STUDIES WITH HYDROGEN ISOTOPES ON THE MECHANISM OF ACTION OF COBAMIDE-DEPENDENT RIBONUCLEOTIDE REDUCTASE.[+]

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The reduction of ribonucleotides to deoxyribonucleotides in bacteria has been the subject of intensive investigation in a number of laboratories over recent years. Two enzyme systems have been described in considerable detail : that of Escherichia coli which catalyses the reduction of ribonucleoside diphosphates according to Reaction 1 (Holmgren, Reichard and Thelander, 1965), and that of Lactobacillus leichmannii which catalyses the reduction of ribonucleoside triphosphates according to Reaction 2 (Blakley, Ghambeer, Nixon and Vitols, 1965).

$$CDP + R(SH)_2 \longrightarrow dCDP + RS_2 \qquad (1)$$

$$ATP + R(SH)_2 \xrightarrow{CoB_{12}} dATP + RS_2 \qquad (2)$$

Dihydrolipoate may act as the dithiol, $R(SH)_2$, in Reactions 1 and 2. However, there is considerable evidence that in E. coli the physiological dithiol reductant is

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thioredoxin, a low molecular weight protein which contains two half-cystine residues (Laurent, Moore and Reichard, 1964). Evidence has been obtained that a similar protein is present in L. Leichmannii and participates in Reaction 2 (Blakley, 1966). Thioredoxin acts catalytically in the bacteria since there is present in the bacteria a flavoprotein, thioredoxin reductase, which catalyses conversion of thioredoxin to its reduced form according to Reaction 3 (Moore, Reichard and Thelander, 1964).

$$\text{Thioredoxin} + \text{NADPH} + H^+ \longrightarrow \text{dihydrothioredoxin} + \text{NADP}^+ \quad (3)$$

Cobamides are not involved in the reduction of CDP according to Reaction 1. This Reaction is catalysed by two proteins which have been partially purified from E. coli B (Holmgren, Reichard and Thelander, 1965). The enzyme catalysing Reaction 2 has been partially purified and shows an absolute dependence on coenzyme B_{12} (5'-deoxyadenosyl cobalamin) but not on other cofactors (Vitols and Blakley, 1965). As yet there is no evidence to suggest that more than one protein is involved in Reaction 2.

In considering the mechanism of Reactions 1 and 2 a point of interest is the manner in which coenzyme B_{12} participates in Reaction 2. The question of whether the participation of coenzyme B_{12} results in a mechanism different from that of Reaction 1 is one to which we have recently directed our attention. Little is known of the mechanism of Reaction 1 but Larrson (1965) has demonstrated that when the reduction of CDP occurs in the presence of tritiated water, tritium becomes incorporated into dCDP at position 2'. If the role of coenzyme B_{12} in Reaction 2 does not result in a mechanism basically different from that of Reaction 1 a similar incorporation of

isotope into position 2' of the product might be expected to occur when Reaction 2 proceeds in the presence of tritiated or deuterated water. On the other hand, the participation of the cobamide might result in a different mechanism. One such mechanism is suggested by the cobamide-dependent glycol dehydrase reaction (Reaction 4) which occurs in Aerobacter aerogenes (Abeles and Lee, 1964).

$$R.CHOH.CH_2OH \xrightarrow{CoB_{12}} R.CH_2.CHO + H_2O \qquad (4)$$

A similar dehydration of the ribose moiety of ribonucleotides might produce a 2'-deoxy-3'-keto-pentose derivative and subsequent reduction of this intermediate would then yield the 2'-deoxyribonucleotide. In such a mechanism the reductive step would result in the incorporation of hydrogen isotope from thiol (which is in rapid equilibrium with water hydrogen) at position 3' of the deoxyribonucleotide.

We wish to report that when Reaction 2 is catalysed by partially purified enzyme from L. leichmannii hydrogen isotope from tritiated or deuterated water is incorporated into the deoxyribonucleotide, and that proton magnetic resonance studies indicate that deuterium incorporated in this manner is attached at position 2' rather than position 3' of the nucleotide.

