Kinetic Studies on the Effect of Uridine Diphosphate Galactose and Manganous Ions on the Reaction between Lactose Synthetase A Protein from Human Milk and *p*-Hydroxymercuribenzoate

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(Received 3 June 1974)

The inhibition of lactose synthetase A protein by *p*-hydroxymercuribenzoate at pH 7.5 and 25°C, which involves the reaction of one molecule of inhibitor with each molecule of enzyme, was decreased in rate by UDP-galactose, especially in the presence of Mn^{2+} . Pseudo-first-order rate constants for the reaction between 0.1 mM *p*-hydroxymercuribenzoate and free enzyme, the enzyme–UDP-galactose complex and the enzyme– Mn^{2+} –UDP-galactose complex were 4.4×10^{-2} , 1.9×10^{-2} and $0.3 \times 10^{-2} \text{ min}^{-1}$ respectively. The results also indicated that dissociation constants for UDP-galactose in the enzyme–UDP-galactose and enzyme– Mn^{2+} –UDP-galactose complexes were 313 and 16 μM respectively, the latter value being similar to the K_m for UDP-galactose in the lactose synthetase reaction. The protective effect of UDP-galactose and the role of Mn^{2+} ions in lactose synthetase are discussed.

Lactose synthetase A protein catalyses the transfer of a galactosyl unit from UDP-galactose to glucose to form lactose. *p*-Hydroxymercuribenzoate reacts with the human milk enzyme and inhibits lactose synthesis, but UDP-galactose has a protective effect, especially in the presence of Mn^{2+} ions (Kitchen & Andrews, 1974). The inhibitor probably reacts with a thiol group which is close to the UDP-galactose-binding site although not an essential part of the enzyme active site, and prevention of UDP-galactose binding is the most likely cause of the inhibition. We have now obtained more information about the reaction between enzyme and inhibitor and about the interaction between enzyme and UDP-galactose from the kinetics of the inactivation reaction with unprotected and protected enzyme.

Theory

Interpretation of the kinetic results

When the inactivation reaction between A protein and *p*-hydroxymercuribenzoate follows first-order kinetics, pseudo-first-order rate constants for the reaction in the absence and presence of UDP-galactose (U), k_1 and k' respectively, may be obtained from plots of $\log[E]_0/[E]_t$ against t , where $[E]_0$ and $[E]_t$ are concentrations of active A protein at zero time and time t respectively. Relationships developed by O'Sullivan & Cohn (1966) can then be applied to the effect of UDP-galactose concentration on inactivation rate to measure both k_2 , the pseudo-first-order rate constant for the reaction between inhibitor and the A protein–UDP-galactose (or A protein–

Mn^{2+} –UDP-galactose) complex, and K_d , the dissociation constant for UDP-galactose in the same complex. The rate constants are related by:

$$k' = k_1[E]/[E]_T + k_2[EU]/[E]_T \quad (1)$$

where $[E]_T$ is the total concentration of active enzyme. Since $[E]_T = [E] + [EU]$ and $K_d = [E][U]/[EU]$, eqn. (1) becomes:

$$k' = \frac{k_1 K_d}{K_d + [U]} + \frac{k_2 [U]}{K_d + [U]} \quad (2)$$

which may be transformed into the form suggested by O'Sullivan & Cohn (1966):

$$\frac{k'}{k_1} = \frac{K_d}{[U]} \left(1 - \frac{k'}{k_1} \right) + \frac{k_2}{k_1} \quad (3)$$

which is convenient to use when $k' < k_1$. A plot of k'/k_1 against $[1 - (k'/k_1)]/[U]$ therefore has intercepts of k_2/k_1 and $-(k_2/k_1)/K_d$ on the ordinate and abscissa respectively, from which k_2 and K_d can be calculated.

Materials and Methods

Materials

Lactose synthetase A protein, isolated from human milk as described by Andrews (1970), was the preparation we used in previous work (Kitchen & Andrews, 1974). When freshly prepared it had a specific activity in the spectrophotometric lactose synthetase assay (see below) of about 6.5 μmol of UDP formed/min

per mg of protein at 25°C. α -Lactalbumin was prepared from human milk as described by Barman (1970) for cows' milk. UDP-galactose was synthesized by the morpholidate procedure (Moffatt, 1966). *p*-Hydroxymercuribenzoate, crude pyruvate kinase (type I) and other chemicals used in the enzyme assay were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Enzyme assay

Lactose synthetase activity was determined by spectrophotometric measurement of the amount of UDP formed in the reaction from UDP-galactose (Brodbeck & Ebner, 1966) at 25°C. The standard assay mixture contained, in a total volume of 1 ml: Tris-HCl buffer, pH 7.5, 50 μ mol; $MnCl_2$, 4 μ mol; UDP-galactose, 0.4 μ mol; glucose, 80 μ mol; human α -lactalbumin, 150 μ g; phosphoenolpyruvate, 1 μ mol; NADH, 0.2 μ mol; ATP, 0.1 μ mol; crude pyruvate kinase, 0.5 mg; an appropriate amount of A protein.

