Investigation of the slow inhibition of almond β-glucosidase and yeast isomaltase by 1-azasugar inhibitors: evidence for the 'direct binding' model

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(−)-1-Azafagomine [(3*R*,4*R*,5*R*)-4,5-dihydroxy-3-hydroxymethylhexahydropyridazine; inhibitor **1**] is a potent glycosidase inhibitor designed to mimic the transition state of a substrate undergoing glycoside cleavage. The inhibition of glycosidases by inhbitor **1** and analogues has been found to be a relatively slow process. This 'slow inhibition' process was investigated in the inhibition of almond β-glucosidase and yeast isomaltase by inhibitor **1** and analogues. Progress-curve experiments established that the time-dependent inhibition of both enzymes by inhibitor **1** was a consequence of relatively slow dissociation and association of the inhibitor from and to the enzyme, and not a result of slow interchanges between protein conformations. A number of hydrazine-containing analogues of inhibitor **1** also inhibited β-glucosidase and isomaltase slowly, while the amine isofagomine [(3*R*,4*R*,5*R*)-3,4-dihydroxy-5-hydroxymethylpiperidine; inhibitor **5**] only inhibited β-glucosidase slowly. Inhibitor **1** and related inhibitors were found to leave almond β-glucosidase with almost identical rate constants, so that the difference in K_i values depended almost entirely on changes in the binding rate constant, k_{on}. The same trend was observed for the inhibition of yeast isomaltase by inhibitor **1** and a related inhibitor. The values of the rate constants were obtained at 25 °C and at pH 6.8.

Key words: non-steady-state kinetics, rate constants, slow binding inhibitors.

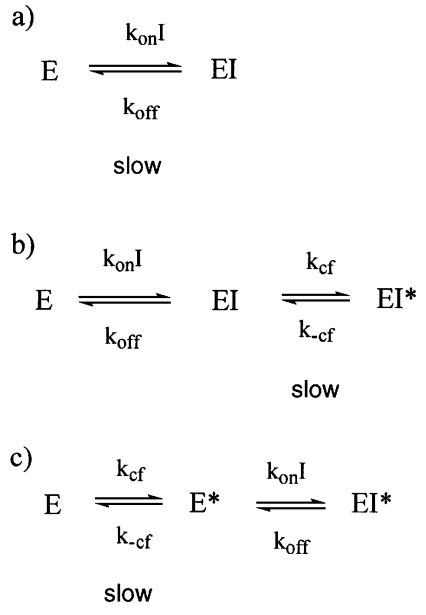
INTRODUCTION

Glycosidases are crucial in many biological processes, including breakdown of edible carbohydrates [1], eukaryotic glycoprotein processing [2], and polysaccharide and glycoconjugate anabolism and catabolism [3]. Glycosidases are also involved in a variety of metabolic disorders and other diseases such as diabetes [4], viral attachment [5], bacterial infection [6], and cancer formation [7]. Thus potent and selective glycosidase inhibitors have many interesting potential applications, such as treatment of AIDS [8], diabetes [4] and cancer [9], as well as crop protection.

Of the many potent glycosidase inhibitors the valieneamine derivatives (acarbose) and monosaccharide analogues, with nitrogen in place of ring oxygen, are the most notable [10]. A vigorous debate whether, or to what extent, the various inhibitor types are transition-state analogues has taken place [11]. It has recently been found that monosaccharide analogues with nitrogen in place of anomeric carbon is a new group of potent glycosidase inhibitors [12], in some cases having K_i values in the nanomolar range. It is of interest for future inhibitor design to understand how these molecules work, particularly if they function as transition-state analogues.

Slow binding, or slow onset of inhibition, is a widespread phenomenon among potent glycosidase inhibitors [10], where the inhibition process is relatively slow, occuring over a period of minutes and not at diffusion-controlled rates [13]. Rather than the subject of detailed study, it has often been considered as a troublesome complication in the measurement of enzyme inhibition constants. However, a study of slow inhibition may give a more detailed insight into the binding mode of potent glycosidase inhibitors. Thus it has been suggested that slow inhibition might be caused by the enzyme having two conformational states: one that binds the substrate in the ground state, and one that binds it in the transition state [13,14]. Transition state-like inhibitors bind to the latter form, which is present in small amounts, and have to be formed from the other state. The slowness of the inhibition is, according to this explanation, due to protein conformational changes which require the repositioning of a large number of atoms.

