Induced Reoxidation and Reactivation of a Reduced Uridine Diphosphate Galactose-4-epimerase Complex*

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SUMMARY

An inactive reduced UDP-galactose-4-epimerase complex, which is prepared by reduction with NaBH₄, in the presence of substrates and is known to contain DPNH and UDP-epimerases in tightly bound form, is largely reoxidized and partially reactivated in the presence of 0.02 to 0.4 M cyclohexanone or cyclohexanol. Upon placing the epimerase-[4-β-3H]DPNH-UDP-hexose complex in the presence of 0.4 M cyclohexanone about 70% of the DPNH is reoxidized and about 40% of the catalytic activity is restored within 90 min at 27° and pH 8.5, while the major radioactive products are 3H₂O and free tritiated DPN⁺, as well as enzyme-bound tritiated DPN⁺, but not tritiated cyclohexanol. There is no evidence that cyclohexanone is reduced, and cyclohexanol at similar concentrations causes a similar reactivation. It is probable that these compounds act by causing the tightly bound UDP-hexose molecules to be released from the reduced complex. The resultant epimerase-DPNH complex then undergoes spontaneous autooxidation and reactivation.

Uridine diphosphate galactose-4-epimerases (EC 5.1.3.2) from bacteria, yeast, and animal tissues all require DPN⁺ as the essential coenzyme. The Escherichia coli and Saccharomyces fragilis enzymes contain 1 mole of tightly bound DPN⁺ per mole of enzyme when purified to near homogeneity, and this appears to constitute a full complement of pyridine nucleotide (1-3). The partially purified mammalian enzymes require added DPN⁺ to activate them, but they also appear to bind the coenzyme fairly tightly as indicated by very small Kᵣ values, in the 10⁻⁷ M range, for added DPN⁺ (4).

Available data from several laboratories strongly support the molecular pathway proposed by Maxwell (4-12), in which the nucleotide sugar is oxidized by the enzyme-bound DPN⁺ to produce DPNH and UDP-4-ketoglucose as enzyme-bound intermediates. Recent experiments, in particular, clearly establish that under certain conditions UDP-4-ketosugars are free intermediates (12).

Reduced inactive epimerase-DPNH complexes are of interest because of their potential relationship to the DPNH containing complexes which occur as intermediates during the catalytic process. Such complexes can be prepared by treating the yeast or bacterial epimerases with NaBH₄, in the presence of an UDP-sugar or with one of several aldohexoses or aldopentoses in the presence of UMP (10, 12, 13). These complexes contain tightly bound UDP-sugar or UMP and they can be reoxidized and reactivated by certain ketones, dTDP- or UDP-4-keto-6-deoxyglucose and by 2-deoxyglucose or myo-inosose-2 (10, 14, 15). The reactivation by nucleoside diphosphate-4-keto-6-deoxyglucoses proceeds by direct transfer of the 4-β-hydrogen of enzyme-bound DPNH to produce the corresponding nucleoside diphosphate-6-deoxyglucose and nucleoside diphosphate-6-deoxyglactose. The reactions of the reduced S. fragilis enzyme with 2-ketoglucone or myo-inosose-2 have not been shown to proceed by direct hydrogen transfer or to involve actual reduction of the ketones.

We present here the results of some studies on the cyclohexanone-induced reoxidation and partial reactivation of an E. coli epimerase-DPNH-UDP-hexose complex, which is found to proceed without direct hydrogen transfer and probably without reduction of the ketone.

EXPERIMENTAL PROCEDURE

Enzymes—UDP-galactose-4-epimerase was purified from E. coli cells as described earlier (12) following the procedure of Wilson and Hogness (2). Purified acetooacetate decarboxylase from Clostridium acetobutylicum was a generous gift from Professor F. H. Westheimer, Department of Chemistry, Harvard University.

The reduced inactive epimerase-DPNH-UDP-hexose complex was prepared essentially as described (12) by reducing UDP-galactose-4-epimerase with 0.005 M NaBH₄ in the presence of 0.002 M UDP-glucose and then reisolating the protein by gel filtration. The activity loss upon reduction was typically 98 to 99%, but 5 to 10% of the activity was restored upon gel filtration. The epimerase-[4-β-3H]DPNH-UDP-hexose complex was prepared by the same procedure with NaBH₄.

Assays—UDP-galactose 4 epimerase was assayed by the published procedure (2). Radiochemical assays were carried out by liquid scintillation counting in a Packard model 3310 Tri-Carb liquid scintillation spectrometer. Radioactive areas on paper chromatograms were located with a Packard model 7201 radiochromatogram scanner.

Coenzymes and Radiochemicals—These materials were purchased from the following commercial suppliers. DPN⁺ from Sigma, DPNH from Boehringer-Mannheim, UDP-glucose and
UDP-galactose from Calbiochem, reagent grade cyclohexanone and cyclohexanol from Mallinckrodt and J. T. Baker, respectively, and NaB\textsubscript{4}H\textsubscript{4} from Amersham-Searle.

