Direct NMR-Spectroscopic Determination of Active-Enzyme Concentration by Titration with a Labeled Inhibitor: Determination of the k_{cat} Value of Almond β-Glucosidase

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*A new method for the determination of active-enzyme concentration of a glucosidase by using ^{13}C NMR spectroscopy is reported. The method consists of quantifying the binding between a ^{13}C-labelled, strong competitive inhibitor, [5-^{13}C]-1-azafagomine (**1**), and the enzyme. The concentration of free inhibitor **1** is measured in a series of binding experiments from the intensity of its NMR signal relative to that of a reference. From a plot of the concentrations of bound vs. free inhibitor **1**, the amount of specifically bound **1**, that is, the amount of active sites, is determined. From this value, active-enzyme concentration and k_{cat} value can be calculated.*

KEYWORDS:

azasugars · enzyme inhibitors · enzyme kinetics · glycosidases · NMR spectroscopy

Introduction

Emil Fischer, a giant of his time, almost single-handedly started research in the fields of carbohydrate and protein chemistry and enzymology. One of his famous discoveries, the lock-and-key principle, was a result of work with two enzymes: almond β-glucosidase and yeast α-glucosidase.[1] Curiously, today—100 years after Fischer's work—the turnover number (k_{cat}) of almond β-glucosidase has still not been securely determined, because both molecular weight and purity of the enzyme is questionable. Here we introduce a method that allows the determination of an enzyme's k_{cat} value without knowledge of purity and molecular weight, and we use it to determine the k_{cat} values for a number of enzymes including that of almond β-glucosidase.

The determination of an enzyme's k_{cat} value requires knowledge of active-enzyme concentration $[E_0]$, since it is calculated from the maximum velocity v_{max} and $[E_0]$ [Eq. (1)].

$$v_{max} = k_{cat}[E_0] \quad (1)$$

Determination of an enzyme's concentration is usually carried out by purifying the protein to homogeneity and determining its molecular weight. Based on the expected purity of the protein and its molecular weight, the molar concentration of a given enzyme solution can be calculated. This approach depends, of course, on whether the enzyme can be isolated in pure form and/or whether the molecular weight can be (accurately) determined . In some cases this is very difficult. In the case of almond β-glucosidase, molecular weight determination and purification has proven difficult because of the presence of different isoenzymes.[2] For some other glycosidases, k_{cat} is undetermined or based on an assumed purity of the enzyme.

We here report a method that allows the direct determination of active-enzyme concentration even in an impure enzyme sample. The method consists of quantifying the binding between a ^{13}C-labelled, strong competitive inhibitor and an enzyme by using ^{13}C NMR spectroscopy. When such a tightly binding inhibitor is bound to a relatively large enzyme molecule, its ^{13}C signal disappears due to line broadening as a result of slow tumbling of the enzyme–inhibitor complex (Figure 1). Therefore, only unbound inhibitor is seen in the ^{13}C NMR spectrum and the intensity of the signal can be used to determine the concentration of free inhibitor. Consequently, the amount of bound inhibitor can be calculated. From a series of

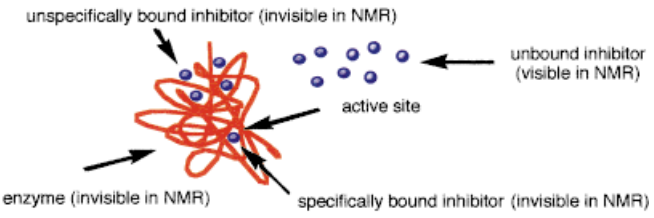

Figure 1. *Schematic illustration of specific and unspecific binding of an inhibitor to an enzyme.*

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spectra at various inhibitor concentrations, the amount of specific and unspecific binding[3] can be determined. If the binding constant K_i is known, and if it is much smaller than [I], then the total enzyme concentration in the sample can be calculated from the concentration of specifically bound inhibitor. From a v_{max} determination, the value of k_{cat} can be determined.

In the conventional methods for the determination of specific binding by titration experiments, the entire binding curve is obtained, including values for bound species at very low concentrations of ligand prior to saturation of the binding site. This is not possible in the present case, since a substantial concentration is necessary to obtain a reliable NMR signal, and substantial enzyme concentrations are consequently also required. It would seem that this limitation is a small price to pay when extensive enzyme purification is not necessary.

