Vol. 214, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 5, 1995 Pages 28-35

THE MECHANISM OF THE ANAEROBIC ESCHERICHA COLI RIBONUCLEOTIDE REDUCTASE INVESTIGATED WITH NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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Received July 11, 1995

Summary. During the reduction of ribonucleotides with [^{3}H]formate by the class III anaerobic ribonucleotide reductase from Escherichia coli tritium appears in water and not in the product deoxyribonucleotide. In D_2O, deuterium replaces the OH-group at carbon-2´ with retention of configuration. In addition we find 1-2 % deuterium in the 3´-position demonstrating a small exchange of this hydrogen with the protons of water during catalysis. Class I and II enzymes catalyze identical reactions. Members of the three classes of reductases apparently use the same chemical mechanism in spite of having completely different protein structures.

The enzyme ribonucleotide reductase catalyzes the first reaction in a metabolic sequence leading exclusively to the synthesis of DNA by reducing ribonucleoside di-(or tri)phosphates to the corresponding deoxyribonucleotides. Surprisingly, three different classes of enzymes carry out

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0006-291X/95 $12.00

this reaction, each with a distinct protein structure (1). Nevertheless all ribonucleotide reductases have common features. The substrate specificity of all enzymes is regulated in an identical way by allosteric effects such that a single protein reduces all four common ribonucleotides. Furthermore, all enzymes employ radical chemistry to transform the ribonucleotide to an activated cation radical intermediate (2,3) by a process involving a radical amino acid of the enzyme (1,2). To this purpose **Class I** reductases, present in all higher organisms and in aerobically growing Escherichia coli contain a stable tyrosyl radical that during catalysis generates a cysteinyl radical (2). Also **class II** enzymes operate via a cysteinyl radical but in their case the radical is generated from adenosyl cobalamin (2,4). Class II enzymes are found in both aerobic and anaerobic microorganisms. **Class III** enzymes are present in anaerobically growing microorganisms. They contain a stable, oxygen-sensitive glycyl radical (5). The possible intermediate formation of a cysteinyl radical during catalysis remains to be established.

One major difference between the various classes concerns the reductant used for the reduction of C´-2. Both class I and II enzymes employ enzyme-bound cysteine-thiols, maintained in the reduced state by transthiolation with small dithiol-proteins (thioredoxin or glutaredoxin) or dithiothreitol (1,2). These dithiols are not active with the E.coli class III reductase. This enzyme uses formate as an external reductant (6) and catalyzes the following reaction:

$$\text{formate} + \text{CTP} + \text{H}^+ \longrightarrow \text{dCTP} + \text{CO}_2 + \text{H}_2\text{O}$$

With [^{3}H]formate in the reaction, tritium was recovered in water and not in the deoxyribonucleotide (6) suggesting

the formation of a reduced intermediate in rapid exchange with the protons of water. Thus the hydrogen that replaces the OH-group at C'-2 is derived from solvent. We have now carried out the reaction in D_2O to investigate with NMR the stereochemistry of the reaction.

MATERIALS AND METHODS

Reduction of CTP to dCTP in D_2O. Incubations were made in eleven separate anaerobic tubes continously flushed with moist (D_2O) argon connected to an anaerobic manifold at room temperature. Each tube contained 30 mM Tris-HCl, pH 8.5, 30 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM sodium formate and 2 mM [^{3}H]-CTP (1800 cpm/nmol) in D_2O in a final volume of 0.1 ml. The reaction was started by the anaerobic addition of 36 μg of enzyme (6) in 3 μl of H_2O to each tube. After 20 min at room temperature the tubes were opened to air to stop the reaction. The amount of dCTP formed was determined in one tube and found to be 110 nmol. The solutions from the remaining ten tubes were combined and treated with 4 mg alkaline phosphatase at 37° for 60 min to transform dCTP to deoxycytidine. After boiling for two minutes and centrifugation the pH of the solution was adjusted to 5 with 1 M acetic acid and deoxycytidine was separated from cytidine by HPLC on a Nucleosil 100-5 C18 column by isocratic elution with 20 mM ammonium acetate, pH 5.0 at 0.5 ml/min. Cytidine appeared after 24 min cleanly separated from deoxycytidine at 34 min. Ammonium acetate was removed from the combined deoxycytidine fractions by repeated evaporation in a vacuum. The dry residue (789 nmol) was then dissolved in 0.5 ml of water and the solution was used directly for NMR analysis.

