Carbohydrate Research, 138 (1985) 127–134
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

MIXED INHIBITION OF β-D-GLUCOSIDASE FROM *Stachybotrys atra* BY SUBSTRATE ANALOGUES

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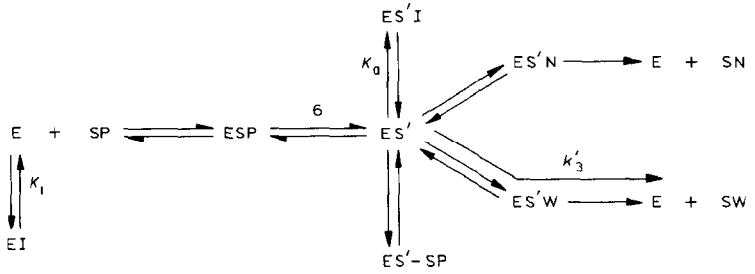
(Received July 3rd, 1984; accepted for publication, September 19th, 1984)

ABSTRACT

The binding of series of alkyl and aryl β-D-glycopyranosides and their 1-thio analogues to the active site of β-D-glucosidase from *Stachybotrys atra* has been investigated. The binding constants for competitive and uncompetitive inhibition clearly demonstrated the existence of a hydrophobic aglycon-binding-site. The correlations found between competitive and uncompetitive inhibition suggest that the latter type of inhibition originates from the unspecific binding of the aglycon group to the aglycon binding-site of the intermediary enzyme–glycosyl complex.

INTRODUCTION

In previous papers[1–4], we have reported on the hydrolysis of β-D-glucopyranosides and β-D-xylopyranosides by an induced β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from *Stachybotrys atra*. For both glucosidase and xylosidase activities, hydrolysis proceeds *via* a common enzyme–glycosyl intermediate (ES′) as depicted in Scheme 1[2–4].



Scheme 1

It has been proposed[2–4] that part of the active site of the enzyme, namely the "aglycon site" is built up from hydrophobic groups. In the enzyme–substrate complex (ESP), these groups, interacting with the aglycon part (P) of aryl and alkyl gluco- and xylo-pyranosides, contribute to the binding of these substrates. However, when the aglycon group of the substrate has been split off, the hydrophobic aglycon-site in ES′ becomes empty and can be occupied by a variety of hydrophobic

0008-6215/85/$ 03.30

groups and molecules. We have used this hypothesis to explain (*1*) substrate inhibition[2] (ES′-SP); (*2*) transfer reactions to nucleophiles (N) such as alcohols[3], phenols[4], and anilines[4] (ES′N); and (*3*) in particular the mixed inhibitory effect of such substrate analogues as *p*-chlorophenyl 1-thio-β-D-xylopyranoside[2]. As could be expected, this thioxyloside (I) did bind to the free enzyme ($E + I \rightleftharpoons EI$) and behaved as a competitive inhibitor. However, it also decreased the maximal rate of hydrolysis of such substrates as *p*-nitrophenyl β-D-glucopyranoside. This uncompetitive type of inhibition was explained by assuming that the thioxyloside did bind, through its aromatic ring, to the aglycon site in ES′ (complex ES′I with association constant K_a). Thus, part of the binding energy in both competitive (K_i) and uncompetitive (K_a) inhibition stems from the interaction of the phenyl ring with the same aglycon-site. If our assumption is correct, the two types of inhibition should also be found with other glycopyranosides having a hydrophobic aglycon and the inhibitory power should be related to the relative hydrophobicity of the aglycon group. Moreover, there should be some correlation between the change in standard Gibbs free energy calculated from K_i (ΔG_i^0) and that calculated from K_a (ΔG_a^0). Therefore, K_i and K_a values for series of alkyl and aryl β-D-glycopyranosides (and their 1-thio analogues) have been determined and compared.

EXPERIMENTAL

The isolation, purification, and standardisation of the β-D-glucosidase from *S. atra* have been described[1]. Substrates and inhibitors were synthesised by literature procedures: alkyl β-D-xylopyranosides[5], alkyl 1-thio-β-D-xylopyranosides[6,7], aryl 1-thio-β-D-galactopyranosides[8], aryl 1-thio-β-D-xylopyranosides[9]. The rate equations, rate coefficients, and methods of data analysis have been reported[2–4]. The determination of the competitive (K_i) and uncompetitive (K_a) inhibition constants was performed as described[2] for *p*-chlorophenyl 1-thio-β-D-xylopyranoside. The substrate used was *p*-nitrophenyl β-D-glucopyranoside.

