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ROLE OF SUGAR HYDROXYL GROUPS IN GLYCOSIDE HYDROLYSIS

CLEAVAGE MECHANISM OF DEOXYGLUCOSIDES AND RELATED SUBSTRATES BY β-GLUCOSIDASE A_3 FROM *ASPERGILLUS WENTII* *

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Summary

The contribution of the hydroxyl groups at C-2 and C-4 and of the hydroxymethyl group at C-5 of β-glucopyranosides to their hydrolysis by β-glucosidase A_3 (β-D-glucoside glucohydrolase, EC 3.2.1.21) from *Aspergillus wentii* was investigated with 4-methylumbelliferyl-β-glucosides with appropriate structural modifications. Relative hydrolysis rates expressed by k_{cat}/k_{cat}(glucoside) are: 2-deoxy, $4 \cdot 10^{-6}$; 2-deoxy-2-amino, $2.4 \cdot 10^{-4}$; 2-deoxy-2-ammonio, $<1 \cdot 10^{-6}$; 4-deoxy, $1.8 \cdot 10^{-4}$; xyloside, $6.3 \cdot 10^{4}$; galactoside, $<1 \cdot 10^{-6}$. Binding to the active site as measured by the K_m value of these substrates or by the K_i value of appropriate inhibitors is only moderately decreased by the above modifications. A temperature study with the 2-deoxyglucoside showed that the decrease in k_{cat} is not due to a change in ΔH^* but to a more negative ΔS^*.

The steady-state hydrolysis of the 2-deoxyglucoside is approached with a 'burst' (rate constant 0.13 min^{-1}) at pH 6 and 1 mM substrate; deglycosylation of the enzyme is partially rate-limiting. Rate constants for glycosylation and deglycosylation calculated from pre-steady-state kinetics were in good agreement with the constants calculated from experiments where the 2-deoxyglucoside was used as an inhibitor for the hydrolysis of the glucoside and where a slow approach to the steady state of the inhibited reaction is observed.

Abbreviation: 4-MeUmb, 4-methylumbelliferyl.

* Supplementary data to this article are deposited with and can be obtained from Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/164/69185/657 (1981) 321—333. The supplementary information includes: 1. Chromatographic profile of pepsin hydrolysate of *p*-nitrophenyl-β-D-2-deoxy[^{3}H]glucoside after incubation with β-glucosidase A_3. 2. Amino acid composition of peptide (peak I) isolated during Sephadex chromatography of pepsin hydrolysate.

** Taken in part from the thesis of K.-R. Roeser, University of Cologne 1979.

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A glycosyl enzyme could be trapped by denaturing the enzyme in the presence of *p*-nitrophenyl-[^{3}H]-β-2-deoxyglucoside. A radioactive peptide was isolated from peptic digests of the labeled enzyme, where the deoxyglucosyl residue was bound to the same aspartate side chain that had previously been shown to be involved in the binding of the active site directed inhibitor conduritol B epoxide and of D-glucal.

Introduction

Specificity of glycosidases with respect to the sugar part of the substrate is usually absolute for α or β configuration at C-1 and of varying tolerance to variations at the other carbon atoms. Discrimination between different glycosides is governed by the adaption of the binding site to the steric and functional requirements of the substrate, which may include the ability of the latter to induce conformational changes in the enzyme that optimize the orientation of the catalytic groups with respect to the bond to be cleaved. Part of the potential binding energy will be 'used' to deform the substrate towards the transition state. Most of this binding energy will come from hydrogen bonds to the hydroxyl groups of the sugar and to hydrophobic and van der Waal's interactions with the pyranose ring. Aglycon binding will contribute only a minor part since aglycon specificity is usually low.

In order to study the contributions of individual parts of the substrate it is necessary to introduce only such changes in substrate structure that do not increase its steric requirements. Even if the changed part of the molecule is not involved in binding the rigidity of the binding site it is expected to prevent the binding of larger analogues. We have, therefore, studied mainly such β-glucosyl derivatives which have a hydroxyl group replaced by hydrogen, or an amino group.

The enzyme we used is β-glucosidase A_3 from *Aspergillus wentii* which is well characterized with respect to its molecular and mechanistic properties [1,2].

Materials and Methods

The following compounds were synthesized according to published procedures: 4-MeUmb-β-D-glucoside [3] and -xyloside [4], α- and β-D-galactosylamine [5] and β-xylosylamine [6]. 4-MeUmb-β-D-2-acetamido-2-deoxyglucoside was a commercial product (Serva, Heidelberg). The synthesis of 4-MeUmb-β-D-2-deoxyglucoside (m.p. 212—3°C, $[\alpha]_{578}$, —126° (c = 1, pyridine), *p*-nitrophenyl-β-D-2-deoxyglucoside (m.p. 168°C (dec.), $[\alpha]_{578}$, —135° (c = 1, pyridine)), β-2-deoxyglucosylpyridinium bromide (m.p. 121—4°C, $[\alpha]_{578}$, +44.7° (c = 2, water)), and 4-MeUmb-β-D-4-deoxyglucoside (m.p. 192—8°C) $[\alpha]_{578}$, —79°, c = 1.5, pyridine)) will be published separately.