Results and Discussion

The inhibitor used in this study is (±)-[5-^{13}C]-1-azafagomine (**1**, Figure 2).[4] This compound has the advantages that it is relatively easy to prepare and that it is also a strong inhibitor of many glycosidases. The (−)-form of **1** (depicted in Figure 2) is a potent competitive inhibitor of almond β-glucosidase ($K_i = 0.33$ μM), yeast α-glucosidase ($K_i = 6$ μM), isomaltase ($K_i = 0.32$ μM), and glucoamylase ($K_i = 10$ μM),[5] whereas the (+)-form is a very weak inhibitor of those enzymes ($K_i \gg 1$ mM).[6] Thus, only the (−)-form of **1** shows significant specific binding at the concentrations used in this study. Importantly, the inhibitor used should be relatively potent so that the specific binding of the inhibitor is essentially complete during the experiments. With a relatively weak inhibitor it is also possible that exchange processes would interfere with the spectra.

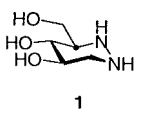

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Figure 2. *[5-^{13}C]-1-azafagomine (**1**). The compound used in this study was racemic, but only the enantiomer shown is active as an inhibitor.*

The inhibitor **1**, at concentrations from 0.6 – 5 mM, was mixed with almond β-glucosidase in D_2O in an NMR tube, and the ^{13}C NMR spectrum was recorded. The peak height of the C5 signal was compared to the peak height of the C5 signal in a reference spectrum of **1**, which was recorded without enzyme present (Figure 3). A clear decrease in intensity of the signal relative to the reference signal was observed.[7] This is a result of the inhibitor being bound to the enzyme; bound inhibitor is not observed. The chemical shift and line width of the peak did not change significantly. This means that the relaxation time T_2 is unchanged and that the peak height is a reliable measure of the area of the peak. From the relative peak heights the concentration of free inhibitor could be calculated. The amount of bound inhibitor was determined by subtraction of this value from the total amount of inhibitor. Note that it is not necessary to take into account that **1** is racemic as unbound antipode is removed by subtraction and is irrelevant. This experiment was done at a series of concentrations of **1**, and the amount of free and bound **1** was calculated per milligram of enzyme preparation (Table 1).

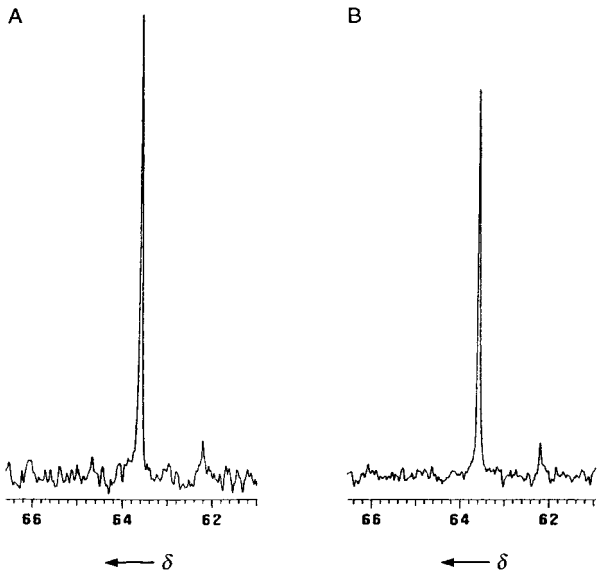

Figure 3. *A 50 MHz ^{13}C NMR spectrum of **1** (60 μg in 600 μL D_2O) without (A) and with 6.9 mg β-glucosidase (B).*

Table 1. *The peak height of the C5 signal of **1** (H_{peak}) relative to that of a reference signal (H_{ref}) in a series of ^{13}C NMR experiments at varied concentrations of **1** and β-glucosidase.[a]*

mg of enzyme	μg of **1**	H_{peak}/H_{ref}	$c(\mathbf{1}_{free})$ [μM][b]	$c(\mathbf{1}_{bound})$ [μM][b]
0.0	53.74	1.00	–	–
6.9	89.50	1.37	119.6	25.47
6.9	179.00	2.88	250.6	39.55
6.9	268.50	4.36	379.5	55.73
4.5	89.57	1.44	192.5	30.11
4.8	152.27	2.50	312.7	42.15
4.8	152.27	2.46	308.3	46.50
5.2	53.74	0.82	94.96	20.64
5.2	53.74	0.83	96.45	19.15
6.8	358.27	5.93	523.9	65.47
6.8	358.27	5.95	526.3	63.02

