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The Concerted Inactivation of *Escherichia coli* Uridine Diphosphate Galactose 4-Epimerase by Sugar Nucleotide together with a Free Sugar

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1. The combined effect of the sugar nucleotides UDP-D-fucose or UDP-D-glucuronic acid together with the free sugars D-fucose or L-arabinose is the inactivation of the *Escherichia coli* enzyme UDP-galactose 4-epimerase (EC 5.1.3.2). The sugar nucleotide or the free sugar alone or the sugar nucleotide plus 5'-UMP do not inactivate the enzyme. 2. The inactivation of the enzyme by its substrate UDP-D-glucose was not affected by the presence of free sugar. 3. In all cases the inactivation observed follows pseudo-first-order kinetics. 4. A comparison of various sugar nucleotides indicates that the hydroxymethyl group at position 6 of the sugar moiety of the natural substrates is important for substrate binding.

The enzyme UDP-galactose 4-epimerase (EC 5.1.3.2) from both yeast (Bertland *et al.*, 1966) and *Escherichia coli* (Kalckar *et al.*, 1970) is reductively inactivated in the presence of 5'-UMP and certain free sugars such as D-glucose, D-galactose, D-fucose and L-arabinose. The most effective sugar in the concerted reductive inactivation is L-arabinose.

Reductive inactivation has been shown to result from a transfer of a hydride ion from either C-1 or C-4 of D-galactose (Ketley & Schellenberg, 1973) and from C-1 of D-glucose (Seyama & Kalckar, 1972; Ketley & Schellenberg, 1973) to the tightly bound NAD^+ moiety of the epimerase.

The natural substrates, UDP-galactose and UDP-glucose, also bring about the reductive inactivation of the *E. coli* UDP-galactose 4-epimerase (Kalckar *et al.*, 1970).

We report here the inactivation of the *E. coli* UDP-galactose 4-epimerase by sugar nucleotides and free sugars, in a concerted process that resembles the inactivation of the enzyme by 5'-UMP and free sugar. The inactivation reported here follows pseudo-first-order kinetics.

Experimental

Materials

5'-UMP, UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid and NAD^+, as their sodium salts, and UDP-glucose dehydrogenase were all purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

UDP-D-fucose, prepared by the method of Nordin *et al.* (1965), was a gift from Dr. Malcolm Spencer, Department of Chemistry, University of Sheffield, Sheffield, U.K.

All other reagents were of analytical grade and purchased from BDH, Poole, Dorset, U.K., or Fisons Fine Chemicals, Loughborough, Leics., U.K.

Methods

UDP-galactose 4-epimerase was prepared from the constitutive strain *E. coli* K12 HfrH 0°81-2 (Buttin, 1963), and purified by the method of Wilson & Hogness (1964). The enzyme was assayed by the coupled method described by Wilson & Hogness (1964). Changes in E_{340} were followed with a Gilford model 222 recording spectrophotometer.

Incubations of the enzyme with the various substrate analogues were carried out at 27°C in plastic-capped glass vials of nominal capacity 1.5ml. The incubations of the enzyme with UDP-D-fucose in the presence of D-fucose, L-arabinose or 5'-UMP were carried out in 10mM-potassium phosphate/1.0mM-EDTA, pH7.0. All other incubations of the enzyme with substrate or analogues were carried out in 10mM-Tris/1.0mM-EDTA, adjusted to pH7.85 with HCl. The enzyme was first preincubated to temperature in the incubation vial. The inactivation of the enzyme was started by the addition of substrate or substrate analogues. A 20μl sample was removed from the incubation vial at zero time and at subsequent intervals, diluted into 10mM-phosphate/1.0mM-EDTA, pH7.0, at 0°C and then assayed for activity. Control vials were incubated under identical conditions, but without one or both of the ligands, and similarly assayed for activity.