4-MeUmb-β-D-2-amino-2-deoxyglucoside hydrochloride. 10 g 2-amino-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucosylbromide hydrobromide [7] and 10.7 g each of 4-methylumbelliferone and its potassium salt were stirred in 150 ml acetone at room temperature overnight. 100 ml 10% aqueous K_2CO_3 were added and the product extracted with 100 ml diethyl ether. 4-MeUmb-tri-*O*-

acetyl-β-D-2-amino-2-deoxyglucoside hydrochloride was precipitated from the dried (solid K_2CO_3) ether solution by the addition of ethanolic HCl and recrystallized from methanol/diethyl ether. The material gave a single spot on TLC (silica gel, precoated foils, Merck, Darmstadt) in benzene/methanol/triethylamine (95 : 5 : 0.5, v/v) when stained with ninhydrin or 30% H_2SO_4 in acetic acid. Yield: 3.7 g; m.p. 192°C; $[\alpha]_{578}$, −50.4 (c = 1, methanol).

For deacetylation 2 g of the triacetyl derivative were dissolved in 30 ml methanol, 20 ml methanolic HCl (prepared from 20 ml methanol/3 ml acetyl chloride) added and the mixture heated to 60°C. Control by TLC in ethyl acetate/methanol/water/acetic acid (4 : 1 : 1 : 1, v/v) showed that the transesterification was complete after 2 h. 4-MeUmb-2-amino-2-deoxyglucoside hydrochloride was precipitated by the addition of 60 ml diethyl ether and recrystallized from hot ethanol. Yield: 1.4 g; m.p. 215°C (dec.); $[\alpha]_{578}$, −69.3° (c = 1, water); pK_s = 6.7 (potentiometric titration). The ultraviolet spectrum in methanol was identical with that of 4-MeUmb-β-glucoside (λ_m 317 nm; ϵ = 13, 100 $cm^{-1} \cdot M^{-1}$); the ^{1}H-NMR-spectrum showed only the signals expected from the structure.

Radioactive p-nitrophenyl-β-D-2-deoxyglucoside. Radioactivity was measured in Bray's solution with a Delta 300 (Searle) liquid scintillation counter. Since counting efficiency varied less than 3% with sample size (0.1 ml or less) and since only dilute aqueous solutions were counted we applied no quench corrections and used cpm as measure for the radioactivity.

14.7 mg 2-deoxyglucose (Koch-Light, Colnbrook, U.K.), 0.3 mCi 2-deoxy-[^{3}H]glucose (Amersham-Buchler, Braunschweig), and 11 μCi 2-deoxy[^{14}C]-glucose (prepared from labeled D-glucal [8]) with a final specific activity of $2.8 \cdot 10^6$ cpm/μmol were acetylated over night at room temperature with 0.5 ml acetic anhydride and 0.5 ml pyridine. (2-Deoxy[^{14}C]glucose was added to faciliate the control of the reactions by TLC and radioautography on X-ray film.) Excess reagents were removed in a desiccator over H_2SO_4/NaOH. All subsequent reactions were carried out at 0°C.

The acetylated 2-deoxyglucose was reacted for 1 h with 0.5 ml 40% HBr in acetic acid, diluted with 2 ml chloroform and the acids removed by washing with water (three times with 3 ml) and 5% $NaHCO_3$ (two times with 3 ml). The chloroform solution was dried with 100 mg $MgSO_4$, cooled to −18°C and concentrated to about 0.7 ml (evaporation to dryness will cause decomposition). After the addition of 12 mg *p*-nitrophenol and 15 mg Proton-Sponge ® (Aldrich-Europe, Beerse, Belgium) the mixture was warmed to room temperature; the hydrobromide of Proton-Sponge started to crystallize after a few minutes. The solution was filtered after 40 min and subjected to preparative TLC in benzene/methanol (98 : 2, v/v), the bands of α- and β-2-deoxyglucoside, localized by comparison with authentic samples, were cut from the foils and eluted with methanol. Yield: α-glucoside $48 \cdot 10^6$ cpm (20%); β-glucoside $138 \cdot 10^6$ cpm (56%). Deacetylation was done in 1 ml 0.02 M sodium methoxide in methanol, 10 min at 0°C. Longer incubation times and higher temperatures caused formation of D-glucal. The sodium methoxide was neutralized with 2 μl acetic acid and the solution taken to dryness.

p-Nitrophenyl-[^{14}C]-β-D-glucoside. 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide was prepared from 41 mg D-[U-^{14}C]glucose with a spec. act. of 0.95 ·

10^6 cpm/μmol as described by Lehmann and Schröter [8]. It was reacted for 6 h at room temperature with 23.5 mg *p*-nitrophenol in 60% aqueous acetone at approx. pH 9 (phenolphthalein added as indicator). pH was adjusted as required by the addition of 2 M NaOH. The mixture was partitioned between chloroform and water and the lower phase subjected to preparative TLC in benzene/methanol (97 : 3, v/v). Further procedures were as described for the deoxyglucoside. Yield: 37%.

