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Note

Direct measurement of the rate of ring opening of D-glucose by enzyme-catalyzed reduction

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The mutarotation of simple sugars has been postulated to proceed *via* pseudoacyclic intermediates[1], rather than the acyclic, carbonyl form, in order to account for the low rate of exchange of ^{18}O for ^{16}O in the anomeric hydroxyl group, and to reconcile the high rates of mutarotation with the extremely low levels of the free carbonyl species detected by various techniques[2]. Indeed, detailed kinetic studies of the mutarotation and ring-opening reactions for sugars containing sulfur in the ring have confirmed the existence of such intermediates for thio sugar tautomerization, as base-catalyzed mutarotation proceeds at a rate that is 500-fold higher than that of ring opening, as measured by chemical trapping of the free thiol group[3]. Calculations for the oxygen-containing sugars, based on several assumptions and the kinetic model developed for the thio sugars, indicated that base-catalyzed mutarotation proceeds through the acyclic form some 94–97% of the time. However, at that time, suitable methods for testing this prediction did not exist.

Recently, confirmatory evidence has been presented, from measurements of ring-opening and closing rates by saturation-transfer, n.m.r.-spectral methods, to implicate the carbonyl form as the obligatory intermediate in the anomerization of simple sugars and sugar phosphates[4]. Advantage has now been taken of the enzymic reduction of D-glucose to D-glucitol, catalyzed by aldose reductase (EC 1.1.1.21), to measure the ring-opening rate for this aldopyranose directly by utilizing levels of the enzyme sufficient to insure that formation of the free carbonyl species is rate-limiting for the overall reaction.

For D-glucose, the reactions under consideration are shown in Eq. *1*.

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$$\alpha\text{-Pyranose} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{R-CHO} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \beta\text{-pyranose} \quad (1)$$

$$\downarrow \text{aldose reductase}$$

$$\text{R-CH}_2\text{OH}$$

The rate constant for ring opening (k_{op}) and for mutarotation (k_{mut}) derived from this model are given by Eqs. *2* and *3*, respectively.

$$k_{op} = \frac{k_1[\alpha\text{-pyranose}] + k_3[\beta\text{-pyranose}]}{[\alpha\text{-pyranose}] + [\beta\text{-pyranose}]} \quad (2)$$

$$k_{mut} = \frac{k_1k_4 + k_2k_3}{k_2 + k_4} \quad (3)$$

At sufficiently high levels of aldose reductase, the conversion of either pyranose into the acyclic form (R-CHO) becomes an irreversible step, and hence, k_2 and k_4 do not appear in Eq. *2*. The mutarotation rate constant is the sum of each ring-opening rate constant multiplied by the partitioning ratio for subsequent reaction of the acyclic intermediate. By determining k_{op} for any two combinations of α- and β-pyranose (*e.g.*, pure $\alpha(+)$ anomer and the equilibrium, α/β mixture), Eq. *2* can be used to solve for the values of k_1 and k_3. Because the overall equilibrium-constant[5] [$K_{eq} = (k_1k_4/k_2k_3) = 1.74$] and the proportion of acyclic, aldehyde form[5,6] {[R-CHO]/([α-pyranose] + [β-pyranose]) = 0.000026} are known, values for k_2 and k_4 can also be calculated.

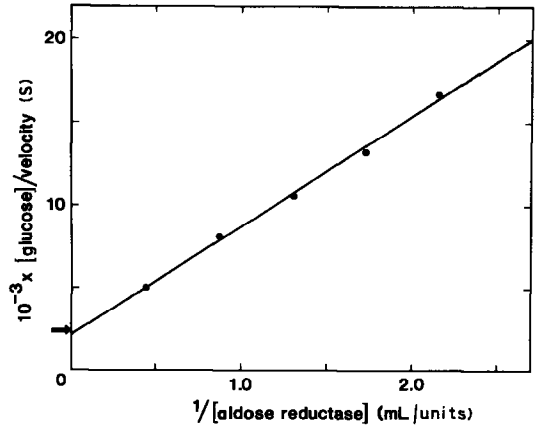

Fig. 1. Enzyme saturation experiment for determination of the ring-opening rate for D-glucose [95% $\alpha(+)$ anomer, 5% $\beta(+)$ anomer]. The arrow indicates the value for $(k_{mut})^{-1}$ measured under identical conditions.

Fig. 1 shows the results of an enzyme-saturation experiment in which [D-glucose]/v_i is plotted *vs.* 1/[aldose reductase] at a constant level of 2.5mM D-glucose [freshly dissolved α(+)-pyranose containing 5% of β(+) anomer]. The extrapolated value of v_i/[D-glucose] at saturating aldose reductase was 0.028 ±0.002 min^{-1} at pH 7.0 and 25.0°. A similar experiment conducted with the equilibrated, anomeric mixture showed k_{op} = 0.025 ±0.003 min^{-1}. Substituting these values into Eq. *2*, and solving yields, k_1 = 0.028 ±0.003 min^{-1}; k_2 = 400 ±40 min^{-1}; k_3 = 0.023 ±0.003 min^{-1}; and k_4 = 570 ±60 min^{-1}. Furthermore, using these k_1–k_4 values in Eq. *3* gives a calculated value for k_{mut} = 0.026 min^{-1}, which is identical to the reported value[7] (0.026 min^{-1}) and the value of 0.026 ±0.002 min^{-1} measured in this study under conditions identical to those employed in the enzyme-saturation experiments.