Fluorescence was monitored with an Aminco–Bowman spectrophotofluorimeter. The cell compartment was maintained at a constant temperature of

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27°C by circulating water. Excitation was at 340nm and emission was measured at 438nm and recorded on a Servoscribe recorder. The instrument was calibrated with standard quinine sulphate solution (2.21 μg/ml) in 0.05M-H_2SO_4, as follows. Excitation and emission slits (1mm) were inserted and the meter multiplier was set to 0.03. The fluorescence reading was then adjusted to 24 units with the sensitivity control. Before use the slits were replaced with a 5mm excitation slit and a 3mm emission slit.

The incubation of enzyme with UDP-D-fucose (0.4mM) and D-fucose (20mM) was carried out in 10mM-Tris/HCl/1.0mM-EDTA buffer, pH7.85.

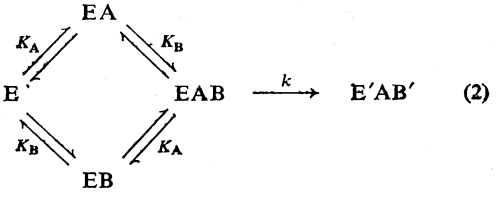
Results

The inactivation of UDP-galactose 4-epimerase by various concentrations of UDP-D-fucose and D-fucose is shown in the semi-log plots of Figs. 1 and 2. Controls with neither UDP-D-fucose nor D-fucose or with only one of the two present gave the same low rate of inactivation, owing to the inherent instability of the enzyme in dilute solutions. The inactivation follows pseudo-first-order kinetics. The apparent first-order rate constants derived from the slopes of Figs. 1 and 2 were replotted as their reciprocals, against the reciprocal of the ligand concentration at fixed concentration of the second ligand (Fig. 3). The linearity of these plots and the fact that they do not pass through the origin indicates that inactivation follows 'saturation' kinetics. We may describe the process by the approximation:

$$\text{Active species} \xrightarrow{k'} \text{inactive species} \qquad (1)$$

where k' is the apparent first-order rate constant for the process. A plot of $\log(\text{activity})_t$ against time gives a straight line of slope $-k'/2.303$.

We assume that the free sugar on the one hand and the nucleotide or sugar nucleotide on the other bind to the enzyme by a rapid random equilibrium process, where the rate-limiting step for inactivation is the hydride-transfer process or some other process occurring after formation of the ternary complex:



(2)

where ligand A is a nucleotide or sugar nucleotide and ligand B is a free sugar, and K_A and K_B are the dissociation constants for the ligands A and B respectively.

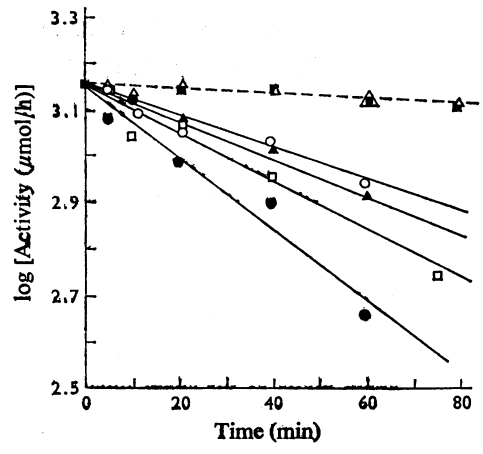


Fig. 1. *Effect of various concentrations of UDP-D-fucose and 20mM-D-fucose on the activity of UDP-galactose 4-epimerase*

Semi-log plots of activity remaining after various times of incubation at 27°C, pH7.0, in 10mM-phosphate buffer containing 1mM-EDTA. Control with neither UDP-D-fucose nor D-fucose, △. Concentrations of UDP-D-fucose: ■, 0.0mM; ○, 0.4mM; ▲, 0.8mM; □, 1.6mM; ●, 3.2mM. After subtracting the control slope, the slope of each line was used to calculate an apparent first-order rate constant k', as described in the text.