β-Glucosidase A_3. The enzyme was isolated from spray-dried culture filtrates of *A. wentii* (Röhm GmbH, Darmstadt) as described previously [1].

Kinetic and inhibition studies. Fluorimetric activity determinations with 4-MeUmb-derivatives were done as previously [9]. For measurements with 4-MeUmb-β-D-2-deoxyglucoside the sensitivity of the fluorimeter was adjusted so that 100% fluorescence corresponded to 5 or 10 nmol product/ml. Initial and steady-state rates were calculated from the slopes of tangents drawn to the recorder traces of fluorescence vs. time.

Labeling of β-glucosidase A_3 with p-nitrophenyl-β-D-2-deoxyglucoside. 60 mg β-glucosidase were added to 0.9 ml 5 mM radioactive glucoside in 0.1 M phosphate buffer, pH 6.0, and incubated for 1 h at room temperature followed by 1 h at 0°C. The incubation mixture was added to 8 ml rapidly stirred 8 M guanidinium chloride and dialyzed at 0°C against several changes of water until radioactivity could no longer be detected in the dialysate. Ultraviolet absorbance at 280 nm and radioactivity of the non-dialysable material showed that 0.8 mol 2-deoxyglucose had been incorporated/mol protein. The solution was freeze-dried and subjected to pepsin digestion as described previously [10]. Purification and characterization of the radioactive peptide was also carried out using proved methods [10].

Results and Discussion

(a) Kinetic measurements

Table I summarizes the results of the steady-state kinetic experiments. The

TABLE I

KINETIC CONSTANTS FOR THE HYDROLYSIS OF 4-METHYLUMBELLIFERYL-β-D-GLYCOPYRANOSIDES BY β-GLUCOSIDASE A_3 AT pH 4.0 AND 25°C

D-Glycose	K_m (mM)	k_{cat} (min^{-1})	$10^3 \cdot \frac{k_{cat}}{k_{cat}\,(Glucose)}$
Glucose	0.57	21 600	1000
	0.071 *	2460 *	1000
Glucosamine	—	<0.05	<0.002
	1.05 *	0.1	0.04
N-Acetylglucosamine	—	<0.05	<0.002
2-Deoxyglucose	1.0	0.09	0.0042
	0.66 *	0.041 *	0.017
4-Deoxyglucose	0.48	4.0	0.18
Galactose	—	<1	<0.05
Xylose	2.9	34	1.6
	2.3 *	23 *	9.4

* pH 6.0.

lack of reactivity of the apparently inert substrates deserves some comment since only estimates for an upper limit are given. The crude filtrate from *A. wentii* used as starting material for β-glucosidase A_3 contains, in addition to several β-glucosidases [11], other glycohydrolases with the following relative activities (total β-glucosidase with 4-MeUmb-β-glucoside = 100): β-galactosidase, 110; β-xylosidase, 20 and β-*N*-acetylglucosaminidase, 3. In order to test whether any of the residual activities were due to contamination or lack of specificity of β-glucosidase A_3 we investigated the inhibition by D-glucono-δ-lactone and conduritol B epoxide. These inhibitors, which are specific for β-glucosidases, showed no or only a slight inhibition of the β-galactosidase and *N*-acetylglucosaminidase activities. The discrimination of β-glucosidase A_3 against an inversion of the hydroxyl group at C-4 or the introduction of an acetyl group at C-2 is, therefore, even higher than the figures in Table I suggest.

Results of the inhibition studies are summarized in Table II. All inhibitors showed mixed competitive/non-competitive inhibition. Only the competitive component is listed, since the non-competitive part appears to result from an indirect effect of the inhibitor on the ES complex [2].

