Ribonucleotide Reductase. Studies with ^{18}O-Labeled Substrates*

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ABSTRACT: Adenosine triphosphates labeled with oxygen-18 have been used as substrates for the deoxyadenosylcobalamin-dependent ribonucleotide reductase of *Lactobacillus leichmannii* in the presence of dihydrolipoate as reductant. The isotope distribution in the deoxyadenosine phosphates formed was analyzed by mass spectrometry after their conversion into deoxyadenosine. Reduction of [2′-^{18}O]adenosine triphosphate to deoxyadenosine triphosphate results in complete loss of the isotope from the nucleotide, whereas [3′-^{18}O]adenosine triphosphate is reduced to [3′-^{18}O]deoxyadenosine triphosphate with the same isotope content. Thus in contrast to the deoxyadenosylcobalamin-dependent diol-dehydrase reaction where migration of a hydroxyl group occurs between two adjacent carbon atoms, no such transfer of oxygen takes place during enzymatic ribonucleotide reduction. If dihydrolipoate is omitted from a reaction mixture, ribonucleotide reductase does not catalyze exchange of heavy oxygen between [2′-^{18}O]adenosine triphosphate and water. These results and earlier studies using isotopes of hydrogen indicate that displacement of the hydroxyl function at C-2′ of a ribonucleotide by hydrogen proceeds in a concerted SN1-type reaction in which the carbon–oxygen bond is not broken without concomitant reduction of the substrate.

The reduction of ribonucleotides to deoxyribonucleotides is catalyzed by two different enzyme systems. Ribonucleoside diphosphate reductase has been purified from *Escherichia coli* and from Novikoff hepatoma, while ribonucleoside triphosphate reductase was isolated from *Lactobacillus leichmannii* and partially purified from several rhizobia (Reichard, 1968; Blakley, 1966; Cowles *et al.*, 1969). The main differences between these two systems are the phosphorylation level of the substrates and the requirement for 5′-deoxyadenosylcobalamin in the *L. leichmannii* enzyme. However, the over-all reduction process is very similar in both cases: TPNH is the ultimate reductant, and the hydrogen is transferred by thioredoxin reductase to thioredoxin which in turn is oxidized by ribonucleotide reductase with the concomitant reduction of the ribonucleotide. In both enzyme systems thioredoxin can be replaced by dithiols such as dihydrolipoate. Furthermore, the mechanism of reaction appears to be identical: the hydroxyl function of a ribonucleotide is replaced by hydrogen under retention of configuration at carbon 2′ during both reductions.

All the reactions in which 5′-deoxyadenosylcobalamin participates as a coenzyme involve a transfer of hydrogen, the displacement of another group (alkyl, hydroxyl, or amino group), and the formation of a new carbon–hydrogen bond (Hogenkamp, 1968). For example, in the dioldehydrase reaction (Lee and Abeles, 1963), a hydrogen is transferred from C-1 to C-2 of propane-1,2-diol *via* the 5′-methylene group of the coenzyme. At the same time, the C-2 hydroxyl function migrates to C-1 under formation of propane-1,1-diol which is then dehydrated to propionaldehyde. In this mechanism, the participation of the two hydroxyl groups in the intramolecular redox reaction has been demonstrated using ^{18}O-labeled propanediol (Retey *et al.*, 1966). Although the reductant (dihydrolipoate) and the oxidant (nucleoside triphosphate) are different compounds in the ribonucleotide reductase system, hydrogen transfer also occurs *via* the 5′-methylene group of deoxyadenosylcobalamin. The structural similarity of the substrates containing a 1,2-diol system or ribose, respectively, suggests that in ribonucleotide reduction both vicinal hydroxyl groups likewise participate in the reaction. This hypothesis appears to be substantiated by the finding of Suhadolnik *et al.* (1968) that 3′-deoxyadenosine 5′-triphosphate is not reduced by a reductase preparation of *L. leichmannii*.

In order to further investigate the mechanism of the reduction of ribonucleotides, we have synthesized ATP labeled with heavy oxygen in either the 2′ or 3′ position. In the present work, these two labeled nucleotides have been used as substrates in the *L. leichmannii* ribonucleotide reductase system, and the isotope distribution in the products has been determined.

