Flavoprotein-catalyzed Direct Hydrogen Transfer between Pyridine Nucleotides*

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(Received for publication, February 16, 1961)

Several flavoproteins which oxidize reduced pyridine nucleotides have been observed to catalyze an exchange reaction between the protons of the medium and the reduced pyridine nucleotide (1, 2) as well as to mediate reduction of analogues of pyridine nucleotides by reduced pyridine nucleotides (3). Weber, Kaplan, San Pietro, and Stolzenbach (2) have made isotope studies of the analogue reduction reaction as well as the exchange reaction, with diaphorase as catalyst. When this enzyme mediates the reduction of the acetylpyridine analogue of diphosphopyridine nucleotide by reduced diphosphopyridine nucleotide (α- or β-D), no isotope appears in the reduced analogue, but when the reaction is carried out with DPNH as reductant in D₂O, deuterium is incorporated into the acetylpyridine-DPNH.

We have now investigated the properties of the DPNH cytochrome b₅ reductase of microsomes which differs from the previously mentioned cases in that it fails to catalyze exchange between DPNH and the protons of the medium, but does carry out a flavin-dependent reduction of acetylpyridine-DPNH by reduced diphosphopyridine nucleotide (α- or β-D). No isotope appears in the reduced analogue, but when the reaction is carried out with DPNH alone. Here the specific activity of the nicotinamide was 0.47 atom D per molecule was oxidized with the reductase with Fe(CN)₆⁻³ as acceptor. The DPN produced in the reaction was cleaved to yield nicotinamide which, when analyzed for deuterium, was found to have retained 0.01 atom D per mole. The reductase, therefore, shows essentially complete stereospecificity for the α side of the dihydropyridine ring of DPNH.

EXPERIMENTAL PROCEDURE

Commercial sources of nucleotides and enzymes and the general method of procedure are described in previous papers (1, 5). The extinction coefficients reported by Siegel, Montgomery, and Boch (6) for the reduced acetylpyridine-DPN at 365 μν and the CN-complex of acetylpyridine-DPN at 340 μν were used. Acetylpyridine-DPN was separated from the nicotinamide of DPN and chromatographed as described in (5); the analogue samples were then diluted with carrier, precipitated with acid acetone, and analyzed directly. Specific details are described in connection with the individual experiments. The flavoprotein was prepared as described previously (7, 8) and the totally resolved apoenzyme was prepared by a method which is described in (9). DPNH (α-D) was prepared as described in (10), with C₂D₅OH purchased from the New England Nuclear Corporation. The DPNH (α-D) was analyzed by oxidizing portions to DPN with glutamic and alcohol dehydrogenases; the DPN was cleaved and the resulting nicotinamide was diluted and crystallized. None of the samples of DPNH (α-D) had appreciable D on the β side of the ring.

RESULTS

Stereospecificity of DPNH Oxidation by Reductase—DPNH (α-D) which had 0.47 atom D per molecule was oxidized with the reductase as acceptor. The DPN produced in the reaction was analyzed to yield nicotinamide which, when analyzed for deuterium, was found to have retained 0.01 atom D per molecule. The reductase, therefore, shows essentially complete stereospecificity for the α side of the dihydropyridine ring of DPNH.

Tests for Tritium Incorporation from Medium—In one experiment, 13 μmoles of DPNH, 3.75 μmoles of EDTA, HTO to give a final specific activity in the medium of 1.3 × 10⁴ c.p.m. per μatom of H, and 0.1 mg of reductase were incubated in 3.0 ml of 0.1 M Tris-chloride buffer at pH 7.9, under anaerobic conditions, for 90 minutes at 15°. The description of the methods used for denaturing the enzyme and preparing the samples for counting as nicotinamide are described in reference (1). In the experiment reported here, portions of the DPNH were oxidized with glutamic dehydrogenase as well as with alcohol dehydrogenase so that isotope which had been in either the α or β position in the DPNH could be detected. The dilutions used were such that the samples counted would have contained approximately 12,000 c.p.m. had complete equilibration of the DPNH with the protons of the medium occurred, so that 1% equilibration could have been readily detected. The radioactivity actually found in the samples in no case exceeded 6 c.p.m. per μmole corrected for dilution, a value based on a very small number of net counts over background, and therefore of dubious significance. This experiment was repeated with a mixture of 6 μmoles of DPNH and 7.5 μmoles of DPN rather than with DPNH alone. Here the specific activity of the nicotinamide samples was again very low, approximately twice the value found.

