JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Determination of Hydrolysis and Mutarotation Rates during the Enzymatic Hydrolysis of Lactose

DANIEL M. JENKINS,*,† MICHAEL A. TERUEL,† JOSÉ I. REYES-DE-CORCUERA,§ AND OWEN YOUNG#

Room 218, Molecular Biosciences and Bioengineering, University of Hawaii, 1955 East West Rosd, Honolulu, Hawaii 96822; Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, Florida 33850; and Food Technology, Auckland University of Technology, 24 Saint Paul Street, Auckland, New Zealand

An experiment is described in which a custom-made glucose electrode is used to directly monitor the enzymatic hydrolysis of lactose to glucose. The transient profile of β-D-glucose can be used to simultaneously determine the rate constants for mutarotation and for enzymatic hydrolysis by applying a dynamic nonlinear regression routine. Due to differences in the mutarotation rate constants between lactose and glucose, the β-D-glucose concentration "overshoots" equilibrium under certain conditions, which can be modeled mathematically. This overshoot can be observed reliably and used to quantify the differences in mutarotational equilibria between glucose and lactose. These observations may be important for the analysis of dairy products and commercial lactase preparations and illustrate an unusual kinetic phenomenon caused by intramolecular forces. This approach may also be important for the accurate determination of a variety of oligosaccharides such as glycogen, which tend to be composed primarily of one stereoisomer.

KEYWORDS: Biosensor; β-galactosidase; glucose; carbohydrate metabolism; anomer

INTRODUCTION

In previous research unexpected local maxima in glucose concentrations have been observed during the enzymatic hydrolysis of lactose (*1*) and glycogen (see the Supporting Information). We were unable to account for these phenomena until we considered the anomerization of the respective saccharides. Glucose in the pyranose (ring) form can spontaneously undergo a ring opening and rotation of the hydroxyl group on the C1 carbon before reclosure of the ring, resulting in a stereoisomer or "anomer" (*2*, pp 421−422) as indicated in **Figure 1**. This also occurs to the glucose subunit conjugated to β-galactose in the disaccharide lactose and can be regarded as a reversible first-order process (*3*−*6*). Hydrolysis of lactose into its monosaccharide components effectively releases glucose in an unchanged anomeric state, but because the equilibria between α and β isoforms may differ for lactose and glucose, the products of hydrolysis may undergo a net mutarotation in a second relaxation process even when the lactose is initially at equilibrium. Because many analytical methods for glucose rely on enzymes that are specific to the β-D-glucose anomer (see, e.g., ref *7*, p 1110), it was hypothesized that mutarotation effects might help to account for the unexpected observations reported above. In this paper we consider a formal treatment of the kinetics of these processes and adapt a glucose electrode to investigate them experimentally. It is anticipated that this analysis will have implications for interpreting enzyme-dependent methods for carbohydrate analysis, as well as for more general understanding of carbohydrate metabolism and transformation in food processing and medicine.

To more thoroughly characterize the kinetics of simultaneous hydrolysis and mutarotation, we required a glucose sensor that was stable, sensitive, reproducible, fast, and capable of continuously monitoring glucose concentration in a reaction mixture. The literature is rich with reports of glucose sensors that meet these criteria in various degrees, including the first reported "biosensor", which incorporated an enzyme onto a polarographic oxygen electrode (*8*), and numerous others that detected glucose through mediated electron exchange from the products of glucose oxidation (see, e.g., refs *9*−*17*). Disposable glucose test strips, while extremely successful commercially and generally predicated on the same principles as those referred to above, use freeze-dried reagents that will wash off if immersed into a solution and are not able to continuously monitor glucose. For this research we adapted protocols to entrap the enzyme glucose oxidase in a thin electrically deposited polymer film of poly(*o*-phenylenediamine) (*18*) on the surface of electrodes that had previously been electroplated with a rough platinum de-

* Corresponding author [telephone (808) 956-6069; fax (808) 956-3542; e-mail danielje@hawaii.edu].
† University of Hawaii.
§ University of Florida.
# Auckland University of Technology.

10.1021/jf801403n CCC: $40.75
Published on Web 08/20/2008

posit (*18, 19*). In principle, the resulting electrodes operated similarly to many other electrochemical glucose sensors: enzymatic oxidation of glucose resulted in coincident reduction of dissolved oxygen to peroxide, which could be detected analytically through electrooxidation back to oxygen at the electrode surface. However, the method we selected had the advantage of being relatively simple, and the resulting electrodes were relatively sensitive and fast.

