions of the enzyme prepared in H_2O and D_2O were used to catalyze the hydrolysis of PNPGlc (in H_2O), identical kinetics were observed. The hydrolysis of PNPGlc at the various pH(D) values was monitored at the isosbestic point for *p*-nitrophenol (λ = 347.5 nm, $\epsilon = 3.5 \times 10^3$ M^{-1} cm^{-1}). The pL (=pH or pD) profiles for the kinetic parameters were fitted to the appropriate equations with the computer programs of Cleland (1979).

Temperature Effects. The pH of each buffer solution was adjusted at the temperature of the reaction (27–47 °C). The enzyme was found to be quite stable at these temperatures. Indeed, when enzyme (3 mg/mL) was incubated at 59 °C (pH 5.6), the half-life for activity loss (assayed at 27 °C) was found to be 55 min.

Results

pH(D) Profile. The hydrolytic activity of β-glucosidase with a variety of substrates (aryl and alkyl β-D-glucopyranosides and *p*-nitrophenyl β-D-glycosides of xylose, galactose, and fucose) was found to fit a bell-shaped pH profile with an optimum near pH 5.6. The data for the second-order rate

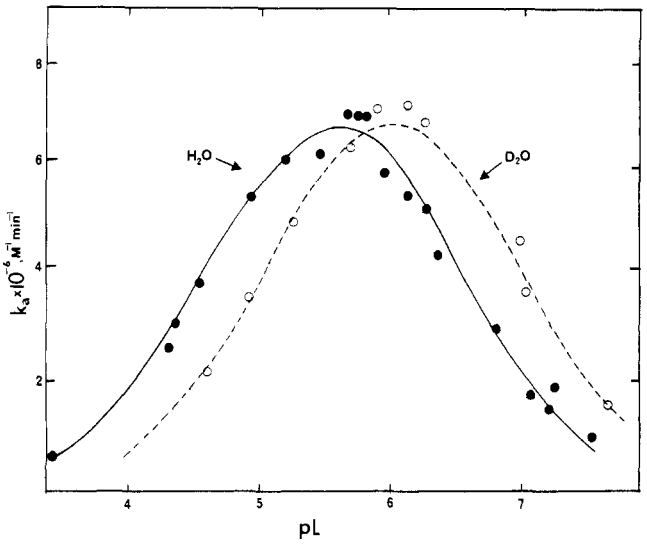


FIGURE 1: Dependence of the second-order rate constant for the β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (=S) on pL (=−log [lyonium ion]). Reaction conditions: T = 27 °C; [S] = 0.14 mM, ~65 μg/mL enzyme; 0.01 M PIPES, 0.02 M sodium acetate, and 0.1 mM EDTA. The lines are the theoretical fit to eq 1 in the text with k_a^{lim} = 7.5 × 10^6 M^{-1} min^{-1}, pK_1 = 4.4, and pK_2 = 6.7 in H_2O and k_a^{lim} = 7.8 × 10^6 M^{-1} min^{-1}, pK_1 = 5.0, and pK_2 = 7.1 in D_2O.

constant k_a (=k_{cat}/K_M) for the β-glucosidase-catalyzed hydrolysis of PNPGlc is shown in Figure 1. The data were fit to the equation:

$$k_a^{obsd} = \frac{k_a^{lim}}{1 + 10^{pK_1-pL} + 10^{pL-pK_2}} \quad (1)$$

where pL = pH or pD and pK_1 and pK_2 are the molecular dissociation constants or "effective pK_as". The pH dependence of k_a was found to be almost entirely due to the pH dependence of k_{cat}. Thus, over the range 4.0 ≤ pH ≤ 7.3, K_M was found to vary by less than a factor of 2.

In H_2O the pK_a values (4.4 and 6.7) appear to be essentially independent of the substrate. For example, at pH 5.6 (27 °C) the k_a value for the β-glucosidase-catalyzed hydrolysis of PNPGlc is 13 times greater than that for hydrolysis of PNPGal. The same ratio [13 (±1)] was found at pH 4.5 and 7.5. Similarly, the k_a value for PNPGlc was found to be 14 (±1) times greater than that for salicin and 250 (±20) times greater than that for phenyl β-D-glucopyranoside at pH 4.5, 5.6, 6.2, and 7.5. The pK_a values were also found to be independent of temperature. Thus, with PNPGlc k_a(37 °C)/k_a(27 °C) = 1.6 (±0.1) and k_a(47 °C)/k_a(27 °C) = 2.2 (±0.2) at pH 4.8, 5.6, 6.2, and 7.5. While k_a varies by over a factor of 2 in the temperature range of 27–47 °C [consistent with a $\Delta H^{\ddagger} \approx 8.7$ (±0.6) kcal/mol^{-1}], the K_M [=2.5 (±0.3) mM] is independent of temperature.

