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Uridine diphosphoglucose 4-epimerase: Kinetic isotope effect*

The epimerization of UDP-[4-^{3}H]Gal by the enzyme UDP-Glc 4-epimerase (E.C. 5.1.3.2) isolated from lactose-grown *Saccharomyces fragilis* has been reported to proceed at 1.5–3 times the rate of the unlabeled compound[1]. This observation permitted certain conclusions to be drawn concerning the mechanism of this enzymatic catalysis. The studies reported here establish that the kinetic isotope effect associated with both the *S. fragilis* and *Escherichia coli* epimerases is in the region of 0.5 and the previously reported reverse isotope effect for the *S. fragilis* epimerase is in error.

E. coli epimerase was purified through step VII of the procedure of WILSON AND HOGNESS[2] from *E. coli* strain B78A, a galactose operon constitutive mutant, and had a specific activity of 160 I.U./mg protein when assayed according to the one-step assay of IMAE *et al.*[3] The *S. fragilis* epimerase preparation used was capable of converting 2.1 μmoles of UDP-Gal to UDP-Glc per min per mg protein under the conditions described below. UDP-Glc dehydrogenase was purified from beef liver through step VI of the method of STRAW[4] and had a specific activity of 0.45 I.U./mg protein.

Two methods were employed for obtaining the data used for the estimation of the kinetic isotope effect.

Method A involved incubating UDP-[4-^{3}H]Gal with the epimerase in 0.1 M glycine buffer (pH 8.8) at 25°. At various appropriate times, samples of a size suitable to allow later manipulations were pipetted into a test tube and placed in a boiling-water bath for 60 sec. After cooling, an aliquot was removed and assayed for UDP-Glc content. The assay cuvette contained, in addition to the enzyme aliquot, 20 μmoles glycine buffer (pH 8.8) and about 32 mI.U. of UDP-Glc dehydrogenase in a total volume of 0.19 ml. At 0 time, 5 μl of 20 mM NAD were added and the absorbance at 340 nm was monitored in a Beckman Model DU spectrophotometer until the reaction reached completion. The contents of the microcuvette were then quantitatively transferred to Whatman No. 1 chromatography paper and developed in abs. ethanol–1 M ammonium acetate (75:25, v/v) pH 7.5, (ref. 5) for 24 h together with UDP-Glc and UDP-GlcUA standards. The area corresponding to UDP-GlcUA was cut out and quantitatively eluted into a scintillation vial. After drying in an oven, the residual solid was dissolved in 0.2 ml of water and 10 ml of scintillation solution (10 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(5-phenyloxazolyl)-benzene in 1.5 liters of toluene and 1.0 liter of abs. ethanol) and the vials were counted in a Nuclear-Chicago Model 720 series liquid scintillation counter. It is evident that the 340-nm absorbance gives a measurement of the extent of reaction of unlabeled molecules, while the count in the UDP-GlcUA is a measure of the extent of reaction of those molecules having tritium at the 4-carbon of the hexose.

Method B was that of BEVILL *et al.*[1].

Fig. 1 and 2 represent data obtained by method A plotted according to the method described by BEVILL *et al.*[1]. A plot of log [1-(G/Geq)·(A/Aeq)] *versus* log (1-G/Geq) (symbols described in the legend to Fig. 1) is expected to produce a straight line passing through the origin whose slope is equal to the kinetic isotope effect

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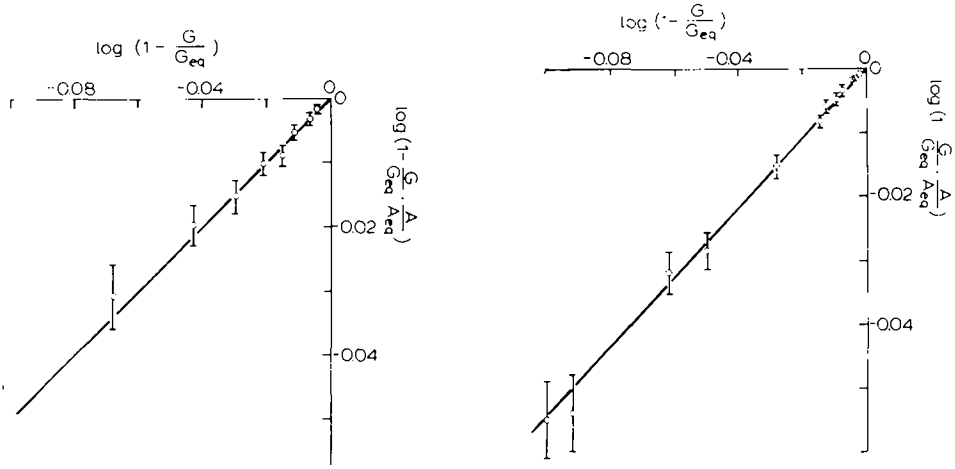