KINETIC MODEL

For simplicity, in our analytical model of the processes in **Figure 1** we assume that the concentration of lactose is much lower than the Michaelis constant K_m so that the hydrolysis of lactose can be approximated as a first-order process. We also assume that the conformation of the glucose subunit on the lactose molecule does not affect the kinetics of hydrolysis, because the enzyme is selective to the β-galactopyranoside ring on the dissaccharide. Indeed, unpublished experiments in our laboratory have shown that the kinetics of the enzyme preparation we used did not differ significantly when α or β anomers of the substrate were hydrolyzed (see the Supporting Information). Mathematically, this system is described by four coupled linear differential equations

$$\frac{dL_\alpha}{dt} = \frac{dx_1}{dt} = -\left(k_1 + \frac{V_{max}}{K_m}\right)x_1 + k_{-1}x_2 \quad (1)$$

$$\frac{dL_\beta}{dt} = \frac{dx_2}{dt} = k_1x_1 - \left(k_{-1} + \frac{V_{max}}{K_m}\right)x_2 \quad (2)$$

$$\frac{dG_\alpha}{dt} = \frac{dx_3}{dt} = \frac{V_{max}}{K_m}x_1 - k_2x_3 + k_{-2}x_4 \quad (3)$$

$$\frac{dG_\beta}{dt} = \frac{dx_4}{dt} = \frac{V_{max}}{K_m}x_2 + k_2x_3 - k_{-2}x_4 \quad (4)$$

where $L_\alpha = x_1$ is the concentration of α-D-lactose, $L_\beta = x_2$ is the concentration of β-D-lactose, $G_\alpha = x_3$ is the concentration of α-D-glucose, $G_\beta = x_4$ is the concentration of β-D-glucose, and V_{max} is the enzyme activity when substrate is in great excess. For simplification, we can assign a new variable $v = V_{max}/K_m$.

This system can be represented in vector form

$$\mathbf{x}' = \begin{bmatrix} -(k_1+v) & k_{-1} & 0 & 0 \\ k_1 & -(k_{-1}+v) & 0 & 0 \\ v & 0 & -k_2 & k_{-2} \\ 0 & v & k_2 & -k_{-2} \end{bmatrix}\mathbf{x} = \mathbf{A}\mathbf{x} \quad (5)$$

and its solutions described by

$$\mathbf{x} = \xi\, e^{rt} \quad (6)$$

where ξ represents the eigenvectors of eq 5 associated with the eigenvalues $r = 0$; $-(k_2 + k_{-2})$, $-v$, and $-(v + k_1 + k_{-1})$.

After solving for the eigenvectors, we apply initial conditions that all of the carbohydrate is initially lactose at equilibrium (no enzyme has been added yet, so that $x_{3(0)} = x_{4(0)} = 0$)

$$k_1x_{1(0)} = k_{-1}x_{2(0)} \quad (7)$$

and the total initial lactose concentration is constant

$$x_{1(0)} + x_{2(0)} = L_0 \quad (8)$$

so that

$$x_{1(0)} = \frac{L_0}{\left(1 + \frac{k_1}{k_{-1}}\right)} \quad (9)$$

and

$$x_{2(0)} = \frac{L_0}{\left(1 + \frac{k_1}{k_{-1}}\right)}\frac{k_1}{k_{-1}} \quad (10)$$

Here we are only interested in the solution for β-D-glucose (x_4), which we can observe with our electrode after mixing the substrate and enzyme:

$$x_4 = L_0\left\{\frac{k_2}{(k_{-2}+k_2)} + \frac{v(k_1k_{-2} - k_2k_{-1})}{(k_2+k_{-2})(k_1+k_{-1})(v-k_2-k_{-2})}e^{-(k_2+k_{-2})t} + \frac{[k_2(k_{-1}+k_1) - vk_1]}{(k_{-1}+k_1)(v-k_2-k_{-2})}e^{-vt}\right\} \quad (11)$$

On the basis of our experience we expect that under certain conditions this model should predict a local maximum in x_4,