The data in Figure 1 show the effect of substituting D_2O for H_2O on the pL–rate profile for the β-glucosidase-catalyzed hydrolysis of PNPGlc. The only significant effect is the 0.5 (±0.1) unit increase in the pK_as. There is no significant solvent kinetic isotope on the limiting second-order rate constant since $k_a^{lim}(H_2O)/k_a^{lim}(D_2O)$ = 0.95 (±0.15). The solvent isotope effects on V_{max} and K_M were determined by measuring these parameters either at pH 5.6 in H_2O or pD 6.1 in D_2O. Again there is no significant isotope effect: ^DV = 1.02 and ^DK_M = 1.06.[2] We find no solvent isotope effect on k_a for any substrate tested. The k_a for 2,4-DNPGlc was measured over a range of pL values in both H_2O and D_2O. The value of $^D(V/K)$ was found to be 0.97 (±0.12).[2] A small solvent kinetic isotope effect on k_{cat} was found at the pL optimum for 2,4-DNPGlc hydrolysis—^DV = 1.5 (±0.2). The solvent kinetic isotope effects on k_a were measured (pH 5.6, pD 6.1) for other substrates (varying over 4 orders of magnitude in reactivity). For *p*-nitrophenyl β-D-fucopyranoside, PNPGal, methyl β-D-glucopyranoside, and *p*-nitrophenyl thio-β-D-glycopyranoside, the solvent kinetic isotope effects were found to be, within experimental error, indistinguishable from unity.

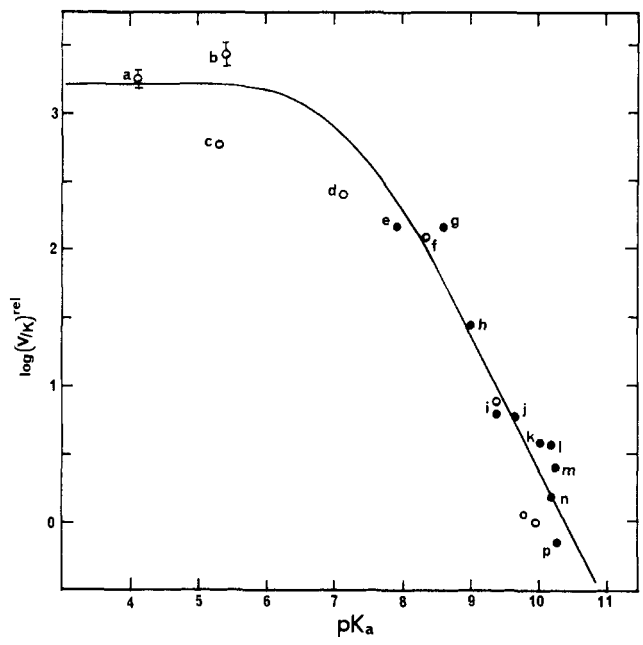


FIGURE 2: Brønsted plot for the β-glucoside-catalyzed hydrolysis of substituted phenyl glucosides. $(V/K)^{rel}$ is the ratio of V_{max}/K_M for the substituted phenyl glucoside to that of phenyl glucoside (point o). The phenyl substituents are (a) 2,4-dinitro, (b) 3,4-dinitro, (c) pentafluoro, (d) 4-nitro, (e) 4-cyano, (f) 3-nitro, (g) 3-cyano (h) 3-chloro, (i) 4-chloro, (j) 3-methyl, (k) 3-methoxy, (l) 4-methoxy, (m) 4-isopropyl, (n) 4-methyl, (o) H, and (p) 4-*tert*-butyl. The solid points are from the data of Nath and Rydon (1954). A line drawn through points e–p has a slope of β_{lg} = −0.97.

Structure–Reactivity Studies. Nath and Rydon (1954) conducted a comprehensive investigation of the β-glucosidase-(emulsin-) catalyzed hydrolysis of 21 substituted phenyl β-glucosides. They found that the V/K value is well correlated with the Hammett σ^- value, yielding a value of ρ near 2. Since the most acidic leaving group examined (*p*-nitrophenol) has a pK_a = 7.1, we extended Nath and Rydon's study by including more acidic leaving groups: 3,4-dinitrophenol (pK_a = 5.4), pentafluorophenol (pK_a = 5.3), and 2,4-dinitrophenol (pK_a = 4.1). Figure 2 illustrates the dependence of the normalized V/K (or, equivalently, of k_a) for the β-glucosidase-catalyzed hydrolysis of a series of substituted phenyl β-glucosides on the pK_a of the phenol leaving group. The rate constants follow a nonlinear Brønsted relationship and conform to the expression

$$1/k_a\ (M^{-1}\ s^{-1}) = 10^{0.97pK_a-12.74} + 10^{-5.81} \quad (2)$$

This is indicative of the two-step process with equal rate constants for the two steps (=$10^{5.81}$ = 6.5 × 10^5 M^{-1} s^{-1}) in the case of a leaving group with pK_a ~7.1.

The data of Nath and Rydon (1954) was obtained under conditions only slightly different from ours (i.e., pH 5.25 vs. pH 5.6 (cf. Figure 1) and 30 °C vs. 27 °C]. These differences have very little effect on the second-order rate constants. In order to facilitate a comparison of the data, the results are

[2] $^DV = k_{cat}(H_2O)/k_{cat}(D_2O)$; $^DK = K_m(H_2O)/K_m(D_2O)$; $^D(V/K) = {}^Dk_a = k_a(H_2O)/k_a(D_2O)$.