Experimental Section

Materials and Methods. Various compounds were obtained from commercial sources as follows: nucleosides and nucleotides, Sigma and P-L Biochemicals; DL-lipoic acid, Sigma; and bacterial alkaline phosphatase, Worthington. Dihydrolipoate was prepared by sodium borohydride reduction of lipoic acid (Gunsalus and Razzell, 1957) without distillation of the product. Deoxyadenosylcobalamin was prepared by the procedure of Hogenkamp and Pailes (1968). Adenosine triphosphates labeled with oxygen-18 were synthesized as described by Follmann and Hogenkamp (1970). Ribonucleotide reductase purified from extracts of *L. leichmannii* (Vitols *et al.*, 1967a,b) was kindly supplied by Dr. R. L. Blakley. This preparation had a specific activity of 70 μmoles/hr per mg of protein.

Absorption spectra were recorded with a Cary Model 15 and other spectral measurements were made with a Zeiss PMQ II spectrophotometer. The purity of nucleosides and

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nucleotides was established by spectral analysis and by paper chromatography in two solvent systems. Chromatography was performed by the descending technique on Whatman No. 1 paper in 1-butanol saturated with water and in ethanol–1 M ammonium acetate (5:2, v/v), respectively. Oxygen-18 analyses were obtained using an Atlas CH-4 low-resolution mass spectrophotometer at 11-eV electron energy and a probe temperature of about 190°.[1] After correction for the small naturally occurring M + 2 peaks, the atom % excess ^{18}O of labeled nucleosides was calculated from the various molecular and fragment ions by using the equation, % ^{18}O = (M + 2) × 100/M + (M + 2).

Enzymic Reduction of Labeled ATP. A reaction mixture containing 0.3 M dimethyl glutarate buffer (pH 7.2), 30 mM dihydrolipoate, 1 mM EDTA, 0.2 mM dGTP as specific effector of ATP reduction, 0.04 mM deoxyadenosylcobalamin, 10 mM [3′-^{18}O]ATP, and 12 mg of ribonucleotide reductase in a total volume of 30 ml was incubated in the dark at 37°. The reaction was initiated by adding the coenzyme, and the rate of dihydrolipoate oxidation was followed by the absorbance increase at 333 mμ. After 12 hr, 10 ml of 0.1 N sodium periodate was added to oxidize residual ATP. After incubation for 30 min at 37°, the excess periodate was destroyed by adding 2.5 ml of 0.5 M glucose and incubating the mixture for 30 min. The solution was adjusted to pH 10 with 1 M glycine buffer (pH 10.2), incubated at 37° for 10 hr to decompose the aldehydic oxidation products, and then applied to a column (45 × 2.5 cm) of Dowex 1-X2 (Cl^-), 50–100 mesh. The column was washed with water and eluted first with 0.01 N HCl–0.08 N LiCl to remove a small amount of adenine resulting from the oxidation of unreacted ATP and next with 0.01 N HCl–0.2 N LiCl to elute the desired dATP (Lehman *et al.*, 1958). The eluate was neutralized with ammonium hydroxide. The pooled fractions containing the deoxyribonucleotide (yield, 193 μmoles, 64%) were adjusted to pH 3.5 with formic acid, treated with 5 g of acid-washed charcoal (Smith and Khorana, 1958), and filtered. The charcoal pad was washed with water adjusted to pH 4.5 with formic acid, and the nucleotide was then eluted with ammoniacal aqueous ethanol (50% ethanol containing 10 ml of concentrated NH_4OH/l.). The yield was 140 μmoles. The solution was evaporated to dryness, the residue dissolved in 30 ml of water, and the pH adjusted to 8.2 with 0.1 N NaOH. Alkaline phosphatase (5 mg) was added to the solution and the pH was maintained at 8.2 with 0.1 N NaOH. After the uptake of alkali had ceased, the solution was freeze dried. The residue was dissolved in 4 ml of water and applied to a column (130 × 1 cm) of Sephadex G-10. After two small protein peaks which also contained the inorganic phosphate, deoxyadenosine was eluted with water in a prominent peak. Freeze drying of the pooled fractions yielded 35 mg (139 μmoles) of fluffy white material.

A second reduction was carried out using 275 μmoles of [2′-^{18}O]ATP. Deoxyadenosine was isolated as described above, yield 28 mg (111 μmoles).