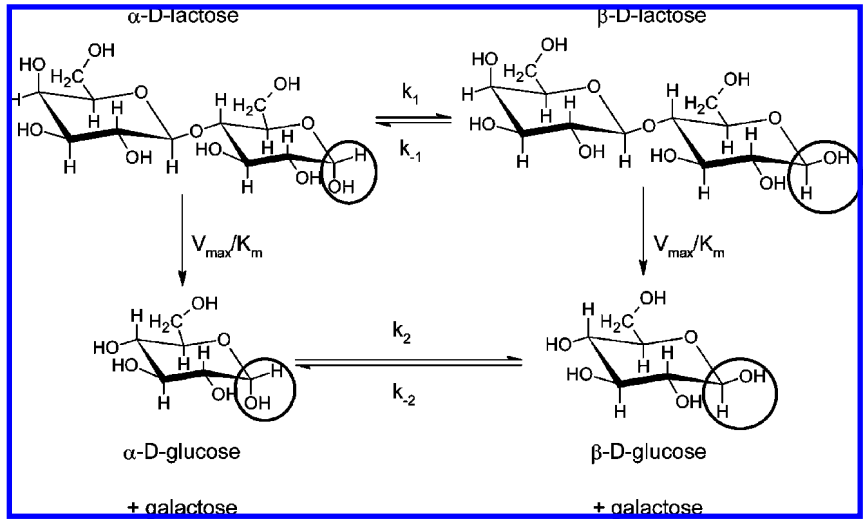

Figure 1. Stereochemistry of α and β anomers of D-lactose and D-glucose and simplified kinetic model showing possible interconversion of the respective saccharides by mutarotation or by enzymatic hydrolysis of lactose. Circles illustrate the locations where mutarotation occurs, where α anomers have the hydroxyl group in the axial orientation and β anomers have the hydroxyl group in the equatorial orientation.

which mathematically occurs when the derivative of eq 11 with respect to time is zero and the second derivative with respect to time is negative. This occurs when

$$t = \frac{\ln\left\{\frac{[vk_1 - k_2(k_{-1} + k_1)]}{(k_1k_{-2} - k_2k_{-1})}\right\}}{(v - k_2 - k_{-2})} \quad (12)$$

For a local maximum to occur in x_4, the hydrolysis rate (v) must exceed the mutarotation rate ($k_2 + k_2$). This solution can occur only when $k_1\,k_{-2} > k_2k_{-1}$ (when the ratio of β to α anomers is higher at equilibrium for lactose than for glucose) and when

$$v > k_2\left(\frac{k_{-1}}{k_1} + 1\right) \quad (13)$$

This is a somewhat intuitive result, as for a maximum to occur the enzyme activity must be fast enough to convert a lot of β-D-lactose to β-D-glucose before the latter mutarotates to α-D-glucose. Structurally speaking, the criteria $k_1k_{-2} > k_2k_{-1}$ is consistent with extra steric crowding from the galactose subunit in lactose exerting stress on the glucose C1 hydroxyl group (**Figure 1**) to force it into the less crowded and more stable equatorial plane (*2*, p 424) and may also occur if the dipole moment is correspondingly enhanced in β-D-lactose, making it more stable in aqueous solutions (*20*).

MATERIALS AND METHODS

Potentiostatic Measurements. All potentiostatic procedures were implemented using a standard three electrode cell configuration interfaced to a commercial potentiostat (Omni-101, Cypress Systems, Chelmsford, MA).

Electrode Platinization. Integrated electrodes containing a 1 mm diameter platinum working electrode, a platinum counter electrode, and a silver/silver chloride reference electrode printed onto alumina were obtained commercially (AC1.W2.R2, BVT Technologies, Brno, Czech Republic). These were cleaned by immersing them overnight in concentrated sulfuric acid and then rinsed with distilled water. To increase the active surface area of the working electrode prior to enzyme immobilization, the electrode was platinized in a solution of 2 mM hexachloroplatinic acid, 1 mM lead acetate, and 100 mM KCl. The active area of the electrode was immersed in the platinizing solution after the latter was deoxygenated by sparging with nitrogen gas for about 5 min, and then −150 mV was applied to the working electrode relative to the reference electrode. The platinizing potential was sustained until a total charge displacement of about 1.0 C/cm^2 was achieved, which required about 30 min. After platinization, the electrode was rinsed with distilled water.