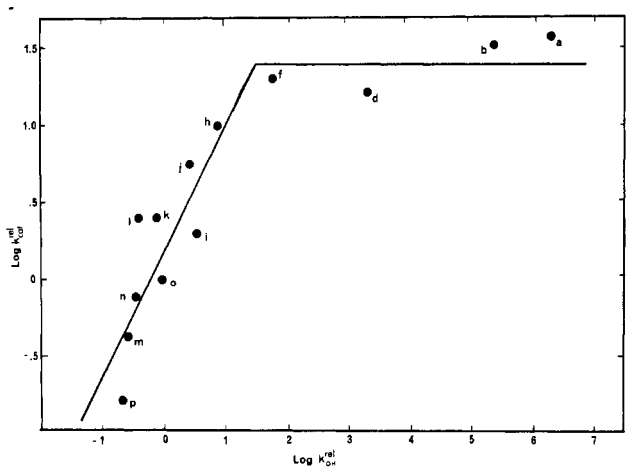


FIGURE 3: Relationship between k_{cat} for the β-glucosidase-catalyzed hydrolysis of substituted phenyl β-D-glucopyranosides and the pseudo first-order rate constant for alkaline hyrolysis (4 N NaOH, 60 °C). The rate constants are given as those relative to the corresponding value for the unsubstituted phenyl glucoside. The labeling of the points is the same as in Figure 2. Points f–p are from Nath and Rydon (1954), and the line drawn through them corresponds to a value of $\beta_{lg} = -1.0$ for k_{cat}. The break in the curve is arbitrarily drawn at a point corresponding to a leaving group of $pK_a \sim 8.5$. The Brønsted plot for alkaline hydrolysis is linear for the entire series.

presented as values relative to the V/K obtained with phenyl β-glucoside. As a check for possible differences in the enzyme preparation used by Nath and Rydon and by us, the $(V/K)^{rel}$ values of several glucosides can be compared. For example, Nath and Rydon (1954) found that V/K for PNPGlc was 233 times greater than V/K for phenyl β-glucoside. With the enzyme preparation used in our study, we find a $(V/K)^{rel}$ value for PNPGlc of 243 (±14). $(V/K)^{rel}$ values for the *o*- and *m*-nitrophenyl β-glucosides and for *p*-chlorophenyl β-glucoside were also found to be nearly identical with the corresponding values reported by Nath and Rydon. This suggests that there is no significant difference in the substrate specificities between the enzyme preparations used in the two studies, and a direct comparison of the results is justified.

It will be noted in Figure 2 that for those points corresponding to leaving groups with pK_as greater than 7.9 there is a linear relationship between log (V/K) and pK_a. The equation for this line is

$$\log (V/K)^{rel} = -0.97pK_a + 10.13; \quad n = 12, r = -0.94 \quad (3)$$

The slope of this line corresponds to a Brønsted β value of −0.97. Since the ρ^- value for phenol ionization is −2.23 (Biggs & Robinson, 1961), this Brønsted coefficient is equivalent to a ρ value of 2.2 [=(−0.97)(−2.23)]. It should be noted, however, that only those points corresponding to meta- and para-substituted phenols were included in this correlation. The ortho-substituted phenols, with $pK_a > 7.1$, show positive deviations (of 3–30-fold) from the line.

The k_{cat} values for the β-glucosidase-catalyzed hydrolysis of most of the phenyl glucosides show a strong dependence on the acidity of the leaving group, as do the rate constants for alkaline hydrolysis, k_{OH}. The correlation between k_{cat} and k_{OH} is shown in Figure 3. As with the V/K data, the extended correlation suggests a nonlinear free energy relationship. The break in the curve is at a point corresponding to a leaving group of $pK_a \sim 8.5$.

Transglucosylation. The nonlinear structure–reactivity correlations (Figures 2 and 3) suggest the possibility of an intermediate (e.g., a covalent glycosyl-enzyme or an electrostatically stabilized glycosyl oxocarbonium ion) in the reactions catalyzed by β-glucosidase. If such an intermediate exists,

Scheme I

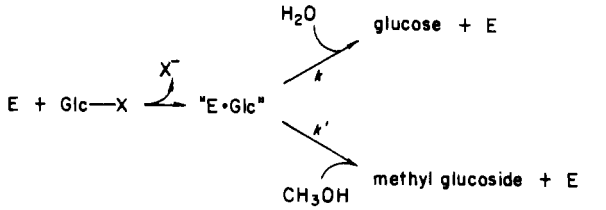


Table I: Methanolysis/Hydrolysis Ratios for Reaction of β-Glucosides with β-Glucosidase[a]

β-glucoside	k_a^{rel} [b]	k'/k [c]
3,4-dinitrophenyl	16300	14.2 (±0.8)
2,4-dinitrophenyl	6000	13.0 (±0.9)
4-methylumbelliferyl	1800	12.5 (±0.9)
4-nitrophenyl	1000	14.7 (±1.0)
2-formylphenyl	740	17.3 (±0.5)
3-nitrophenyl	740	15.9 (±1.5)
4-(nitrothio)phenyl	1	10.2 (±1.2)[d]

[a] pH = 5.6, T = 27 °C, $[CH_3OH]$ usually ~3.5 M. [b] Second-order rate constant ($=k_{cat}/K_M$) for hydrolysis, relative to that for 4-(nitrothio)phenyl β-glucoside. [c] Partition ratio calculated from eq 4 in the text. [d] This value is less accurate than the others due to corrections involved in its estimation. The k_a for hydrolysis of this substrate is only about twice that for methyl glucoside. It was thus necessary to correct the observed k'/k value for the hydrolysis of the methyl glucoside product.