An evaluation of the contribution of individual parts of the substrate to non-covalent binding in the ES-complex should, ideally, be based on true binding constants of non-reacting substrate or sugar analogs. Equilibrium methods are not suitable for the determination of these constants because of the low affinity ($K_d > 1$ mM) or instability of these compounds and one has to make use of kinetically determined constants like K_i or K_m. In order to extend the rather limited range of $[I]/K_i$ for sugars with their low affinity we have included glycosylamines, which have a much higher affinity [2]. It is seen from Table II that the contribution to the free energy of binding $\Delta\Delta G^\circ$ as calculated from the ratio of inhibition constants $K_i^c/K_i^c(\text{ref})$ with $\Delta\Delta G^\circ = -RT \ln K_i^c/K_i^c(\text{ref})$ amounts to −12 kJ/mol for the hydroxyl group at C-2 and to −14 kJ/mol for the hydroxymethyl group at C-5. In contrast to these moderate effects on the binding process we observe a much larger influence of the structural changes on the catalytic step(s) as seen from the data in Table I.

The most striking result is the extraordinary influence of the hydroxyl group

TABLE II

COMPETITIVE INHIBITION OF β-GLUCOSIDASE A_3 BY β-GLYCOSYL DERIVATIVES AT pH 4.0 AND 25°C. SUBSTRATE: 4-METHYLUMBELLIFERYL-β-D-GLUCOSIDE

Inhibitor	K_i^c (mM)	$\frac{K_i^c}{K_i^c(\text{ref})}$ *
Glucose	2.8	—
2-Amino-2-deoxyglucose [2]	18	6.3
2-Deoxyglucose	>50	>20
Glucosylpyridinium ion [2]	0.30	—
2-Deoxyglucosylpyridinium ion	33	110
Glucosylamine [2]	0.0016	—
Galactosylamine	7.4	4600
Xylosamine	0.53	330

* Reference compound is the corresponding β-glucosyl derivative.

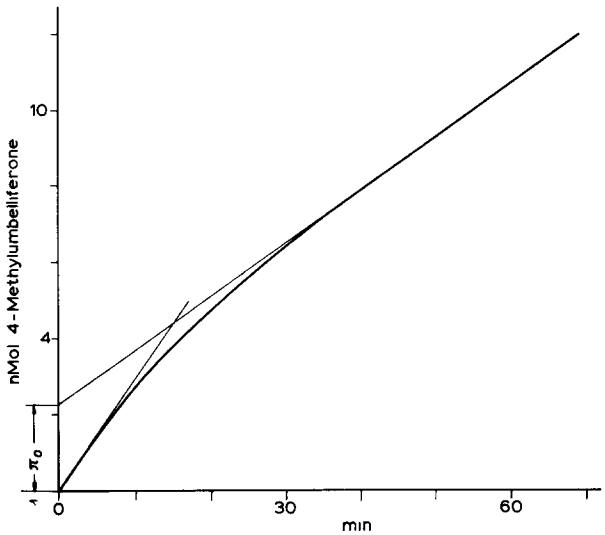

Fig. 1. Hydrolysis of 1.12 mM 4-MeUmb-β-D-2-deoxyglucoside by β-glucosidase A_3 (7.5 nmol) at pH 6.0 and 25°C.

at C-2. Its absence reduces the rate constants for glycosylation (k_2, scheme I) and deglycosylation (k_3) by four to five orders of magnitude so that both become measurable by manual techniques. The reduction is more pronounced for k_3 so that deglycosylation becomes rate limiting and 'burst'-kinetics are observed in the time-scale of minutes (Fig. 1). For β-glucosides the rate-limiting step is governed by k_2 [2].

The non-linear product release shown in Fig. 1 with the asymptote intercept π_0 proportional to the enzyme concentration indicates that in the two-step reaction (Scheme I) the deglycosylation step (k_3) has become rate limiting.

$$E + AB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot AB \xrightarrow[BH]{k_2} E - A \xrightarrow[H_2O]{k_3} E + AOH$$

Scheme I A, glycosyl residue; B, aglycon.

A straightforward evaluation of the rate constants for glycosylation (k_2) and deglycosylation (k_3) [12] was not possible since the limited solubility of the 2-deoxyglucoside did not permit experiments under conditions where the enzyme is saturated with substrate. It can be shown [13] that, with a rapid equilibrium for the E · AB complex, the release of the first product obeys the following rate law (Eqn. 1):

$$[BH] = v_\infty \cdot t + \frac{v_0 - v_\infty}{\frac{k_2[AB]}{K'_m + [AB]} + k_3} \cdot [1 + e^{-(k_2[AB]/(K'_m+[AB])+k_3)\cdot t}] \qquad (1)$$

v_0 and v_∞ are the initial and final rates for the formation of BH; $K'_m = k_{-1} + k_2/k_1$. The initial and final rates are related to the respective rates under the condi-

tion of saturating substrate concentration $v_{0(max)}$ and $v_{\infty(max)}$ by Eqns. 2 and 3:

$$v_0 = \frac{v_{0(max)} \cdot [AB]}{K'_m + [AB]} \tag{2}$$

with $v_{0(max)} = k_2 e$ and e = total molar concentration of enzyme

$$v_\infty = \frac{v_{\infty(max)} \cdot [AB]}{K_m + [AB]} \tag{3}$$

with

$$v_{\infty(max)} = \frac{k_2 \cdot k_3}{k_2 + k_3} \cdot e$$

and

$$K_m = \frac{k_{-1} + k_2}{k_1} \cdot \frac{k_3}{k_2 + k_3}$$

It can be seen that Eqn. 1 simplifies to the rate law given by Gutfreund [12] when $[AB] >> K'_m$.