In order to validate the method, and to confirm that the enzyme was acting only as a trapping reagent and was not also catalyzing the ring-opening reaction, a similar experiment was conducted using 5-thio-D-glucose, for which the ring-opening rate is known[3]. The extrapolated value for v_i/[5-thio-D-glucose] at saturating aldose reductase was 5.1 ±0.6 × 10^{-4} min^{-1} and 6.4 ±0.6 × 10^{-4} min^{-1} for data obtained at a fixed level of 5-thio-D-glucose of 25mM and 50mM (equilibrated anomeric mixture), respectively. These values agree quite well with the value of 6.6 × 10^{-4} min^{-1} calculated from the chemical-trapping experiments[3], and demonstrate that the observed rate at saturating enzyme is proportional to the concentration of 5-thio-D-glucose, as expected if the first-order, ring-opening process is rate-limiting for the overall reduction-reaction.

The results reported here for the direct measurement of the ring-opening rate of D-glucose and 5-thio-D-glucose confirm the prediction that base-catalyzed mutarotation of simple sugars proceeds primarily through the acyclic, carbonyl intermediate. This conclusion is consistent with recent results obtained by using n.m.r.-spectral techniques[4], and points up the contrast between the situation for normal, oxygen-containing sugars and thio sugars, where there is good evidence in the latter case for the involvement in the mutarotation process of a non-covalently bonded intermediate in which the thiolate anion is in intimate contact with the carbonyl carbon atom[3]. For the oxygen-containing sugars (*e.g.*, D-glucose), it is simply the lower stability of this non-covalently bonded intermediate with regard to spontaneous opening or protonation to the free carbonyl form, relative to the thio sugar case, which results in a common pathway for ring opening and mutarotation. Quantitative analysis of the k_1–k_4 values for D-glucose indicates that the difference in ring-closing rate constants (k_4/k_2 = 1.43 ±0.23) is more important than ring opening (k_1/k_3 = 1.22 ±0.20) in determining the overall K_{eq} value. A similar conclusion had been deduced from a detailed, kinetic analysis of α/β-pyranose interconversion for D-galactose[8]. It further appears that ring closing is the major factor in determining the relative levels of acyclic intermediate for D-glucose and D-galactose. Thus, both k_1 and k_3 are within a factor of 1.5 for the two sugars, whereas k_2 and k_4 are 5.7- to 8-fold higher for D-glucose, a factor roughly equal to the observed[5,6] 8- to 10-fold difference in content of free aldehyde species.

EXPERIMENTAL

5-Thio-D-glucose, α(+)-D-glucose, and all other chemical compounds were obtained from Sigma. Bovine-kidney aldose reductase was prepared as previously reported[9]. Enzymic assays were conducted in 1.0-cm quartz cuvets containing 50mM MOPS [3-(4-morpholinyl)propanesulfonic acid] buffer (pH 7.0), 0.1mM EDTA, 0.16mM NADPH, and the indicated concentration of D-glucose or 5-thio-D-glucose in a total volume of 0.4 and 1.0 mL, respectively. The cell compartment was thermostatted at 25.0 ±0.1°. Sugar substrate was added to start the reaction, and the disappearance of NADPH at 340 nm (ε 340 nm = 6,220 $M^{-1}cm^{-1}$), monitored by using a Beckman DU monochromator, a Gilford model 262 optical-absorbance converter, and a 50-mV recorder, was used to calculate the initial velocity. Blanks containing no sugar substrate were run in all cases, and used to correct the initial velocities observed. Mutarotations were measured for a solution (40 mg/mL) in 50mM MOPS, 0.1mM EDTA buffer (pH 7.0), with a Perkin–Elmer model 141 spectropolarimeter. Reactions were conducted in a 1.0-dm, water-jacketed cell at 25°. Sugar stock-solutions were calibrated by enzymic coupling for the conversion of hexose + ATP into hexose 6-phosphate + ADP, to NADH disappearance using hexokinase, pyruvate kinase, and lactate dehydrogenase[10]. Ring-opening rates were calculated from least-squares fits to the equation $v_{obs} = VA/(K + A)$, where $v_{obs} = v_i/[\text{sugar}]$, v_i is the initial velocity, A is enzyme activity, and V is the ring-opening rate at infinite enzyme activity. K is the enzyme activity at which half the maximal rate is observed, and has no meaning for these experiments. The mutarotation rate for D-glucose was calculated from fits to the equation $(\alpha_0 - \alpha_t) = (\alpha_0 - \alpha_\infty)(1 - e^{-k_{mut}t})$, where α_0, α_t, and α_∞ are the optical rotations observed at zero time, at time t, and at equilibrium.

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