Enzymic in vitro repair and chemical nature of DNA chain breaks induced by incorporated phosphorus- ^{32}P decay

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ABSTRACT

In vitro repair of single strand breaks in T4 and λ phage DNA caused by ^{32}P decay was studied. Zone centrifugation procedure showed that polynucleotide kinase, ligase enzyme system failed to repair ^{32}P -damages. It was found that damaged DNA contained gaps and could be repaired by DNA-polymerase I, polynucleotide ligase treatment.

INTRODUCTION

Good evidences exist that every decay of incorporated ^{32}P atom causes mostly a single strand breakage in the DNA chain which can be efficiently repaired in bacteria¹. Only a certain fraction of the disintegration of ^{32}P atoms in DNA is lethal to microorganisms due to the double strand breakage². The chemical nature of the broken strands, as well as the mechanism of enzymic repair are not known with certainty. Tomizawa and Ogawa postulated that the radioactive transmutation results in the release of the terminal phosphate group. Therefore phosphorylation of 5'-termini by polynucleotide kinase and DNA ligase reaction should repair the damaged chains. Recent in vivo experiments suggest an important role of DNA polymerase I in repair of ^{32}P induced damages³. It was also shown that kinase was not a critical factor in this process⁴. The experimental data reported here concern with the conditions of in vitro repair of single strand breaks of phage DNA caused by ^{32}P decay and the role of different enzyme systems in this process. From the results obtained data might be deduced concerning the chemical nature of the induced damage. Some data were presented earlier⁵.

METHODS

Polynucleotide kinase was prepared from E.coli B infect-

ed at a cell density 1×10^9 /ml with L-tryptophan added to the concentration of 10 μ g/ml shortly before infection with bacteriophage T4 am'N 82 at multiplicity of 4. M 9 medium supplemented with casein hydrolysate (1,5% Difco) was used. Cells were harvested for 2 hours after infection. Frozen cells were disrupted using French press and enzyme was purified by the procedure of Richardson⁶. Phosphocellulose eluate fraction of polynucleotide kinase containing 50% glycerol was stored at -20°C for over a year without appreciable loss of activity. In purification procedure the conversion of a nucleoside - 3'-monophosphate into its nucleoside-5', 3'-diphosphate was used as a test for enzyme activity. Incubation mixture of 0,3 ml contained: 0,02 M tris-HCl, pH 7,6, 0,01 M MgCl_2 , 0,01 M 2-mercaptoethanol, (Koch-Light), 10 μ Moles of 2',3'-AMP (Reanal) and 20 μ Moles of (γ - ^{32}P)-ATP-(1-5 $\times$ 10^5 cpm). Samples were incubated for 30 min at 37°C . Reaction was stopped by addition of 1,2 ml 1N HCl and mixture was boiled at 100°C for 10 min. After cooling in cold water bath 0,1 ml of 30% Norit A (Serva) suspension was added to the mixture. Samples were shaken using vibrator, and Norit with adsorbed nucleotides was collected on membrane filter then washed with 5 portions of 5 ml 0,02 M orthophosphate. Filters were dried under infrared lamp and were counted in gasflow counter (Protoka) or in SL 30 (Intertechnique) scintillation counter in empty vials by Cerenkov counting. Radiation from filter containing Norit on the surface with ^{32}P can be easily counted in empty vials with good efficiency. The purified enzyme was identical to the enzyme described by Richardson⁶.

Phosphorylation of DNA samples were described⁶. (γ - ^{32}P)-ATP was prepared by the method of Glynn, Chapell⁷ in total volume of incubation mixture 5 ml. 3-phosphoglyceraldehyde dehydrogenase was obtained from Reanal (Hungary), 3-phosphoglycerate from Koch-Light. Phosphoglycerate kinase was purified from bakers yeast as described lower or obtained from Koch-Light. The mixture for ATP- ^{32}P synthesis was incubated at 37°C for 30 min, cooled, and then applied to Dowex 1x8, 200-400 mesh (Serva) column (1x2 cm). After washing

with water AMP, ADP and inorganic orthophosphate were eluted with 0,01 N HCl + 0,08 M NaCl, ATP- ^{32}P of specific radioactivity of 0,4C/mM was eluted in volume of 10 ml with 0,25 N HCl. The solution was neutralized immediately with solid tris (Calbiochem), pH was adjusted to 8,0. (γ - ^{32}P)-ATP solution of 350 mM/ml was stored frozen at -20°C .