Enzyme Immobilization. A prepolymer solution containing 5 mM *o*-phenylenediamine was prepared in 200 mM acetate buffer of pH 5.2 and sparged with nitrogen gas for about 5 min. Immediately prior to electropolymerization onto the working electrode, approximately 2 mg of glucose oxidase (type X, 180 units/mg, Sigma-Aldrich, St. Louis, MO) was dissolved in 1 mL of the prepolymer solution, and the active area of the platinized electrode was immersed into the resulting solution for 5 min with zero applied potential to allow the enzyme and monomer to diffuse into the pores of the working electrode. Subsequently, electropolymerization was achieved by applying +650 mV to the working electrode for about 30 min.

Electrode Calibration and Testing. The electrodes were tested with solutions of various compositions of glucose, lactose, and β-galactosidase (lactase), all prepared in 50 mM citrate buffer, pH 4.5. All lactose and glucose stock solutions were allowed to equilibrate for at least 12 h prior to use, unless noted otherwise. To calibrate the electrode, it was immersed in 10 mL of glucose-free buffer, stirred with a magnetic stirrer, and allowed to equilibrate with +700 mV applied to the working electrode relative to the reference (approximately 10−15 min). After equilibration, 100 μL aliquots of 100 mM glucose stock were added sequentially to introduce different glucose concentrations, and the equilibrium current signal was recorded for each step. For analyzing the activity of the commercial β-galactosidase (lactaid fast act caplets, McNeil Nutraceuticals, Ft. Washington, PA) isolated from *Aspergillus oryzae* (*21*), stock solutions of enzyme were prepared by dissolving caplets into buffer and allowing the solid material to settle. The supernatants of these solutions were diluted as required for further analysis.

To estimate the enzyme activity, the electrode was equilibrated at +700 mV as above in 10 mL of dilute enzyme solution (typically the equivalent of one lactaid caplet in 2500 mL buffer), and then the initial rate of current increase was recorded after the addition of an aliquot of lactose stock (typically 10 mL of 100 mM stock in citrate buffer). To observe the relaxation of excess β-D-glucose to α-D-glucose following the hydrolysis of lactose, the experiment was repeated with higher enzyme and lower substrate concentrations by adding a 100 μL aliquot of 100 mM lactose stock to 10 mL of enzyme solution from the equivalent of one caplet dissolved in 25 mL.

Data Analysis. Electrode currents observed during the lactose hydrolysis experiment were first corrected by subtracting the average current observed for 1 min prior to addition of lactose. A dynamic fitting tool from commercial software (SigmaPlot 10.0, SysStat Software, San Jose, CA) was used to fit the background-corrected data to a double-exponential equation as predicted in eq 11. To ensure a good fit with the physical model, a constraint was applied such that the sum of the coefficients preceding the exponents was zero (thereby ensuring that the initial sensor response was zero in the fitted equation, just as in the observed data). The observed and fitted data were then normalized by dividing by the value of the first fitted coefficient, which represented the value of the electrode current at equilibrium (infinite time).

To estimate the degree to which β-D-lactose is favored at equilibrium compared to β-D-glucose, the coefficients from the normalized fitted equation

$$y = 1 + a\,e^{-bt} + c\,e^{-dt} \quad (14)$$

can be related to the similarly normalized version of the physical model in eq 11, and thereafter it can be shown that

$$1 + \frac{a(d-b)}{d} = \frac{k_1(k_2 + k_{-2})}{(k_1 + k_{-1})k_2} \quad (15)$$

The right-hand side of eq 15 is the ratio of the respective ratios of β anomer to total saccharide for lactose and glucose at equilibrium and can be estimated directly from the fitted coefficients. A value of >1 implies that, at equilibrium, the ratio of β-D-lactose to total lactose is higher than the corresponding ratio for glucose.

The observed Michaelis constant K_m is determined as the ratio of the enzyme activity V_{max} to the rate constant v (equivalent to the fitted coefficient d). The mutarotation rate ($k_2 + k_{-2}$) for glucose is taken directly as the fitted coefficient b. Standard errors for the parameters fitted to eq 14 were determined in the software using a reduced chi squares method, and the standard error in the estimate for the ratio espressed in eq 15 was determined using a general uncertainty analysis assuming that the errors of each fitted parameter were independent (*22*, p 53).