then it may be possible to partition this intermediate between alternate acceptors. The partitioning of a common intermediate between water and an alternate acceptor such as methanol is illustrated in the simplified mechanism of Scheme I. If the transfer of the glucosyl residue from the intermediate to the acceptor is first-order in the acceptor concentration, then the product ratio is given by eq 4. Although methanol acts

$$[\text{methyl glucoside}]/[\text{glucose}] = k'[CH_3OH]/k[H_2O] \quad (4)$$

as an inhibitor of the β-glucosidase reaction (Dale et al., 1985), perhaps via a "medium effect", it was found to be a suitable acceptor when PNPGlc was used as the glucose donor. The partition ratio k'/k was found to be 15 (±1). Consistent with eq 4, this value was found to be independent of the methanol concentration (from 2 to 5.6 M). The partition ratios obtained with a variety of glucosides are summarized in Table I. The partition ratio is independent of the leaving group. The partition ratio, however, is dependent on the glycon moiety. When PNPGal was used as the donor, a value of $k'/k = 35$ (±5) was found. Glucose transfer (from PNPGlc) was found to occur with other acceptors in addition to methanol. These include ethanol ($k'/k = 9$) and β-mercaptoethanol ($k'/k = 20$).

Viscosity Variation. The effects of viscosity in sucrose-containing buffer on the k_a values for the β-glucosidase-catalyzed hydrolysis of a variety of substrates is summarized in Table II. In general, the effect of sucrose appears to be more pronounced as the reactivity of the substrate increases. The sucrose effect on the second-order rate constant is predominantly due to an increase in K_M. 2,4-DNPGlc, in the absence of added sucrose, has a K_M = 1.2 mM (95% confidence interval = 1.0–1.5 mM). In the presence of 24% (w/w) sucrose, K_M increased 3-fold and k_{cat} remains unchanged.

An increase in the viscosity of the medium is expected to slow down the diffusion-controlled steps in the reaction (i.e., substrate association with the enzyme or product dissociation from the enzyme). These steps are most likely to be rate determining for the most reactive substrate. It, therefore, was surprising to find a "viscosity" effect for the slower substrates such as *m*-nitrophenyl glucoside. This, however, can be ex-

Table II: Dependence of Viscosity Effect on Nature of Substrate[a]

β-D-glycoside	k_a (M^{-1} s^{-1}) (for glucoside)	k_a(Glc)/k_a(Gal)[b]	k_a(Fuc)/k_a(Glc)[c]	$k_a(\eta_{rel}=1)/k_a(\eta_{rel}=2.2)$ galactoside	glucoside	fucoside
3,4-dinitrophenyl	1.77 (±0.05) × 10^6	7.8 (±0.5)		1.70 (±0.09)	2.56 (±0.15)	
2,4-dinitrophenyl	6.5 (±0.6) × 10^5	8.0 (±0.5)	1.0 (±0.1)	2.36 (±0.21)	3.04 (±0.18)	2.54 (±0.09)
4-nitrophenyl	1.1 (±0.2) × 10^5	13.1 (±0.9)	2.9 (±0.2)	1.00 (±0.04)	1.25 (±0.04)	1.68 (0.08)
3-nitrophenyl	8.0 (±0.7) × 10^4				1.36 (±0.05)	

[a] T = 27 °C, pH = 5.6 (0.01 M PIPES, 0.02 M sodium acetate, and 0.1 mM EDTA). Buffers with a relative viscosity of 2.2 contain 24% (w/w) sucrose. [b] Ratio of second-order rate constants (in the absence of sucrose) for reaction of glucoside and galactoside. [c] Ratio of rate constants for reaction of fucoside and glucoside.

Table III: Dependence of Inhibition on Nature of Substrate

β-glucoside	$k_a(-I)/k_a(+I)$, pH 5.6 3-phenylpropylamine (1 mM)	2-deoxy-2-aminoglucose (5 mM)
3,4-dinitrophenyl	1.28 (±0.11)	1.59 (±0.09)
2,4-dinitrophenyl	1.33 (±0.09)	1.48 (±0.10)
4-nitrophenyl	1.39 (±0.09)	1.35 (±0.10)
3-nitrophenyl	1.49 (±0.05)	1.33 (±0.07)
phenyl	1.27 (±0.02)	1.45 (±0.07)

plined by assuming that sucrose not only increases the viscosity of the medium but also acts as a competitive inhibitor. Similar effects were observed with D-mannose as a viscosogen. It would be desirable to find a viscosogenic agent that does not competitively inhibit the enzyme. This, however, is difficult because (1) almond β-glucosidase is inhibited by a wide variety of compounds (Dale et al., 1985) and (2) there is a possibility of translgucosylation with viscosogens such as glycerol. Viscosogenic agents that are polymers (e.g., ficoll) generally do not show a substantial retardation of diffusion of small hydrophilic species at concentrations that result in a substantial increase in the macroscopic viscosity of the medium (Biancheria & Kegeles, 1957; Stokes & Weeks, 1964; Hardy & Kirsch, 1984). Consistent with this, we see only slight effects of polymeric viscosogenic agents on the reactions catalyzed by β-glucosidase. Thus, the k_a values for 2,4-DNPGlc, 2,4-DNPGal, 3,4-DNPGlc, and 3,4-DNPGal were decreased by only 25% (±5%) in the presence of ficoll (η_{rel} = 2.2). Hyaluronic acid (1.5 mg/mL, $\eta_{rel} \approx 5$) had no effect on the k_a values.