Slow-binding enzyme inhibition has been thoroughly reviewed in [13]; since this review a number of studies have appeared [15–19]. From the kinetic point of view, three possible mechanisms have been considered for the slow inhibition phenomena (Scheme 1). Binding between enzyme and inhibitor



Scheme 1 Kinetic schemes for three models of slow inhibition

(**a**) 'Direct binding' model, (**b**, **c**) two 'conformational change' models

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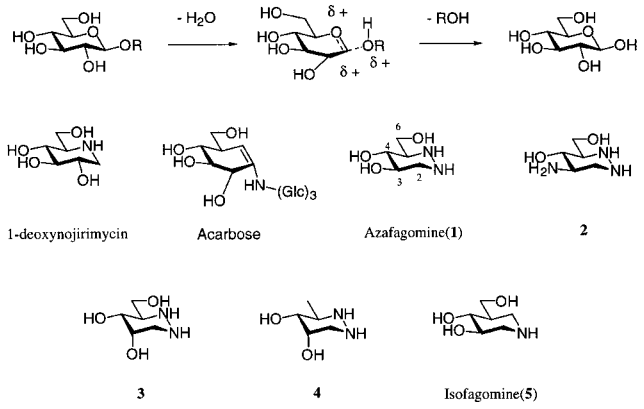

Figure 1 The strucure of the substrate, transition state and product in hydrolysis of a β-glucoside and the structures of some glycosidase inhibitors

may either involve a single step, having slow on and off rates (k_{on}[I] and k_{off} being small, Scheme 1a); have an initial fast-binding step, followed by a slow reversible transformation of EI to another entity, EI* (Scheme 1b); or have an initial slow interconversion of the enzyme E into another form E*, which binds the inhibitor by a fast step.

In our study of 1-azasugar inhibitors, we noticed that one of the inhibitors, 1-azafagomine (inhibitor **1**, see Figure 1], exhibited a time-dependent inhibition of almond β-glucosidase and isomaltase. As a study of the slow inhibition process might give more insight into the binding mode of inhibitor **1** and analogues, we report here an investigation of the slow-inhibiton process of the two enzymes by inhibitors **1**–**5**. We report the finding that almond β-glucosidase and isomaltase are slowly inhibited by compounds **1**–**5** in a manner that agrees with the direct-binding model (Scheme 1a).

MATERIALS AND METHODS

Inhibitors

Compound (−)-**1** was synthesized optically pure from L-xylose [20], compounds (±)-**2**–**4** were synthesized as racemates as previously described [21,22], and racemic **5** was synthesized from arecoline [23]. All the compounds have previously been found to be competitive inhibitors of isomaltase and almond β-glucosidase [20,22].

Substrates

4-Nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-β-D-glucopyranoside were purchased from Sigma.

Enzymes

Chromatographically purified sweet almond β-glucosidase (21–25 units/mg) was obtained from Sigma. SDS/PAGE showed essentially pure protein (purity > 90 %) as a 67 kDa band. This accords with the subunits of the type A isoenzyme, which has a molecular mass of 135 kDa [24,25]. Partially purified isomaltase (Sigma) was used without further treatment.

Enzyme kinetics: general conditions

All reactions were carried out in 0.05 M sodium phosphate buffer (pH 6.8) at 25 °C. When β-glucosidase was the catalyst, 4-nitrophenyl-β-D-glucopyranoside was used as substrate; when isomaltase was the catalyst, 4-nitrophenyl-α-D-glucopyranoside was used as substrate. Formation of the product, 4-nitrophenol, was measured continually at 400 nm using a Milton Roy Genesys 5 spectrometer.

Measurement of K_m

Measurements were performed in 2 ml solutions with 0.05 M buffer. Substrates were added at various concentrations (0.5, 2, 8 or 16 mM), the enzyme was mixed, and the reaction immediately monitored. From experiments ($n = 4$) with varied substrate concentrations, initial reaction rates were calculated as the slope of the product absorption versus reaction time. K_m and V_{max} were calculated by non-linear regression of the reaction rate versus substrate concentration using the graphics program Easy Plot.