RESULTS AND DISCUSSION

Alkyl β-D-glycopyranosides. — Alkyl β-D-xylopyranosides (but not the 1-thio analogues) are hydrolysed by β-D-glucosidase from *S. atra*. However, their maximal rate of hydrolysis at pH 6.7 and 30° is very low (k_{cat} ~0.03 s^{-1}) compared to that of a fast substrate such as *p*-nitrophenyl β-D-glucopyranoside (k_{cat} = 33 s^{-1})[3,4]. Moreover, the k_{cat} values are nearly independent of the length of the alkyl chain: propyl, 0.030; butyl, 0.044; pentyl, 0.033; octyl, 0.032 s^{-1}. Therefore, these alkyl β-D-xylopyranosides can be regarded as pseudo-inhibitors. Thus, by the same methods as described previously[2] for *p*-chlorophenyl 1-thio-β-D-xylopyranoside, the competitive (K_i) and uncompetitive (K_a) binding constants were determined for eight *n*-alkyl β-D-xylopyranosides and the corresponding 1-thio derivatives. The standard-free-energy changes ΔG_i^0 (from K_i) and ΔG_a^0 (from K_a), calculated for the

TABLE I

ΔG_i^0 AND ΔG_a^0 VALUES FOR ALKYL β-D-XYLOPYRANOSIDES AND ALKYL 1-THIO-β-D-XYLOPYRANOSIDES (pH 6.7, 30°)

No.	*Aglycon group*	π^a	$-\Delta G_i^0$ (*kJ.mol*$^{-1}$)		$-\Delta G_a^0$ (*kJ.mol*$^{-1}$)	
			1-oxygen	*1-thio*	*1-oxygen*	*1-thio*
1	Methyl	0.5	2.78	5.51	6.26	10.90
2	Ethyl	1.0	6.49	9.57	7.54	12.01
3	Propyl	1.5	10.61	13.00	8.24	12.76
4	Butyl	2.0	14.32	15.66	9.80	14.44
5	Pentyl	2.5	16.18	18.73	10.96	16.30
6	Hexyl	3.0	19.02	20.64	12.76	18.79
7	Heptyl	3.5	22.33	23.25	14.50	20.42
8	Octyl	4.0	23.83	25.05	15.78	22.33

[a]From ref. 10.

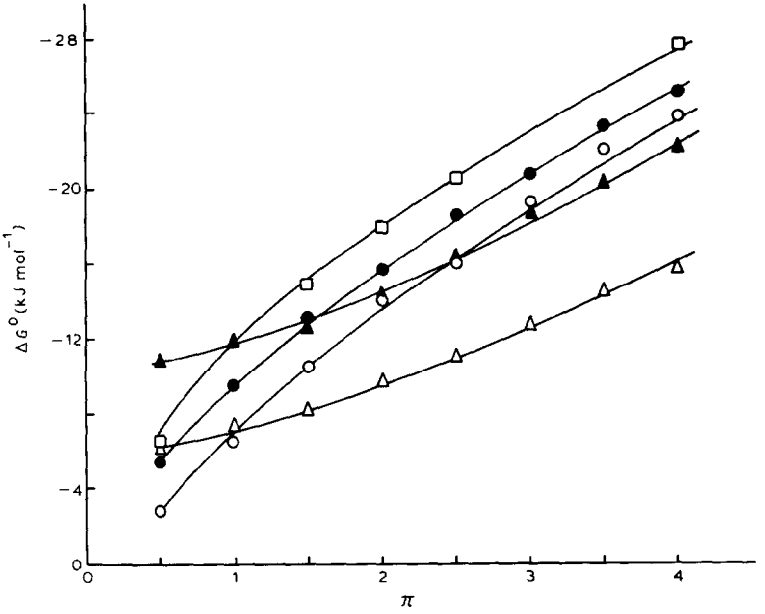


Fig. 1. Plot of ΔG^0 *versus* the Hansch hydrophobicity parameter π: ΔG_i^0, alkyl β-D-xylopyranosides (—○—); ΔG_i^0, alkyl 1-thio-β-D-xylopyranosides (—●—); ΔG_a^0, alkyl β-D-xylopyranosides (—△—); ΔG_a^0, alkyl 1-thio-β-D-xylopyranosides (—▲—); ΔG^0 (K_1), alkyl β-D-glucopyranosides (—□—).

association process, are given in Table I. The experimental K_i and ΔG_i^0 values for the monosaccharides (measured with α/β equilibrium mixtures) are: (*1*) D-glucose, K_i = 80 M^{-1} and ΔG_i^0 = −11 kJ.mol^{-1}; (*2*) D-xylose, K_i = 4 M^{-1} and ΔG_i^0 = −3.5 kJ.mol^{-1}; (*3*) D-galactose, K_i ~0.

