The Intermediate in the Uridine Diphosphate Galactose 4-Epimerase Reaction

RESOLUTION OF AN APPARENT AMBIGUITY*

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SUMMARY

Incubation of UDP-galactose with substrate amounts of UDP-galactose 4-epimerase (EC 5.1.3.2) from Escherichia coli for different times between 2 and 120 min, followed by reduction with NaB³H₄, invariably yields a mixture of labeled UDP-glucose and UDP-galactose. Chemical degradation of tritiated hexoses, isolated after NaB³H₄ reduction of 2-min incubation mixtures, shows all of the tritium associated with carbon 4, in agreement with the results presented earlier using longer incubation times (MAITRA, U. S., AND ANKEL, H. (1972) Proc. Nat. Acad. Sci. U. S. A. 68, 2660). The total amount of label introduced after 2 min of incubation, when enzyme activity is practically unchanged, is considerably less than that after 120 min of incubation, when enzyme activity is reduced to about 50%. The excess of labeled UDP-hexoses after 120 min of incubation can be quantitatively related to the amount of inactive enzyme formed, assuming that during inactivation additional UDP-4-ketohexose is produced in amounts equivalent to the amounts of inactive enzyme.

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The interconversion of UDP-glucose and UDP-galactose is catalyzed by UDP-galactose 4-epimerase. Depending on the source of the enzyme, the epimerase either requires catalytic amounts of exogenous DPN for activity or contains 1 mole of DPN bound per mole of enzyme (1). There is convincing evidence supporting the view that the inversion of the configuration at carbon 4 of the hexosyl moiety takes place by an intramolecular oxidation-reduction mechanism in which DPN participates as the hydrogen carrier (1). Thus, epimerization takes place in a two-step reaction: oxidation of C-4 of the glycosyl moiety of the sugar nucleotide to a keto group by DPN, followed by stereospecific reduction to the corresponding epimeric form by intermittently produced DPNH. In our previous communication, we demonstrated the existence of UDP-4-ketogalactose as an intermediate in the epimerization reaction by using NaB³H₄ as a trapping agent (2). However, recently our experiment has been subjected to criticism by Wee et al. on the following grounds. (a) The time employed during incubation of the substrate with the enzyme used in our experiment was much longer than the time required for reaching equilibrium, and (b) during extended incubation with substrate the activity of the enzyme is progressively reduced (3). In the present communication we report on the quantitative aspects of the formation of the intermediary UDP-4-ketoglucose in dependence on the time of incubation of enzyme and substrate.

The UDP-galactose 4-epimerase preparation from Escherichia coli that has been employed was purified and assayed according to Wilson and Hogness (4) as described before (2). The specific activity of the preparation used was 185 μmoles of UDP-glucose formed per min per mg of protein (185 units per mg). From the specific activity presented by Wilson and Hogness for their essentially homogenous enzyme preparation, we calculate that the enzyme preparation used by us was 80% pure.

When the purified enzyme is incubated for different times with UDP-galactose and then reduced with NaB³H₄, we find substantial amounts of tritium incorporated into UDP-hexoses already after 2 min of incubation, namely 0.14 mole per mole of enzyme (Table I). This amount is in good agreement with the concentration of enzyme-bound DPNH as calculated from the spectrophotometric data of Wilson and Hogness (4). At this time UDP-glucose and UDP-galactose are in equilibrium, as we have confirmed by enzymatic determination of their concentrations. As can be seen in Table I, the substrate-induced inactivation is less than 5% during the first 5 min of incubation. Therefore, the considerable amount of tritium incorporated after 2 min cannot be attributed to a "fortunate consequence of the substrate-induced change in catalytic properties of the enzyme (3)," but must be related to a true intermediate of a fully active enzyme.

An aliquot of the tritiated UDP-hexoses (0.2 × 10⁶ cpm) isolated after 2 min of incubation, was hydrolyzed with 0.1 N HCl at 100°C for 30 min and subjected to chromatography in propanol-ethyl acetate-water (7:1:2, v/v). The mixture of tritiated hexoses was eluted from the paper and converted to the corresponding mixture of methyl glycosides by the method of...
The activity of 960 cpm per pmole. The sodium periodate degraded substrate (8).