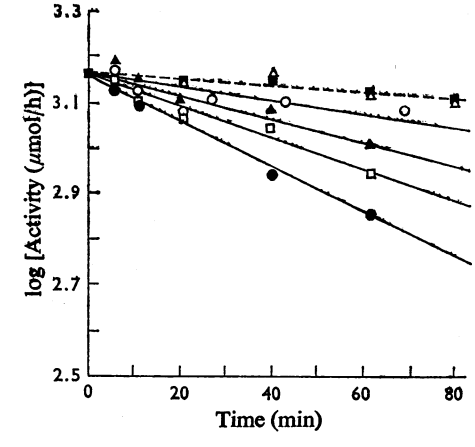


Fig. 2. *Effect of various concentrations of D-fucose and 0.4mM-UDP-D-fucose on the activity of UDP-galactose 4-epimerase*

Semi-log plots of activity remaining after various times of incubation at 27°C, pH7.0, in 10mM-phosphate buffer containing 1mM-EDTA. Control with neither UDP-D-fucose nor D-fucose, △. Concentrations of D-fucose: ■, 0mM; ○, 5mM; ▲, 10mM; □, 20mM; ●, 35mM. Apparent first-order rate constants were calculated as described in the legend of Fig. 1.

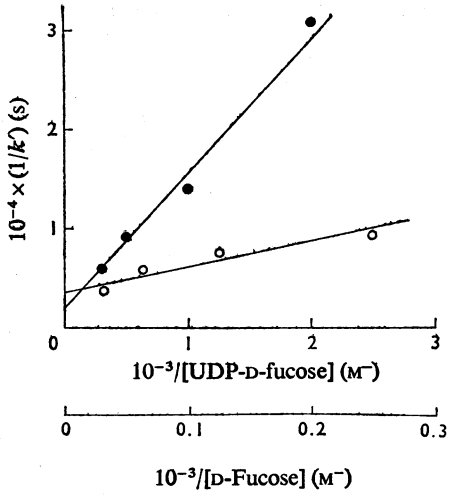


Fig. 3. *Double-reciprocal plot of apparent first-order rate constants (k') versus concentration of UDP-D-fucose* (○) *at 20mM-D-fucose, and versus concentration of D-fucose* (●) *at 0.4mM-UDP-D-fucose, for the inactivation of UDP-galactose 4-epimerase*

The apparent first-order rate constants were calculated from the slopes of the plots shown in Figs. 1 and 2.

The rate of inactivation is thus given by $v = k[\text{EAB}]$ and also, from eqn. (1), by $v = k'(\text{activity})$. Therefore:

$$k'(\text{activity}) = k[\text{EAB}]$$

and

$$k' = \frac{k[\text{EAB}]}{[\text{E}]+[\text{EA}]+[\text{EB}]+[\text{EAB}]}$$

since all the denominator terms represent active forms of the enzyme. By inverting:

$$\frac{1}{k'} = \frac{1}{k}\left(1+\frac{K_B}{[B]}\right)+\frac{K_A}{k[A]}\left(1+\frac{K_B}{[B]}\right) \qquad (3)$$

A plot of $1/k'$ against $1/[A]$ at constant [B] gives a straight line of slope $\frac{K_A}{k}\left(1+\frac{K_B}{[B]}\right)$ and intercept on the ordinate of $\frac{1}{k}\left(1+\frac{K_B}{[B]}\right)$.

Dividing the term for the slope by that of the intercept gives K_A, the dissociation constant for ligand A. Similarly, K_B may be obtained by repeating the experiment at constant concentration of A. By substituting the values for the dissociation constants K_A and K_B into the terms for the slope and intercept derived above (eqn. 3), an estimate of the first-order rate constant k for the reductive inactivation may be obtained.