There is a possibility that the different rate constant ratios $k_a(\eta_{rel}=1)/k_a(\eta_{rel}=2.2)$ obtained with different substrates in the presence of sucrose (Table II) are due not to viscosity variation but exclusively to competitive inhibition (e.g., different isozymes that act on the different substrates and have different K_i values for sucrose). To rule out this unlikely possibility, we examined the effect of a couple of known competitive inhibitors (Dale et al., 1985) on the β-glucosidase-catalyzed hydrolysis of several phenyl glucosides. These are summarized in Table III. The inhibition by 3-phenylpropylamine and by 2-deoxy-2-amino-D-glucose shows little dependence on the glucoside substrate. Similar K_i values were also found with PNPGal, PNPGlc, and *p*-nitrophenyl β-D-fucoside for 3-phenylpropylamine [K_i = 3.0 (±0.8) mM] and for 2-deoxy-2-amino-D-glucose [K_i = 12 (±3) mM].

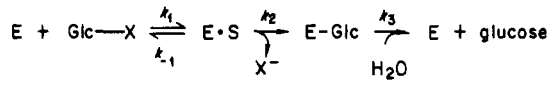
Discussion

pL Dependence of k_a. The bell-shaped curve for the pH(D) dependence of k_a (Figure 1) for the β-glucosidase reaction is typical of the pH dependence seen for most enzymic reactions. The simplest kinetic model accommodating this pH dependence (with a nonionizing substrate) is one in which two groups on the enzyme can ionize, but activity is seen only when one is protonated and the other is not. This model is described algebraically by eq 1. The apparent pK_a values (in H_2O) that describe the pH dependence of k_a for PNPGlc hydrolysis are pK_1 = 4.4 and pK_2 = 6.7. While there is a danger in assigning these values to the pK_as of groups on the enzyme [see, e.g., Knowles (1976) and Cleland (1977)], several properties of these apparent pK_a values are noteworthy: (1) Similar pH profiles are observed for substrates varying over a wide range of reactivity (e.g., PNPGlc and phenyl β-glucoside). (2) Although there is no solvent isotope effect on the pH-corrected second-order rate constant k_a^{lim}, the apparent pK_a values increase in D_2O by amounts (~0.5 pK unit) typical of those seen for solvent isotope effects on ionizations of weak acids with $4 < pK_a < 7$ (Laughton & Robertson, 1969). (3) Although k_a increases with temperature (corresponding to an activation enthalpy of ~9 kcal/mol), the apparent pK_a values are temperature-independent. These observations indicate that the apparent pK_as are not significantly distorted by rate constants. Also, consistent with this is the observation that k_a for PNPGlc is not significantly affected by viscosogenic agents and, therefore, is not significantly limited by diffusion. The ΔH_{ion} values near zero hint at the possibility that the two ionizable groups on β-glucosidase, which influence the catalytic activity, are carboxylic acids. Results with reversible inhibitors suggest that the protonated group with a pK_a = 6.7 is a neutral acid (Dale et al., 1985). Using the irreversible inhibitor 6-bromo-6-deoxyconduritol B epoxide, Legler (1970) demonstrated the presence of a carboxylic acid residue at the active site of sweet almond β-glucosidase. The participation of two carboxylic acid residues in the mechanism of action of other glucosidases has been well established, most noteworthy of which is lysozyme (Phillips, 1967; Parsons & Raftery, 1969).

A significant point of interest from the pH study of the β-glucosidase-catalyzed hydrolysis of PNPGlc is that while k_a varies by over an order of magnitude in the range of $4.0 \leq pH \leq 7.3$, the K_M is only slightly (less than a factor of 2) affected. Thus, the predominant influence of pH is on k_{cat} and not on K_M. This is precisely the expected result if K_M is, in fact, a true thermodynamic dissociation constant, i.e., $K_M = K_S$ (Haldane, 1930; Cornish-Bowden, 1976). This conclusion is further substantiated by the observation that K_M is invariant to temperature, whereas k_{cat} is temperature-dependent.

Structure–Reactivity Studies. The data of Nath and Rydon (1954) show a substantial dependence of k_a on aryl substituents in the β-glucosidase-catalyzed hydrolysis of substituted phenyl glucosides. Hansch et al. (1965) have shown that this effect is predominantly electronic in origin. Thus, there is essentially no influence of hydrophobicity of meta or para substituents on k_a. We find a reasonably good correlation between log k_a and the single parameter pK_a. The pK_a of the leaving group accounts for over 88% ($=r^2 \times 100\%$) of the variance in the data for phenols with $pK_a > 7.1$. The slope of the Brønsted plot obtained with these more basic phenols, $\beta_{lg} = -0.97$, suggests that bond breaking is far advanced in the rate-determining transition state, the leaving group being the phenoxide. This is consistent with the large ^{18}O kinetic isotope observed by Rosenberg and Kirsch (1981) on k_a, indicating

Scheme II



~90% C–O cleavage in the transition state for PNPGlc hydrolysis. The lack of a solvent isotope effect on k_a is also consistent with a transition state involving little, if any, proton transfer to the leaving group.

