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Studies on the Thiol Group of Lactose Synthetase A Protein from Human Milk and on the Binding of Uridine Diphosphate Galactose to the Enzyme

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The lactose synthetase activity of A protein from human milk was much decreased but not abolished by reaction with thiol-group reagents. Protection experiments indicated that a free thiol group on the enzyme is situated near the UDP-galactose binding site and inactivation of the enzyme with *p*-hydroxymercuribenzoate was probably due to prevention of UDP-galactose binding. Affinity chromatography showed that the mercuribenzoate substituent also decreased the affinity of A protein for *N*-acetylglucosamine but complex-formation between A protein–*N*-acetylglucosamine and α-lactalbumin was relatively unaffected. UDP-galactose appears to be bound to the enzyme mainly through its pyrophosphate group with Mn^{2+} ion and through the *cis* hydroxyls of ribose, whereas its hexose moiety has little if any affinity for the enzyme. Lactose synthetase activity remaining after the reaction with thiol-group reagents indicates that a free thiol group is not an essential part of the A protein active site.

The A protein of lactose synthetase (UDP-galactose–D-glucose 4-galactosyltransferase; EC 2.4.1.22) occurs in a variety of animal tissues attached to particulate material (Fitzgerald *et al.*, 1971) and in milk as the free enzyme (Babad & Hassid, 1966; Brodbeck *et al.*, 1967). The probable function of the particulate form is to catalyse the transfer of galactosyl units from UDP-galactose to terminal *N*-acetylglucosaminyl units in the synthesis of glycoprotein oligosaccharide chains (Brew, 1970). The A protein also catalyses transfer of galactosyl units to free *N*-acetylglucosamine, forming *N*-acetyl-lactosamine, and to glucose, forming lactose, but when acting alone its affinity for glucose is so low that lactose synthesis is very slow at physiological concentrations of glucose (Brew *et al.*, 1968; Klee & Klee, 1970; Fitzgerald *et al.*, 1970). In the presence of α-lactalbumin (the B protein of lactose synthetase; Brodbeck *et al.*, 1967) the reaction with free *N*-acetylglucosamine is partly inhibited but affinity between A protein and the free monosaccharide substrates is increased so that, as occurs in the mammary gland, the rate of lactose synthesis at physiological concentrations of glucose is greatly increased.

A report that lactose synthetase from cow's milk was inhibited by *p*-chloromercuribenzene-sulphonate (Babad & Hassid, 1966), indicating the presence of a cysteine residue, is so far the only information to identify an amino acid at or near the active site of the A protein. We have investigated the reactivity of a thiol group on the A protein from human milk in the absence and presence of substrates and other ligands, and examined the effect of blocking the thiol group on the interaction between A protein, *N*-acetylglucosamine and α-lactalbumin. A brief report of this work has been published (Kitchen & Andrews, 1972).

Materials and Methods

Materials

Lactose synthetase A protein was isolated from human milk as described by Andrews (1970*a*) and when freshly prepared it had a specific activity in the spectrophotometric lactose synthetase assay (see below) of 6–7 μmol of UDP formed/min per mg of protein at 25°C. The enzyme was eluted from a Bio-Gel P-200 column as a single peak in which E_{230} and lactose synthetase activity coincided (Andrews, 1970*a*), and in the analytical ultracentrifuge its distribution at sedimentation equilibrium indicated homogeneity (P. Andrews, unpublished work). α-Lactalbumin was prepared from human and cow's milk by the procedure described by Barman (1970) for cow's milk. α-D-Galactose 1-phosphate was prepared by the method of MacDonald (1962) and used to synthesize UDP-galactose by the morpholidate procedure (Moffatt, 1966). UDP-glucose, UDP-*N*-acetylglucosamine, uridine, deoxyuridine, uracil, *p*-hydroxymercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoate) and crude pyruvate kinase (type I) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. and *N*-ethylmaleimide, iodoacetic acid (recrystallized from 50% ethanol before use) and iodoacetamide (recrystallized from *n*-heptane before use) from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bio-Gel P-200 (100–200 mesh, wet) and Sepharose 6B were obtained from Calbiochem Ltd., London, W.1, U.K., and Pharmacia (Great Britain) Ltd., London, W.5, U.K., respectively.