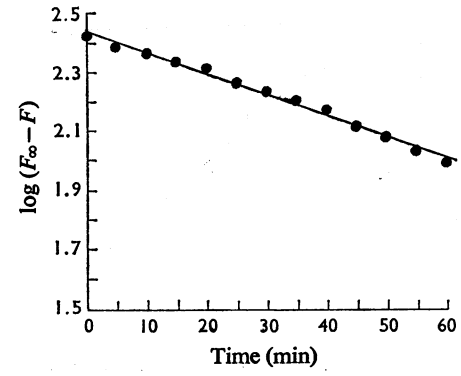


Fig. 4. *Effect of UDP-D-fucose and D-fucose on the 438nm fluorescence of UDP-galactose 4-epimerase*

The semi-log plot of the effect of 0.4mM-UDP-D-fucose and 20mM-D-fucose on the 438nm fluorescence of epimerase incubated at 27°C in 10mM-Tris/HCl/1.0mM-EDTA, pH7.85 (exciting wavelength 340nm). F is fluorescence emission.

From the slopes and intercepts of Fig. 3, estimates of the dissociation constants for UDP-D-fucose and D-fucose were obtained and found to be 0.76×10^{-3}M and 6.43×10^{-2}M respectively. The first-order rate constant for the process was determined by substituting these values for K_A and K_B into the terms for the slope and intercept derived from eqn. (3) and found to be $1.28\times10^{-3}s^{-1}$ from the plot at constant D-fucose concentration and $1.45\times10^{-3}s^{-1}$ from the other plot.

The inactivation of the enzyme brought about by the concerted action of UDP-D-fucose and D-fucose is accompanied by the reduction of the protein-bound NAD^+ moiety. Fig. 4 shows the increase in fluorescence emission at 438nm of the reduced NAD^+ moiety when excited at 340nm. The increase in fluorescence follows pseudo-first-order kinetics, as does the inactivation of the enzyme by the same substrate analogues, and, when due allowance is made for the difference in pH, the apparent first-order rate constants for the two processes are identical. Hence the concerted effect of UDP-D-fucose and D-fucose on the activity of the epimerase is to bring about a reductive inactivation of the enzyme, similar to that caused by 5'-UMP and free D-fucose (Kalckar *et al.*, 1970).

The combined effect of UDP-D-fucose and L-arabinose on the activity of the UDP-galactose 4-epimerase was briefly investigated. The inactivation, under conditions identical with those used for investigating the effect of UDP-D-fucose and D-fucose, was very rapid; 0.4mM-UDP-D-fucose and 10mM-L-arabinose, incubated with 1500 units (μmol/h) of the enzyme, produced more than 90%

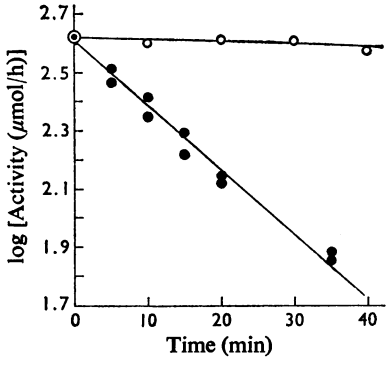


Fig. 5. *Effect of* 0.5 mM-*UDP-*D-*glucuronic acid and* 25 mM-L-*arabinose on the activity of UDP-galactose* 4-*epimerase*

Semi-log plots of activity remaining after various times of incubation at 27°C in 10mM-Tris/1mM-EDTA, adjusted to pH7.85 with HCl. ○, Control without arabinose; ●, with arabinose.

inactivation within the first few minutes of incubation. The apparent first-order rate constant was higher than $8.3\times10^{-3}s^{-1}$.

The effect of UDP-D-fucose with and without 5'-UMP on the activity of the epimerase was also investigated. Concentrations of UDP-D-fucose up to 3.2mM, in the presence or absence of 1.0mM-5'-UMP, in 0.01M-phosphate/1mM-EDTA, pH7.0, 27°C, for up to 2h, had no effect on the activity of the enzyme.