RESULTS AND DISCUSSION

Platinization of electrodes and enzyme entrapment in poly(*o*-phenylenediamine) proceeded as reported in previous research (*18*). Initially, platinum plating currents diminished as the electrodes polarized and diffusion limitations were introduced, and then they gradually increased as the electrode roughness increased (**Figure 2**). Oxidation current for the polymerization of phenylenediamine gradually diminished as the nonconductive polymer more completely coated the platinum surfaces (**Figure 3**).

Calibration of the electrodes (**Figure 4**) demonstrated that they possessed the requisite speed, sensitivity, range, and linearity for successfully monitoring the proposed reactions. The risetime for the sensors was on the order of 1 s and appeared to be limited

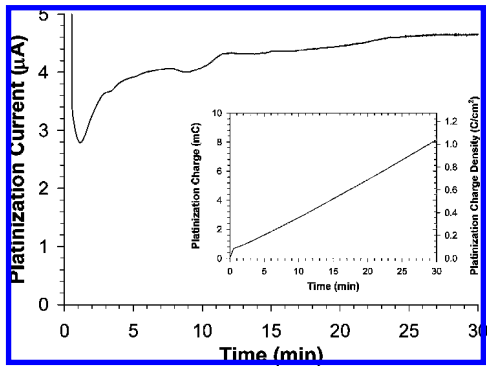

Figure 2. Reduction current observed during platinization of working electrode and corresponding platinization charge and charge density on working electrode (inset).

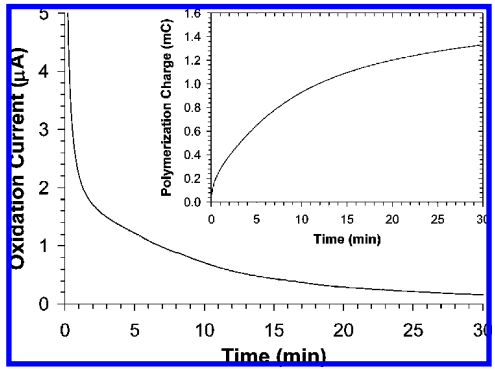

Figure 3. Oxidation current observed during electropolymerization of *o*-phenylenediamine and total charge displacement during polymerization step (inset).

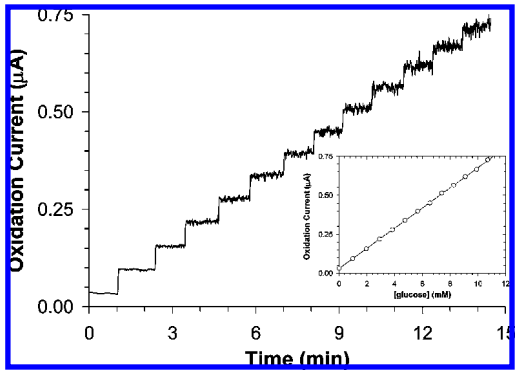

Figure 4. Sensor output recorded during sequential 100 μL aliquots of 100 mM glucose to 10 mL of glucose free buffer. Regression of equilibrium sensor output to glucose concentration (inset) results in a calibration equation of I (μA) = 0.065 [glucose] (mM) + 0.03, R^2 = 0.9999, and standard error of 0.04 mM glucose.

only by the rate of mixing in solution. Absolute noise increased with increasing glucose concentrations (**Figure 4**), presumably due to increased oxygen gas partial pressure and random adhesion and desorption of gas on the electrode surface (*19*). Even so, the accuracy and stability were adequate to observe transitions in glucose concentration of well below 1 mM, especially in solutions containing 1 mM total glucose or less. By recording the enzyme reaction rate in the presence of excess substrate and using the definition of 1 unit as the quantity of enzyme converting 1 μmol of substrate to product per minute, these sensors could be applied to the determination of the activity of commercial preparations of β-galactosidase (**Figure 5**). Estimated β-galactosidase activities (2227 units per caplet) were well below specifications (9000 units