* The abbreviation used is: EDTA, ethylenediaminetetraacetic acid.
The acetylpyridine-*DPN formed when deuterated acetylpyridine-*DPN is oxidized with glutamic dehydrogenase will contain the D which had been on the alpha side of the dihydropyridine ring of the reduced analogue, and the acetylpyridine-*DPN produced by reduction with alcohol dehydrogenase will contain the D which had been on the beta side of the ring in the reduced analogue.

It was assumed that the acetylpyridine-*DPN had been prepared as the monohydrate and that, therefore, 3.33 atom % excess would correspond to 1 atom D per molecule. These values are uncorrected for purity. The samples were 95 to 98% pure based on absorption at 260 mμ, and 70 to 75% pure based on the absorption of the CN complex at 340 mμ. This difference was noted also with the initial commercial sample of acetylpyridine-*DPN.

### Table I

<table>
<thead>
<tr>
<th>Steric position in acetylpyridine-*DPN, D formed</th>
<th>Corrected atoms D per molecule</th>
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</thead>
</table>
| DPNH (α-D), 20.3 μmoles, with 0.87 atom D per molecule in the α position of the dihydronicotinamide ring, acetylpyridine-*DPN, 20.2 μmoles, EDTA, 50 μmoles, and reductase, 1.8 mg, were incubated anaerobically in 34 ml of 0.085 M Tris-chloride buffer at pH 7.9 for 6 hours at 22°. At this time 16.3 μmoles of the reduced analogue were divided into two equal portions; 1 mmole of CH₂CHO and 0.2 mg of yeast alcohol dehydrogenase were added to one portion and the pH was lowered to 7.1. The other portion was treated with 300 μmoles of NH₄Cl, 20 μmoles of α-ketoglutarate, and 1.0 mg of liver glutamic dehydrogenase. Under these conditions, the reduced nucleotide mixtures were oxidized almost instantaneously. The oxidized nucleotides were treated with Neurospora DPNase which removed nicotinamide from the DPN, instantaneously. The oxidized nucleotides were treated with alcohol dehydrogenase will retain the D which had been on the alpha side of the dihydropyridine ring of the reduced analogue, and the acetylpyridine-*DPN produced by reduction with alcohol dehydrogenase will contain the D which had been on the beta side of the ring in the reduced analogue.

The reduction conditions were the same as in 4 above, except that the reductase was omitted. After 6 hours, 3.4 μmoles of acetylpyridine-*DPN had been formed. The mixture was divided into portions and treated exactly as in 4.

The reaction conditions were the same as in 4 above except that the reductase was omitted. After 2 hours, 0.90 μ mole of acetylpyridine-*DPN had been formed. The sample was then treated exactly as in 4.

The reaction conditions were the same as in 6 above except that the reductase was omitted. After 2 hours, 0.90 μ mole of acetylpyridine-*DPN had been formed. The sample was then treated exactly as in 6.

**Notes:**
- 4. Reductase...
- 5. Reductase...
- 6. No enzyme...

**References:**
1. This experiment was performed on one-half the scale used for 4 above with DPNH (α-D) which had 0.75 atom D per molecule in the α position of the dihydronicotinamide ring. In 3 hours 5.2 μmoles of acetylpyridine-*DPN were formed. The entire reaction mixture was treated with glutamic dehydrogenase, α-ketoglutarate, and ammonia, and the acetylpyridine-*DPN was isolated for analysis as in 4.

**Deuterium Transfer from DPNH (α-D) to Acetylpyridine-*DPN**

The results of two experiments performed to determine whether or not direct hydrogen transfer occurs in the flavoprotein-catalyzed analogue reduction are shown in Table I. It was necessary to use low concentrations of DPNH (α-D) and acetylpyridine-*DPN to minimize interference from the nonenzymic reaction between these substances, which proceeds by direct transfer (5). The values shown in the last column of Table I are corrected for the amount of analogue actually reduced and for the fact that the DPNH (α-D) contained less than the theoretically expected 1 atom of D per molecule. Line 1 of Table I shows that an amount of deuterium corresponding to 0.36 atom of deuterium when DPNH alone was incubated with the enzyme. The difference is accounted for by incorporation in the control containing no enzyme, and presumably results from incorporation of tritium from the medium into position 2 of the nicotinamide of DPN in the course of the incubation (11). In a third type of experiment, a mixture of DPNH and acetylpyridine-*DPN was used. One μ mole each of DPNH and acetylpyridine-*DPN, 1.25 μ moles of EDTA, 1.0 μ mole of H₂O from which aliquots were removed for spectrophotometric analysis and for counting. The acetylpyridine-*DPN had no significant counts over the background although if all of the H used to reduce the analogue had been derived from the medium, the sample would have shown approximately 4000 c.p.m.