Reduction of Ribonucleotide Reductase. Ribonucleotide reductase (12 mg) was incubated in 0.3 M dimethyl glutarate buffer (pH 7.2, 1.0 ml) containing 50 mM dihydrolipoate and 1 mM EDTA at 0° for 30 min. The enzyme solution was then dialyzed against 250 ml of deaerated 0.05 M dimethyl glutarate buffer containing 1 mM EDTA for 90 min. This dialysis was repeated nine times to ensure complete removal of reductant. The reduced enzyme was fully active in the catalysis of hydrogen exchange from [5′-$^3H_2$]deoxyadenosylcobalamin to water in a complete system (Hogenkamp *et al.*, 1968).

Reisolation of ATP. A reaction mixture containing 0.3 M dimethyl glutarate buffer (pH 7.2), 1 mM EDTA, 0.2 mM dGTP, 0.04 mM deoxyadenosylcobalamin, 15 mM [2′-^{18}O]-ATP, and 12 mg of reduced ribonucleotide reductase in a total volume of 10 ml was incubated anaerobically in the dark at 37°. After 2 hr, the solution was boiled for 5 min and the coagulated protein was removed by centrifugation. The supernatant was diluted fivefold and applied to a column (50 × 2 cm) of DEAE-cellulose (HCO_3^-). The column was washed with 0.05 M triethylammonium bicarbonate and nucleotides were then eluted with a gradient of triethylammonium bicarbonate (0.05–0.45 M). After a small amount of dATP (5 μmoles), 145 μmoles of ATP was recovered and rendered salt free by repeated evaporation *in vacuo*. Adenosine triphosphate was dephosphorylated with phosphatase and adenosine was desalted on Sephadex G-10 as described above, yield 30 mg (112 μmoles).

Results

Deoxyadenosine triphosphate formed by enzymatic reduction of ^{18}O-labeled ATP was converted into deoxyadenosine and the deoxyriboside was analyzed by mass spectrometry at low electron energy. In Table I are presented the isotope-containing fragments obtained from [2′-^{18}O]- and [3′-^{18}O]-adenosine and from both 2′-deoxyadenosine preparations derived from [2′-^{18}O]- and [3′-^{18}O]ATP. The fragmentation pattern is in accord with the mass spectra of adenine nucleosides reported before (Biemann and McCloskey, 1962; Caprioli and Rittenberg, 1968). Large isotope peaks appear at two mass units higher than the normal ion if ^{18}O is present in a fragment. The naturally occurring M + 2 isotope peaks of the unlabeled fragments were too small to be measured, and in Table I the calculated values are given. It is evident from these data that the adenosine preparations used for the synthesis of 2′- and [3′-^{18}O]ATP were labeled solely in the 2′ and 3′ position and that only the deoxyadenosine preparation derived from [3′-^{18}O]ATP was labeled. In Table II are shown the per cent excess heavy oxygen in the substrates and products of the ribonucleotide reductase reactions. It is clear that with [2′-^{18}O]ATP as substrate, all the label is lost from the 2′ position during reduction and that deoxyadenosine derived from [3′-^{18}O]ATP has retained virtually all of the heavy oxygen in the 3′ position.

In another experiment, [2′-^{18}O]ATP was incubated with ribonucleotide reductase in the presence of all reaction components but dihydrolipoate, and the substrate was recovered and converted into adenosine. Because in the standard reaction mixture dihydrolipoate also serves to reduce ribonucleotide reductase to its active form (Vitols *et al.*, 1967a,b), in this experiment an enzyme preparation was used which had been reduced separately and dialyzed before incubation with substrate. The data included in Table II demonstrate that the reduced enzyme does not promote an

[1] We are indebted to Dr. K. L. Rinehart, University of Illinois, Urbana, Ill., for his assistance.

TABLE I: ^{18}O Distribution and Relative Peak Heights of Fragments in the Mass Spectra of Adenine Nucleosides (Intensities as Per Cent of the Molecular Ion M^+).