Fig. 1 and Table I show that both ΔG_i^0 and ΔG_a^0 increase regularly but non-

linearly with the number of carbon atoms in the alkyl chain and with the Hansch[10] hydrophobicity parameter π. The function lines for oxygen and thio derivatives run parallel, the nearly constant difference being $\Delta\Delta G_i^0 = (2 \pm 0.8)$ kJ.mol^{-1} and $\Delta\Delta G_a^0 = (5.3 \pm 0.8)$ kJ.mol^{-1}.

A pattern analogous to that shown in Fig. 1 has previously been found for the binding of alkyl β-D-galactopyranosides to β-D-galactosidase from *E. coli*[11] and for that of alkyl β-D-xylopyranosides to β-D-xylosidase from *P. wortmanni*[7]. Thus, for β-D-glucosidase from *S. atra*, the same conclusion can be drawn. There exists, next to the glycon site on the enzyme, a hydrophobic micro-region sufficiently large to accommodate a flexible unbranched chain of up to at least eight carbon atoms. When the glycon part of an alkyl β-D-xylopyranoside binds to the glycon site of the free enzyme, the orientation of the chain must be such that at least eight methylene groups can be transferred from the bulk-water phase to this micro-region and partially desolvated. Thus, binding of the aglycon group is probably unspecific, the main part of the aglycon-binding energy resulting from the return of the water molecules from the ordered layer around the alkyl chain to the less-ordered bulk-water phase.

In the enzyme–glycosyl complex ES′, the glycon site is occupied by the glycosyl residue of the substrate and a competitive-type specific binding of an alkyl xyloside through its glycon group is impossible. However, because the hydrophobic aglycon-site is now empty, unspecific binding through the alkyl chain is still possible. In this uncompetitive-type of binding, the xylose part of the inhibitor does not provide the orientation of the aglycon, and the alkyl chain can interact more freely with the hydrophobic site. Therefore, we assume that the hydrophobic skeleton only is extracted from the water phase, whereas the polar sugar residue remains in its initial hydrated state. But even if the glycon interacts with the enzyme, this would be through unspecific contacts of only secondary importance in determining the tightness of binding. Thus, the regular increase of the free energy of binding (ΔG_a^0; Table I; Fig. 1) would merely represent the transfer of alkyl chains with regularly increasing hydrophobic character from water to the hydrophobic micro-region on the enzyme.

Alkyl β-D-glucopyranosides are better substrates than the corresponding β-D-xylopyranosides, and k_{cat} and K_{app} can be determined (Table II). The overall rate constant (k_3') for the reaction of the enzyme–glucosyl complex[2] with water is 33 s^{-1}. Thus, values for k_6 (bond-breaking and desorption of the aglycon) and $K_1 = k_1/[k_{-1} + k_6]$ (steady-state constant for the formation of the enzyme–substrate complex, ESP) can be calculated from the equations[2-4] $k_{cat} = k_3'k_6/[k_3' + k_6]$ and $K_{app} = K_1[k_3' + k_6]/k_3'$ (Table II).

As shown in Fig. 1, ΔG^0 (K_1) increases regularly with π, and the function line runs parallel with those for ΔG_i^0 of alkyl β-D-xylopyranosides and the 1-thio analogues. Thus, K_1 is probably a good approximation of the binding constant for alkyl β-D-glucopyranosides. Glucosides bind somewhat better than the corresponding xylosides, the nearly constant difference being $\Delta G_i^0 = (4 \pm 0.4)$ kJ.mol^{-1}.

TABLE II

RATE COEFFICIENTS FOR ALKYL β-D-GLUCOPYRANOSIDES (pH 6.7, 30°)

Aglycon group	k_{cat} (s^{-1})	K_{app} (M^{-1})	k_6 (s^{-1})	K_1 (M^{-1})	$-\Delta G^0$ (K_1) ($kJ.mol^{-1}$)	$k_{cat} \times K_{app}$ ($s^{-1}.M^{-1}$)
Methyl	5	14	6	12	6.26	70
Propyl	18	800	37	380	14.95	1.4×10^4
Butyl	24	4 000	82	1 180	17.83	9.6×10^4
Pentyl	23	11 000	70	3 500	20.59	2.5×10^5
Octyl	15	110 000	26	61 500	27.78	1.6×10^6

The product $k_{cat} \times K_{app}$ ($= K_1 k_6$), which has the dimensions of a second-order rate constant, can be used as a measure of the enzyme's overall preference[12] for a particular substrate. For alkyl β-D-glucopyranosides, this preference increases strongly with increasing chain-length (Table II). However, it is clear from the values of K_1 and k_6 that the main reason for the increase in substrate specificity is the better binding (K_1) which results from the higher hydrophobicity. The lengthening of the chain has only a very limited effect on the bond-breaking ($\sim k_6$).