Methods

Enzyme assay. Lactose synthetase activity of the A protein was determined by spectrophotometric estimation 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 1ml: Tris–HCl buffer, pH7.5, 50μmol; $MnCl_2$, 4μmol; UDP-galactose, 0.4μmol; glucose, 80μmol; human α-lactalbumin, 150μg; phosphoenolpyruvate, 1μmol; NADH, 0.2μmol; crude pyruvate kinase, 0.5mg; an appropriate amount of A protein. The A protein was assayed in the presence of *N*-acetylglucosamine, as in the effluent from the affinity chromatography column, by determination of its *N*-acetyl-lactosamine synthetase activity in an assay mixture containing *N*-acetylglucosamine (6μmol or the amount added with the enzyme, whichever was the greater) instead of glucose and α-lactalbumin. The activity of A protein which had been inactivated with *p*-hydroxy-mercuribenzoate was regenerated by the inclusion of 2.5μmol of 2-mercaptoethanol in the assay mixture.

Inhibition and protection experiments. Enzyme concentration in the incubation mixtures was about 100μg/ml. Thiol-group reagents were dissolved in 0.25M-Tris–HCl buffer, pH7.5, and the pH checked and readjusted to 7.5 if necessary. In protection experiments, the thiol-group reagent was the last component added to the mixture. Enzyme activity was estimated on 10μl or 20μl samples. The various thiol-group reagents did not inhibit the enzyme or affect the measurement of its activity when present in assay mixtures at the concentrations obtained when samples from inhibition experiments were added.

Thiol-group estimation. The reaction between native A protein and 1mM-5,5′-dithiobis-(2-nitrobenzoate) (Ellman, 1959) in 75mM-Tris–HCl buffer, pH8.0, and 7.5mM-EDTA at 25°C was followed spectrophotometrically at 412nm. Reaction with denatured A protein was measured under similar conditions but the reaction mixture also contained 7.5M-urea. The molarity of A protein solutions was calculated from extinction measurements at 280nm by using a value for $E_{1cm}^{1\%}$ of 10 and a molecular weight of 44000 (determined by dry-weight measurements and sedimentation-equilibrium analysis respectively; P. Andrews, unpublished work). A molar extinction coefficient of 13600cm^{-1} at 412nm (Gething & Davidson, 1972) was used to calculate the amount of 2-nitro-5-thiobenzoate anion formed in the reaction.

Gel filtration of native and modified A protein. A column (2.5cm×42cm) of Bio-Gel P-200 was equilibrated at 8°C with 10mM-Tris–HCl buffer, pH7.5, containing 40mM-KCl (Tris–KCl buffer) and calibrated for molecular-weight estimation with a mixture of myoglobin (0.5mg; mol.wt. 17800), myoglobin dimer (0.5mg; mol.wt. 35600) and bovine serum albumin (5mg; mol.wt. 67000) (Andrews, 1970*a,b*). A mixture of native and modified A protein was prepared by incubating A protein (200μg) in the Tris–KCl buffer (3ml) containing 0.1mM-*p*-hydroxymercuribenzoate at 25°C until about half the initial activity remained. Then the solution was cooled in ice and UDP-galactose and $MnCl_2$ added to 1mM and 8mM concentration respectively to stop further inactivation. The three reference proteins were then added in the amounts given above and the resultant solution was applied to the Bio-Gel column. The column was eluted with the Tris–KCl buffer. Native A protein in the effluent was determined with the standard assay mixture and native and modified A protein together with the assay mixture containing 2-mercaptoethanol.

Preparation of Sepharose–α-lactalbumin. Sepharose 6B (30ml settled volume) was activated with CNBr (1g) at pH11 (Porath *et al.*, 1967), washed thoroughly with cold water and cold 0.1M-$NaHCO_3$, then stirred with a solution of bovine α-lactalbumin (75mg) in 0.1M-$NaHCO_3$ (30ml) for 18h at 5°C. The gel was filtered off and washed with 0.1M-$NaHCO_3$ (1.0litre) containing 1M-NaCl and then with water to remove the salts. Spectrophotometric estimation indicated that even the first washings of the gel contained little, if any, α-lactalbumin. The substituted gel was mixed with an equal volume of unsubstituted gel before use, giving a preparation containing about 1.2mg of α-lactalbumin/ml settled volume of gel.