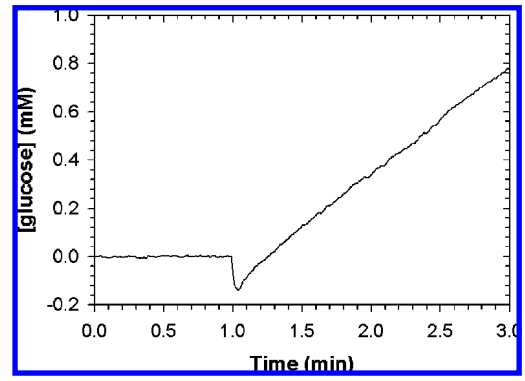

Figure 5. Lactase activity assay: 10 mL of 100 mM lactose stock added to 10 mL of lactase solution (from 1 lactaid caplet diluted in the equivalent of 2500 mL of citrate buffer, pH 4.5) at minute 1. Rate of hydrolysis is approximately 0.45 mM/min, corresponding to an enzyme activity of 2227 units/caplet (compared to specifications of 9000 units/caplet).

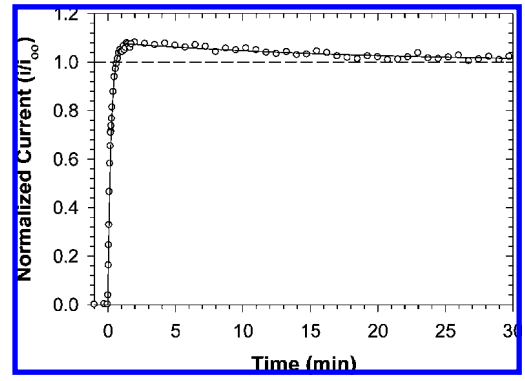

Figure 6. Hydrolysis of lactose to galactose and glucose by addition of 100 μL of 100 mM lactose standard to 10 mL of lactase stock (1 lactaid caplet dissolved in 25 mL of citrate buffer, pH 4.5) at time 0: observed (○) and fitted (—; $i/i_\infty = 1 + 0.0815\ e^{-0.055t} - 1.0815\ e^{-4.24t}$; R^2 = 0.9788) currents, normalized to the equilibrium current for the system (- - -).

per caplet), perhaps due to activity loss during storage or during the approximately 20−30 min equilibration step prior to assay and perhaps partly due to the use of a different substrate from that used by the manufacturer for product validation.

Mutarotation from an initial amount of α-D-glucose (**Figure 6**) approached an equilibrium with a rate constant of 0.055 min^{-1} (time constant of about 18 min), roughly 8 times faster than published data for glucose in pH 6.9 phosphate buffer (*23*). The discrepancy in the mutarotation rates between these data may be explained by differences in pH, as acidic or alkaline solutions tend to accelerate mutarotation (*23, 24*), and likewise mutarotation rates of sugars are highly dependent on the chemical composition of the solution (*3, 5*). Even so, the process is slow enough to observe local maxima in the β-D-glucose concentration during hydrolysis of lactose even with moderate activities of β-galactosidase. Indeed, under the conditions used in these experiments, a local maximum in the β-D-glucose concentration was clearly visible within 2 min of adding lactose to the enzyme solution (**Figure 6**). The observed data fit the double-exponential form of eq 11 with a high degree of correlation (R^2 = 0.9788), and the standard errors of the fitted parameters are relatively small (**Table 1**), suggesting that the modeling of the processes involved is reasonably accurate.

The Michaelis constant K_m for the β-galactosidase can be estimated by dividing the predicted enzyme activity by the rate constant determined from **Figure 6** corresponding to v in eq 11. The value for K_m estimated in this manner was about 10

Table 1. Summary of Fitted Parameters for the Appearance of β-Glucose during the Hydrolysis of Lactose and Corresponding Estimates of Rate Constants for Hydrolysis and Mutarotational Equilibria

	estimate ± standard error
a	0.0815 ± 0.0009
$b = (k_2 + k_{-2})$	0.055 ± 0.0019 min^{-1}
c	−1.0815 ± 0.003
$d = (v)$	4.24 ± 0.02 min^{-1}
$1 + \frac{a(d-b)}{d} = \frac{k_1(k_2+k_{-2})}{(k_1+k_{-1})k_2}$	1.08 ± 0.002

mM, which compares favorably to values reported in the literature for the same enzyme/substrate pair (*21*). The relatively high value of K_m lends validity to the modeling assumption that the lactose concentrations in the mutarotation experiment (**Figure 6**) were sufficently below K_m to treat the hydrolysis as a first-order process, although it also suggests that the amount of lactose added in the activity assay (**Figure 5**) was not sufficiently high to reflect the true maximum enzyme activity, leading to a possible underestimation of enzyme activity of 15−20%. Likewise, because our estimate of K_m is related to the assumed enzyme activity, our estimated value of this parameter may be correspondingly low.