Initial and final rates under saturating conditions and the constants K'_m and K_m were taken from double-reciprocal plots of these rates against substrate concentration (Fig. 2) and Eqns. 2 and 3 solved for the rate constants k_2 and k_3. The results are summarized in Table III. The large increase in error with decreasing pH is due to the decreased sensitivity of the fluorimetric assay (decreasing quantum yield of the product) and the increase in K'_m which causes the difference between v_0 and v_∞ to become smaller.

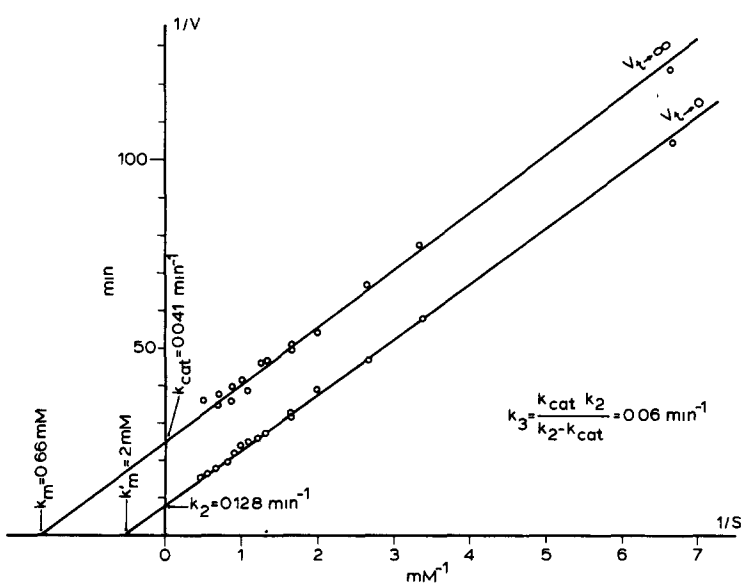

Fig. 2. Substrate dependence of the initial (v_0) and final rates (v_∞) of hydrolysis of 4-MeUmb-β-D-2-deoxyglucoside by β-glucosidase A_3 at pH 6.0 and 27°C. Ordinate is the apparent turnover number $v/[E]$.

TABLE III

RATE CONSTANTS FOR GLUCOSYLATION (k_2) AND DEGLUCOSYLATION (k_3) FOR THE HYDROLYSIS OF 4-MeUmb-β-D-2-DEOXYGLUCOSIDE BY β-GLUCOSIDASE A_3 at 27°C

pH	k_2 (min^{-1})	k_3 (min^{-1})
4.0	0.4 ± 0.1	0.12 ± 0.01
5.0	0.4 ± 0.08	0.09 ± 0.01
6.0	0.13 ± 0.01	0.06 ± 0.006

An independent check of these results is possible by using the 2-deoxyglucoside as an inhibitor for the hydrolysis of a good substrate, e.g., the corresponding glucoside. The initial competitive inhibition should be governed by $K_i^c = K'_m$ since the concentration of free enzyme is diminished only by the formation of E · AB. After the exponential approach to the steady state the free enzyme is diminished by E · AB plus E-A and we should have $K_i^c = K_m$. This is verified by the results shown in Fig. 3a.

The time constant for the exponential approach to the steady state of the inhibited reaction depends in a complicated way on the individual rate constants and cannot be used directly for their evaluation. It is, however, possible to determine k_3 directly if the enzyme is preincubated with 4-MeUmb-2-deoxyglucoside and then diluted into a solution of the good substrate. The initial rate is then only due to that part of the enzyme which is not present as E-A. The rate increase with time is governed by the rate of breakdown of E-A; i.e., the observed rate increase (Fig. 3b) should be a first-order process with the rate constant k_3. Since the initial enzyme/2-deoxyglucoside solution is diluted 50 000-fold we can neglect any influence of the remaining 2-deoxyglucoside. If we take account of the different temperatures of the experiments shown in Fig. 3 and the measurements for Table III, we have a good agreement between the two values for k_3. This, together with the results of the inhibition constants (Fig. 3a), shows that our interpretation of the kinetic experiments is correct.