The inactivation of the epimerase by 0.5mM-UDP-D-glucose, a substrate for the enzyme, was investigated in the presence and absence of 2.5mM-L-arabinose. Pseudo-first-order kinetics were again observed. The apparent first-order rate constants in the presence and absence of L-arabinose were $1.1\times10^{-4}s^{-1}$ and $1.3\times10^{-4}s^{-1}$ respectively, the difference being within experimental error. The apparent inhibition by L-arabinose of the inactivation brought about by UDP-D-glucose was not considered significant, and it was concluded that L-arabinose has no effect on the reductive inactivation by the substrate.

The effect of UDP-D-glucuronic acid on the activity of the epimerase is shown in Fig. 5. By itself, 0.5mM-UDP-D-glucuronic acid, like UDP-D-fucose, had no effect on the activity of the enzyme. However, in the presence of 25mM-L-arabinose the enzyme was inactivated, the process once again being pseudo-first-order with an apparent first-order rate constant of $7.9\times10^{-4}s^{-1}$.

Discussion

UDP-D-fucose was shown by Spencer *et al.* (1973) to be a competitive inhibitor of *E. coli* UDP-galactose 4-epimerase, having a K_i of 1.3×10^{-3}M. They also showed that UDP-D-fucose was a poor substrate for the enzyme, extending previous observations by Druzhinina *et al.* (1969), who found only 4% epimerization of UDP-D-quinovose to UDP-D-fucose by the enzymes from calf liver and *Phaseolus aureus*. Spencer *et al.* (1973) carried out preliminary studies, which suggested that the reason for the low extent of epimerization of UDP-D-fucose by the *E. coli* enzyme might be due to inactivation of the enzyme by UDP-D-fucose. Those preliminary studies (though not the others) were carried out with a sample of UDP-D-fucose which was estimated to be more than 96% pure, but which had not been treated with charcoal, the last step in purification. We now find that pure UDP-D-fucose alone does not inactivate the enzyme, nor does the addition of 5'-UMP together with UDP-D-fucose. On the other hand, UDP-D-fucose together with D-fucose or L-arabinose brings about rapid inactivation. Similarly, UDP-D-glucuronic acid alone will not inactivate the enzyme, except in the presence of L-arabinose.

We conclude that the nucleotide moiety of UDP-D-fucose or UDP-D-glucuronic acid is able to bind to the enzyme in a manner that mimics 5'-UMP, but that the sugar moiety of these sugar nucleotides is either weakly bound or not bound at all, so that free sugar (D-fucose or L-arabinose) molecules are able to bind near to the NAD prosthetic group of the enzyme and bring about reductive inactivation. The fact that L-arabinose brings about no increase in the rate of reductive inactivation caused by UDP-D-glucose leads us to suppose that in this case the sugar moiety is tightly bound, as well as the nucleotide moiety, and cannot be displaced by L-arabinose. Indeed, the K_m values for UDP-D-glucose (2.2×10^{-4}M at pH8.5; Wilson & Hogness, 1964) and UDP-D-galactose (1.6×10^{-4}M; Wilson & Hogness, 1964; 1.3×10^{-4}M; Spencer *et al.*, 1973, both at pH8.5) are about an order of magnitude lower than the K_i for UDP-D-fucose (1.3×10^{-3}M at pH8.5; Spencer *et al.*, 1973) or its K_s, as found in the present work (0.76×10^{-3}M at pH7.0). The slight difference between the last two values may be due to the different pH values at which they were determined. It seems reasonable to suppose that the hydroxyl group at C-6 in the hexose ring is largely responsible for the ability of the hexose moiety to bind, since this is the only difference between UDP-D-galactose, which binds tightly, and UDP-D-fucose, which binds much less tightly. That some binding of the fucose moiety of UDP-D-fucose does occur is indicated by the small extent of epimerization found by both Spencer *et al.* (1973) and Druzhinina *et al.* (1969). These conclusions are at variance with those of Nelsestuen & Kirkwood (1971), who found that the hydroxyl group on C-6 of the hexose was unimportant for its interaction with the enzyme from *E. coli* B. Our own conclusions, however, agree with those of Druzhinina *et al.* (1969), who