[a] The total volume was 0.6 mL. From the relative peak heights the amount of unbound **1** can be calculated. The amount of bound **1** is determined as the missing fraction of the total amount of **1**. Both sets of values are divided by the amount of enzyme. [b] Per milligram of enzyme.

A plot of the amounts of bound versus free **1** is shown in Figure 4 A. The amount of bound inhibitor per milligram of protein increases linearly with increasing concentration of free **1**. This must be due to unspecific binding between the protein and **1**. Unspecific binding between ligands and proteins is known to occur, and the amount of unspecifically bound inhibitor (B) is known to be proportional to the free inhibitor concentration [I], (i.e., $B = k[I]$).[8] The amount of specifically bound **1** is independent of inhibitor concentration for $[I] \gg K_i$, since the specific binding sites are saturated under these conditions. Thus the amount of specifically bound **1** is found as the intercept between a linear regression of the data and the ordinate.

A similar series of experiments was carried out for yeast α-glucosidase, *Aspergillus niger* glucoamylase, and yeast isomaltase

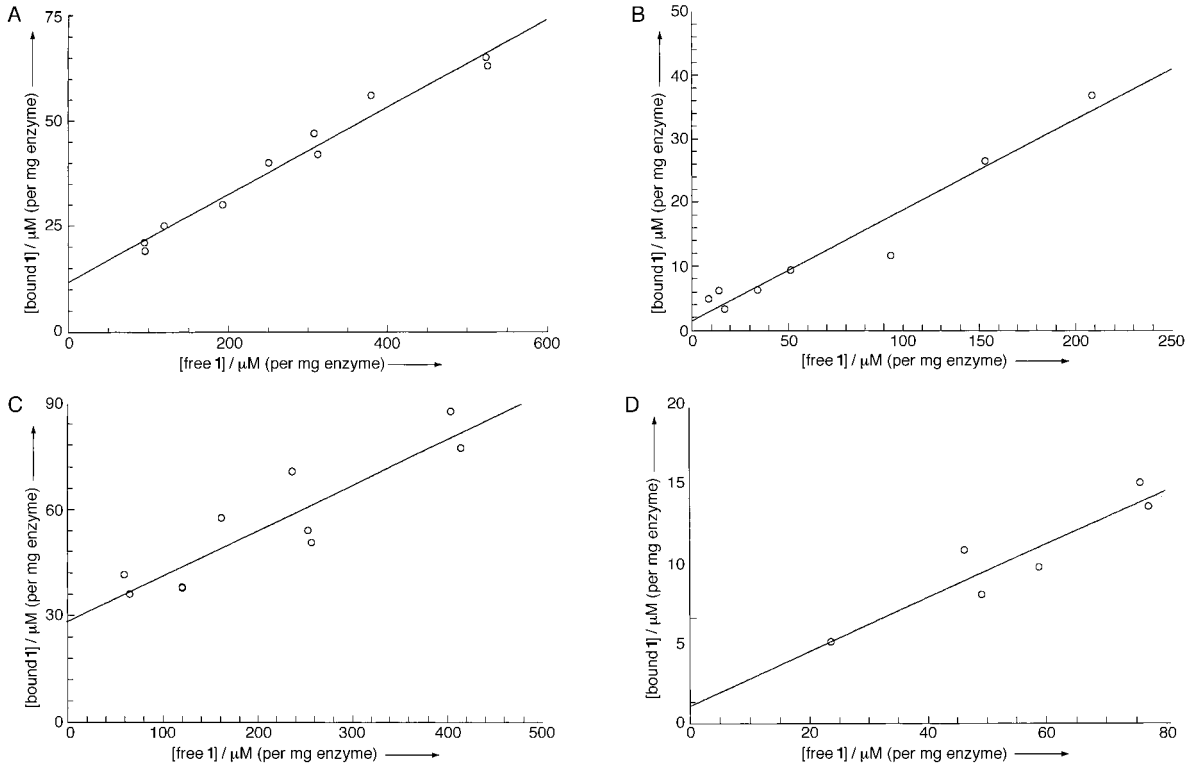

Figure 4. *A plot of the concentrations of bound **1** vs. free **1** per milligram of β-glucosidase (A), α-glucosidase (B), glucoamylase (C), and isomaltase preparation (D). Linear regressions of the data are also shown (A: $y = 0.104x + 11.7$, maximal deviation: 4.89, $r^2 = 0.974$, $\sigma_a = 0.00601$, $\sigma_b = 1.92$; B: $y = 0.157x + 1.41$, max. dev.: 6.16, $r^2 = 0.945$, $\sigma_a = 0.0155$, $\sigma_b = 1.57$; C: $y = 0.126x + 28.3$, max. dev.: 12.1, $r^2 = 0.810$, $\sigma_a = 0.0216$, $\sigma_b = 5.25$; D: $y = 0.167x + 1.07$, max. dev.: 1.93, $r^2 = 0.900$, $\sigma_a = 0.0249$, $\sigma_b = 0.0249$). The intercepts show the concentration of specifically bound **1** per milligram of β-glucosidase, α-glucosidase, glucoamylase, and isomaltase preparation to be 11.7 ± 1.9, 1.4 ± 1.6, 28.3 ± 5.3, and 1.1 ± 1.4 μM mg^{-1}, respectively.*