Two explanations may be proposed for the large effect caused by replacing the hydroxyl group at C-2 by hydrogen: (1) The hydroxyl group is essential to hydrolysis because it participates in the reaction as a nucleophile by forming an 1,2-anhydroglucosyl intermediate as in the base catalyzed hydrolysis of aryl-β-glucosides [14] and as discussed for a β-glucosidase from almonds by Dahlquist et al. [15]. (2) Interaction of the hydroxyl group with the enzyme is necessary in order to have the substrate deformed towards the transition state and to induce the optimal orientation of the catalytic groups with respect to C-1 and the glucosyl oxygen.

The first possibility appears highly unlikely on account of our results of the secondary α-deuterium (D) kinetic isotope effect [2]. The observed isotope effects for a variety of substrates ranged from $k_H/k_D = 1.14$ to 1.08. They are, on the whole, slightly smaller than the effects for the acid catalyzed hydrolysis ($k_H/k_D = 1.13$ to 1.15), and much larger than for the base catalyzed reaction ($k_H/k_D = 1.03$). This shows that any nucleophilic participation in the transition state is not larger than with lysozyme ($k_H/k_D = 1.11$), where the substituent at C-2 of the hexapyranose at the cleavage site has been shown not to be involved in the hydrolysis of the glycosidic bond [16].

