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N-phenylglucosylamine hydrolysis: A mechanistic probe of β-glucosidase

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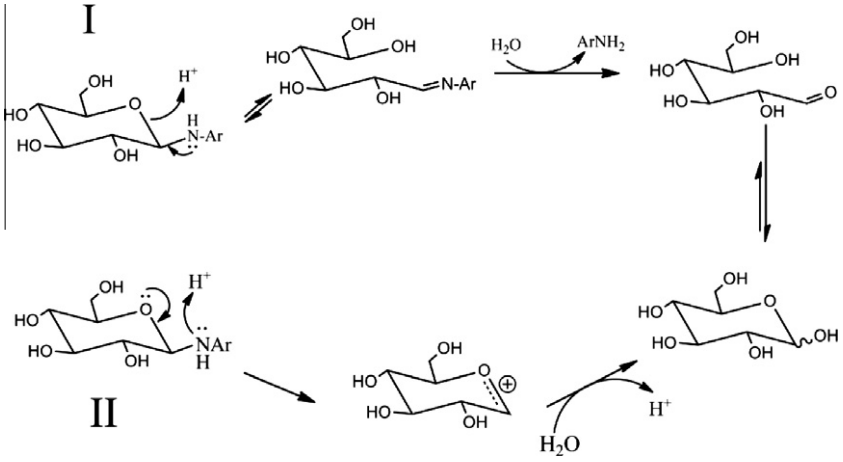
ABSTRACT

The spontaneous hydrolysis of glycosylamines, where the aglycone is either a primary amine or ammonia, is over a hundred million-times faster than that of *O*- or *S*-glycosides. The reason for this (as pointed out by Capon and Connett in 1965) is that, in contrast to the mechanism for *O*- or *S*-glycoside hydrolysis, hydrolysis of these *N*-glycosides (e.g., glc-NHR) involves an *endo*cyclic C–O bond cleavage resulting in formation of an imine (iminium ion) which then reacts with water. Since ring-opening is kinetically favored with glycosylamines, compounds such as phenylglucosylamine can be a useful probes of enzymes that have been suggested to possibly follow this mechanism. With β-glucosidase from sweet almonds, the enzyme is highly efficient in catalyzing the hydrolysis of phenyl glucoside ($k_{cat}/k_{non} \sim 10^{14}$) and phenyl thioglucoside ($k_{cat}/k_{non} \sim 10^{10}$) while with either the almond or the *Aspergillus niger* enzyme or with yeast α-glucosidase, there is no detectable catalysis of phenylglucosylamine hydrolysis ($k_{cat}/k_{non} < 20$). These results are consistent with the generally accepted mechanism involving *exo*cyclic bond cleavage by these enzymes.

1. Introduction

The non-enzymic hydrolysis of glycosylamines (glycosylated ammonia or primary amines) is a much more rapid process than hydrolysis of glycosides or thioglycosides [1]. The reason for this is due to the effective "push" by the non-bonding electrons on nitrogen, resulting in a ring-opening mechanism [2] yielding a Schiff base (imine) intermediate (Mech. I), in contrast to the "classical" mechanism for nucleophilic substitution at the anomeric center (Mech. II) usually seen with *O*-glycosides:



For some glycosides (especially conformationally strained ones) there is direct experimental evidence for *endo*cyclic C–O bond cleavage in the acid-catalyzed hydrolysis reactions (e.g., [3,4]). However, for the majority of acid, and enzyme, catalyzed hydrolysis of glycosides, the overwhelming body of evidence is consistent

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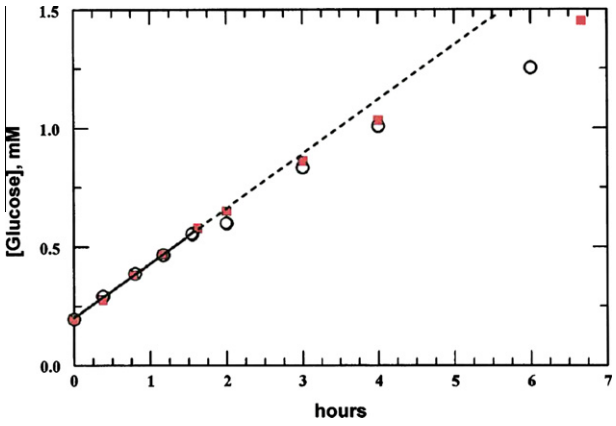