Progress curve determinations

Reactions with inhibitor (compounds **1**–**3**) present were carried out with and without preincubation of enzyme and inhibitor. Experiments without preincubation were carried out with 2 ml solutions (0.05 M buffer) with substrate [S] (4 mM for 4-nitrophenyl-β-D-glucopyranoside and 2 mM for 4-nitrophenyl-α-D-glucopyranoside), and the inhibitor [I] at various concentrations (0.7–14 K_i). The enzyme was mixed, and the reaction immediately monitored spectrophotometrically for 8–10 min. Experiments with preincubation were carried out with 50 μl solutions of inhibitor (10 K_i), with the enzyme being kept at 25 °C for 60 min. The reaction was started by diluting to 2 ml (0.05 M buffer) with substrate solution containing variable amounts of inhibitor, so that [I] = $K_i - 20\ K_i$ and [S] = 4 M or 16 M. No more than 5 % of the substrate was consumed in any single run.

Data treatment

The progress curves were fitted to eqn. (1), in the form $P(t) = at + b[\exp(-\beta t) - 1]$, using the program INRATE in the software package Simfit (developed by W. G. Bardsley, University of Manchester, U.K.).

RESULTS

Observation of slow inhibition

It was found that inhibitors **1**–**5** showed a non-instantaneous onset of inhibition of β-glucosidase, while inhibitors **1**–**4** showed similar behaviour towards isomaltase. Some representative examples of progress curves are shown in Figure 2.

For β-glucosidase, the slow inhibition was more apparent when the enzyme was incubated with the inhibitors **1**–**5** at elevated inhibitor concentrations for a period of 30 min or more. When the incubated enzyme and inhibitor were diluted with substrate and buffer, the progress curves (examples of which are shown in Figures 2C and 2D) were obtained. The time-dependent increase in reaction rate, resulting from slow release of enzyme from inhibitor, is readily recognized.

For isomaltase, preincubation was not necessary. In fact best results were obtained by directly monitoring the progress curve without preincubation.

Kinetics of slow inhibition: models and data analysis

There are in principle three ways in which a slow-binding competitive inhibitor **1** may interact with an enzyme (Scheme 1):