Inhibition and protection experiments

Concentrations of enzyme (mol.wt. about 44000 from sedimentation-equilibrium analysis; P. Andrews, unpublished work), $MnCl_2$ (when present) and Tris-HCl buffer, pH 7.5, in the incubation mixtures were about 1 μ M (50 μ g/ml), 8 and 50 mM respectively. In protection experiments with UDP-galactose, *p*-hydroxymercuribenzoate dissolved in 0.25 M-Tris-HCl buffer, pH 7.5, was the last component added to the mixture. Incubation was at 25°C and enzyme

Fig. 1. Inactivation of lactose synthetase A protein by various concentrations of *p*-hydroxymercuribenzoate

The A protein (1 μ M) was incubated at pH 7.5 and 25°C with *p*-hydroxymercuribenzoate concentrations as follows: $\circ$, 1.0 mM; Δ , 0.6 mM; $\square$, 0.5 mM; $\bullet$, 0.4 mM; $\blacktriangle$, 0.3 mM; $\blacksquare$, 0.2 mM. 'Inhibitor-sensitive activity' is explained in the text.

Fig. 2. Determination of the order of the reaction between lactose synthetase A protein and *p*-hydroxymercuribenzoate with respect to the latter

Values for *p*-hydroxymercuribenzoate concentration and half-time of inactivation ($t_{1/2}$) were taken from Fig. 1 and plotted as indicated in the text.

activity was determined on 50 μ l samples. The amount of inhibitor present in the samples had no effect on the measurement of enzyme activity when added separately to assay mixtures.

Results and Discussion

Inhibition with *p*-hydroxymercuribenzoate

As described previously, lactose synthetase activity of the A protein was not completely inhibited by *p*-hydroxymercuribenzoate (Kitchen & Andrews, 1974). When the enzyme was incubated at 25°C with this reagent (1.0 mM), 84% of its activity was lost within 5 min, but thereafter the activity remained constant. In the following kinetic studies of the inhibition reaction, therefore, the activity resistant to inhibitor (16% of initial activity) was subtracted from measured activities to give the activity values used in the calculations (inhibitor-sensitive activity).

Fig. 1 shows that loss of at least 90% of the inhibitor-sensitive activity followed first-order kinetics in the presence of *p*-hydroxymercuribenzoate concentrations ranging from 200 to 1000 times the enzyme concentration. Measurements of the reaction between enzyme and 5,5'-dithiobis-(2-nitrobenzoate) indicated the presence of one thiol group on each inhibitor-sensitive enzyme molecule (Kitchen & Andrews, 1974) and, in agreement with this, the logarithmic plot (Fig. 2) of values obtained from Fig. 1 for $1/t_0$ against inhibitor concentration (Levy *et al.*, 1963) was linear with a slope of 1.07.

Fig. 3. Inactivation of lactose synthetase A protein by p -hydroxymercuribenzoate in the absence and presence of Mn^{2+} ions and UDP-galactose

The A protein (1 μ M) was incubated at pH 7.5 and 25°C with 0.1 mM- p -hydroxymercuribenzoate and either (a) UDP-galactose alone at the following concentrations: $\circ$, zero; Δ , 0.2 mM; $\square$, 0.5 mM; $\bullet$, 1.0 mM; $\blacktriangle$, 2.0 mM; or (b) 8 mM- $MnCl_2$ and UDP-galactose at the following concentrations: $\circ$, zero; Δ , 20 μ M; $\square$, 50 μ M; $\bullet$, 100 μ M; $\blacktriangle$, 500 μ M. The results are plotted in the form indicated in the text to obtain rate constants.

Protection with UDP-galactose

Loss of the inhibitor-sensitive A protein activity in 0.1 mM- p -hydroxymercuribenzoate, in the absence and presence of UDP-galactose, also followed first-order kinetics (Fig. 3), at least over the investigated range. Much longer incubations were avoided, since trial experiments showed that these usually resulted in small but variable losses of activity, probably owing to enzyme instability. UDP-galactose was clearly more effective in decreasing the inactivation rate in the presence (Fig. 3b) than in the absence (Fig. 3a) of Mn^{2+} ions. The UDP-galactose concentrations, ranging from 20 to 2000 times the enzyme concentration, were assumed to be high enough for the amount bound to enzyme to be negligible. The concentration of Mn^{2+} ions used in the experiments (8 mM) was chosen with regard to the dissociation constant of about 1 mM derived from kinetic studies for the interaction between Mn^{2+} ions and the cows'-milk enzyme (Morrison & Ebner, 1971) and the fact that increasing the Mn^{2+} concentration above that normally used in the lactose synthetase assay (4 mM; see above) had no appreciable effect on activity measurements.

The pseudo-first-order rate constants k_1 and k' obtained from Fig. 3 for inactivation of the unprotected and protected enzyme respectively are given in Table 1. Figs. 4(a) and 4(b) show information from Table 1 plotted in the form indicated by eqn. (3) to obtain rate constants (k_2) for inactivation of A protein in the enzyme-UDP-galactose and enzyme- Mn^{2+} -UDP-galactose complexes respectively. The values of k_2 so obtained are included in Table 1. Dissociation constants (K_d) for UDP-galactose in the enzyme-UDP-galactose and enzyme- Mn^{2+} -UDP-galactose complexes, calculated from the intercepts in Figs. 4(a) and 4(b), are 313 and 16 μ M respectively.