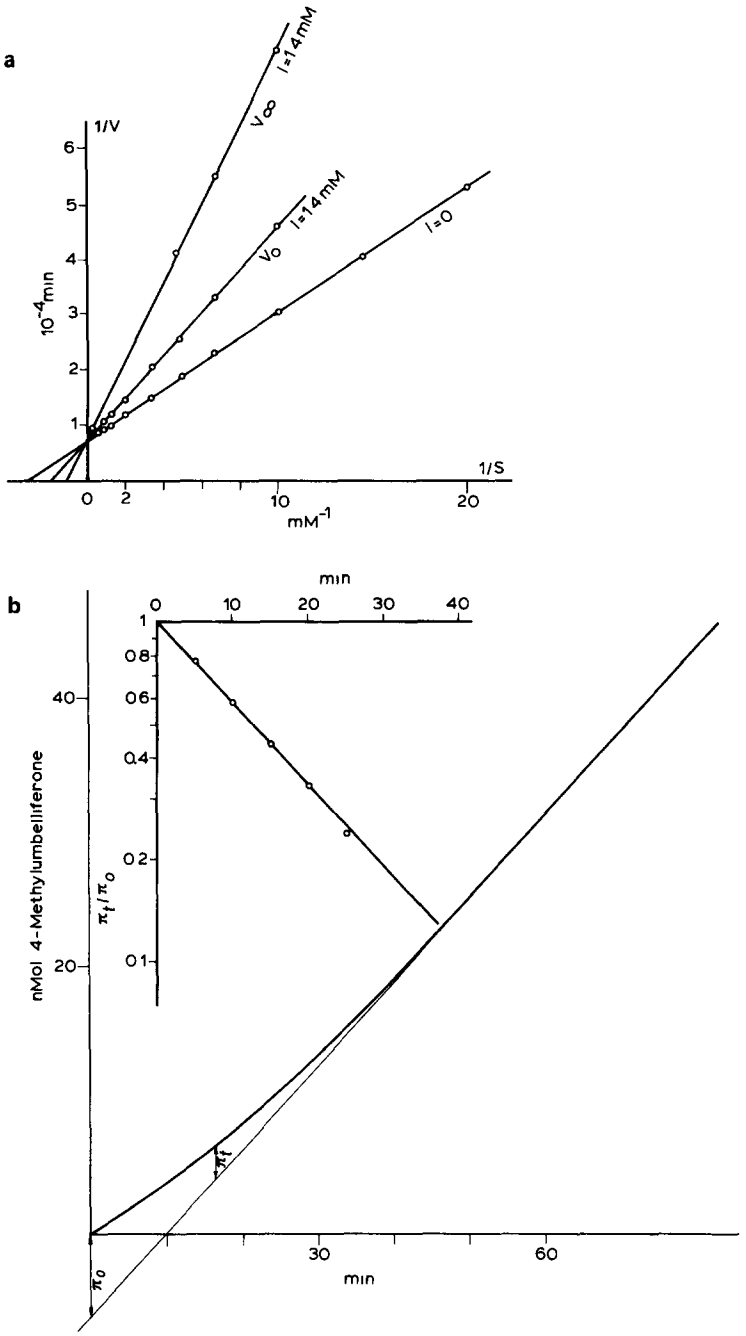

Fig. 3. Inhibition of the hydrolysis of 4-MeUmb-β-glucoside by 4-MeUmb-β-D-2-deoxyglucoside. (a) Double-reciprocal plots of the initial (v_0) and final (v_∞) rates of 4-MeUmb-β-D-glucoside hydrolysis in the presence of 1.4 mM 4-MeUmb-β-D-2-deoxyglucoside at pH 5.0 and 27°C. Ordinate as in Fig. 2. v_∞ was calculated from the slope of the fluorescent trace measured after approx. 60 min reaction time and corrected for substrate depletion with the results of the uninhibited reaction. The following figures are calculated from the data shown: K_m 0.31 mM; K_f^c(initial) 3 mM; K_f^c(final) 0.85 mM. The hydrolysis of the 2-deoxyglucoside under these conditions proceeds with K'_m 4 mM and K_m 0.87 mM (see Fig. 2 and text for explanations). (b) Hydrolysis of 2 mM 4-MeUmb-β-D-glucoside by β-glucosidase A_3 that was preincubated for 60 min with 1.5 mM 4-MeUmb-β-D-2-deoxyglucoside at pH 6.0 and 25°C. The enzyme was diluted $5 \cdot 10^4$-fold for the assay. Insert: plot of lg π_t/π_0 against time. Rate constant for the approach to the steady state: 0.052 min^{-1}.

The second explanation requires that the binding energy contributed by a single hydroxyl group reduces the free energy of activation ΔG^* by 28.6 kJ/mol (6.8 kcal/mol), if the $2.5 \cdot 10^5$-fold reduction of k_{cat} is considered. 28.6 kJ/mol may appear large for two (or at most three) hydrogen bonds which could be formed with complementary groups of the substrate binding site, especially if we take into account the energy needed to desolvate the hydroxyl group when the substrate is transferred from the fully hydrated state to the bound state.

There are, however, precedents for an energy contribution of a single hydroxyl group of this magnitude. An example is the activity of tyrosine-specific aminoacyl-*t*-RNA synthetase with tyrosine and phenylalanine, respectively [17]. The ratio of k_{cat}/K_m for the ATP-pyrophosphate exchange with the two substrates was found to be $1 : 1.6 \cdot 10^5$ which corresponds to a contribution to ΔG^* by the hydroxyl group of −27.3 kJ/mol. The large effect observed here and in the cited example is probably due to a highly specific orientation of hydrogen bond donors and acceptors in a fairly rigid site.

Additional support for a transition state with a highly specific orientation of the substrate with respect to the active site comes from measurements of 2-deoxy-glucoside hydrolysis at 37°C and pH 6, where the following rate constants for glycosylation and deglycosylation were found: k_2 0.32 min^{-1} and k_3 0.02 min^{-1}. From these values and those measured at 27°C we can calculate 68 kJ/mol and 38 kJ/mol for the enthalpy of activation for the first and second step, respectively. The former is identical with ΔH^* for the hydrolysis of 4-MeUmb-β-glucoside by β-glucosidase A_3 (68.5 kJ/mol [18]), where k_2 is rate limiting. This shows that the absence of the hydroxyl group at C-2 has no influence on the enthalpy of activation but causes the entropy of activation to be more negative by 82 J/mol · K (at 25°C).

A point which may indicate an even larger contribution of the C-2 hydroxyl group to ΔG^* is the intrinsic difference in hydrolysis between glucosides and 2-deoxyglucosides. The latter are hydrolyzed 2000 to 10 000-times more rapidly than the former under acidic conditions [19] and 10-times more rapidly at pH 4—6 (Legler, unpublished data). This is mainly due to the destabilisation of the oxocarbonium ion transition state by the electron withdrawing effect of the hydroxyl group at C-2 [19]. Since our isotope effect studies indicate a considerable carbonium ion character of the enzymic transition state and since proton transfer to the glucosyl oxygen is important for catalysis [2], k_{cat} for the 2-deoxyglucoside might well be much smaller were it not for the accelerating effect of the missing hydroxyl group. Such differences in the electronic properties also cause difficulties in the proper evaluation of the protonated 2-amino-2-deoxyglucoside. In addition to different hydrogen bonding properties its inertness might be due to a further destabilisation (compared to the glucoside) of the ionic transition state: acid catalyzed hydrolysis of 2-amino-2-deoxyglycosides is up to 200-times slower than that of the corresponding glycosides [19].

Inversion of the hydroxyl group at C-4 to the galactoside gives a substrate with $k_{cat} < 1 \cdot 10^{-5}$-times k_{cat} for the glucoside. This is in marked contrast to the β-glucosidases from almonds [20] and from the snail *Helix pomatia* [21], where the configuration at C-4 is of little or no consequence to the enzymic hydrolysis.

Replacement of the hydroxyl group at C-4 by hydrogen has practically no influence on the K_m value but causes a 5000-fold reduction of k_{cat}. The arguments discussed for the slow reduction of the 2-deoxyglucoside are probably applicable here, too.

The hydroxyl group at C-6 is of minor importance; earlier experiments [11] had shown that phenyl-β-D-6-deoxy-glucoside is hydrolyzed at about one-tenth the rate of the corresponding glucoside.