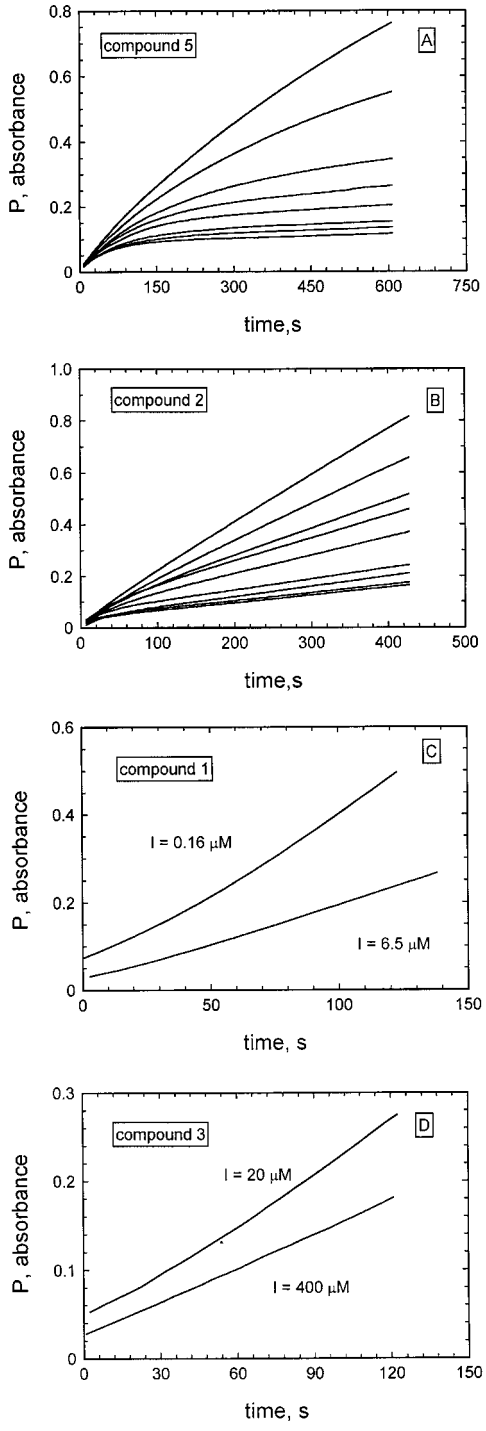

Figure 2 Representative examples of progress curves

(**A**, **B**) Solutions of phosphate-buffer (0.05 M, pH 6.8), substrate (4-nitrophenyl-β-D-glucoside), inhibitor and almond β-glucosidase were mixed to yield a final solution with [S] = 3.9 M and variable inhibitor concentrations between 0.35 μM and 4.8 μM (**A**) or 24 μM and 450 μM (**B**). (**C**, **D**) Phosphate-buffer (0.05 M, pH 6.8), almond β-glucosidase and inhibitor ([I] = 6.5 μM) were preincubated 60 min. Phosphate-buffer (0.05 M, pH 6.8), substrate (4-nitrophenyl-β-D-glucoside) and, in (D) inhibitor, were added to yield a final solution with [S] = 3.9 M and the inhibitor concentration shown.

in the direct-binding model (a), the slow step is the inhibitor binding and release steps, characterized by the rate constants k_{on} and k_{off}; in model (b), the inhibitor binds rapidly to the enzyme, forming the complex EI which in turn, by a slow step, undergoes a conformational change to the species EI*; and in model (c), the slow rate is caused by a slow conformational change of the enzyme prior to binding the inhibitor. For model (a) the equations describing the time evolution are the simultaneous differential equations $dP(t)/dt$ and $dE(t)/dt$. Under steady-state conditions, with respect to substrate, these are solved in closed form to yield the progress curve:

$$P(t) = A(1-\alpha/\beta)t+(AC/\beta)[\exp(-\beta t)-1] \quad (1)$$

where,

$$A = V_m \times s/(1+s),\ s = [S]/K_m,\ \alpha = k_{on} \times [I]/(1+s),\ \beta = \alpha + k_{off},\ C = (ei)_{t=0} - \alpha/\beta,\ ei = [EI]/[E_0] \quad (2)$$

Eqn. (1) has, in slightly different notation, been derived previously [13]. For model (b), the progress curve for eqn. (1) is also valid, but with altered A, α and β coefficients:

$$A = V_m \times s/(1+s+i),\ \alpha = k_{cf} \times i/(1+s+i),\ \beta = \alpha + k_{-cf},\ i = [I]/K_i \quad (3)$$

Also, model (c) has a progress curve that follows eqn. (1), but the A, α and β coefficients are:

$$A = V_m \times s/(1+s),\ \alpha = k_{on} \times [I]/(1+s),\ \beta = [k_{cf}/(1+s)] + [k_{-cf}/(1+i)] \quad (4)$$

For all the models the intrinsic $K_i = k_{off}/k_{on}$. Thus the progress curve data is given by the same equation for the three models, but they can be distinguished by the dependence of the coefficients on the inhibitor concentration. By fitting the data to eqn. (1) we can determine, by non-linear regression, the coefficient of the linear term, the coefficient of the exponential term, and the apparent rate constant β.

Note that the appearance of the progress curve will depend on the way the kinetic experiments are carried out. If enzyme and sufficient inhibitor are first preincubated, after which substrate is added to initiate reaction, the term $(ei)_{t=0}$ is close to 1, and the constant $C > 0$. As a result the progress curve will be upwards concave (Figures 2C and 2D). In contrast, if no preincubation is performed, $C < 0$, and the progress curve will be upwards convex (Figures 3A and 3B).

From eqns (2) and (3) it is seen that for model (a) the apparent rate constant is a linear function of the inhibitor concentration, having the form:

$$\beta = k_{on} \times [I]/(1+s) + k_{off} \quad (5)$$

From this rectilinear relationship the rate constants k_{on} and k_{off} may be determined. In contrast, from eqn. (3) we see that for model (b):

$$\beta = k_{cf} \times [I]/\{(1+s)K_i + [I]\} + k_{-cf} \quad (6)$$

The apparent rate constant is a monotonically increasing hyperbolic function of the inhibitor concentration. Finally, it can be seen from eqn. 4, that for model (c) β will also be a hyperbolic function of [I], but a decreasing one. We may thus expect that a study of the apparent rate constant (β), determined from a fit of the data to eqn. (1) can provide us with a diagnostic tool allowing a distinction between the models and a subsequent calculation of the rate constants.