Fig. 1. Rate of hydrolysis of N-phenylglucosylamine (6 mM, pH 6.3, 25 °C) in the absence of enzyme (○) and the presence of 80 μM almond β-glucosidase (■). The line is the linear regression fit of the (separate) data through the initial velocity range (<8% completion).

with a process analogous to Mech. II involving varying extents of nucleophilic participation in the transition state [5–8]. Nevertheless, there have been occasional proposals of enzymic mechanisms for *O*-glycoside hydrolysis involving initial cleavage of the *endo*cyclic, rather than the *exo*cyclic, C–O bond (analogous to Mech. I). These suggestions were based mainly inhibition analysis [9] and/or structural and computational approaches [10–12]. While for most enzymes involving glycosyl transfer (i.e., glycohydrolases and glycotransferases), physical organic studies are more consistent with mechanism II than with mechanism I (e.g., multiple atom kinetic isotope effects [13]), proposals occasionally reappear, raising the possibility of *endo*cyclic cleavage, especially for β-glycosidases [12]. To test this possibility, we examined the substrate potential of N-phenyl-D-glucosylamine with β-glucosidases from sweet almonds (a family 1 glycohydrolase) and from *Aspergillus niger* (a family 3 glycohydrolase) as well as with yeast α-glucosidase. The β-glucosidases have a fairly relaxed specificity for the leaving group, cleaving a wide variety of *O*-glycosides (e.g., [14]) and the almond enzyme has been shown to cleave *S*-glycosides [15]. If these enzymes operate *via* an *endo*cyclic cleavage mechanism, than phenylglucosylamine should be an even better substrate for the enzyme than is phenylglucoside.

2. Results and discussion

While phenyl β-glucoside and phenyl β-thioglucoside are stable in aqueous solution ($t_{1/2}$ half a million years for spontaneous hydrolysis at 25 °C [16]), phenyl glucosylamine is relatively unstable. The half-life for spontaneous hydrolysis of a 10 mM solution (pH = 6.3) of an anomeric equilibrium mixture of PhNHGlc is 21 (±2) h, corresponding to a hydrolysis rate constant which is about 10^8-times larger than that for either the corresponding *O* or *S* glucoside [16]. We were unable to increase the rate of hydrolysis of phenylglucosylamine, even in the presence of large amounts of enzyme. For example, with a 6 mM solution of phenylglucosylamine, there was no detectable difference in the amount of glucose released (over an 8 h time period) in the presence or absence of 5.2 mg/ml (130 units/ml) of almond β-glucoside (=80 μM). Fig. 1 shows the initial velocity data. The linear regression fit of the data in this range yield the following equations:

$$\text{with enzyme}: \ [\text{Glc}] = 0.240\ (\pm.003)\ \text{mM/h} + 0.200\ (\pm0.002)\ \text{mM} \quad (1)$$

$$\text{without enzyme}: \ [\text{Glc}] = 0.234\ (\pm.004)\ \text{mM/h} + 0.200\ (\pm0.004)\ \text{mM} \quad (2)$$

The spontaneous rate of glucose production was 2×10^{-4} M/h. Thus, the enzymic reaction must occur at a rate that is less than 10% of this value (i.e., $<2 \times 10^{-5}$ M/h). Based on a substrate concentration (6 mM = $0.6K_m$), this indicates the V_{max} must be less than 5.3×10^{-5} M/h (=1.5×10^{-8} M/s) and, therefore, $k_{cat} < 2 \times 10^{-4}$ s^{-1}. The kinetic parameters for this "substrate" and those of its *O* and *S* analogs are summarized in Table 1. Clearly the rate of the enzyme-catalyzed hydrolysis of phenylglucosylamine (if it occurs at all) is much less than that of the oxygen analog. While the upper limit of k_{cat} for phenylglucosylamine hydrolysis is comparable to the measured value of k_{cat} for phenyl thioglucoside hydrolysis, this corresponds to a very poor catalytic proficiency ($k_{cat}/k_n < 20$) compared to that for it's thiol analog (k_{cat}/k_n 10^{10}) at 25 °C. This catalytic "proficiency" for phenylglucosylamine hydrolysis is even more negligible when compared to that for that for phenyl glucoside hydrolysis (k_{cat}/k_n 10^{14}). Similarly, no enhancement could be detected in the hydrolysis rate of 40 mM phenylglucosylamine (pH 6.3) when incubated in the presence of β-glucosidase from either almonds or *A. niger* (15 units/ml, K_i for phenylglucosylamine = 9.3 mM) or α-glucosidase from yeast (100 units/ml, K_i for phenylglucosylamine = 8.6 mM). If the catalytic mechanism of these glucosidases did involve an *endo*cyclic C–O cleavage step, phenylglucosylamine would be expected to be a very good substrate for the enzyme. Our results indicate that this is *not* the case. Contrary to the proposal of Frank [12] there appear to be at least two β-glucosidases, one from sweet almonds (a family 1 glycohydrolase) and one from *A. niger* (a family 3 glycohydrolase), that do not catalyze the hydrolysis of β-glucosides *via* a ring-opening mechanism. Considering the relative ease in which phenylglycosylamines can be prepared, these compounds can serve as useful probes of possible *endo*cyclic cleavage with other glycohydrolases.