The following conclusions can be drawn with respect to the interaction between substrate and the active site. The glucosyl moiety is deeply buried in the ES complex since strong interactions are seen with the hydroxyl groups at C-2, C-4, and the hydroxymethyl groups at C-5. These interactions manifest themselves not only in decreased binding per se, if they cannot occur, but also in an even more pronounced reduction of the catalytic power against the altered substrates. Since differences in binding energy were calculated from inhibition constants a possible explanation for the greater influence of structural changes on catalysis compared to binding would be the existence of additional non-productive binding modes for the altered substrates that do not occur with glucosides. In that case catalysis as expressed by k_{cat}/K_m will be more inhibited by the structural change than binding.

An additional feature of enzyme substrate interaction is revealed by the influence of substrate structure on k_{cat}. It is generally assumed that a part of the binding energy can be 'used' by the enzyme to distort the substrate towards the transition state and to effect the correct orientation of the catalytic groups with respect to the bond to be broken, and to enforce on the active site a conformation that is optimal for catalysis (for a review see Ref. 22). The former contribution can be estimated from the increased binding energy shown by so-called transition state analogs and by changes in ΔH^* in a series of related substrates. The orientational and conformational influence will manifest itself in a more negative entropy of activation when the ability of the substrate to interact properly with the complementary groups on the enzyme is diminished by structural changes. This is well documented for the 2-deoxyglucoside, where ΔH^* for the glucosylation step is the same as for the glucoside but where ΔS^* is more negative by 82 J/mol · K. Since deglucosylation is not rate limiting for glucosides a direct comparison is not possible for this step. We can see, however, that, for the 2-deoxyglucoside, this step has a much more negative ΔS^* since its ΔH^* is smaller by 30 kJ/mol than ΔH^* for glucosylation which is faster. This is plausible since the enzyme substrate interactions involve the whole substrate in the first step while they are restricted to the glucosyl residue during the second. A greater flexibility in the ES complex due to the lack of the OH-group at C-2 will thus have a much greater influence on k_3 than on k_2.

The influence of the hydroxyl at C-2 on hydrolysis by this enzyme is much larger than with other glycosidases where such studies have been made. The hydrolysis of 2-deoxyglucosides by β-glucosidase A_3 from sweet almonds is 700—2000-fold slower than that of the corresponding glucosides [23]. In view of this comparable small influence the proposal made by Dahlquist et al. [15] about nucleophilic participation of the C-2 hydroxyl group during hydrolysis of glucosides by almond β-glucosidase should be reinvestigated.

(b) Isolation and identification of a 2-deoxyglucosyl enzyme

Since for the 2-deoxyglucoside the glycosylation is faster than deglucosylation we thought it possible to trap the intermediate 2-deoxyglucosyl enzyme E-A. In order to work with substrate concentrations which would saturate the enzyme we used *p*-nitrophenyl-2-deoxyglucoside which has a much higher solubility than the 4-MeUmb-derivative.

Incubation of β-glucosidase A_3 with 5 mM *p*-nitrophenyl-β-2-deoxy-[^{3}H]-glucoside gave, after denaturation and dialysis, a radioactive protein that appeared to have retained 0.8 mol 2-deoxyglucose per mol enzyme. On hydrolysis with pepsin and chromatography on Sephadex G-25 three radioactive peaks were obtained (see BBA Data Deposition Bank).

Peak I with 30% of the bound radioactivity was identified as the same peptide that had been found previously by labeling the enzyme with conduritol B epoxide [24] and D-glucal [10] by the following criteria: (1) Its properties during chromatography on SP-Sephadex C-25 and thin layer electrophoresis were identical to those of the peptides obtained with the other labels. (2) Its N-terminal amino acid was found to be valine contaminated by small amounts of phenylalanine and isoleucine. This, too, is in agreement with the peptides isolated previously. (3) Its amino acid composition (see BBA Data Deposition Bank) is identical, within the limits of error, to that of the peptide from the enzyme with D-glucal as label.

Peak II containing 35% of the radioactivity was identified by radioautography with authentic material as 2-deoxyglucose.

Peak III, eluted from Sephadex G-25 with strong retardation, could not be identified. It was only slightly retarded on SP-Sephadex at pH 3.15; i.e., it had zero or only a small fractional positive charge. It remained at the origin on TLC in butanol/acetic acid/water. Amino acid analysis showed small amounds of various amino acids in non-stoichiometric proportions.

All attempts to trap the corresponding glucosyl intermediate with *p*-nitrophenyl-β-D-[^{14}C]glucoside under conditions where such an intermediate should have been observable, were unsuccessful. This is to be expected since glycosylation is rate limiting with glucosides [2] and E-A does not accumulate in the steady state.

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