giving data similar to those shown in Table 1 (see Supporting Information). The plots of the concentrations of bound versus free **1** for these enzymes are shown in Figures 4B, C, and D, respectively. The amount of specifically bound **1** in each case is shown in Table 2. In the case of α-glucosidase and isomaltase these values are so small that the standard deviation actually exceeds the value for the specific binding. Thus, the values for these two enzymes are not well determined. The purity of the enzymes in these cases is less than 10%, which may be the lower limit for the present method to be applicable.

Based on the expected molecular weight of the enzymes the purity of each enzyme preparation can be calculated assuming that only one active site is present. The value is critically dependent on the certainty of the molecular weight, for which in the case of β-glucosidase several values are reported (135 000 Da[9] and 117 000 Da[10]). For glucoamylase 16.8 nmol of **1** bind to one milligram of the enzyme, and based on the reported molecular weight[11] (Table 2) this means that **1** must bind to more than one site. Glucoamylase is known to have, in addition to the catalytic site, a starch-binding site to which small glucose-type molecules can bind.[12] The result can be explained by assuming that **1** also binds to this site. The purity calculated for glucoamylase thus assumes specific binding to both sites.

The low purities of α-glucosidase and isomaltase also suggest an explanation as to why the specific binding to these enzymes is poorly determined. The enzyme preparations obviously

Table 2. *Purity and k_{cat} values for the four glycosidases tested.*

Enzyme[a]	$c(\mathbf{1}_{bound})$ [μM][b]	$n(\mathbf{1}_{bound})$ [nmol][b]	M [g mol^{-1}]	Purity [%]	[E] [mg L^{-1}]	v_{max} at [E] [μM s^{-1}]	k_{cat} [s^{-1}]
β-glucosidase (25 U mg^{-1})	11.7 ± 1.9	7.0 ± 1.1	135 000[9]	94.5	10.6	1.31	17.6
α-glucosidase (4.5 U mg^{-1})	1.4 ± 1.6	0.84 ± 0.96	68 500[10]	5.8	21.6	0.314	17.5
glucoamylase (40 U mg^{-1})	28.3 ± 5.3	16.8 ± 3.0	80 000[11]	67.2	29	0.114	0.46
isomaltase (47 U mg^{-1})	1.1 ± 1.4	0.67 ± 0.82	110 000	7.4	44.6	1.04	35.1

[a] The specific activity is given in brackets. [b] Per milligram of enzyme.

contain a large amount of protein impurities, which will be responsible for increased unspecific relative to specific binding. This was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples, which showed the presence of several proteins.