Applying the fitted coefficients from **Figure 6** to eq 15, we find that at equilibrium the ratio of β anomer to total sugar is about 8% higher in a solution of lactose compared to glucose (**Table 1**). We have estimated the ratio of β-D-glucose anomer to total D-glucose at equilibrium to be approximately 0.63−0.64 (*25*), which is consistent with other published data (*2*, p 422; *23, 26, 27*). It follows from our observations then that under the conditions of our experiments the ratio of β-D-lactose anomer to total D-lactose at equilibrium is about 0.68−0.69. This last figure contrasts with other published values in the range of 0.62−0.63 (*6*, p 450; *28*). Because our locally observed maxima have been reproduced in a variety of conditions as long as the hydrolysis rate exceeded the mutarotation rate, and simple structural considerations would suggest that D-lactose more readily assumes the β conformation than D-glucose, we have some degree of confidence that our observations more accurately reflect the true equilibria.

Understanding of the true mutarotational equilibria can be important in designing protocols and in interpreting glucose-based assays for lactose concentrations in dairy products or in quantifying the enzyme activity in commercial preparations of β-galactosidase. Likewise, the principles used in this research can be important for the accurate determination of carbohydrate polymers such as glycogen, which are composed primarily of a single stereoisomer that can mutarotate following cleavage from the parent molecule.

SAFETY

Proper care, such as use of protective clothing, gloves, and goggles, should be taken, especially during the preparation of glucose electrodes, and the reagents should be disposed of in accordance with local and federal regulations. The buffers and other reagents used in the analytical work are not hazardous and can be disposed of in the sink after adjustment to a neutral pH.

Supporting Information Available: Representative data showing overshoot in glucose concentration during the hydrolysis of glycogen by amyloglucosidose and information showing that, for the purposes of this research, differences in enzyme kinetics for hydrolysis of lactose are negligible whether the substrate is in the α or β conformation. The tools used to derive eq 11 are summarized, and the derivation of eq 15 is shown explicitly. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Jenkins, D. M.; Delwiche, M. J. Adaptation of a manometric biosensor to measure glucose and lactose. *Biosensors Bioelectronics* **2003**, *18*, 101–107.

(2) McMurry, J. *Fundamentals of Organic Chemistry*, 3rd ed.; Brooks/Cole Publishing: Pacific Grove, CA, 1994.

(3) Herrington, B. L. Some physico-chemical properties of lactose: IV. The influence of salts and acids upon the mutarotation velocity of lactose. *J. Dairy Sci.* **1934**, *17*, 659–670.

(4) Haase, G.; Nickerson, T. A. Kinetic reactions of α and β lactose. I) Mutarotation. *J. Dairy Sci.* **1966**, *49*, 127–132.

(5) Patel, K. N.; Nickerson, T. A. Influence of sucrose on the mutarotation velocity of lactose. *J. Dairy Sci.* **1970**, *53*, 1654–1658.

(6) Walstra, P.; Jenness, R. *Dairy Chemistry and Physics*; Wiley: New York, 1984.

(7) *Sigma-Aldrich Biochemicals, Reagents, & Kits for Life Science Research*; Sigma-Aldrich: St. Louis, MO, 2006−2007.

(8) Clark, L. C.; Lyons, C. Electrode systems for continuous monitoring in cardiovascular surgery. *Ann. N.Y. Acad. Sci.* **1962**, *102*, 29–45.

(9) Shu, F. R.; Wilson, G. S. Rotating ring-disk enzyme electrode for surface catalysis studies. *Anal. Chem.* **1976**, *48*, 1679–1686.

(10) Cass, A. E. G.; Davis, G.; Francis, G. D.; Hill, H. A. O.; Aston, W. J.; Higgins, I. J.; Plotkin, E. V.; Scott, L. D. L.; Turner, A. P. F. Ferrocene-mediated enzyme electrode for amperometric determination of glucose. *Anal. Chem.* **1984**, *56*, 667–671.

(11) Bourdillon, C.; Bourgeois, J. P.; Thomas, D. Covalent linkage of glucose-oxidase on modified glassy-carbon electrodes—kinetic phenomena. *J. Am. Chem. Soc.* **1980**, *102*, 4231–4235.