Affinity chromatography on Sepharose–α-lactalbumin. A column (1cm×5cm) of the Sepharose–α-lactalbumin preparation was equilibrated with 10mM-Tris–HCl buffer, pH7.5, containing 40mM-KCl and different concentrations of *N*-acetylglucosamine as required. Samples applied to the column in 1ml of equilibrium medium consisted of either native A protein (100μg) or A protein (100μg) inactivated by incubation in 1mM-*p*-hydroxymercuribenzoate at 25°C. The equilibration medium was used for elution at a flow rate of 30ml/h and the effluent collected in 3ml fractions. The column was run at 8°C. Native and modified A protein in the effluent were determined by the *N*-acetyllactosamine synthetase assay, 2-mercaptoethanol being added where necessary. Elution volumes were estimated to the nearest 1ml and the elution volume of A protein in the absence of *N*-acetylglucosamine subtracted from the elution volume obtained in its presence to obtain values for retardation due to complex formation (Andrews *et al.*, 1973).

Results

Effect of thiol-group reagents on A protein activity

The A protein lost 84% of its lactose synthetase activity in 1mM-*p*-hydroxymercuribenzoate at pH7.5 and 25°C after 5min, but thereafter the activity

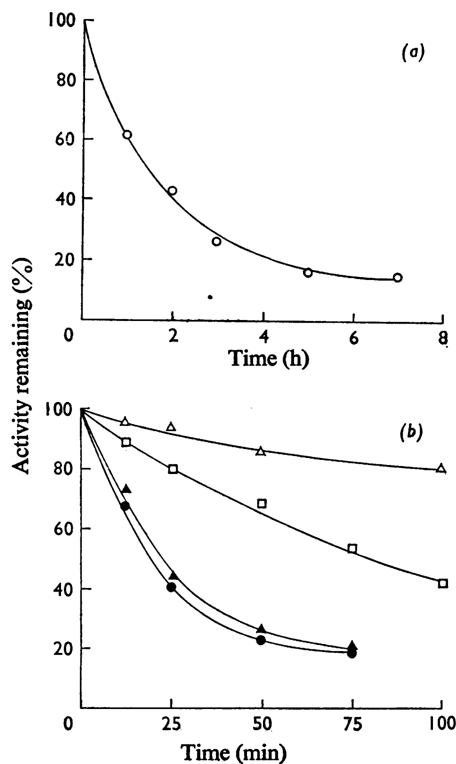

Fig. 1. *Effect of thiol-group reagents on lactose synthetase A protein activity*

The A protein (100 µg/ml) was incubated at 25°C in 0.25 M-Tris–HCl buffer, pH 7.5, containing either (*a*) 1 mM-5,5′-dithiobis-(2-nitrobenzoate) or (*b*) another thiol-group reagent as follows: ●, 0.1 mM-*p*-hydroxymercuribenzoate; △, 0.1 M-iodoacetic acid; □, 0.1 M-iodoacetamide; ▲, 0.1 M-*N*-ethylmaleimide.

remained unchanged. The residual activity was not increased when determined in assay mixtures containing $MnCl_2$, UDP-galactose, glucose or α-lactalbumin at five times the concentrations used in the standard assay. A similar end point was reached in 1 mM-5,5′-dithiobis-(2-nitrobenzoate) after 7 h (Fig. 1*a*). The time-course of inhibition by 0.1 mM-*p*-hydroxymercuribenzoate and other thiol-group reagents at 100 mM concentration is shown in Fig. 1(*b*). No inhibition occurred in 1 h in 1 mM-*N*-ethylmaleimide, 1 mM-iodoacetic acid or 5 mM-iodoacetamide. Initial activity was restored when enzyme inhibited with *p*-hydroxymercuribenzoate was assayed in the presence of 2.5 mM-2-mercaptoethanol.

N-Acetylglucosamine and the substances involved in lactose synthesis were tested, both individually and in various combinations, for their ability to protect the A protein against inactivation by 0.1 mM-*p*-hydroxymercuribenzoate in 30 min at 25°C and pH 7.5. The results are shown in Table 1. Only UDP-galactose and the mixture of UDP-galactose and Mn^{2+} ions had much effect. Concentration-dependence of the protection afforded by UDP-galactose, in the absence and presence of Mn^{2+} ions is shown in Fig. 2. Other compounds, chemically related to UDP-galactose, were tested similarly in place of UDP-galactose. From the results of these experiments the protective effects were assessed as the concentration of each compound that limited the inhibition of A protein activity by 0.1 mM-*p*-hydroxymercuribenzoate in 30 min to 50% (Table 2).

