EFFECT OF pH ON INCORPORATION OF RIBONUCLEOTIDES INTO DNA BY DNA POLYMERASE I

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1. Introduction

Incorporation of ribonucleotides into DNA by DNA polymerase I from *E. Coli* in the presence of Mn^{2+} was first described by Berg et al. in 1963 [1]. Since that time other groups have studied the reaction in more details [2, 3]. Most recently synthetic DNAs of defined sequence have been used to study fidelity and completion of repair [4]. The repair process was found to proceed essentially to completion; some misincorporation was however observed.

Repair with ribonucleotides has proven to be a valuable tool for sequence determination of DNA [5–7]. It is, however, highly desirable to use optimal conditions for incorporation in such studies. In the present work we describe the effect of pH and some other parameters on the repair reaction with ribonucleotides. Maximum rate of repair was found to occur at pH 9.1. At this pH value, rUMP was also incorporated.

2. Materials and methods

2.1. *Enzymes, DNA and nucleoside triphosphates*

DNA polymerase I from *E. Coli* was purified according to Jovin et al. [8]. The specific activity was 6000 units/mg assayed in the absence of exonuclease III. Exonuclease III was obtained from the same procedure.

T_7 DNA was isolated from T_7 phage by the method of Grossman [9] and ^{3}H-labelled DNA was prepared according to a published procedure [2]. Exonuclease III treatment of T_7 DNA was performed essentially as described by Richardson et al. [2]. Usually approximately 20% was degraded away by this treatment. Poly[d(A-T) · d(A-T)] was a gift from Dr. I.F. Nes, Bergen.

Unlabelled and labelled deoxy- and ribonucleoside triphosphates were obtained from Sigma Chemical Company and the Radiochemical Centre Amersham respectively. All nucleoside triphosphates were checked for purity by the solvent systems previously described [10] and in addition the following systems were used: Solvent I, isopropanol, concentrated NH_3, 0.1 M H_3BO_4, 60:10:30; Solvent II *n*-butanol, formic acid, water, 77:10:13. In some cases the nucleoside triphosphates were contaminated and had to be purified employing chromatography in the solvent systems described above.

2.2. *Assay for repair synthesis*

All assays were performed at 37°C and the reaction mixture contained 3 deoxynucleoside triphosphates at a concentration of 33 μM each, one ribonucleoside triphosphate at a concentration of 0.33 mM, 0.9 mM $MnCl_2$, 7 μg/ml exonuclease III treated T_7 DNA and 13 units of DNA polymerase I/ml. Other details are given in the legends to each figure. The kinetics were followed by withdrawing aliquots from the reaction mixtures and precipitating the DNA on Whatman 3 MM filter discs as previously described [10]. When ^{3}H-labelled nucleotides were used, aliquots were precipitated with 5% trichloroacetic acid using 5 μg/ml of calf thymus DNA as coprecipitant. The DNA was then collected on glass fiber filters.

2.3. *Base analyses*

Acid hydrolyses of DNA was carried out as described by Bendich [11].

3. Results

3.1. *Effect of pH*

Previous studies on the incorporation of ribonucleotides into DNA have been carried out essentially at one pH value, namely pH 7.6 [1, 2, 4,]. It has now been well documented that in the presence of Mg^{2+} and four deoxynucleoside triphosphates repair with DNA polymerase I proceeds optimally at pH 7.6 [12]. When Mn^{2+} was substituted for Mg^{2+} was substituted for Mg^{2+} and four deoxynucleotide triphosphates were employed, we found that optimal incorporation occurred at the same pH value. However, in the presence of one ribonucleotide triphosphate, three deoxynucleotide triphosphates and Mn^{2+}, fig. 1, two distinct pH optima were observed, one at approximately 7.5 and the other at approximately 9.1. The activity at the latter pH value using rGTP was approximately 1 fold higher than at pH 7.5. Similar results were also obtained when rCTP or rATP was substituted for rGTP. With rUTP very little incorporation was found at pH 7.5 in agreements with earlier findings [1]. At pH 9.1 however, considerable incorporation was observed. The incorporation was not due to impurities of deoxynucleoside triphosphates. The DNAs repaired with various ribonucleoside triphosphates were isolated by gelfiltration and subjected to base analysis and susceptibility to alkaline treatment, i. e. 0.3 M KOH at 37°C for 18 hr. In all cases correspondence was seen between radioactivity and the base expected and furthermore the radioactive DNA was sensitive to KOH treatment. The results for the base analysis in the case of rUTP is shown in fig. 2. At least 95% of the radioactivity was found in U.

The optimal concentration of Mn^{2+} was found to be the same as at pH 7.6 namely approximately 0.9 mM. Spermine at a concentration of 0.1 mM also gave an increase in reaction rate of approximately 10%.

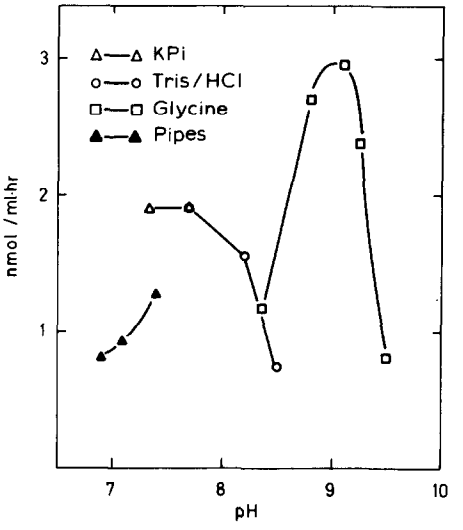

Fig. 1. Effect of pH on incorporation of rGMP into exonuclease III treated T_7 DNA. The concentration of the buffers were 67 mM in all cases. The specific activity of [^{3}H]rGTP was 750 cpm/nmoles. The pH was measured directly in the reaction mixtures. Further experimental details are given in Materials and methods.