Labeled UDP-hexose was isolated and purified as described under Table I. Periodate oxidation and further analyses were carried out as described in the text.

| Incubation time | (Moles of tritium in UDP-hexose)/(moles of epimerase) | Activity%
<table>
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<tbody>
<tr>
<td>min</td>
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</tr>
<tr>
<td>30</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>120</td>
<td>0.55</td>
<td>51</td>
</tr>
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</table>

*a* Incubation mixtures contained 1150 units of *Escherichia coli* UDP-galactose 4-epimerase (6.2 mg), 1.2 μmoles of UDP-galactose, and 200 μmoles of glycine-KOH buffer (pH 8.6) in a total volume of 1 ml at 27°C. At the indicated times, 20 μCi of NaBH₄ (7.2 Ci per mmole) were added. Enzymatic activity decreased to about 50% of the original within 30 s after NaBH₄ addition. Incubation with NaBH₄ was carried out for 5 min, and then excess NaBH₄ was destroyed by addition of 100 μmoles of acetone. The isolation procedure for the UDP-hexoses was the same as described before (2), except that no carrier UDP-hexose was added. After purification by charcoal treatment, the UDP-hexoses were further purified by paper chromatography by using ethanol-1 M ammonium acetate, pH 7.3 (7:3), followed by paper electrophoresis in 0.15 M ammonium acetate (pH 8.5) at a potential gradient of 32 volts per cm. Aliquots of the tritiated UDP-hexoses were incubated with 2 μmoles of DPN, 250 μmoles of glycine-KOH buffer (pH 8.6), 0.5 units of UDP-galactose 4-epimerase, and 1000 units of UDP-glucose dehydrogenase (EC 1.1.1.22, Sigma) in a total volume of 1.5 ml. The reaction was monitored at 340 nm until completed in a Gilford spectrophotometer. The reaction mixtures were subjected to electrophoresis as described above. The radioactive material corresponding to UDP-glucuronic acid was eluted and evaporated to dryness, and radioactivity was determined in a liquid scintillation counter as described before (2).

The concentration of UDP-glucuronate was determined from the ultraviolet absorption by using the extinction coefficient 9.7 × 10⁴ M⁻¹ cm⁻¹. Hydride incorporation from NaBH₄ into UDP-hexoses was calculated, based on a specific activity of one-fourth that of the NaBH₄ used, and was corrected for final recovery (about 70 to 75% of original UDP-hexoses). The molar concentration of the epimerase was calculated by assuming that our preparation was 100% pure and that the enzyme has a molecular weight of 78,000 (4). Controls with either enzyme or 1.2 μmoles of UDP-galactose were treated in the same manner. Insignificant amounts of radioactivity were found in the final isolates of the control reaction mixtures.

Reaction mixtures contained enzyme, UDP-galactose, and buffer as described under footnote a. After incubation at 27°C for the indicated times, samples were quickly diluted and assayed by the method of Wilson and Hogness (4). The activity of the untreated enzyme was taken as 100%. The experiment was repeated twice with the same enzyme and showed identical results. Likewise, using a different enzyme preparation from *E. coli* (specific activity, 40 units per mg), at a concentration of 1150 units per ml gave essentially the same results. This preparation was kindly donated by O. Gabriel, Georgetown University, Washington, D. C.

Frahn and Mills (5). The methylated glycosides were purified by paper chromatography in the above solvent. The radioactive methyl glycosides were eluted together from the paper and diluted with α-methyl glucoside (500 μ mole) to a final specific activity of 960 cpm per μmole. The sodium periodate degradation was carried out by the methods of Gabriel (6): C-3 was isolated as ammonium formate; ethylene glycol (from C-1 and C-2) and glyceraldehyde from C-4, C-5 and C-6) were obtained after NaBH₄ reduction of the periodate-treated material followed by acid hydrolysis and a second reduction with NaBH₄. Ethylene glycol and glyceraldehyde were separated by paper chromatography in water-saturated butanol. The concentration of glyceraldehyde was determined as formaldehyde (7). The possibility that the tritium that was found exclusively in glyceraldehyde was associated with C-5 or C-6 of the original hexoses, was eliminated by bromination of a part of the original n'-methoxy-n-hydroxymethyl diglycic aldehyde to n'-methoxy-n-hydroxymethyl diglycic acid. The results of the periodate degradation are presented in Table II. The fact that all of the radioactivity is associated with glyceraldehyde, but none with n'-methoxy-n-hydroxymethyl diglycic acid, shows that tritium is exclusively associated with C-4 of the original hexosyl moieties. These data confirm the interpretation of our earlier experiments and exclude that the formation of UDP-4-ketoglucone under these conditions is a consequence of the inactivation of the enzyme by substrate.