Progress curve measurement

As seen earlier, the equations show that it is possible to distinguish between the three models (a), (b) and (c) by measuring β as a function of [I]. For each of the inhibitors **1–5** a set of progress

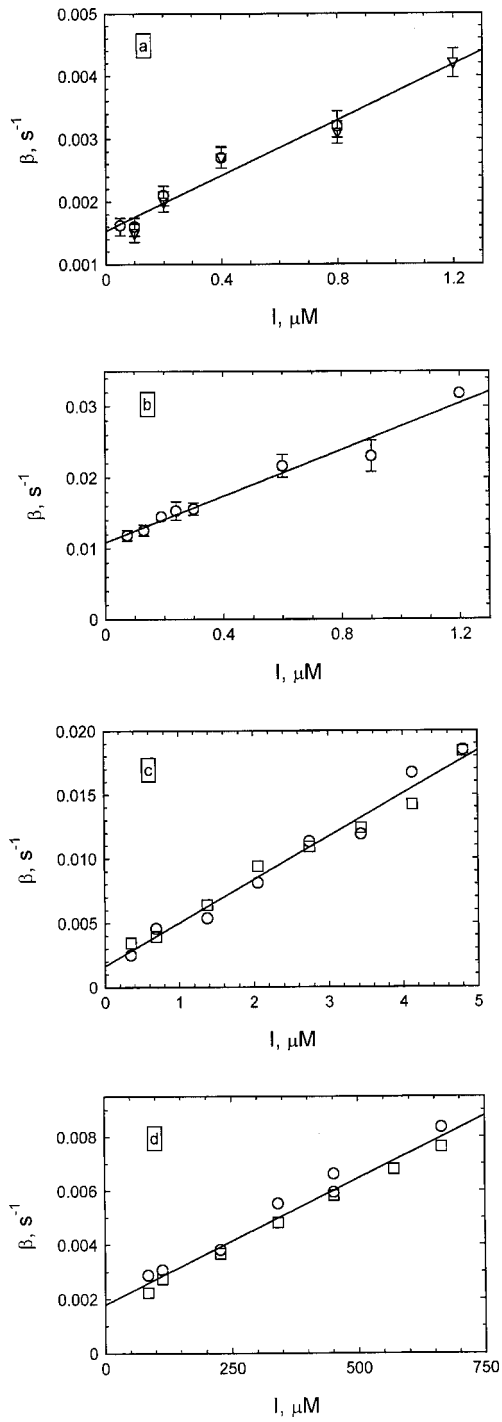

Figure 3 Representative examples of plots of the apparent rate constants β [cf. eqn. (1)] as a function of inhibitor concentration

The rate constants k_{on} and k_{off} are obtained from the slope and intercept, respectively, of the straight lines fitting the data. (**A**) Isomaltase inhibited by **1**. (**B**) β-glucosidase inhibited by **1**. (**C**) β-glucosidase inhibited by **5**. (**D**) Isomaltase inhibited by **2**.

curves was determined at different concentrations of inhibitor. This was done with (Figures 2C and 2D) and without (Figures 2A and 2B) a preincubation of the inhibitor with the enzyme prior to experiment. If preincubation gave more curved progress curves these were used for data extraction; this was the case for inhibitor **1**. For each of the progress curves thus obtained, β values were determined. Plots of β versus [I] were made for inhibitors **1**–**5**. Typical plots of β versus [I] are shown in Figure 3, and the rate constants and inhibition constants determined from fitting the plots to eqn. (4) are collected in Table 1.

Table 1 Dissociation constants and rate constants for inhibitors of almond β-glucosidase and isomaltase at temperature 25 °C and pH 6.8

The rate constants are the means of parameters of 2–4 weighted linear plots of β versus [I] (as in Figure 3). The weights used are determined by the S.D. of β obtained from non-linear fitting of the progress data [see eqn. (1)]. The K_i are calculated as k_{off}/k_{on} with S.D. obtained by propagation of errors.