In calculating the specific radioactivity of the product it was assumed that the quantity of ATP recovered was equaled to that one put into the reaction mixture as ATP: this was checked by autoradiography. Samples from preparative reaction were analysed on precoated TLC plates Ionex-25 SB, AC-form developed by Macherey-Nagel and Co. These plates gave good separation of all products: orthophosphate, nucleoside mono-, di- and triphosphates. Radioactive ATP- ^{32}P was checked by autoradiography with Röntgen film ORWO PF44. Time of exposure - about 1 hr. Less than 1 μl of (γ - ^{32}P)-ATP solution can be analysed. The efficiency of ATP- ^{32}P synthesis was usually high, at least 70% of the $^{32}\text{PO}_4$ being transferred to ATP.

Phosphoglycerate kinase was isolated from bakers yeast, as described by Bucher⁸, with some modifications. The fresh paste of yeast obtained from the plant was divided into small pieces, air-dried for 1-2 days at room temperature with vigorous ventilation and stored at room temperature. Other deviations from original method are as follows. Dry powder in step 3 was prepared by lyophilisation. Centrifugation at $4500 \times g$ for 30 min was used instead of filtration procedure in step 6. Use of aluminium hydroxide as well as crystallization of purified protein in step 11 were omitted. The enzyme was stored in saturated ammonium sulphate solution containing 0,05 M $\text{Na}_4\text{P}_2\text{O}_7$, pH 7,0 at 4°C and did not lose its activity for at least one year.

For dephosphorylations of DNA preparations alkaline phosphatase was purified from E.coli⁹. DNA ligase was purified as described¹⁰. DNA polymerase I was purified from E. coli according to the described method¹¹ and obtained from Romaschenko A.G. S1 nuclease was isolated from Takadiastase as described by Ando¹². Conditions of DNA enzyme treatment

described by Shishida and Ikeda¹³ were used. Other enzymes: pancreatic DNase and micrococcal nuclease were obtained from Koch-Light, glyceraldehyde phosphate dehydrogenase was obtained from Reanal.

Samples of DNA were digested with pancreatic DNase and incubated with alkaline phosphatase of *E. coli* as described by Weiss et al.¹⁴. For deproteinization phenol treatment with subsequent extensive dialysis was used.

Conditions of phosphorylation reaction with polynucleotide kinase were essentially the same as described by Richardson⁶. Usually kinase reaction was stopped by phenol treatment and extensive (2-3 days with several changes) dialysis was used to free the solution from phenol traces and ATP. DNA recovered after this procedure did not change its structure checked by gradient centrifugation and was treated by DNA ligase as described elsewhere¹⁵. Sometimes ligase treatment of DNA followed kinase reaction without previous phenol deproteinization. Before alkaline gradient centrifugations all enzymic reactions here and below were stopped by addition of solutions of EDTA (Reanal) and NaOH to concentration 0,01 M and 0,1, respectively and then within a few minutes samples of DNA (0,15-0,2 ml) were applied to the top of gradients.

The techniques of cell growing, lysis, purification of highly ³²P-labeled T4, and λ phages, and isolation of DNA-³²P are described elsewhere¹⁵. The specific activity of the growth medium was about 50 mC/mg P. Samples of DNA-³²P distributed into many small test tubes were stored at -20°C. Marker ³H-DNA of phage λ was purified as described¹⁶.

To measure the effect of ³²P disintegration on viability of *E. coli* different strains were used: pol A⁺, pol A⁻¹⁷; AB 1157 (rec A⁺) AB 2463 (rec A⁻)¹⁸; KS 100 (lex⁺), KS 104 (lex⁻) - were obtained from G. Smirnov, lex mutation is derived from strain AB 2494¹⁹; 1071 (lig⁺), 1254 (lig⁻)²⁰. The specific radioactivity of the growth medium was about 200 mC/mg P.