As seen in Table I, the amount of labeled UDP-hexose found after NaBH₄ reduction increases with time of incubation of enzyme and substrate. The amount of label after 120 min is about 4 times that found after 2 min of incubation. The increased amount of tritium incorporated after 2 hours of incubation can best be explained by assuming that the inactivation of epimerase by substrate is due to dissociation of intermediary UDP-4-ketoglucone yielding inactive DPNH-epimerase, or to the production of an inactive conformational species of the epimerase-PTP-UDP 4-ketoglucone complex (abruptive complex), while the remaining active population of the enzyme still contains 0.14 mole per mole of reactive epimerase-DPNH-UDP 4-ketoglucone complex. Assuming 49% of the enzyme is inactivated after 120 min, this would yield 0.49 mole of abortive UDP 4-ketoglucone per mole of total enzyme present. To this would be added 0.14 mole per mole of the remaining 51% of active enzyme, which would be 0.07 mole per mole of total enzyme present. The sum of both is 0.56 mole per mole of total enzyme present, which is in excellent agreement with the value observed.

Further support of the above assumptions comes from the experiments of Nelsestuen and Kirkwood, which show a direct correlation between reduction of enzyme-bound DPN and loss of catalytic activity upon prolonged incubation of enzyme with substrate (8). The reactivation of inactive enzyme by TDP-
4-keto-6-deoxyglucose indicates that the inactive enzyme is the epimerase-DPNH complex, as it has been clearly established that UDP-4-keto-6-deoxyglucose is specifically reduced by enzyme-bound DPN (8, 9).

The ratio of tritiated UDP-glucose to UDP-galactose obtained from short time incubation mixtures differs drastically from that obtained after 120 min of incubation (Table III), suggesting that NaBH₄ reduction of the active epimerase-DPNH-UDP-4-ketoglucose complex proceeds with a stereospecificity different from that of free UDP-4-ketoglucose (or of the abortive epimerase-DPNNH-UDP-4-ketoglucose complex). The ratio of UDP-glucose to UDP-galactose decreases with time and approaches the ratio of the corresponding glucose and galactose analogs obtained by NaBH₄ reduction of 4-keto-2,3,6-tribenzyol methyl glucoside (2:1; Reference 6) or of TDP-4-keto-6-deoxyglucose (1:711, Reference 10) which might be considered as model compounds for the reduction of free UDP-4-ketoglucose. This difference in stereospecificity upon NaBH₄ reduction of short and long time incubation mixtures indicates that in the former case UDP-4-ketoglucose is bound to the enzyme, whereas in the latter case the majority of it is available for NaBH₄ reduction in a fashion similar to that expected for dissociated UDP-4-ketoglucose. A similar phenomenon has been observed by Lehmann and Pfeiffer in the NaBH₄ reduction of TDP-4-ketoglucose bound to TDP-glucose oxidoreductase, which, only, resulted in TDP-[4-3H]glucose (11).

The results presented in this paper clearly show that UDP-4-ketoglucose is the intermediate in the UDP-galactose 4-epimerase reaction. Davis and Glaser, using the reduction of enzyme-bound DPN in the presence of UMP and free sugars as a model system for the first step in the epimerization, observed a pronounced isotope effect with [3-2H]glucose, but not with [4-2H]glucose or [5-2H]glucose (12). From these observations they suggested that, in fact, UDP-3-ketoglucose is the intermediate. However, Seyama and Kalckar have shown recently that in this system C-1 of the free sugar is oxidized, yielding the corresponding aldonic acid (13). Thus, the assumption that this system is a true representative of the half reaction is not correct. Unpublished data from our laboratory (14), as well as those by others (15, 16), show a normal isotope effect in the case of 4-tritiated glycosyl nucleotides, but no isotope effect with 3-tritiated glycosyl nucleotides. This also indicates that the C-H bond at C-4 of the glycosyl moieties of UDP-glucose and UDP-galactose is involved in the reaction mechanism. The evidence for such mechanism is strengthened by the findings of Nelsestuen and Kirkwood, who were able to show the transfer of tritium from E. coli epimerase containing chemically reduced [4-3H]DPN to TDP-4-keto-6-deoxyglucose, to form the substrate analogs TDP-6-deoxy[4-3H]glucose and TDP-6-deoxy[4-3H]galactose (8). Furthermore, direct transfer of tritium from 6-deoxy[4-3H]glycosyl- and [4-3H]glycosyl nucleotides to enzyme-bound DPN, contrasting lack of such transfer with TDP-[3-3H]glucose rules out a 3-keto intermediate (8, 17).

Finally, the objection raised by Wee et al. to our previous demonstration of the chemical nature of the intermediate is valid with respect to the extent of quantitation. However, the carefully repeated experiments reported in this communication provide good additional evidence that UDP-4-ketoglucose is the intermediate of the epimerization reaction and show that its concentration immediately after reaching equilibrium is in good agreement with that expected from the data of Wilson and Hogness (4). There is, indeed, an increase of the amount of UDP-4-ketoglucose upon prolonged incubation, which directly correlates with the amount of inactivated enzyme.

### TABLE III

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<th>Incubation time</th>
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</table>

REFERENCES

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