In the first two of these experiments, if the DPNH had been exchanging with the medium at the same rate per mole of enzyme flavin that was observed previously for the DPNH-cytochrome c reductase of mitochondria (1), the nicotinamide would have had 4000 c.p.m. per μ mole. In the third experiment, the DPNH would have been completely exchanged over almost the entire experimental interval, so that the acetylpyridine-*DPN would have incorporated a large amount of isotope from the medium either directly or indirectly. If the cytochrome b₅, reductase catalyzed exchange at the same rate, relative to its maximal turnover number, that the cytochrome c reductase does, complete exchange would have been observed in all three experiments. If the enzyme formed a complex in the reaction which did not reverse to give free DPNH and enzyme, this would explain the failure of the first two experiments to show exchange, but not that of the third. Although the results of the third experiment raised the possibility that the reduction of acetylpyridine-*DPN proceeds by direct transfer, an exchange with the medium which differs from the one observed for other flavoproteins, either by being intrinsically very slow or by being subject to a large tritium rate effect, may also occur. Recent experiments with the threonine synthetase have shown that, when homoserine phosphate is converted to threonine, protons are incorporated into the threonine at 40 times the rate at which homoserine phosphate is converted to threonine, protons are incorporated into the threonine at 40 times.
per molecule of acetylpyridine-*DPNH formed in the reaction was recovered in the acetylpyridine-*DPN sample which had been prepared for analysis by oxidation with glutamic dehydrogenase. This dehydrogenase is specific for the H on the β side of the dihydropyridine ring and thus leaves in position 4 of the oxidized pyridine nucleotide the H which had been on the α side in the reduced compound. No significant excess of deuterium was found in the acetylpyridine-*DPN produced by oxidation of the reduced analogue with alcohol dehydrogenase so as to retain the β-H of the dihydro compound (Table I, Line 2).

Lines 3 and 4 of Table I show the results of a control experiment in which the reductase was omitted. This experiment excludes the possibility that contamination of the analogue samples with DPN-4-D or nicotinamide-4-D can be responsible for the results obtained in the enzyme experiments. The small amounts of deuterium found in the acetylpyridine-4-DPN samples are of the order to be expected from the nonenzymic reaction (Table II). From the results it can be concluded that: (a) the DPNH cytochrome b₅ reductase catalyzes a direct transfer of deuterium from DPNH (α-D) to acetylpyridine-*DPN; (b) the reaction is stereospecific both for the donor and acceptor; and (c) in the reaction the same steric position is reduced in the analogue as is oxidized in the DPNH.

Although this experiment is satisfactory from a qualitative standpoint, it is inadequate from a quantitative one. If the reaction is a stereospecific direct transfer and does not pass through an intermediate which can exchange hydrogen with the protone of the medium, then, in theory, 1 atom of D should appear in acetylpyridine-4-DPN for each molecule of DPNH (α-D) oxidized. It can be seen in Table I that even after correction is made for the fact that the DPNH (α-D) contained 0.84 atom D per molecule only 0.36 atom D per molecule of acetylpyridine-*DPN is found. An experiment was performed to minimize several possible causes of the discrepancy. The time of incubation was shortened and the fraction of the acetylpyridine-*DPN formed in the reaction was recovered in the acetylpyridine-*DPN sample which had been prepared for analysis by oxidation with glutamic dehydrogenase as described in footnote a to Table I.

Deuterium Transfer from DPNH (α-D) in Nonenzymic Reaction—The distribution of deuterium between the α and β sides of the dihydropyridine ring in the nonenzymic reaction serves to differentiate it from the reductase-catalyzed reduction.