These values for K_d emphasize the role of Mn^{2+} ions in enhancing the binding of UDP-galactose to A protein, effecting as it does a 20-fold increase in affinity between the two. The value for K_d in the presence of Mn^{2+} ions is similar to the K_m of 20 μ M for UDP-

Table 1. Effect of UDP-galactose and Mn^{2+} on the rate of inactivation of lactose synthetase A protein by 0.1 mM- p -hydroxymercuribenzoate at pH 7.5 and 25°C

Pseudo-first-order rate constants k_1 and k' in the absence of Mn^{2+} were obtained from Fig. 3(a) and those in the presence of Mn^{2+} from Fig. 3(b); rate constants k_2 were obtained from Fig. 4(a) (Mn^{2+} absent) and Fig. 4(b) (Mn^{2+} present). The rate constants are defined in the text.

Mn ²⁺ absent				8 mM-MnCl ₂ added			
Concentration of UDP-galactose (mM)	10 ² × k_1 (min ⁻¹)	10 ² × k' (min ⁻¹)	10 ² × k_2 (min ⁻¹)	Concentration of UDP-galactose (mM)	10 ² × k_1 (min ⁻¹)	10 ² × k' (min ⁻¹)	10 ² × k_2 (min ⁻¹)
0	4.4	—	—	0	4.7	—	—
0.2	—	3.4	—	0.02	—	2.4	—
0.5	—	2.85	—	0.05	—	1.4	—
1.0	—	2.6	—	0.10	—	0.9	—
2.0	—	2.2	—	0.50	—	0.5	—
Saturating	—	—	1.9	Saturating	—	—	0.3

Fig. 4. Plots to determine rate constants for inactivation of lactose synthetase A protein by 0.1 mM-*p*-hydroxymercuribenzoate and dissociation constants for UDP-galactose in (a) the A protein-UDP-galactose complex and (b) the A protein- Mn^{2+} -UDP-galactose complex

Rate constants k_1 and k' for (a) and (b) were obtained from Figs. 3(a) and 3(b) respectively and are defined in the text. The values are plotted as indicated by eqn. (3) to obtain the required information from the intercepts.

galactose in the lactose synthetase reaction (Andrews, 1968). The results also show that UDP-galactose binds to the enzyme even in the absence of Mn^{2+} ions, as suggested by the detection of complex-formation between the cows'-milk enzyme and α -lactalbumin in the presence of UDP-galactose alone (Mawal *et al.*, 1971; Klee & Klee, 1972).

Although the enzyme catalyses the hydrolysis of UDP-galactose to UDP and galactose at a very slow rate in the absence of Mn^{2+} ions (P. Andrews, unpublished work) it has lactose synthetase activity only when Mn^{2+} ions are present. Protection experiments with compounds structurally related to UDP-galactose indicated that Mn^{2+} ions enhance the binding of UDP-galactose to the enzyme by complexing with the pyrophosphate group (Kitchen & Andrews, 1974), so this may be necessary to hold the UDP-galactose firmly in the correct position for galactosyl transfer to occur. The ability of Mn^{2+} ions to increase the blocking power of UDP-galactose on the reaction between enzyme and *p*-hydroxymercuribenzoate can be explained on a steric basis in a similar way. In the absence of Mn^{2+} ions, UDP-galactose is probably

bound to the enzyme only through the nucleoside end of the molecule (Kitchen & Andrews, 1974) and flexing of the remainder may then be such that the rate of the inhibition reaction is only halved. On the other hand, when flexing of the molecule is minimized by the firm binding through Mn^{2+} ion and pyrophosphate the rate of inhibition is decreased by more than 90%, although it is not stopped altogether. This suggests that the thiol group is situated near the galactosyl end of the UDP-galactose binding site on the A protein, and since the galactosyl residue and an acceptor monosaccharide on the enzyme must of necessity be contiguous, this is consistent with the impairment of *N*-acetylglucosamine binding owing to the covalent attachment of a mercuribenzoate residue to the thiol group (Kitchen & Andrews, 1974).

Some of the effects of Mn^{2+} ions, however, could be due to conformational changes in the enzyme. Addition of Mn^{2+} ions causes a conformational change in the cows'-milk enzyme (Klee & Klee, 1972) and such a change in the human enzyme, if it resulted in an alteration in the position of the thiol group, might account for the effect of Mn^{2+} ions on the rate of the inhibition reaction in the presence of UDP-galactose, even though the metal had no effect on the rate in its absence (Table 1). A further possibility is that the enzyme catalyses the transferase reaction only when in the conformation induced by Mn^{2+} ions, in which case the role of Mn^{2+} ions in lactose synthetase would be more complex than indicated by our experiments with UDP-galactose.

We thank the Australian Dairy Produce Board for a postgraduate studentship held by B. J. K. during the period 1971-1972, when he was on leave from the Otto Madsen Dairy Research Laboratory, Hamilton, Queensland 4007, Australia.

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