METHODS

Extracts of L. leichmannii grown under optimum conditions for the production of ribonucleotide reductase were prepared by the use of the Ribi pressure cell. The reductase was purified by protamine sulfate treatment, ammonium sulfate fractionation, ethanol fractionation and chromatography on hydroxylapatite. The partially purified enzyme had a specific activity of 70-100 μmoles of ATP reduced per mg of protein per

hour at 37° under standard conditions. Enzyme reaction mixtures for experiments with D_2O contained 10 or 20 mM ATP (total of 200 μmoles), 0.3 M 3,3-dimethylglutarate buffer, pH 7.3, 30 mM dihydrolipoate, 1 mM EDTA, 8 μM deoxyadenosyl cobalamin and 6-30 mg of enzyme preparation in a total volume of 10 or 20 ml. In experiments with tritiated water the latter had a specific activity of 9×10^5 cpm per μmole in Bray's scintillation fluid (Bray, 1960) in a Packard Tricarb scintillation counter. Reaction mixtures contained 0.52 mM ATP, 0.3 M 3,3-dimethylglutarate buffer, pH 7.3, 30 mM dihydrolipoate, 1 mM EDTA, 0.5 to 1 mg of enzyme and either 8 μM or 0.8 mM deoxyadenosyl cobalamin in a total volume of 0.25 ml. In experiments with deuterated water the solution of all reaction components except enzyme was freeze-dried and dissolved in 99.7% D_2O. After standing to allow exchange of hydrogen with the solvent the solution was again freeze-dried. This process was repeated three times. Water in the enzyme solution was replaced by D_2O either by repeated dialysis against 2 volumes of D_2O, or by freeze-drying once and dissolving the residue in D_2O. About 75% of the enzymic activity was retained after freeze-drying, but the yield of dATP when the reaction was carried out in D_2O was much lower than expected (20 μmoles from 200 μmoles of ATP). The cause of these low yields is being investigated.

The deoxyadenosine from the enzymically produced dATP was isolated as follows. The reaction mixture was freeze-dried and the residue dissolved in water. In some experiments cobamides were then removed by phenol extraction and residual phenol was removed by ether extraction. The nucleotides were then dephosphorylated by incubation with E. coli phosphatase (Worthington, chromatographically purified). Salts were

removed by adsorbing the nucleoside on a column (5.5 x 1 cm) of acid-washed Nuchar. After washing with water until the effluent no longer contained significant amounts of salt as determined by conductivity measurements, the nucleoside was eluted by 40% (v/v) ethanol. The solution was evaporated to dryness under reduced pressure, the residue dissolved in 0.01 M borax and the solution passed through a column of Dowex-1-chloride (x2, 200-400 mesh, 24 x 1.5 cm). Deoxyadenosine was eluted by 0.02 M borax containing 0.03 M KCl. The deoxyadenosine was freed of salt as before and in the case of deuterated samples freeze-dried from D_2O three times.

Proton magnetic resonance spectra of solutions in D_2O were determined at 60 Mc/sec. and 33.5° on a Perkin-Elmer R10 Spectrometer. Chemical shifts, measured from 3-trimethylsilyl-1-propanesulphonic acid (sodium salt) as internal standard are quoted in τ units. With 2'-deoxyadenosine, two drops of trifluoroacetic acid were added to the solution to shift the HDO peak and reveal the H-3' multiplet. Unfortunately, similar treatment of the very dilute solution of deuterated deoxyadenosine caused the resolution to deteriorate so that useful results could not be obtained.

RESULTS AND DISCUSSION

When the enzymic reduction of ATP was carried out in the presence of tritiated water the deoxyadenosine moiety of the product was found in four experiments to have incorporated an average of 0.23 atom of tritium from the solvent per molecule of deoxyadenosine. This result is similar to that reported by Larrson (1965) for the *E. coli* system in which 0.3 atom of tritium was incorporated per molecule of dCDP.

Since it has been suggested that deoxyadenosyl cobal-

amin acts as a hydrogen carrier in some enzymic reactions, we also investigated the effect of greatly increasing the concentration of deoxyadenosyl cobalamin on the incorporation of hydrogen isotope from water into the solvent. In the presence of 8 μM coenzyme the tritium incorporation into dATP was 0.25 atom per molecule; with 0.8 mM coenzyme incorporation was 0.22 atom per molecule. This result therefore indicates that the hydrogen transferred to the nucleotide is not incorporated into a non-exchanging position on the free cobamide during the transfer process.

In order to demonstrate the location of the tritium label in the deoxyribonucleoside, it is necessary to resort to a relatively lengthy degradation procedure which converts the deoxyribose moiety to a number of products in which hydrogen cannot exchange with the solvent. Such a procedure has been worked out by Larrson (1965). We have used the simple procedure of carrying out the enzymic reduction of ATP in the presence of D_2O and establishing the location of deuterium in the product by determining the p.m.r. spectrum of deoxyadenosine derived from the enzymically formed dATP.