Deuterium Rate Effect in DPNH (α-D) Oxidation—The effects of substituting deuterium for hydrogen in the α position on the rates of DPNH oxidation with Fe(CN)₆³⁻ or O₂ as acceptor and on the rate of acetylpyridine-4-DPN reduction are shown in Table III. These rates have been calculated as DPNH disappearance per unit amount of enzyme to facilitate comparison. The acetylpyridine-4-DPN reduction was measured under aerobic conditions in which competition between analogue and O₂ for DPNH as well as reoxidation of the reduced analogue by O₂ occur. The reaction is slower with DPNH (α-D) as substrate when Fe(CN)₆³⁻ or acetylpyridine-4-DPN are used as acceptors, but the apparent rate effect is smaller with the analogue than with Fe(CN)₆³⁻. When the acceptor is O₂ there is no rate effect.

Because the amounts of DPNH and acetylpyridine-4-DPN used were equal and the deuterium content of the initial DPNH was 0.87 atom D per molecule.
TABLE III

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>DPNH rate</th>
<th>DPNH(a-D) rate</th>
<th>DPNH rate</th>
<th>DPNH(a-D) rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(CN)_6^3⁻</td>
<td>28,200</td>
<td>7,700</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>Acetylpyridine-*DPN</td>
<td>3.0</td>
<td>1.7</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>O_2</td>
<td>1.4</td>
<td>1.3</td>
<td>1.06</td>
<td></td>
</tr>
</tbody>
</table>

* Absorbancy changes at 340 mp were read at 15-second intervals in systems containing 0.02 µmole of reduced pyridine nucleotide, 10⁻⁸ M cytochrome b₅ reductase, and 0.06 µmole of potassium ferricyanide aerobically in 0.2 ml of 0.1 M Tris-acetate and 0.001 M EDTA buffer, pH 8.1.

* Assay systems were the same as for a except that 5 X 10⁻⁶ M enzyme was used and 0.02 µmole of acetylpyridine-*DPN was used for the potassium ferricyanide. The absorbancy changes at 460 mp were recorded.

* Assay systems were the same as for a except that 5 X 10⁻⁴ M enzyme was used and the potassium ferricyanide was omitted.

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**DISCUSSION**

This is the first report of a direct transfer of hydrogen which is dependent upon the presence of a flavin coenzyme. Vennesland (14) reported results indicating the transfer of deuterium from acetaldehyde-1-D to DPN by xanthine oxidase. There are several examples of reduction of the oxidized form of one pyridine nucleotide by the reduced form of another which proceed by a direct transfer or succession of such transfers. The *Pseudomonas* transhydrogenase catalyzes a direct transfer from DPNH (β-D) to TPN (15), but as far as is known this enzyme is not a flavoprotein. The steroid-mediated transhydrogenations, whatever the details of their mechanism, involve a direct transfer (16), which is presumably of the type familiar in pyridine nucleotide-linked dehydrogenases. The nonenzymic reduction of acetylpyridine-*DPN* by DPNH involves direct transfer of the hydrogen (5).

Several of the properties of the DPNH cytochrome b₅ reductase are pertinent to a consideration of the mechanism of the hydrogen transfer which it catalyzes. Previously reported experiments (8, 13, 17) support a hypothesis for the course of the analogue reduction which is indicated schematically as follows:

\[ \text{SH} \quad \text{Enzyme} - \text{FAD} + \text{DPNH (a-D)} \rightarrow \text{Enzyme} - \text{FAD} + \text{H}^+ \quad \text{Enzyme} - \text{FADH}_2 (D) \]

Complex A

\[ \text{S ...DPNH (a-D)} \quad \text{S ...DPN} \]

Complex B

\[ \text{acetylpyridine-*DPN} \]

\[ \text{Enzyme} - \text{FADH}_2 (D) + \text{DPN} \]

The enzyme binds tightly 1 mole of DPNH per mole of flavin. Spectrophotometric titrations show that as DPNH is bound to the flavoprotein, there is a stoichiometric disappearance of the absorbance peaks of the oxidized FAD and of the reduced pyridine nucleotide, with the concomitant appearance of a new peak at 317 mp. This substance with the spectral properties of a reduced flavin, oxidized pyridine nucleotide, enzyme complex is represented as Complex B. The binding of DPNH, the oxidative reaction, and the analogue reduction are all blocked by titration of a single sulfhydryl group of the protein. Recent experiments (9, 18) indicate that FAD is not required for the DPNH binding but that the sulfhydryl group is essential; this suggests that DPNH is bound at the same site in the apoprotein as in the complete enzyme. There is no evidence for the presence on the enzyme of a binding site specific for oxidized pyridine nucleotides.