For alkyl β-D-xylopyranosides[3], $k_3' > 3$ s^{-1} and thus $k_{cat} \simeq k_6 \simeq 0.03$ s^{-1}. The ratio k_6 (glucosides)/k_6 (xylosides) seems to depend somewhat on the alkyl group. The exact numerical value is uncertain, but the order of magnitude must be $\sim 10^3$. With $k_6 \sim 0.03$ s^{-1} and $K_{app} \simeq K_i \simeq K_1$, a rough estimate of $k_{cat} \times K_{app}$ for alkyl β-D-xylopyranosides can be calculated. The product $k_{cat} \times K_{app}$ increases from 0.09 $s^{-1}.M^{-1}$ for the methyl to 390 $s^{-1}.M^{-1}$ for the octyl derivative. The enzyme's overall preference for an alkyl β-D-glucopyranoside is much higher than for the corresponding β-D-xylopyranoside. From the values of k_6 and K_1, it follows that the higher preference results as well from the better binding as from the faster bond-breaking.

Aryl 1-thio-β-D-glycopyranosides. — For a number of substituted phenyl 1-thio-β-D-glycopyranosides, the ΔG^0 values (association; pH 6.7, 30°) calculated from K_i (ΔG_i^0) and K_a (ΔG_a^0) are given in Table III. For each of the series in Table III, a highly significant multiple correlation between ΔG^0 and (*1*) the Hammett electronic substituent parameter σ_H and (*2*) the Hansch hydrophobicity parameter π could be calculated. The regression coefficients for the general equation $\Delta G^0 = P_0 + P_1(\sigma_H) + P_2(\pi)$, the multiple correlation coefficient R, the partial correlation products (PCP) on σ_H and π, and the F test for significance of regression are given in Table IV. The analogy between the regression equations is a first indication that the series themselves are intercorrelated (see below).

For aryl 1-thio-β-D-xylopyranosides, a comparison of the ΔG_i^0 values in Table III with ΔG_i^0 for D-xylose proves that the aromatic ring contributes significantly to the binding of these thioxylosides. This means that, when the xylose moiety of the inhibitor binds specifically to the free enzyme, the phenyl ring is positioned so that it can interact with the hydrophobic aglycon-site. Moreover, the nature of this

TABLE III

SUBSTITUTED PHENYL 1-THIO-β-D-GLYCOPYRANOSIDES CHANGE IN STANDARD FREE ENERGY (pH 6 7, 30°) FOR BINDING TO FREE ENZYME (ΔG_i^0) OR ENZYME-GLUCOSYL COMPLEX (ΔG_a^0).

No.	*Substituent group*	$-\Delta G_i^0$ *(kJ.mol^{-1})*		$-\Delta G_a^0$ *(kJ.mol^{-1})*			σ_H[a]	π[b]
		Xylosides	*Glucosides*	*Xylosides*	*Glucosides*	*Galactosides*		
1	*p*-Nitro	21 83	—	20.64	18.38	17.86	0.78	0.50
2	*p*-Bromo	20.06	19.68	19.44	16.36	15.16	0.27	1 13
3	*p*-Chloro	19.83	18.92	18.73	15.99	14 11	0.24	0.93
4	*p*-Fluoro	17 36	—	15.94	14.37	11.73	0.06	0.31
5	*p*-Methyl	17.50	16 93	16.03	13.53	11.47	−0.13	0.48
6	None	16.75	—	15 19	13.15	11.19	0	0
7	*p*-Methoxy	15.90	—	13.70	11.84	8.11	−0.27	−0.12

[a]From ref. 13. [b]From ref. 14 for phenols.

TABLE IV

RESULTS OF CORRELATION OF ΔG^0 (TABLE III) WITH σ AND π

	ΔG_i^0 *Xylosides*	ΔG_a^0		
		Xylosides	*Glucosides*	*Galactosides*
P_0	−16.77	−15.17	−13.38	−10.8
P_1	−5.18	−5.36	−5.50	−7 79
P_2	−1.99	−2.52	−1.42	−1.98
R	0 993	0 999	0.998	0.993
PCP (σ_H)	0.743	0.668	0.815	0.808
PCP (π)	0.242	0.330	0.181	0.177
F	331	1932	1119	334

interaction must be such that the resulting change in free energy ($\sim\Delta G_i^0$) depends on the substituent groups, according to the regression equation for ΔG_i^0 (thioxylosides) in Table IV.