RESULTS AND DISCUSSION

We used the model λ DNA-³H with a limited number of single strand breaks introduced by pancreatic DNase according

Fig.1 Sedimentation patterns of λ DNA- ^3H digested with pancreatic DNase, incubated with alkaline phosphatase and after removal of the phosphatase incubated with (γ - ^{32}P)-ATP and polynucleotide kinase (a); control incubation contained the same components except of kinase (b). Alkaline glycerol gradients (10-30% w/v), SW 50.1. Spinco L2-65 K, 30000 rmp, 300 min, 10° . Arrows show the direction of centrifugation. Details are described in Methods.

to the scheme elaborated by groups of C.C.Richardson and J. Hurwitz^{14,21} and have demonstrated the efficiency of the used enzymes in repair. Preparations of DNA- ^3H were treated by purified polynucleotide kinase and polynucleotide ligase.

After DNase treatment λ DNA- ^3H failed to be phosphorylated in the polynucleotide kinase reaction. When DNA- ^3H was incubated with purified alkaline phosphatase from E.coli after the removal of phosphatase DNA was incubated with (γ - ^{32}P)-ATP in the kinase reaction. In order to identify

the phosphorylation reaction the kinase product was subjected to zone sedimentation on a glycerol gradient. It was noted that there was an increase in the extent of phosphorylation by polynucleotide kinase (Fig.1a). The control incubation, without kinase, showed a very low extent of phosphorylation of DNA (Fig.1b). It should be noted that there was no significant increase in the amount of the material of lower molecular weight indicating that relatively few endonucleolytic scissions occurred during the kinase incubation. SDS-gel-electrophoresis²² of kinase also supported the purity of the enzyme preparation, the band of protein was found in a position corresponding to mol wt 31800 ± 3150 daltons.

Purified polynucleotide ligase was also free of deoxyribonuclease activity, since incubation of DNA-³H with the enzyme did not change its sedimentation profile in glycerol gradient. As shown in fig.2 polynucleotide ligase can completely repair λ DNA-³H. A sample of λ DNA whose sedimentation

Fig.2 Sedimentation patterns of the intact λ DNA-³H (a); treated with DNase (b); and repaired with ligase (c) alkaline glycerol gradient as for fig.1.

rate in alkali had been reduced by incubation with pancreatic DNase was incubated with polynucleotide ligase and ATP. At least 90% of the fragmentized DNA increased in sedimentation rate and acquired sedimentation properties indistinguishable from those of intact strands of λ DNA (Fig. 2c). Then we used this enzyme system to study in vitro repair of DNA- ^{32}P damaged by incorporated ^{32}P .

In order to obtain the samples of intensively labelled phage DNA- ^{32}P cells of *E. coli* were lysed with T4 or λ bacteriophages in the media contained high specific radioactivity of $^{32}\text{P}^{15}$. Phages were purified and DNA- ^{32}P were extracted by phenol treatment¹⁵. Sedimentation profiles showed that with the progress of disintegration of ^{32}P the average molecular weight decreases, since for every ^{32}P decay a single strand break occurs¹⁵. Samples of T4 DNA- ^{32}P with induced singles-strand breaks were treated with kinase and ligase or

Fig.3 Sedimentation patterns of T4 DNA- ^{32}P damaged by decay of incorporated ^{32}P (A) and treated with ligase (b); and treated with kinase and ligase (c). Alkaline glycerol gradient as for fig.1.

only with ligase and analysis by zone centrifugation procedure. In several experiments the sedimentation patterns showed no significant differences (Fig.3). From these experiments we can conclude that kinase, ligase enzyme system fails to repair in vitro DNA- ^{32}P breaks caused by ^{32}P decay. In view of the known specificity of these enzymes we conclude that ^{32}P -decay does not break polynucleotide chains at a phosphate bond such as to leave a 3'-hydroxyl group and a 5'-phosphate group in juxtaposition or terminal nucleotides are chemically modified and can not be the substrates for enzymes.