Nucleoside	Ion	Mass	Containing O atoms	Nucleoside labeled in 2′	Nucleoside labeled in 3′
Adenosine[a]	M^+	267	2′, 3′, 4′, 5′	100	100
	$(M + 2)^+$	269		37	52
	$(M - 30)^+$	237	2′, 3′, 4′	150	135
	$[(M - 30) + 2]^+$	239		58	70
	$(M - 89)^+$	178	2′	163	120
	$[(M - 89) + 2]^+$	180		60	(0.62)[b]
Deoxyadenosine	M^+	251	3′, 4′, 5′	100	100
	$(M + 2)^+$	253		(1.39)[b]	24
	$(M - 30)^+$	221	3′, 4′	100	150
	$[(M - 30) + 2]^+$	223		(1.05)[b]	37

[a] The [^{18}O]ATP synthesized from these two nucleosides was diluted with unlabeled ATP prior to enzymatic reduction. [b] Calculated values for the naturally occurring M + 2 isotope peak.

exchange of labeled oxygen from the ribonucleotide in the absence of substrate reduction.

Discussion

Earlier studies of enzymatic ribonucleotide reduction using isotopes of hydrogen (Batterham *et al.*, 1967; Durham *et al.*, 1967) and our present results from the experiments using heavy oxygen permit better insight into the reactions involving the ribonucleotide substrate. If the enzymatic reduction is carried out in deuterated or tritiated water, only the 2′α hydrogen atom of the 2′-deoxyribonucleotide is labeled, indicating that the reaction proceeds with retention of configuration at C-2′ of a nucleotide. These results exclude reaction mechanisms involving elimination of water and introduction of a 2′,3′ or 1′,2′ double bond into the ribose ring, followed by hydrogenation of the double bond. They also eliminate a classical bimolecular nucleophilic substitution (SN2) of the hydroxyl function at C-2′ by a hydride ion which would result in inversion of configuration at that carbon atom. More recent electron paramagnetic resonance studies by Hamilton and Blakley (1969) seem to indicate that a ribonucleotide radical is not involved in the reduction process.

TABLE II: Atom % Excess ^{18}O in Nucleotides before and after Reduction with Ribonucleotide Reductase from *L. leichmannii*.[a]

Substrate	%	Product	%
[2′-^{18}O]ATP	16.1	dATP	0.0
[3′-^{18}O]ATP	19.1	[3′-^{18}O]dATP	18.6
[2′-^{18}O]ATP	18.0	[2′-^{18}O]ATP	18.8

[a] The values were calculated from the mass spectra of the corresponding nucleosides (Table I) and are an average from six to ten different spectra. Average error, ±0.5%.

In Scheme I, two alternate ionic mechanisms for the reduction of ribonucleotides to deoxyribonucleotides are summarized. The first mechanism depicts a SN1 reaction at C-2′, resulting in direct displacement of the hydroxyl group by hydrogen, possibly as a hydride ion provided by the reducing agent. Such a reaction is unlikely without prior activation of the 2′-hydroxyl group, but protonation would decrease its basicity and thus increase its ability as a leaving group. Verheyden and Moffatt (1964) proposed that displacement of the hydroxyl group could also be facilitated by phosphorylation; however, cytidine 2′-phosphate 5′-diphosphate synthesized by these workers did not serve as a substrate for ribonucleoside diphosphate reductase of *E. coli*.

The second mechanism is suggested by the similarity between ribonucleotide reduction and the oxidation–reduction reaction catalyzed by dioldehydrase. This mechanism differs from the preceding one in the initial dissociation of the protonated 3′- rather than the 2′-hydroxyl function; the resulting carbonium ion at C-3′ is then attacked by the neighboring hydroxyl group to yield a protonated epoxide as an intermediate or transition state. 2′,3′-Anhydronucleosides of this type have been described (Benitez *et al.*, 1960). Reductive ring opening would lead to a deoxyribonucleotide in which the 3′-hydroxyl function is derived from the 2′-oxygen of the ribonucleotide. Our results are clearly not consistent with such a reaction sequence, since reduction is not accompanied by a distribution of label between the 2′ and 3′ positions. Thus the dioldehydrase and ribonucleotide reductase reactions proceed by basically different mechanisms.