From the ΔG_a^0 values in Table III, it follows that aryl 1-thio-β-D-glycopyranosides, regardless of their glycon structure, can bind to the enzyme–glycosyl complex (ES′). In this ternary enzyme–glycosyl–inhibitor complex, the aromatic ring cannot be oriented in the same way as in the binary enzyme–inhibitor complex. Nevertheless, the analogy between the multiple correlations (Table IV) indicates that, in both complexes, the free energy of binding (ΔG_a^0 and ΔG_i^0) depends in the same way on the characteristics of the substituent. This is further illustrated by the following highly significant, linear correlations between the ΔG^0 values of the series in Table III:

(1) ΔG_a^0 (thioxylosides) = 4.58 + 1.17 ΔG_i^0 (thioxylosides), with correlation coefficient r = 0.992, F = 330, t = 18;

(2) ΔG_a^0 (thioglucosides) = 4.25 + 1.03 ΔG_i^0 (thioxylosides), with r = 0.987, F = 192, t = 14;

TABLE V

THERMODYNAMIC EQUILIBRIUM PARAMETERS FOR *p*-CHLOROPHENYL AND PENTYL 1-THIO-β-D-XYLOPYRANOSIDE (30°)

Parameter	p-*Chlorophenyl xyloside*		*Pentyl xyloside*	
	Competitive	*Uncompetitive*	*Competitive*	*Uncompetitive*
ΔH^0 (kJ.mol^{-1})	−44.4 ±3.8	~0	−30.7 ±1.8	~0
ΔG^0 (kJ.mol^{-1}	−19.9 ±0.4	−18.2 ±0.2	−18.9 ±0.2	−16.4 ±0.3
ΔS^0 (J.K^{-1}.mol^{-1})	−82 ±13	+61 ±5	−40 ±6	+55 ±6

(*3*) ΔG_a^0 (thiogalactosides) = 14 + 1.45 ΔG_i^0 (thioxylosides), with r = 0.98, F = 116, t = 11.

Therefore, we assume that this uncompetitive type of binding must be attributed to a rather unspecific interaction of the phenyl ring with the hydrophobic aglycon-site. The sugar residue may contribute to the binding by unspecific contacts with some groups of the enzyme.

Thermodynamic equilibrium parameters. — For *p*-chlorophenyl and pentyl 1-thio-β-D-xylopyranoside, K_i and K_a were determined at five temperatures (in the range 20–40°). Whereas ln K_i was linearly related with 1/T, K_a was virtually independent of the temperature in the interval used. The thermodynamic equilibrium parameters calculated from K_i and K_a are collected in Table V. For the binding of D-xylose itself to β-D-glucosidase from *S. atra,* values for ΔH^0 and ΔS^0 are not available. However, these parameters are known for the binding of D-xylose to β-D-xylosidase from *B. pumilus*[15]: ΔG^0 = −8 ±1 kJ.mol^{-1}, ΔH^0 = −51 ±3 kJ.mol^{-1}, and ΔS^0 = −142 ±10 J.K^{-1}.mol^{-1}.

In the competitive type of binding, part of the overall ΔG_i^0 (Table V) originates from interactions of the correctly positioned xylose moiety with the glycon site of the enzyme. This process may be compared to the binding of D-xylose: a favourable negative enthalpy term, but an unfavourable decrease in entropy resulting from the formation of a binary complex. The other part of the overall ΔG_i^0 stems from the unspecific transfer of the aliphatic or aromatic aglycon to the hydrophobic micro-region and is an entropically favourable ($\Delta S^0 > 0$) process. As a result, the overall ΔS^0 may become less negative or even positive. The values in Table V show that ΔS_i^0 is still negative and thus that the drive for competitive binding originates from the decrease in enthalpy.

For the formation of the uncompetitive enzyme–glycosyl–inhibitor complex ES'I, the sole driving-force is an increase in entropy (Table V). This observation is in agreement with our assumptions that, in this case, the alkyl chain or the aromatic ring is transferred to the hydrophobic site ($\Delta S^0 > 0$), whereas the xylose moiety remains in its initial hydrated form and does not contribute significantly to the binding ($\Delta H^0 \sim 0$).

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