NMR spectroscopy (7). ^{1}H NMR spectra were recorded at 600 MHz ^{1}H frequency on a Bruker DM-600 NMR spectrometer. The enzymatically prepared sample was compared with a more concentrated sample of deoxycytidine at natural isotopic abundance. D_2O was added for field/frequency locking to a final concentration of about 10 %. The pH of the samples was adjusted to 6.4 by adding minute amounts of acetic acid and NaOH. All spectra were recorded at 25°C. The stereospecific resonance assignments of the 2' and 2" protons under these conditions were verified by a NOESY spectrum of the unlabeled deoxycytidine using a mixing time of 1 s and 4 s relaxation delay. The spectrum of the partially deuterated deoxycytidine was recorded in about 45 min using 512 scans and a relaxation delay of about 2 s. The water resonance was suppressed by selective presaturation.

RESULTS AND DISCUSSION

Fig 1 shows the ^{1}H NMR spectra recorded with enzymatically deuterated and unlabeled deoxycytidine. The stereospecific resonance assignment of the 2' and 2" protons were verified by a NOESY experiment where the 1'and 4'

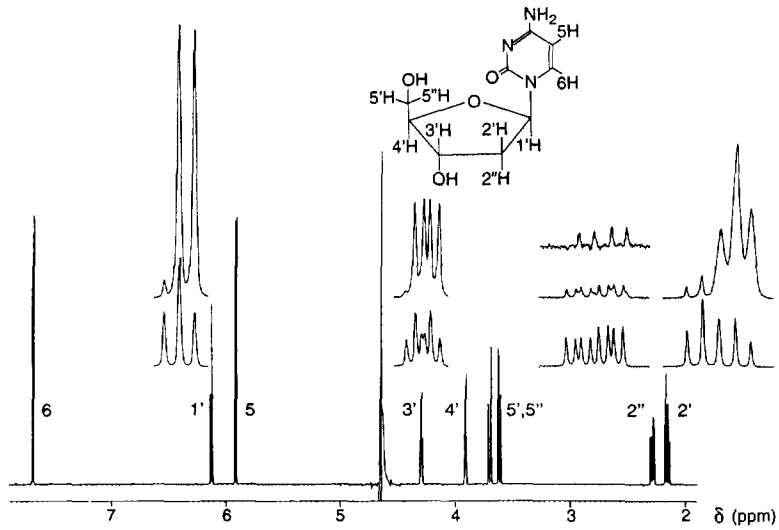

Fig. 1. ^{1}H NMR spectra of deoxycytidine at 25°C, pH 6.4. The bottom trace shows the spectrum of deoxycytidine at natural isotopic abundance with the proton resonance assignments. The residual water signal is observed at about 4.75 ppm. Expansions of the multiplets of the 1´, 3´, 2" and 2´ protons are plotted in the trace above the one-dimensional spectrum. The third trace shows the corresponding multiplets in the spectrum of the partially deuterated deoxycytidine. The much lower intensity of the 2"H compared to the 2´H signal indicates selective deuteration at the 2" site. The residual multiplet is a superposition of the eight lines observed for the 2" proton in the unlabeled sample and a doublet of doublets (shown above) arising from a small fraction of deoxycytidine carrying a proton in the 2" position and a deuterium in the 3´ position (see text). The subspectrum of the doublet of doublets was obtained by subtracting the multiplet fine-structure of the 2" proton in unlabeled deoxycytidine (trace 2) from the corresponding resonance in the spectrum of deuterated deoxycytidine (trace 3) with appropriate scaling and is plotted on a 4 times larger vertical scale.

resonances showed stronger NOEs to the low-field (2"H) resonance thant to the 2' proton, whereas the 6, 3', 5' and 5" resonances showed stronger NOEs to the high-field (2'H) resonance. No stereospecific assignments were attempted for the 5' and 5" resonances. The spectrum of the enzymatically prepared deoxycytidine shows a marked decrease in intensity for the 2" proton signal compared to the unlabeled nucleoside indicating the incorporation of deuterium in the 2" position. The intensity of the residual 2" signal is about 10 % of the other proton signals. The intensities of the other proton

signals are identical within about 2 % (the estimated measurement error in integrating the signals) emphasizing the selectivity of the deuteration at the 2" site.