Table 1
Hydrolysis[a] of phenyl β-glucopyranoside derivatives, Ph-X-Glc.

X	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m, (M^{-1} s^{-1})
O	1.2 (±0.1)	32.0 (±0.7)	39 (±3)
S	$3.2\ (\pm0.5) \times 10^{-4}$	8.7 (±0.5)	$3.7\ (\pm0.6) \times 10^{-2}$
NH[b]	$<2 \times 10^{-4}$	10.0 (±0.2)[c]	$<2 \times 10^{-2}$

[a] Almond β-glucosidase, pH 6.3, 25 °C.
[b] Anomeric mixture.
[c] K_i value.

3. Experimental

3.1. Materials

The chromatographically purified almond β-glucosidase (specific activity 25 units/mg) was obtained from Sigma–Aldrich Chemical Co., St. Louis, MO. Enzyme concentration was determined by absorbance at 278 nm using a molar extinction coefficient of $\varepsilon = 1.22 \times 10^5$ $M^{-1}cm^{-1}$, based on the percent (1 g/100 ml) extinction coefficient (=18.8) of Legler [17] and a subunit molecular weight of 65,000 [18,19]. Yeast (*Saccharomyces cerevisiae*) α-glucosidase (specific activity = 170 units/mg) was also obtained from Sigma–Aldrich. The β-glucosidase from *A. niger* was prepared from crude "cellulase" powder, obtained from Fluka (Sigma–Aldrich) essentially as described by Seidle et al. [20]. The substrates, both α and β *p*-nitrophenyl-D-glucopyranoside (pNPG) and phenyl β-D-glucopyranoside (GlcOPh), were obtained from Sigma–Aldrich as were buffers and all other reagents.

3.1.1. Phenyl β-D-thioglucopyranoside

GlcSPh was prepared by reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with thiophenol in acetone (containing

potassium carbonate). The acetylated glucoside was deacetylated in dry methanol containing catalytic amounts of sodium methoxide. Crystallization from ethanol yielded phenyl β-thioglucoside (400 MHz ^{1}H NMR: δ_{H1} = 4.63 ppm, $J_{1,2}$ = 9.9 Hz, mp = 130–131°, lit mp = 135° [21]).

3.1.2. Phenyl glucosylamine

GlcNHPh was prepared essentially as described by Capon and Connett [22]. ^{1}H NMR (CD_3OD) revealed a 3:2 mixture of α:β anomers. Since the rate of anomerization in aqueous solution is faster than the hydrolysis rate [2], no attempt was made to separate the anomers.

3.2. Kinetics

Reactions were carried out in buffer solutions [0.01 M MES (morpholino-ethansulfonate), 0.01 M NaCl, pH 6.3] at 25 °C. Stock enzyme solutions were prepared by dissolving ~10 mg/ml of protein in the MES buffer. The reaction was monitored by removal of an aliquot of the reaction mixture and then determining the glucose concentration using the coupled enzyme assay of hexokinase (yeast) and glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), in a buffered solution (0.2 M triethanolamine, pH 7.5), containing 10 mM ATP, 5 mM $MgSO_4$, 1 mM NAD^+ and measuring the increase in absorbance at 340 nm due to the formation of NADH ($\varepsilon = 6.32 \times 10^3\ M^{-1}\ cm^{-1}$ [23]). The K_m (or K_i) values for the compounds PhXGlc (X = O, S, NH) were determined by the inhibition of the enzyme-catalyzed hydrolysis of pNPG, as previously described for methyl glucoside hydrolysis [19].

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