reported that hydroxyl groups on C-3 and C-6 were important for the correct binding of substrate to the enzymes from calf liver and *P. aureus*. It may be significant in this respect that UDP-D-glucuronic acid exhibited no activity as a substrate for the enzyme from yeast (Salo *et al.*, 1968), suggesting that its hexose moiety is bound even less tightly than that of UDP-D-fucose. This is supported by the K_i values for UDP-D-glucuronic acid of 2.6×10^{-3}M and 2.2×10^{-3}M for the enzymes from calf liver and *P. aureus* (Druzhinina *et al.*, 1969), which were even higher than those found for UDP-D-fucose.

Because of the absence of the hydroxymethyl group at C-6 of the hexose moiety of the substrate, and its replacement by either a methyl group or a carboxyl group, free sugar molecules are able to enter the active site of the enzyme and bring about reductive inactivation of the enzyme. This is presumably due to the hexose moiety of the sugar nucleotide swinging free from the active site of the enzyme by rotation about the phosphodiester bridge while leaving the nucleotide portion still bound. The stereospecific requirements for epimerization on the one hand and reductive inactivation on the other are obviously quite different, although both require a nucleotide to be bound to the enzyme.

The finding that the nucleotide portion of a sugar nucleotide can replace 5'-UMP in the reductive inactivation of the enzyme sheds new light on the mechanism of action of this enzyme, and, in particular, highlights the need for caution in experiments with various nucleotide sugars, since, if they are contaminated by even low concentrations of free sugars, reductive inactivation could ensue.

Since this work was completed, Kang *et al.* (1975) have published their study of the reductive inactivation of the enzyme by 5'-UMP and D-glucose, from which they conclude that the nucleotide and free sugar probably do bind to the enzyme by a rapid random-equilibrium process, as we have assumed. Other possible models that would also fit the kinetic data include an ordered sequential binding, but Kang *et al.* (1975) considered this to be highly unlikely from estimates of the rate constants for binding and release of the ligands. Kang *et al.* (1975) found that, although rapid random equilibrium occurs, the binding of UMP decreases the dissociation constant for glucose by a factor of about six, and vice versa. They considered it probable that ligand binding stabilizes a particular conformation of the enzyme. Their estimate for the dissociation constant of 5'-UMP from the E–UMP complex at pH8.5 and 27°C is $1.86\times10^{-3}\pm0.72\times10^{-3}$M, in fair agreement with our estimate of 0.76×10^{-3}M for UDP-D-fucose at pH7.0 and 27°C. However, their value for the dissociation constant of the E–D-glucose complex is 3.12 ± 1.85M, nearly fiftyfold higher than our value for the dissociation constant of E–D-fucose, whereas their estimate of k, the first-order rate constant for inactivation of the ternary complex, was $0.105\pm0.013\,s^{-1}$, more than eightyfold higher than the corresponding value in our case. Although it may be true that interaction between the binding of UDP-D-fucose and D-fucose could exist in our case also, and might affect the absolute values of the constants as estimated in this work, the fact that the interaction was only modest (a factor of six) for 5'-UMP and D-glucose suggests that our values are unlikely to be more than one order of magnitude in error. Indeed the good agreement between their K_s value for 5'-UMP and ours for UDP-D-fucose suggests that any error would be much less than an order of magnitude. We therefore conclude that D-fucose binds more tightly to the enzyme than does D-glucose, but that the first-order rate constant for inactivation of the E–UDP-D-fucose and D-fucose ternary complex is much smaller than that for E–UMP-D-glucose.

Further study of the binding and inactivation phenomena reported here and by Kang *et al.* (1975) will be required to reveal the significance of these differences.

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