The structure–reactivity correlations, extended to include the more acidic phenol leaving groups ($pK_a < 7.1$), appear to be nonlinear (Figures 2 and 3). This is most readily interpreted in terms of a mechanism involving at least two steps. One of these steps is strongly sensitive to substituent effects (i.e., $-\beta_{lg} \sim 1$) and is rate-limiting in the enzymic reaction for substrates with the more basic leaving groups. As the leaving group becomes more acidic, there is a change in rate-limiting step so that now the overall process becomes independent of substituent effects (i.e., $\beta_{lg} \sim 0$). There are two explanations for this multistep process. (1) The two teps correspond to parallel pathways for substrate hydrolysis. This implies different mechanisms for the hydrolysis of the "good" substrates (i.e., 2,4-DNPGlc, 3,4-DNPGlc, and pentafluorophenyl β-glucoside) and the "poor" substrates. (2) The two steps occur sequentially in a single mechanism that is common to all substrates. This mechanism (illustrated in Scheme I) requires a common intermediate (e.g., a covalent glucosyl enzyme or an electrostatically stabilized oxocarbonium ion). This mechanism is strongly supported by the results of the partitioning experiments (Table I). Methanol serves as an alternate glucose acceptor. The constant product partitioning (between methyl glucoside and glucose) obtained with substrates varying over 4 orders of magnitude in reactivity is consistent with a mechanism involving a common intermediate. This intermediate, and therefore its partitioning, is independent of how it is formed (i.e., whether from the good substrates or from the poor substrates). If a parallel pathway mechanism occurred, then the partitioning would have been of the substrate, not a common intermediate, and would be expected to depend on the nature of the leaving group [e.g., see Fersht (1977) and Sinnott & Viratelle (1973)]. The existence of an intermediate in the β-glucosidase reaction is consistent with the results of Fink and co-workers, who demonstrated "burst" kinetics with PNPGlc at subzero temperatures (Fink & Good, 1974; Weber & Fink, 1980).

The nonlinear structure–reactivity correlation of k_{cat}, the transglucosylation results, and the burst kinetics strongly suggest an enzyme–"glucose" intermediate in the β-glucosidase reaction. While these results do not distinguish between a nucleophilic (double-displacement) mechanism and a mechanism involving a glucosyl oxocarbonium ion intermediate, the results of Dahlquist et al. (1969) are consistent with the former. Only a small secondary deuterium isotope effect ($^Dk_a = 1.01$) was observed for the β-glucosidase-catalyzed hydrolysis of phenyl glucoside, indicating no hybridization change at C-1 in the rate-determining step. The nucleophilic mechanism implies attack by a group on the enzyme[3] to form a covalent glucosyl-enzyme intermediate (possibly an acylal) rather than direct attack by water. This is because the reaction proceeds with retention of configuration at the anomeric carbon (Eveleigh & Perlin, 1969).

On the basis of the results discussed so far, the minimal mechanism shown in Scheme II can be proposed for β-glucosidase. For such a mechanism, $k_{cat} = k_2k_3/(k_2 + k_3)$. For the more basic phenols, $k_3 \gg k_2$, and therefore, $k_{cat} = k_2$. In this case, $-\beta_{lg} \approx 1$. As the leaving group becomes more acidic ($pK_a < 7$), $k_2 \gg k_3$, and $k_{cat} = k_3$ and is independent of the leaving group (i.e., $\beta_{lg} \approx 0$). This is consistent with the nonlinear structure–reactivity correlation illustrated in Figure 3. The k_3 step may be limited by (a) hydrolysis of a covalent α-glucosyl-enzyme intermediate, (b) product dissociation, or (c) a conformation change.

Solvent Kinetic Isotope Effects (SKIEs). There is essentially no effect of substituting D_2O for H_2O on the pH-independent second-order rate constant k_a^{lim} for any of the β-glucosidase reactions examined. Some care must be taken in the interpretation of such small SKIEs since this may reflect a cancellation of effects. However, since the SKIE was found to be ~1 for substrates varying over 30 000-fold in reactivity (k_a), it seems likely that (1) there is no proton transfer to the leaving group in, or before, the first irreversible step (even with such poor substrates as methyl glucoside) and (2) water is not involved in, or before, the first irreversible step (even with such good substrates as 2,4-DNPGlc). The modest SKIE on k_{cat} obtained with 2,4-DNPGlc ($^DV = 1.5$) indicates that the rate-determining step is limited, at least partially, by the rate of one or more proton transfers. The absence of a SKIE on k_a with this (or any other) substrate and the absence of a SKIE on k_{cat} with PNPGlc suggest that the moderate SKIE on k_{cat} for 2,4-DNPGlc does not arise only from changes in protein or substrate hydration. These results are most readily interpreted in terms of a mechanism involving at least one intermediate subsequent to substrate binding [e.g., see Hardy & Kirsch (1984b)] such as that illustrated in Scheme II. If the k_2 step is indeed a nucleophilic displacement, then a SKIE of ~1 is expected with those substrates (with leaving group $pK_a > 7.1$) for which $k_{cat} = k_2$. The acylation of chymotrypsin, for example, shows a SKIE near unity (Bender et al., 1964). For the more reactive substrates, such as 2,4-DNPGlc, the rate-determining step is the k_3 step. If this step is limited, at least partially, by hydrolysis of an intermediate, then this would explain the observed SKIE being greater than 1. Due to the magnitude of this modest SKIE, however, we cannot rule out the possibility of kinetically significant conformation changes in the k_3 step.