The introduction of deuterium at a level below the aforementioned integration error is revealed by closer analysis of the multiplet finestructure of the residual 2" proton signal which demonstrates the occurence of about 1 to 2 % deuteration at the 3´site but not at the 1´ or 2´ site. Two-bond and three-bond scalar coupling constants are resolved. In general, the deuteration of the 2" position simplifies the observed multiplet patterns because the couplings to the deuterium are not resolved. For example, the 1' resonance changes from a triplet to a doublet, the 3' resonance changes from a doublet of triplets to a doublet of doublets and the 2' resonance becomes a triplet. The 1', 2', and 3' resonances are shifted to higher field because of the isotope effect exerted on the proton chemical shift by the presence of deuterium. The residual 2"H signal in the sample containing 2"-deuterated deoxycytidine shows the superposition of two multiplets, one of which corresponds to the signal in unlabeled deoxycytidine while the other represents a doublet of doublets with coupling constants of 14.3 and 6.7 Hz corresponding to couplings with the 2' and 1' proton, respectively (Fig 1). The coupling to the 3' proton (4.3 Hz) is removed. This demonstrates the presence of deuterium in the 3'-position. Accordingly the doublet of doublets is shifted to high field by the deuterium isotope effect on the proton chemical shift. The intensity of this multiplet is about 1 to 2 % of the intensitiy observed for the fully protonated sites. The residual 2"H signal intensity in the enzymatically deuterated deoxycytidine is completely

explained by the superposition of the multiplets of undeuterated and 3'-deuterated deoxycytidine. In particular, there is no signal intensity left which would hint at deuteration in the 1'-position.

DISCUSSION

The NMR analysis showed that the 2"-position is deuterated with high specificity in the course of the reduction of the ribonucleotide to the deoxyribonucleotide. The hydrogen from the solvent thus replaces the OH-group with retention of configuration. In addition we found that the product of the reaction contained deuterium at the 1 % level also in position 3' suggesting that also the hydrogen at this position exchanged to a small degree with the solvent.

The results concerning position 2" are identical to those obtained earlier with class I (8) and class II (9) enzymes. In their case it was shown that each reduction event is accompanied by the oxidation of two cysteines within the active site to a disulfide. When the reaction is carried out in D_2O, deuterium equilibrates with the SH-groups of the active cysteine-thiols and is incorporated into the deoxyribonucleotide. Fig 2 shows the reaction mechanism for class I and II enzymes proposed by Stubbe (2). The high yield

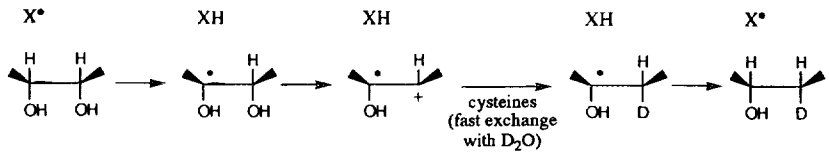

Fig 2. Mechanism for reduction of ribonucleotides by class I and II enzymes (2). A cysteinyl radical (X·) abstracts the 3´-H. After elimination of 2"-OH, cysteine-thiols, in rapid exchange with the solvent, reduce the intermediate radical cation with retention of configuration. In the last step, H is returned from X-H to the 3´-position. X-H is in slow exchange with the solvent. The present results suggest that a similar mechanism may also operate with class III enzymes.

of D incorporation into the product indicates that the rate of exchange with these specific cysteines is faster than the reduction of the intermediate cation radical.

For the class III ribonucleotide reductase there is as yet no evidence for a similar involvement of enzyme-bound cysteine-thiols, but the present results could be explained on this basis. It then remains to be understood how the cystine-disulfide formed during reduction of the ribosyl moiety is reduced by formate. One possibility is that the Fe-S center of the class III reductases (5) is involved in this process.

The small amount of D found in the 3' position is reminiscent of earlier results by Stubbe et al. (10) with class I and class II enzymes. After reduction of ribonucleotides labeled with tritium in the 3'-position about 1 % of the label was recovered in the solvent. This result was part of the evidence for the postulated mechanism in Fig 2 for class I and class II enzymes that involves the abstraction of the 3'-hydrogen by a cysteinyl radical (X· in Fig 2) of these enzymes. This cysteine is also able to exchange to bulk solvent but at a rate that does not compete efficiently with the rate of return of the 3´-H abstracted from the substrate to the 3´-position of the product. The presence of a small amount of D at position 3' in our present experiment suggests that a similar mechanism is at play with class III enzymes.

Our results provide further strong evidence for the conclusion that the chemical mechanism at the nucleotide level is similar for the three classes of enzymes, in spite of their different protein structures. If also with class III reductases cysteines are inovolved both in the abstraction of

the 3'-hydrogen and in the reduction of the 2'-carbon, this would extend the similarities to the protein level.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (to P.R.), from the Centre National de la Recherche Scientifique (to E.M. and M.F.), the European Union (to P.R. and M.F.), the Swedish Natural Science Research Council (project 10161) and the Wallenberg foundation (to E.L. and G.O.).

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