Table 1. *Inhibition of lactose synthetase A protein by p-hydroxymercuribenzoate in the absence or presence of N-acetylglucosamine and components of the lactose synthetase complex*

The A protein was incubated in 0.25 M-Tris–HCl buffer, pH 7.5, with 0.1 mM-*p*-hydroxymercuribenzoate and with or without other substances as indicated, for 30 min at 25°C. Samples were then assayed for lactose synthetase activity in the standard reaction mixture (see the text for details). Activity stable in the presence of inhibitor after prolonged incubation was subtracted from the total remaining activity.

Additions	Inhibitor-sensitive activity remaining (%)
None	22
8 mM-$MnCl_2$	21
80 mM-Glucose ± 8 mM-$MnCl_2$	21
6 mM-*N*-Acetylglucosamine ± 8 mM-$MnCl_2$	24
50 mM-*N*-Acetylglucosamine	24
100 mM-*N*-Acetylglucosamine	25
10 µM-α-Lactalbumin ± 8 mM-$MnCl_2$	25
40 µM-α-Lactalbumin	28
80 mM-Glucose + 10 µM-α-lactalbumin ± 8 mM-$MnCl_2$	22
6 mM-*N*-Acetylglucosamine + 10 µM-α-lactalbumin ± 8 mM-$MnCl_2$	23
50 mM-*N*-Acetylglucosamine + 40 µM-α-lactalbumin	29
1 mM-UDP-galactose	45
1 mM-UDP-galactose + 8 mM-$MnCl_2$	100

Estimation of thiol-group content of A protein

The reaction between A protein and 1 mM-5,5′-dithiobis-(2-nitrobenzoate) at pH 8 and 25°C (Fig. 3*a*) and loss of lactose synthetase activity under similar conditions at pH 7.5 (Fig. 1*a*) proceeded at about the same slow rate. In 7.5 M-urea the end point was reached in less than 1 min (Fig. 3*a*). Varying the concentration of A protein in the denaturing-reaction mixture produced a proportional change in the yield of 2-nitro-5-thiobenzoate anion (Fig. 3*b*),

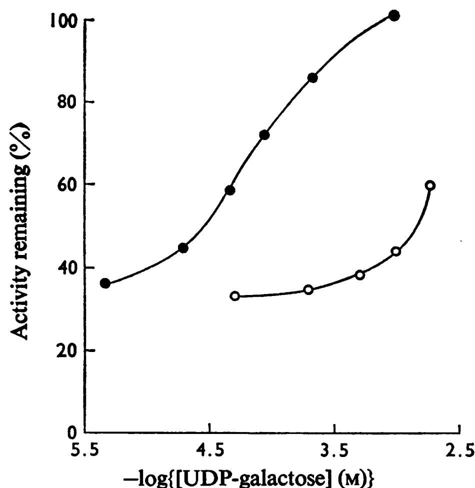

Fig. 2. *Effect of p-hydroxymercuribenzoate on lactose synthetase A protein activity in the presence of UDP-galactose*

Activity remaining was determined after A protein (100μg/ml) had been incubated at 25°C for 30min in 0.25M-Tris–HCl buffer, pH7.5, containing 0.1mM-*p*-hydroxymercuribenzoate and UDP-galactose as indicated, and either with 8mM-$MnCl_2$ (●) or without Mn^{2+} (○).

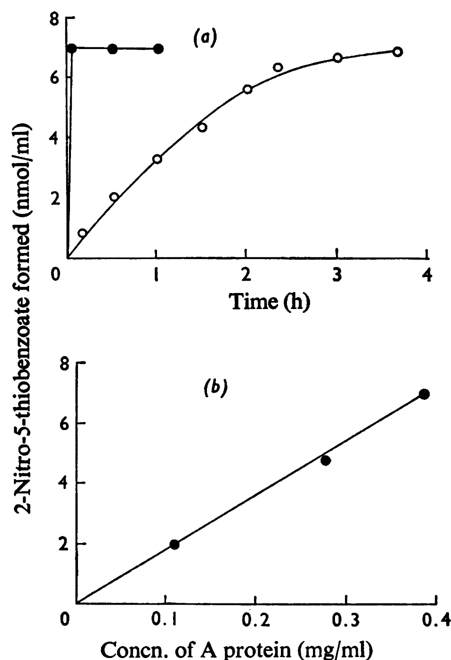

Fig. 3. *Reaction between lactose synthetase A protein and 5,5′-dithiobis-(2-nitrobenzoate)*

(*a*) Time-course of reaction between A protein (385μg/ml) and 1mM-5,5′dithiobis-(2-nitrobenzoate) in 75mM-Tris–HCl buffer, pH8.0, containing 7.5mM-EDTA: ○, native A protein; ●, in the presence of 7.5M-urea. (*b*) Yield of 2-nitro-5-thiobenzoate anion from reaction between A protein and the thiol group reagent as in (*a*) in the presence of 7.5M-urea.