The mechanism illustrated in the scheme, in which DPN is displaced from the oxidized nucleotide, reduced flavin Complex B by acetylpyridine-*DPN*, would account for the observed stereospecificity of the hydrogen transfer, the requirement for the integrity of the sulfhydryl group which is also necessary for DPNH binding and the normal oxidative reaction, and the requirement for the presence of the flavin. When a reduced flavin intermediate is postulated for the reaction, it becomes necessary to account for the fact that appreciable exchange does not occur between the N—H (or O—H) hydrogens of the reduced flavin...
Fig. 1. Reduction of acetylpyridine-*DPN by DPNH and apoenzyme in presence and absence of FAD. The formation of acetylpyridine-*DPN was measured by the increase in absorbancy at 400 mp during aerobic incubations at 25°. Each tube contained 0.02 amole each of DPNH and acetylpyridine-*DPN in 0.20 ml of 0.1 M Tris-acetate buffer at pH 8.1. In addition, for Curve 1, the tube contained 0.004 amole of FAD and 0.0004 amole of aporeductase; for Curve 2, 0.0004 amole of aporeductase; and for Curve 3, 0.004 amole of FAD. After 3 minutes, 0.004 amole of FAD was added to trial 2.

and the protons of the medium, because rapid exchange of these hydrogens of reduced free flavins is generally assumed but has not been tested. In the reduced enzyme-pyridine nucleotide complex, one or both of the hydrogens may be stabilized by hydrogen bonding, may be “buried” out of easy contact with the solvent, or may be perhaps directly involved in bonding in the stable complex between the pyridine and the flavin nucleotides. The information so far available is so meager that it would be premature to postulate a detailed structure for the complex.

One alternative to this mechanism would involve the flavin only as a structural part of the protein, which would provide the configuration necessary for direct hydrogen transfer from bound reduced nucleotide either by collision with the oxidized analogue, or by reaction with acetylpyridine-*DPN bound on an unknown second binding site specific for oxidized nucleotides. If a collision mechanism is assumed, it is difficult to account for the observed stereospecificity of the reduction of the analogue; there is no evidence with this enzyme for the existence of the oxidized pyridine nucleotide-binding site which would be required by the second formulation. Both of these mechanisms, which assume a merely structural role for FAD and do not require a displacement of the oxidized pyridine nucleotide, presuppose reaction of Complex A, which could constitute only a minute fraction of the protein, since in the presence of the reduced nucleotide the enzyme is virtually completely in the form of Complex B, in which the spectral changes strongly suggest that the flavin is completely reduced and the pyridine nucleotide oxidized. If the spectral characteristics of Complex B for some unknown reason do not in fact represent a situation in which the hydrogen is no longer bound to the pyridine nucleotide in covalent linkage, this complex could participate in these alternative mechanisms.

If the mechanism shown in the scheme is involved in the analogue reduction reaction, it would follow that the hydrogen is directly transferred to flavin in the course of the oxidative reaction catalyzed by the reductase. This hydrogen would then be lost as a proton when the reduced flavin is oxidized by Fe(CN)₆³⁻ or cytochrome b₅.

This enzyme is not necessarily a special case among flavoproteins with regard to the catalysis of direct transfer, except that in this example direct transfer seems to occur to the apparent exclusion of exchange. It is obvious that it might be very difficult to detect hydrogen transfer, when it is catalyzed by enzymes which involve reduced intermediates, the hydrogens of which can exchange with the medium, because if exchange of the intermediate occurs rapidly enough, no transfer will be observed. On the other hand, if any hydrogen can be proved to have passed from reactant to product, transfer, in the sense used in this paper, can be assumed.

**SUMMARY**

The reduction of the acetylpyridine analogue of diphosphopyridine nucleotide by reduced diphosphopyridine nucleotide, catalyzed by the microsomal cytochrome b₅ reductase, has been shown to involve a direct and stereospecific hydrogen transfer from the α side of reduced diphosphopyridine nucleotide to the α side of the acetylpyridine analogue.

The analogue reduction, and, therefore, the direct hydrogen transfer, is completely dependent on the presence of flavin in the enzyme.

To account for the flavin dependence, the stereospecificity, and the direct transfer observed in this reaction, a mechanism involving nucleotide displacement from the previously described pyridine nucleotide-enzyme complex has been considered.

**REFERENCES**

10. Colowick, S. P., and Kaplan, N. O., in S. P. Colowick and