Viscosity Effects. One possible explanation of the nonlinear structure–reactivity correlation for k_{cat} (Figure 3) is that with the very good substrates the rate is no longer limited by chemical steps but rather by a physical process such as product dissociation. Product dissociation, however, cannot be rate determining with 2,4-DNPGlc since k_{cat} is not affected by the viscosity of the solvent.

A similar explanation might apply to the nonlinear structure–reactivity correlation for k_a. For the mechanism described in Scheme II, the expression for the second-order rate constant is $k_a = k_1k_2/(k_{-1} + k_2)$. Clearly, a point can be reached, in principle, where the chemical reactivity of the substrate is so great that $k_2 \gg k_{-1}$, and therefore, $k_a = k_1$, the rate constant for diffusion together of the enzyme and substrate. If the k_1 step is rate-limiting, then it would not be expected to show a significant dependence on the structure of the substrate. This is consistent with the observation that, for the good substrates, k_a appears to be independent of the pK_a of the leaving group (Figure 2). Also, with the good substrates k_a is independent

[3] An alternate nucleophilic mechanism consistent with the overall retention of configuration involves intramolecular attack by the C-2 oxygen atom of the substrate. This mechanism is unlikely, however, in view of the fact that sweet almond β-glucosidase catalyzes the hydrolysis of 2-deoxyglucosides with significant catalytic efficiency. The k_a values for hydrolysis of 2-deoxyglucosides are less than those for hydrolysis of the corresponding glucosides by only a factor of ~10^3 (Legler, 1975).

of the nature of the glycon. Thus, with the *p*-nitrophenyl glycosides, k_a is 2.9 times higher for the fucoside than for the glucoside, but with the more reactive 2,4-dinitrophenyl derivatives the k_a values are equal (Table II). The conclusion that k_a for the good substrates is equal to k_1 is also suggested by the observation that k_a, but not k_{cat}, is reduced by viscosity in sucrose-containing buffer. Recently, Kirsch et al. have elegantly demonstrated the use of viscosity variation in analyzing to what extent k_a is limited by diffusion in the reactions catalyzed by chymotrypsin (Brouwer & Kirsch, 1982) and by β-lactamase (Hardy & Kirsch, 1984a). According to the Stokes–Einstein equation, the diffusion coefficient, and therefore the rate constant for a diffusion-controlled reaction, is inversely proportional to the viscosity of the medium (Hiromi, 1979). Thus, in an ideal case, when the viscosity is increased 2.2-fold, k_1 should be reduced by a factor of 2.2. In those reactions for which $k_a = k_1$, the second-order rate constant should be reduced 2.2-fold, and in those reactions for which $k_{-1} \gg k_2$, k_a ($=k_2/K_s$) should not be affected by an increase in the viscosity of the medium. While not in strict quantitative agreement with this, the results in Table II are in qualitative agreement. Thus, for the poorer substrates (PNPGal, *m*-nitrophenyl glucoside, and PNPGlc) a 2.2-fold increase in the viscosity of the medium results in only a small decrease (≤36%) in k_a. *p*-Nitrophenyl fucoside, which has a slightly larger k_a value than that of PNPGlc, shows a somewhat larger sensitivity to viscosity. For the more reactive substrates (2,4-DNPGlc, 3,4-DNPGlc, and 2,4-dinitrophenyl fucoside), a pronounced sensitivity of k_a to viscosity is seen. While the effect on k_a of increasing the viscosity 2.2-fold is slightly larger than is predicted, it is a 2.2- (±0.2) fold larger effect than is seen with PNPGlc. This is consistent with the idea that a 25% rate reduction is due to competitive inhibition. The results with the galactosides are more puzzling. 2,4-DNPGal, for example, is less reactive than PNPGlc, yet k_a is reduced by a viscosity increase more for the former than for the latter. A possible explanation is that k_a for 2,4-DNPGal is difussion-limited but k_1 does depend on the nature of the glycon. A similar situation was observed by Brouwer and Kirsch (1982), who found a dependence of k_1 on the nature of the substrate for chymotrypsin. A clearer analysis of the extent to which the β-glucosidase reactions are diffusion-limited and an accurate evaluation of the k_1 values, however, must await the availability of a suitable viscosogen that is free of additional inhibitory properties. Nevertheless, the observed viscosity effects do have some bearing on the interpretation of the nonlinear structure–reactivity correlations. In Figure 2, the k_a values in the flat portion of the curve show a significantly more pronounced sensitivity to viscosity than the points on the linear portion of the curve. This suggests that the nonlinearity is real. In Figure 3, the k_{cat} values in the flat portion of the curve show no dependence on viscosity (and a small but significant SKIE), indicating that while there is a change in rate-limiting steps, the rate-determining step with the better substrates is not product dissociation.