Table 2. *Protection of lactose synthetase A protein against inhibition by p-hydroxymercuribenzoate*

The A protein was incubated in 0.25M-Tris–HCl buffer, pH7.5, with 0.1mM-*p*-hydroxymercuribenzoate and various concentrations of each compound listed, for 30min at 25°C. Samples were then assayed for lactose synthetase activity in the standard reaction mixture (see the text for details). The concentration of each compound that resulted in 50% of the A protein activity remaining after incubation was obtained by plotting activity remaining against log of concentration, as shown in Fig. 2 for UDP-galactose.

	Concentration resulting in 50% of A protein activity remaining after incubation (mM)	
Compound tested	No Mn^{2+} added	8mM-$MnCl_2$ added
UDP-galactose	1.5	0.028
UDP-galactose	2.0	0.026
UDP-*N*-acetyl-glucosamine	5.5	1.0
UDP	2.0	0.035
Uridine	2.5	2.4
Deoxyuridine	~30	~30
Uracil	—	~100

corresponding on average to the formation of 0.8mol of anion/mol of A protein. No reaction was observed between 5,5′-dithiobis-(2-nitrobenzoate) and A protein which had been incubated with 1mM-*p*-hydroxymercuribenzoate for 5min at 25°C.

Behaviour of native and modified A protein in gel filtration and affinity chromatography

In the gel-filtration experiment with the mixture of native and modified A protein and reference proteins, the elution volume of both forms of the A protein was 85ml and those of serum albumin, myoglobin dimer and myoglobin were 73, 91 and 116ml respectively. Modification evidently caused no major conformational change in the A protein; the molecular weight estimated for both native and modified forms was 44000.

Elution of both native and modified A protein from the Sepharose–α-lactalbumin column was retarded by the presence of *N*-acetylglucosamine in the eluting medium. The reciprocal plots in Fig. 4

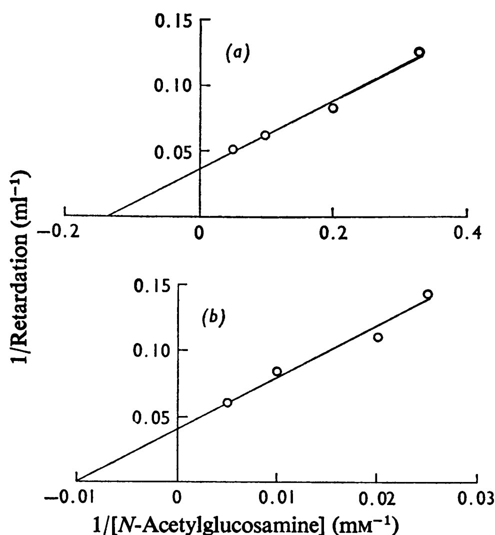

Fig. 4. *Effect of N-acetylglucosamine concentration on the behaviour of (a) native and (b) modified lactose synthetase A protein in affinity chromatography on a Sepharose–α-lactalbumin column*

The A protein was modified by incubation with *p*-hydroxymercuribenzoate. *N*-Acetylglucosamine was dissolved in the column eluent. Further details are given in the text. The lines were fitted to the points by the least-squares method.

show the relationship in each case between retardation and *N*-acetylglucosamine concentration. Intercepts on the abscissae and ordinates respectively gave K_a 7±3mM and maximum retardation 28±10ml for native A protein (Fig. 4*a*) and K_a 100±40mM and maximum retardation 24±8ml for modified A protein (Fig. 4*b*). No activity was detected in column fractions containing the modified enzyme when they were assayed without addition of 2-mercaptoethanol.