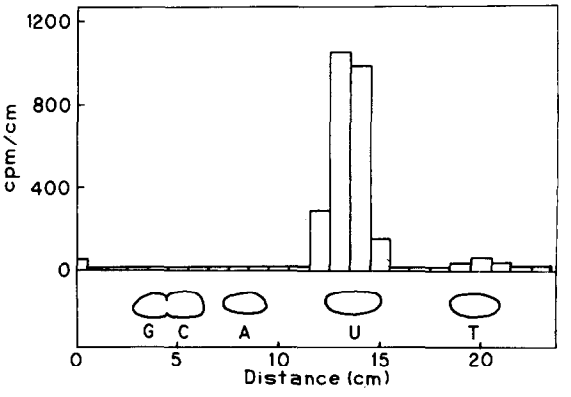

Fig. 2. Paperchromatographic separation of bases after acid hydrolysis of T_7 DNA repaired in the presence of [^{3}H] rUTP. The repaired DNA was separated from excess nucleoside triphosphates by gelfiltration on a column of Sephadex G-50 (50 × 1 cm) equilibrated with 50 mM triethylammoniumbicarbonate. The DNA was then subjected to acid hydrolysis as described in Methods and the bases separated by chromatography in solvent system II. After drying the chromatogram was cut into 1 cm pieces, each piece was shaken with 1ml 0.1 M NH_4OH, then counted in an aqueous scintillation system.

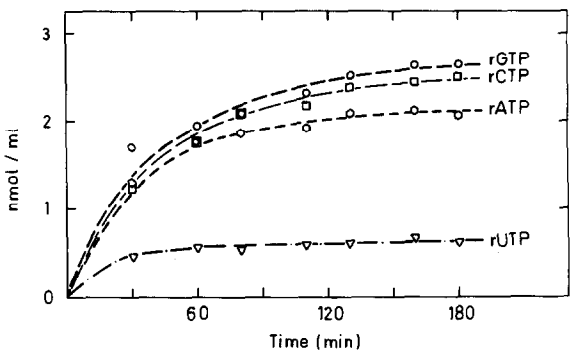

Fig. 3. Kinetics of incorporation of different ribonucleotides at pH 9.1. The labelled nucleotide was [^{14}C]dATP with a specific activity of 550 cpm/nmoles and the buffer 67 mM glycine pH 9.1. Other conditions as described in Materials and methods.

3.2. *Kinetics of incorporation at pH 9.1*

The incorporation of the various ribonucleotides at pH 9.1 are shown in fig. 3. In this case labelled nucleotide was dATP. Similar results were obtained when labelled ribonucleotide triphosphates were used. Both the initial rates and plateau values were higher for rCTP and rGTP than for rATP and rUTP. The incorporation of rUMP varied somewhat depending on the DNA preparation, i. e. the amount degraded away by exonuclease III. Maximum incorporation, however, were in most cases approximately 20% of that obtained with rGTP and rCTP. The plateau values obtained with rGTP and rCTP corresponded to that of complete repair as jugded by several criteria. First, a good correspondance was observed between the amound degraded by exonuclease III and nucleotides incorporated based on radioactivity. Second, the plateau values reached when the repair synthesis was carried out in the presence of four deoxynucleoside triphosphates and Mg^{2+} at low temperatures were the same as in the presence of rGTP or rCTP and pH 6.8. Under the latter conditions it has been shown that repair synthesis proceeds to completion [10]. Finally, the same plateau values as obtained with rGTP or rCTP were found when repair was allowed to proceed in the presence of four deoxynucleoside triphosphates and Mn^{2+}. These results are in agreement with those of Van de Sande et al. [4] concerning completion of repair.

Several experiments were carried out to test the nuclease activity in the presence of Mn^{2+} at pH 9.1. Some degradation took place in the absence of nucleoside triphosphates. In the presence of the latter little er no degradation was seen of the template T_7 DNA. Since all repair expiriments were carried out in the presence of large excess nucleoside triphosphates it is unlikely that the nuclease activity has affected the results to any extent.

4. Discussion

The present results show that maximum incorporation of ribonucleotides into DNA catalyzed by DNA polymerase I occurs at approximately pH 9.1 rather than at pH 7.6 which is the pH optimum found when only deoxynucleotides are used [12]. This rather dramatic change in pH optimum is not an artifact caused by the nuclease activity of the polymerase, even though the nuclease activity of the enzyme also has a pH optimum at pH 9.1 in the presence of Mn^{2+} [13]. One possible explanation for this finding is that with increasing pH, groups on both DNA and the nucleotides become ionized [14]. This most certainly leads to change in the DNA structure, i. e. the forces between the two DNA strands become weaker and the DNA may even in certain regions be partially denatured. At any rate such a 'loosening up' of the DNA structure may facilitate an easier incorporation of ribonucleotides into the DNA. pH induced alteration in the enzyme structure or DNA-protein complex cannot, of course, be ruled out as a possible explanation. It seems unlikely that the effect observed is caused by changes in the Mn^{2+} chelating properties since maximum repair in the presence of deoxynucleotides and Mn^{2+} is found at pH 7.6.

The low plateau values observed with rUTP as compared with rGTP or rCTP may be due to an inability of DNA polymerase I to repair certain sequences in the presence of rUTP such that when the polymerase reaches this sequence on the template strand it stops. Attempts to incorporate rUTP at pH 9.1 using poly[d(A-T) · d(A-T)] were unsuccessful. Experiments are now in progress in this laboratory to determine the base sequence of the region where the repair stops as well as studying the fidelity

of incorporation.

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