Fig. 1. Kinetic isotope effect for *E. coli* epimerase. The reaction mixture was 0.252 mM in UDP-[4-^{3}H]Gal (0.191 mC/mmole measured as UDP-GlcUA) with a total volume of 1.32 ml. At 0 time 0.24 units of epimerase were added, and samples were removed between 0.5 and 13 min which correspond to 9.4 to 67% attainment of equilibrium. G is the concentration of UDP-Glc at the time of assay, Geq is the concentration of UDP-Glc at equilibrium, A and Aeq are the corresponding values of the specific activities of UDP-GlcUA. The quantity of UDP-GlcUA produced in the individual experiments ranged from 3–24 nmoles.

Fig. 2. Kinetic isotope effect for *S. fragilis* epimerase. Two reaction mixtures were run, one yielding points in the 4.7–28% of equilibrium range, the other those in the 48–91% of equilibrium range. Both reactions were 0.36 mM in UDP-[4-^{3}H]Gal (0.10 mC/mmole measured as UDP-GlcUA. Symbols and other conditions are as described for the *E. coli* epimerase determinations in Fig. 1.

(k^3H/k_H). Each value of G/Geq was calculated from the corresponding 340-nm absorbance and the 340-nm absorbance characteristic of the reaction at equilibrium. Similarly, each value of A/Aeq was calculated from the corresponding specific radioactivity of UDP-GlcUA and the specific radioactivity of UDP-GlcUA characteristic of the reaction at equilibrium.

It was found that 0 time reaction mixtures contained no detectable UDP-Glc, but did have a small amount of radioactivity chromatographing in the UDP-GlcUA area. This was determined to be directly proportional to the total radioactivity spotted on the chromatogram (4.0 ± 0.8 (S.E.) %) and a correction was made for each experimental point. However, this correction was significant for only the earlier points in the reaction. Averaging the individual determinations in Fig. 1 and 2, the kinetic isotope effect (k^3H/k_H) was found to be 0.54 ± 0.08 for the *S. fragilis* epimerase and 0.50 ± 0.1 (95% confidence intervals[6]) for the *E. coli* enzyme. The kinetic isotope effect determined using method B on the *E. coli* epimerase indicated a value between 0.3 and 0.6 but much more scattering of the experimental points was observed.

It is the opinion of the authors that the value previously reported[1] for the kinetic isotope effect associated with the *S. fragilis* enzyme is in error. Method A, which was used to obtain the new values, would be expected to yield more precise data than Method B, which was used to obtain the previously reported value. Each point in Figs. 1 and 2 is established by a determination of ^{3}H radioactivity and a chemical determination of UDP-Glc concentration in the same sample. Method B

involves a simultaneous count of both ^{3}H and ^{14}C in the same sample and these data are manipulated in such a way in achieving the plot that the original error is multiplied. Indeed Method B, in our hands, gives a much greater scatter of points than Method A, although the isotope effect obtained by these data is in the same region as that given by Method A. We have found that very low count rates, such as those reported in the previous work, sometimes give anomalous results when Method B is used. It is our feeling, then, that the large error in the original value reported was due to errors resulting from the use of low count rates coupled with the fact that Method B resulted in the multiplication of these errors.

The value of approximately 0.5 of the tritium isotope effect of both the *E. coli* and *S. fragilis* epimerase reactions is consistent with its being a primary isotope effect[7] where the hydrogen atom at the 4 position of the hexose participates directly in the reaction mechanism. This, in turn, is in accord with a mechanism involving removal of the 4-hydrogen with formation of a carbonyl at the 4 position of the hexose followed by reduction of the carbonyl to form either of the two possible hexoses[9]. An isotope effect of similar magnitude and direction has been observed in at least one NAD-linked dehydrogenase reaction[8].

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