Discussion

Reaction with 5,5′-dithiobis-(2-nitrobenzoate) indicated that A protein molecules inactivated by thiol-group reagents possessed one free thiol group. The greatly increased rate at which this group reacted when the protein was denatured suggested that it was not exposed at the surface of the molecule but in a position where steric or electrostatic factors decreased its reactivity. However, reaction with thiol-group reagents until an end point was reached did not completely abolish the lactose synthetase activity of the A protein. This residual activity was not increased when assayed in the presence of increased substrate concentrations nor was activity detected in the enzyme with changed properties in affinity chromatography after reaction with *p*-hydroxymercuribenzoate It seems more likely therefore that the residual activity was a property of A protein molecules which had not reacted with the thiol reagent than of modified A protein with decreased substrate affinity. In any case, a free thiol group is evidently not an essential part of the A protein active site. Possible explanations of the suggested heterogeneity are that the thiol group in some A protein molecules had been altered without destroying the lactose synthetase activity or that some molecules lacked the cysteine residue altogether. Preparations of A protein from human milk may resemble those from cow's milk in containing more than one molecular species (Barker *et al.*, 1972) although in the latter case the heterogeneity seems to have little, if any, effect on the amino acid composition.

Protection experiments (Table 1) indicated that the thiol group on the A protein is closer to the UDP-galactose binding site than to the monosaccharide binding site (Kitchen & Andrews, 1973) or that part of the A protein that is contiguous with α-lactalbumin in the lactose synthetase complex. However, it is unlikely to be an important part of the UDP-galactose site because a form of the enzyme exists which must bind UDP-galactose but which apparently has no free thiol group. The protection experiments also indicated that UDP-galactose binds much more strongly to A protein in the presence of Mn^{2+} ions than in their absence. This is in accord with kinetic studies on the A protein from cow's milk which showed that Mn^{2+} ions and UDP-galactose bind to the enzyme in that order (Morrison & Ebner, 1971*a*,*b*). Since Mn^{2+} ions alone afforded the A protein no protection against inhibition by *p*-hydroxymercuribenzoate, the free thiol group is presumably not involved in metal binding. *N*-Acetylglucosamine also afforded the enzyme little protection but affinity chromatography showed that the covalently attached mercuribenzoate group caused a large decrease in affinity between the A protein and the amino sugar. The binding sites for UDP-galactose and *N*-acetylglucosamine are no doubt adjacent, to permit transfer of a galactosyl moiety from the one molecule to the other. The influence of the mercuribenzoate group on both sites is therefore readily visualized. In contrast, affinity between the A protein–*N*-acetylglucosamine complex and α-lactalbumin attached to Sepharose was changed much less, if at all, when the A protein was modified with *p*-hydroxymercuribenzoate. As the decreased affinity for acceptor substrate is unlikely to account for the loss of enzymic activity, the probable cause is prevention of UDP-galactose binding, either sterically or electrostatically by the attached group or as a result of a local conformational change in the enzyme.

In kinetic studies, UDP-glucose behaved as a

competitive inhibitor of lactose synthetase with about the same affinity as UDP-galactose for the A protein (Morrison & Ebner, 1971*a*,*b*). The similar affinity was confirmed, and extended to include UDP, by the protective effect of these compounds against inhibition by *p*-hydroxymercuribenzoate (Table 2). The three compounds seem to fit equally well at the UDP-galactose binding site and little if any affinity between the hexose moiety and the enzyme is apparent. On the other hand, UDP-*N*-acetylglucosamine fits less well and the different hexose moiety prevents to some extent the stronger binding mediated by Mn^{2+} ions. UDP and its derivatives possess the group involved in complex formation with Mn^{2+} ions, whereas uridine does not. Evidently the group is pyrophosphate, a conclusion in accord with physical studies on metal ion–nucleotide complexes (Cohn & Hughes, 1962). Although the enzyme has no lactose synthetase activity unless Mn^{2+} ions are added, the protection experiments indicated that its affinity for UDP-galactose is still appreciable under these conditions. This affinity is not mediated through pyrophosphate since the result with uridine was similar. The experiment with deoxyuridine suggested that, in the main, the *cis* hydroxyls of ribose are involved. Uracil, if bound to any extent, is possibly too small to have much protective effect. The overall picture is of UDP-galactose binding to the enzyme through the nucleotide portion of the molecule, with complex formation involving the pyrophosphate group and Mn^{2+} ion important both for strengthening the binding and for orientating the molecule correctly relative to the acceptor substrate site.

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