(12) Dicks, J. M.; Aston, W. J.; Davis, G.; Turner, A. P. F. Mediated amperometric biosensors for D-galactose, glycolate and L-amino-acids based on a ferrocene-modified carbon paste electrode. *Anal. Chim. Acta* **1986**, *182*, 103–112.

(13) Ikeda, T.; Hamada, H.; Miki, K.; Senda, M. Glucose-oxidase immobilized benzoquinone carbon paste electrode as a glucose sensor. *Agric. Biol. Chem.* **1985**, *49*, 541–543.

(14) Ikeda, T.; Hamada, H.; Senda, M. Electrocatalytic oxidation of glucose at a glucose oxidase-immobilized benzoquinone mixed carbon paste electrode. *Agric. Biol. Chem.* **1986**, *50*, 883–890.

(15) Sadik, O. A.; Brenda, S.; Joasil, P.; Lord, J. Electropolymerized conducting polymers as glucose sensors—an undergraduate analytical chemistry laboratory experiment. *J. Chem. Educ.* **1999**, *76*, 967–970.

(16) Fiorito, P. A.; Córdoba de Torresi, S. I. Glucose amperometric biosensor based on the co-immobilization of glucose oxidase (GOx) and ferrocene in poly(pyrrole) generated from ethanol/water mixtures. *J. Braz. Chem. Soc.* **2001**, *12*, 729–733.

(17) Hammerle, M.; Schuhmann, W.; Schmidt, H. L. Amperometric polypyrrole enzyme electrodes—effect of permeability and enzyme location. *Sensors Actuators B: Chem.* **1992**, *6*, 106–112.

(18) Reyes De Corcuera, J. I.; Cavalieri, R. P.; Powers, J. R. Improved platinization conditions produce a 60-fold increase in sensitivity of amperometric biosensors using glucose oxidase immobilized in poly-o-phenylenediamine. *J. Electroanal. Chem.* **2005**, *575*, 229–241.

(19) Kim, C. S.; Oh, S. M. Enzyme sensors prepared by electrodeposition on platinized platinum electrodes. *Electrochim. Acta* **1996**, *41*, 2433–2439.

(20) Silva, A. M.; Silva, E. C. d.; Silva, C. O. d. A theoretical study of glucose mutarotation in aqueous solution. *Carbohydr. Res.* **2006**, *341*, 1029–1040.

(21) Tanaka, Y.; Kagamiishi, A.; Kiuchi, A.; Horiuchi, T. Purification and properties of β-galactosidase from *Aspergillus oryzae*. *J. Biochem.* **1975**, *77*, 241247.
(22) Holman, J. P. *Experimental Methods for Engineers*, 7th ed.;McGraw Hill: Boston, MA, 2001.
(23) Pigman, W.; Isbell, H. S. Mutarotation of sugars in solution: part I. *Adv. Carbohydr. Chem.* **1968**, *23*, 11–57.
(24) El Khadem, H. S. *Carbohydrate Chemistry: Monosaccharides and Their Oligomers*; Academic Press: San Diego, CA, 1988.
(25) Teruel, M. A.; Jenkins, D. M.; Reyes De Corcuera, J. I. Crystallization of β-D-glucose and analysis with a simple glucose biosensor. *J. Chem. Educ.* **2008**, under review.
(26) Ballash, N. M.; Robertson, E. B. The mutarotation of glucose in dimethylsulfoxide and water mixtures. *Can. J. Chem.* **1973**, *51*, 556–564.
(27) Gram, F.; Hveding, J. A.; Reine, A. The mutarotation of D-glucose and its dependence on solvent. I. Studies of reaction and equilibrium in the mutarotation catalyzed by water. *Acta Chem. Scand.* **1973**, *27*, 3616–3624.
(28) Holsinger, V. H. Lactose. In *Fundamentals of Dairy Chemistry*; Wong, N. P., Ed.; Von Nostrund Reinhold: New York, 1988; pp 279−342.

Received for review May 6, 2008. Revised manuscript received July 11, 2008. Accepted July 18, 2008. We gratefully acknowledge financial support from the USDA (TSTAR award HAW00559-04G and the Alaska Native/Native Hawaiian serving institutions education program).

JF801403N