RESULTS AND DISCUSSION

Cyclohexanone-induced Reactivation and Partial Reactivation of Reduced Complex—An inactive reduced form of E. coli UDP-galactose-4-epimerase prepared by NaB\textsubscript{4}H\textsubscript{4} reduction in the presence of substrates is described in earlier publications (10, 12). The protein, specific activity 1400 units per mg of protein, was then combined with cyclohexanone and buffer in a reaction mixture consisting of 1.31 mg of protein per ml, 0.087 M sodium bicarbonate buffer at pH 8.5, and 0.4 M cyclohexanone at 27\textdegree. The decrease in \(A_{345}\) and increase in specific activity were measured over a period of 180 min. Symbols: \(\circ\,\circ\,\circ\), specific activity; \(\bullet\,\bullet\,\bullet\,\bullet\,\bullet\), \(A_{345}\).

UDP-galactose-4-epimerase induced by cyclohexanone. A sample of UDP-galactose-4-epimerase, specific activity 8300 units per mg of protein, was reduced and isolated as described under “Experimental Procedure.” The protein, specific activity 1400 units per mg of protein, was then combined with cyclohexanone and buffer in a reaction mixture consisting of 1.31 mg of protein per ml, 0.087 M sodium bicarbonate buffer at pH 8.5, and 0.4 M cyclohexanone at 27\textdegree. The decrease in \(A_{345}\) and increase in specific activity were measured over a period of 180 min. Symbols: \(\circ\,\circ\,\circ\), specific activity; \(\bullet\,\bullet\,\bullet\,\bullet\,\bullet\), \(A_{345}\).

Cyclohexanone-induced reactivation, it appears that they act to induce the dissociation of enzyme-bound tritiated DPN\textsuperscript{+} and the remaining 80\% was found in the small molecular weight fraction. Tritiated cyclohexanol could not be detected in this fraction; specifically the radioactivity could not be extracted into cyclohexanol. Moreover, when carrier cyclohexanol was added and reisolated as its crystalline 3,5-dinitrobenzoate, the derivative contained less than 2\% of the radioactivity (16). The small molecular weight radioactivity was identified as tritiated DPN\textsuperscript{+} and \(^3\text{H}_2\text{O}\) which were present in variable but comparable amounts (16). The \(^3\text{H}_2\text{O}\) was identified by its volatility and by the fact that the tritium was fully exchangeable with the protons of acetone in the presence of acetacetate deacetylase which catalyzes this reaction (17). The balance of the radioactivity chromatographed with DPN\textsuperscript{+} on paper chromatograms and columns of Bio-Gel P-2 and with DPN\textsuperscript{+} upon reduction with ethanol in the presence of alcohol dehydrogenase (16).

Autoxidation Induced by Cyclohexanone and Cyclohexanol—The absence of tritiated cyclohexanol together with the finding that tritiated DPN\textsuperscript{+} is released from the complex suggest that the action of cyclohexanol may not be based upon its reactivity as a hydride acceptor. DPN\textsuperscript{+} does not dissociate from this enzyme except under denaturing conditions or in the presence of p-hydroxymercuribenzoate; we believe that the partial release of DPN\textsuperscript{+} is probably caused by some alteration in the structure of the protein induced by cyclohexanone under the reaction conditions. If so, it is very likely that substrate molecules would also dissociate under these conditions, probably on a shorter time scale than the dissociation of DPN\textsuperscript{+} which is almost certainly bound more tightly. The resulting epimerase-DPN\textsuperscript{+} complex can be expected to undergo spontaneous autoxidation to active epimerase-DPN\textsuperscript{+} complex (10, 18). This interpretation is supported by Fig. 2, which shows that 0.076 M and 0.15 M cyclohexanol cause a reactivation similar to that induced by cyclohexanone. Inasmuch as cyclohexanol is very unlikely to be reduced by DPN\textsuperscript{+} and hydrogen transfer to cyclohexanone cannot be detected, yet both molecules promote reactivation, it appears that they act to induce the dissociation of UDP-hexose molecules. The epimerase-DPN\textsuperscript{+} complex then undergoes autoxidation and reactivation. That the en-
zyme is never fully reactivated is explained by the fact that DPN$^+$ is also released from the protein, probably very slowly and subsequent to UDP-hexose dissociation and autoxidation. This interpretation is further supported by the fact that both cyclohexanone and cyclohexanol at 0.3 M cause a very gradual decrease in the activity of the native enzyme.1

It has recently been proposed (19) that autoxidation of the catalytic intermediate, the epimerase-DPNH-UDP-4-ketoglucose complex, to epimerase.DPN+ concomitant with the dissociation of UDP-4-ketoglucose can account for our earlier observation that the appearance of UDP-4-ketoglucose, in a form reducible by NaBH$_4$, is a slow process by comparison with the catalytic time scale (11). This interpretation does not account for the fact that the activity of the enzyme is greatly decreased in this process (11), nor is it consistent with several other facts more recently reported and accounted for on the basis that free UDP-4-ketoglucose appears slowly as a result of the slow reversible appearance of an abortive complex (12). In addition, as shown here and elsewhere (10) the epimerase-DPNH-UDP-hexose complexes are not subject to autoxidation at a significant rate.

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