More interesting than the purity is the relationship that has been determined between enzyme activity and molarity. Thus the results show that β-glucosidase has 0.28 nmoles per unit,[13] α-glucosidase has 0.19 nmol U^{-1}, glucoamylase 0.21 nmol U^{-1}, and isomaltase 0.014 nmol U^{-1}. From these values it is now possible to calculate the molarity of an enzyme based on its activity. From the determined number of active sites present per milligram of protein, the concentration of active sites in solution can be calculated, and hence k_{cat} can be calculated from v_{max} values. For almond β-glucosidase a k_{cat} value of 17.6 s^{-1} was found at 25 °C and pH 6.8 for the hydrolysis of 4-nitrophenyl-β-D-glucopyranoside. This is a relatively low value, given that certain glycosidases have k_{cat} values of up to 1000 s^{-1} [14] and that the k_{cat} value for *Agrobacterium* β-glucosidase was reported to be 141 s^{-1} at 37 °C for the same substrate.[15] However, a k_{cat} value of this magnitude is not untypical.

The k_{cat} value for yeast α-glucosidase was found to be 17.5 s^{-1} at 25 °C and pH 6.8 for the hydrolysis of 4-nitrophenyl-α-D-glucopyranoside. This value has previously been determined to be 20 s^{-1} under the assumption that a pure enzyme was at hand,[16] so our value is close and confirms the previous value. The k_{cat} value for *A. niger* glucoamylase was found to be 0.46 s^{-1} at 45 °C and pH 4.5 for the hydrolysis of 4-nitrophenyl-α-D-glucopyranoside, whereas the previously reported value under the same conditions is 0.25 s^{-1}.[17] Thus, the value determined in this work is relatively close to the previous one. The relatively small k_{cat} values are due to the fact that aryl glycosides are poor substrates for glucoamylase. The k_{cat} value of the enzyme for the hydrolysis of the substrate maltose is 6.8 s^{-1}.[17] Finally, yeast isomaltase was found to have a k_{cat} value of 35.1 s^{-1} at 25 °C and pH 6.8 for the hydrolysis of 4-nitrophenyl-α-D-glucopyranoside. Thus, this enzyme is apparently a slightly better catalyst for this reaction than α-glucosidase.

In the present work we have introduced a method for determination of active-enzyme concentration based on NMR titration experiments, and we have used it to determine the k_{cat} values of four glycosidases. The method should be generally applicable; all that is required is a labeled strong competitive inhibitor. With this method it is now possible to relate enzyme activity to enzyme concentration, and it is now possible to specify the concentration of enzyme preparations in moles per gram.

Experimental Section

NMR spectroscopic experiments: All NMR experiments were carried out on a Varian Gemini 2000 instrument at 50 MHz and 296 K. Vertical scaling was used and set to 80000. The solvent was D_2O and the sample volume was 0.6 mL. The samples were freshly prepared to a final volume of 0.6 mL. All spectra were made from 15000 – 20000 scans.

Measurement of v_{max}: These values were obtained by using nitrophenyl glycosides as substrates: 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-α-D-glucopyranoside were used as substrates for β-glucosidase and isomaltase, respectively. Formation of the product, 4-nitrophenol, was measured continually at 400 nm using a Milton Roy Genesys 5 spectrometer. To calculate the molar formation of product (4-nitrophenol), $\varepsilon = 6.41 \times 10^3\,M^{-1}cm^{-1}$ was used as the value for the extinction coefficient (pH 6.8, 25 °C).[16] All reactions were carried out in a sodium phosphate buffer (0.05 M, pH 6.8) at 25 °C (thermostat). Two thermostated solutions (1: 1 mL of 0.1 M buffer, 800 μL substrate at varied concentration (1.25, 5, 20, or 40 mM), and 100 μL water; 2: 100 μL enzyme (0.015 mg mL^{-1})) were mixed, and the reaction was immediately monitored. From four experiments with varied substrate concentration, initial reaction rates were calculated as the slope of the plot of product absorption vs. reaction time. K_M and v_{max} values were calculated by nonlinear regression analysis of plots of reaction rate vs. substrate concentration with the graphics program EasyPlot (Spiral Software, Chinle (AZ), USA).

We thank Ms. Anne Bülow for assistance with v_{max} determinations, Dr. Mikael Esmann for a helpful discussion regarding unspecific binding, and the Danish Research Councils for financial support through the THOR program.

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Received: February 28, 2000
Revised version: June 13, 2000 [F 5]