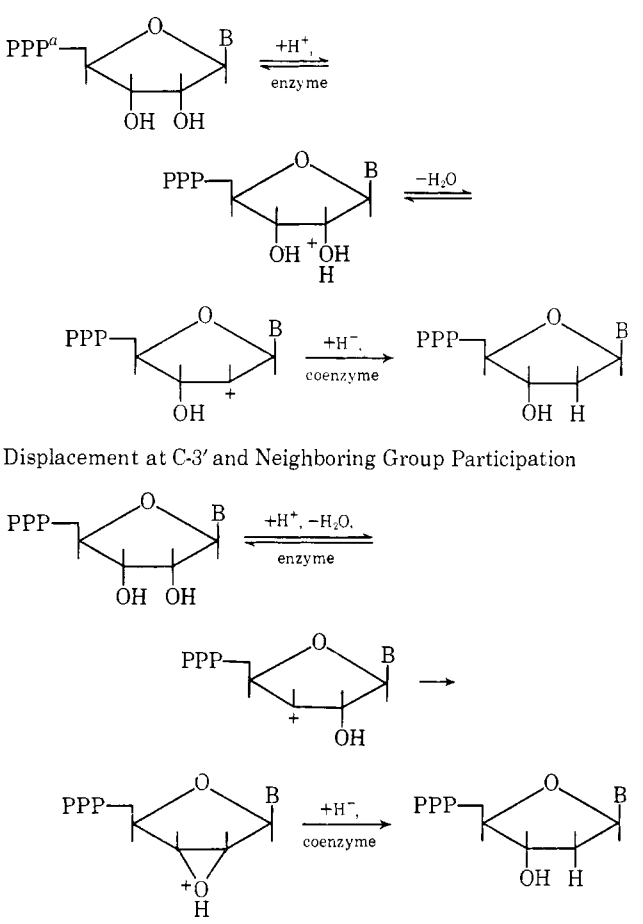
The labeling pattern of ribonucleotide reduction products with both oxygen and hydrogen isotopes is in agreement with a monomolecular nucleophilic substitution reaction such as shown in the first mechanism of Scheme I. In a classical SN1 reaction, a reversible dissociation of the carbon–oxygen bond would be the rate-determining step, followed by nucleophilic attack of a hydride ion on the carbonium ion. Thus if all reaction components except the attacking nucleophile are included in a reaction mixture, an exchange of the nucleotide 2′-hydroxyl group with water should result without reduction

of the substrate. However, all label is retained in [2′-^{18}O]ATP when it is incubated with only the enzyme and coenzyme for a period of time sufficient for reduction in a complete system. Therefore, under these conditions, a carbonium ion is not formed to any detectable extent. Apparently the formation of deoxyribonucleotides proceeds in a concerted substitution reaction of the SN1 type in which the leaving group dissociates only when the attacking nucleophile is present.

Such a mechanism requires a highly ordered situation around C-2′ if an enzyme group is responsible for protonation and removal of the hydroxyl group and the coenzyme functions as the reducing agent as previously proposed (Hogenkamp *et al.*, 1968). In this context, the requirement of the 3′-hydroxyl group in the reaction (Suhadolnik *et al.*, 1968) is best explained in that it provides an additional binding site for the enzyme, thus holding the substrate in a defined position for the reduction to occur. Another possible role for the 3′-hydroxyl group could be to stabilize the postulated carbonium ion at C-2′ by distributing the charge in an epoxide-like structure. The observed retention of configuration at C-2′ rather than complete or partial racemization also indicates a high degree of binding or shielding of the reaction site by other molecules.

Recent studies on the origin of pentoses in *E. coli* (Caprioli and Rittenberg, 1968) indicate that the ribonucleotide reductase of this organism catalyzes nucleotide reduction in a way basically similar to that catalyzed by the *L. leichmannii* enzyme. Adenosine derived from the RNA of *E. coli* that had been grown on media containing [1-^{18}O]glucose or [2-^{18}O]-fructose as the sole carbon source was labeled with heavy oxygen in the 2′, 4′, and 5′ positions. Deoxyadenosine isolated from DNA contained corresponding amounts of label in the 4′ and 5′ positions, showing that the 2′-hydroxyl group had been lost completely and no transfer of oxygen had occurred during reduction. A final elucidation of the reaction mechanisms of both enzymes must now await identification of the reducing species and of the amino acids participating in the active sites of these proteins.

SCHEME I: Reaction Mechanisms for Ribonucleotide Reduction.



[a] PPP = $P_3O_{10}^{4-}$; B = nucleobase.

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