Registry No. PNPGal, 3150-24-1; PNPGlc, 2492-87-7; 2,4-DNPGlc, 25775-97-7; 2,4-DNPGal, 25775-96-6; 3,4-DNPGlc, 65887-60-7; 3,4-DNPGal, 85668-54-8; CH_3OH, 67-56-1; β-glucosidase, 9001-22-3; glucose, 50-99-7; ethanol, 64-17-5; β-mercaptoethanol, 60-24-2; 3-phenylpropylamine, 2038-57-5; 2-deoxy-2-amino-D-glucose, 3416-24-8; 4-methylumbelliferyl β-D-glucopyranoside, 18997-57-4; 2-formylphenyl β-D-glucopyranoside, 618-65-5; 3-nitrophenyl β-D-glucopyranoside, 20838-44-2; 4-(nitrothio)-phenyl β-D-glucopyranoside, 100899-02-3; 2,4-dinitrophenyl β-D-fucopyranoside, 52628-69-0; 4-nitrophenyl β-D-fucopyranoside, 1226-39-7; phenyl β-D-glucopyranoside, 1464-44-4; pentafluorophenyl β-D-glucopyranoside, 7234-27-7; 4-cyanophenyl β-D-glucopyranoside, 28217-36-9; 3-cyanophenyl β-D-glucopyranoside, 100899-03-4; 3-chlorophenyl β-D-glucopyranoside, 28217-28-9; 4-chlorophenyl β-D-glucopyranoside, 4756-30-3; 3-methylphenyl β-D-glucopyranoside, 6092-25-7; 4-methoxyphenyl β-D-glucopyranoside, 6032-32-2; 4-isopropylphenyl β-D-glucopyranoside, 66957-68-4; 4-methylphenyl β-D-glucopyranoside, 20274-94-6; 4-*tert*-butylphenyl β-D-glucopyranoside, 20772-27-4.

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Energetics of Proline Racemase: Racemization of Unlabeled Proline in the Unsaturated, Saturated, and Oversaturated Regimes†

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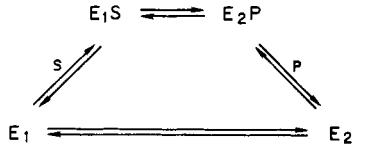
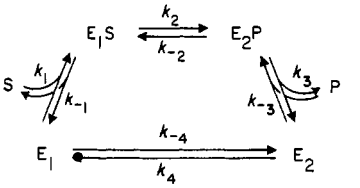
ABSTRACT: The interconversion of L- and D-proline catalyzed by proline racemase has been studied. The entire time course of the approach to equilibrium has been followed. After a short time the product concentration is significant, and the reaction runs under reversible conditions. As the total substrate concentration is increased, the system moves from the unsaturated regime into the saturated regime. At very high substrate levels under the reversible conditions used, the rate constant for substrate racemization *falls*, as the system moves into the "oversaturated" regime. Here, the net rate of the enzyme-catalyzed reaction is limited by the rate of return of the free enzyme from the form that liberates product back to the form that binds substrate. The results are analyzed in terms of the simple mechanism



and illustrate the additional information that is available from reactions studied under reversible conditions. In the unsaturated region the value of the second-order rate constant k_U (equivalent to k_{cat}/K_m) is 9×10^5 M^{-1} s^{-1} in each direction. In the saturated region, $\vec{k}_{cat} = \overleftarrow{k}_{cat} = 2600$ s^{-1} and $K_m = 2.9$ mM. In the oversaturated region, the rate constant k_O is 81 M s^{-1}. The substrate concentration at which unsaturated and saturated terms contribute equally is 2.9 mM, and the substrate concentration at which saturated and oversaturated terms contribute equally is 125 mM.

This series of papers describes a number of experiments that have been designed to elucidate the mechanism and energetics of the reaction catalyzed by proline racemase. While amino acid racemases usually require the cofactor pyridoxal phosphate, which by aldimine formation with the α-amino group labilizes the proton at the chiral center, such a pathway is not followed for imino acids, and two elegant studies from Abeles' group (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975) have illuminated the reaction pathway followed in the enzyme-catalyzed racemization of proline. Proline racemase is a dimeric enzyme of subunit M_r 38 000, and it contains one substrate binding site for every two subunits. It appears that one enzymic base abstracts the α-proton from the substrate's chiral center and the conjugate acid of another enzymic base protonates the substrate from the opposite side. The rate of enzyme-catalyzed tritium release from DL-[2-^{3}H]proline decreases with increasing proline concentration at high proline levels, which indicates that the substrate-derived proton is not released to the medium until after the release of the product

Scheme I: Reaction Scheme for an Isomerase (E) That Catalyzes the Interconversion of S and P



(Rudnick & Abeles, 1975). Since the rate of tritium release from L-[2-^{3}H]proline is not reduced by increasing L-proline concentration, the enzyme-bound proton derived from one enantiomer is only captured by the other enantiomer (Rudnick & Abeles, 1975). When the isomerization of unlabeled proline is studied in D_2O, the initial rate of deuterium incorporation into proline is the same as the rate of product formation in either direction (Cardinale & Abeles, 1968).

These experiments allowed Abeles and co-workers to conclude that there are two forms of the free enzyme, one that binds D-proline and one that binds L-proline. Release of product is faster than release of the substrate-derived proton, and release of this proton is at least as fast as the rate of interconversion of the two forms of the enzyme. At its simplest, therefore, proline racemase appears to fit Scheme I. The particular case of the racemase reaction has the further advantages of simplicity, of having overall symmetry, and of

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0006-2960/86/0425-2529$01.50/0