Enzyme	Inhibitor	K_i (μM)	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})
β-Glucosidase	**1**	0.33 ± 0.06	$(3.3 \pm 0.2) \times 10^4$	$(1.1 \pm 0.2) \times 10^{-2}$
β-Glucosidase	(±)-**1**	0.72 ± 0.12	$(1.4 \pm 0.2) \times 10^4$	$(1.0 \pm 0.1) \times 10^{-2}$
β-Glucosidase	(±)-**2**	56 ± 6	$(1.50 \pm 0.09) \times 10^2$	$(8.4 \pm 0.7) \times 10^{-3}$
β-Glucosidase	(±)-**3**	128 ± 17	$(1.4 \pm 0.4) \times 10^2$	$(1.8 \pm 0.4) \times 10^{-2}$
β-Glucosidase	(±)-**4**	28 ± 6	$(2.4 \pm 0.5) \times 10^3$	$(6.7 \pm 0.1) \times 10^{-2}$
β-Glucosidase	(±)-**5**	0.22 ± 0.06	$(7.3 \pm 0.3) \times 10^3$	$(1.6 \pm 0.4) \times 10^{-3}$
Isomaltase	**1**	0.27 ± 0.03	$(5.33 \pm 0.4) \times 10^3$	$(1.43 \pm 0.09) \times 10^{-3}$
Isomaltase	(±)-**2**	79 ± 10	23 ± 1	$(1.8 \pm 0.2) \times 10^{-3}$
Isomaltase	(±)-**5**	5.8	Not slow	Not slow

DISCUSSION

It is clear that the plots of β versus [I] for the inhibition of β-glucosidase or isomaltase show a linear relationship with all the inhibitors (Figure 3); there is no indication of a hyperbolic relationship. The data therefore exclusively suggest that the inhibition of the two enzymes by the inhibitors studied follows model (a) (Scheme 1). This is corroborated by the property observed for the progress curves without preincubation (Figures 2A and 2B): the slope of these curves at $t = 0$ is independant of [I]. If, as in model (b), the inhibitor were binding rapidly to the enzyme, the initial rate would decrease as the concentration of inhibitor increased. This means that the slow steps are the binding of the inhibitor to the enzyme and its release, and not a conformational change of the enzyme or enzyme–inhibitor complex. This seems at first sight extraordinary, because binding is commonly believed to take place nearly at diffusion-controlled rates, and indeed previous work suggests that most slow-inhibition phenomena are caused by slow conformational changes. However, a number of recent studies of slow inhibition on other systems also support model (a) [15,16,19], so there is reason to believe that this model may be widespread.

The binding constants k_{on} and k_{off} extracted from the model show some remarkable trends (Table 1). First of all, k_{on} is remarkably small even for a potent inhibitor like (−)-**1**. Furthermore, inhibitors **1**–**4** are released from almond β-glucosidase with approximately the same k_{off}. Similarly, **1** and **2** are released from isomaltase with the same rate, though k_{off} in this case is about 10 times smaller than that for β-glucosidase. So inhibitors of the same overall type, in this case hydrazines, are released from either enzyme with a fixed rate, even though their K_i values differ widely, and this variation is due exclusively to differences in the k_{on}. A similar property was observed by Hanozet et al. [26] for the inhibition of sucrase by acarbose, nojirimycin and deoxynojirimycin. Note that inhibitors (−)-**1** and (±)-**1** also have the same k_{off} from β-glucosidase, but differ in k_{on}. This value is also expected, since the (+)-**1** enantiomer has been found not to be an inhibitor [20]. Therefore twice as much racemic **1** as

optically active **1** is required to get the same rates, and the k_{on}, which is multiplied with [I], will be half that of pure inhibitor.

It is, however, also clear that the release rate is not identical for all azasugar inhibitors. An inhibitor of different type, isofagomine (inhibitor **5**), has a 10-fold smaller k_{off} constant than the hydrazines, inhibitors **1**–**4** (Table 1). The inhibition of isomaltase by inhibitor **5** is not slow, which means that with a K_i value of 5.8 μM the release constant k_{off} must be larger than 10^{-3} s^{-1}. It is not necessarily much larger, because with a k_{off} value between 0.1 and 1 s^{-1}, the equilibration time will be less than 10–20 s, and therefore too fast to observe with the present method.

The fact that the release constant of the related inhibitors **1**–**4** does not vary suggests that the stereochemistry or presence of a hydroxyl group does not play a significant role in the release of the inhibitor from the enzyme.

Furthermore, the data in Table 1 show that the azasugars bind tightly and perhaps in a unique orientation with respect to the protein, compared to the possible varied orientation of binding of simple sugars, which bind with lower affinity [27]. Also the 3-hydroxyl group is important and not effectively substituted by an amino group; 3-amino-3-deoxy-D-glucose, a relatively strong inhibitor compared to glucose [27], is suggested to be oriented differently when bound to the enzyme.

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