The general features of the p.m.r. spectra of the sugar moieties in 2'deoxyribosides have been firmly established (Jardetsky, 1960; Schweizer, Chan and Ts'o, 1965; Gatlin and Davis, 1962) and assignment of protons to the observed multiplets can be made with certainty. The p.m.r. spectrum of unlabeled 2'deoxyadenosine in D_2O is shown in Figure 1 (II) with peak assignments indicated. H-1' gives rise to a triplet (τ 3.56, J = 6.5 c/sec.) in this spectrum but only a doublet (τ 3.50, J = 6.8 c/sec.) in that of the deuterated compound (Figure 1 (I)), clearly demonstrating that only one proton is

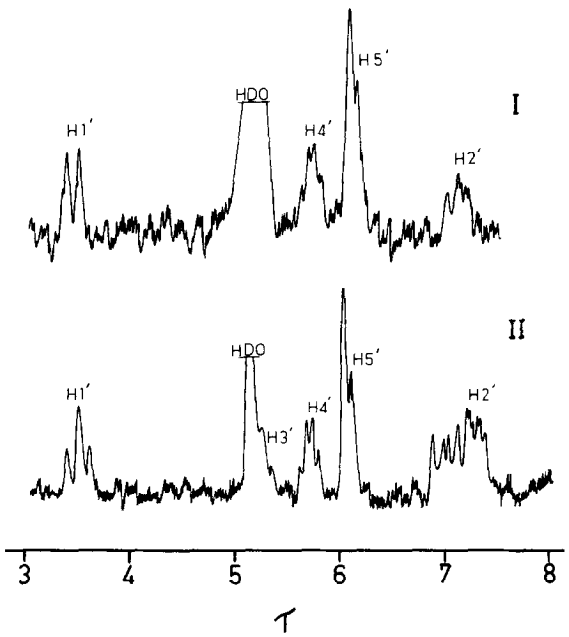

FIGURE 1. Proton magnetic resonance spectra of 2'-deoxyadenosine (II) and deuterated 2'-deoxyadenosine (I) in D_2O.

present on C-2' in the deuterated compound. This is confirmed by the decrease, on deuteration, in the intensity of the multiplet near τ 7.20, assigned to H-2'. Unfortunately, the multiplet due to H-3' in the spectrum of the deuterated deoxyadenosine coincides with the large HDO peak and cannot be seen. However, deuteration at C-3' could not give rise to the observed changes in the spectrum.

Although the data clearly indicate that the enzymically synthesised compound contains deuterium at position 2', it remains to be considered whether deuterium is also incorporated into position 3'. This is a particularly important point, since it is conceivable that the incorporation into position 2' might occur by enolization of the hypothetical 2'-deoxy-3'-ketopentose while reduction of the latter would lead to

deuterium incorporation at position 3' as well. Although the peak due to H-3' is obscured in Figure 1, the fact that the splitting of the peak assigned to H-4' is unchanged in the deuterated compound indicates that there has been no replacement of hydrogen by deuterium at position 3'. This has been confirmed by comparison of the p.m.r. spectra of the unlabeled and the deuterated deoxyadenosine in dimethylsulfoxide. In this solvent the H-3' multiplet is clearly separated from other peaks and is unchanged in the deuterated compound.

This result clearly indicates that the cobamide-dependent ribonucleoside triphosphate reductase reaction does not proceed via a 2'-deoxy-3'-keto-pentose derivative which is subsequently reduced to the corresponding 2'-deoxyribonucleotide. Although analogy with the glycol dehydrase reaction might suggest such a mechanism, the ribonucleotide reductase mechanism appears on the contrary to be similar for the L. leichmannii enzyme to that for the non-cobamide E. coli enzyme since in both cases hydrogen from water is incorporated into position 2' of the deoxyribonucleotide. Our results do not completely exclude the possibility that the cobamide-dependent ribonucleotide reductase catalyses an intramolecular hydrogen transfer from the 3' to the 2' position. The occurrence of such a hydrogen transfer would provide a parallel between the action of this enzyme and a number of other cobamide-dependent enzymes such as glutamate mutase (Barker, Suzuki, Iodice and Rooze, 1964), methylmalonyl CoA mutase (Erfle, Clark and Johnson, 1964) and glycol dehydrase (Brownstein and Abeles, 1961) which catalyse such intramolecular hydrogen transfers. Although our present data make it appear improbable that such an intramolecular hydrogen transfer does occur in the ribonucleotide

reductase reaction we are examining this possibility further.

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