It has been suggested that DNA-polymerase I was needed for filling or for excision and refilling of damaged regions. It was shown that DNA- ^{32}P of bacteriophage T4 damaged by ^{32}P decay could be repaired by DNA-polymerase I, polynucleotide ligase treatment¹⁵. Approximately 20-30% decrease in the number of the single strand breaks per molecule of DNA was observed¹⁵. Fig.4 shows the typical sedimentation profiles of

Fig.4 Repair of λ DNA chain breaks induced by ^{32}P decay (a) with DNA-polymerase I, ligase (b). Alkaline glycerol gradient as for fig.1.

damaged λ DNA- ^{32}P (a), partly repaired λ DNA- ^{32}P after DNA-polymerase-polynucleotide ligase treatment (b). DNA-polymerase is, presumably, needed for the removal of chemically altered nucleotides or/and for refilling of gaps formed as the result of ^{32}P decay. Chemically altered nucleotides can be formed in DNA after ^{32}P decay and they are to be removed before repair synthesis through the action of 5'-3'-nuclease activity of DNA-polymerase I. Besides the strand breakage, chemical modification of nucleotides due to β -irradiation, and formation of gaps are supposed. The appearance of the low molecular weight fraction in sedimentogram of DNA- ^{32}P after ^{32}P decay (Fig. 3a, b) and the results of the following experiments strongly support this supposition. For the detection of single strand regions (gaps) we used purified S1 nuclease

Fig. 5 Sedimentation patterns of T4 DNA- ^{32}P damaged by ^{32}P decay (a) after nuclease S1 treatment (b) and DNA- ^3H treated with DNase (c) and then treated with nuclease S1 (d). Neutral glycerol gradient. Details are described in Methods. Vertical arrows show the position of the intact DNA.

from takadiastase which specifically attack single strand but not double strand molecules of DNA¹².

In order to produce random single strand breaks λ DNA was partially digested with pancreatic DNase. After subsequent S1 nuclease treatment it was found that DNA had not changed markedly the sedimentation profile in neutral gradient (Fig. 5c, d). When ^{32}P -damaged DNA was treated with S1 nuclease the sedimentation patterns in several experiments, one of which was presented in Fig. 5a, b, showed the fragmentation process of DNA- ^{32}P molecules suggesting the presence of single strand regions caused by ^{32}P -decay. The control profiles of T4 DNA before the effects of ^{32}P -decay did not change.

A number of E.coli K-12 strains were tested for its ability to survive the decay of incorporated ^{32}P . Bacterial cultures grown for several generations in highly labelled media were frozen and stored at -79°C . The effect on cell viability of intracellular ^{32}P disintegrations which occurred, was then assessed by periodically thawing aliquots and assaying them for the number of cells able to form colonies on nutrient broth agar. The results of experiments are summarized in Fig.6. It is evident that DNA-polymerase I

Fig.6 Survival of different mutants of E.coli after decay of incorporated ^{32}P atoms. Details are described in Methods.

deficient mutant (pol A⁻) of E.coli as well as rec A⁻ and lex A⁻ mutants are highly sensitive to ³²P decay.

The viability of ligase deficient mutant and its wild type did not differ significantly. The loss of viability of rec⁻ mutant was shown earlier². The results of experiments with lig⁻ mutant suggest that the low level of enzyme in cell can ensure the repair process.

CONCLUSIONS

It is evident that DNA-polymerase play an important role in repair of DNA damages induces by ³²P decay. It has been demonstrated in the experiments of in vitro and in vivo. It has been shown that DNA polymerase is needed for refilling of gaps formed as the result of ³²P-decay and possibly for repair of chemically altered nucleotides. Earlier repair in vitro of X-ray-induced chain breaks in DNA was investigated^{23,24}. It was found that breaks produced by the X-irradiation of DNA of bacteria were not directly repairable by ligase²² but DNA polymerase I, polynucleotide ligase system and possibly additional enzyme or enzymes were efficient in repair of damages²⁴. It is likely that the nature of both kinds of damages (X-ray and ³²P decay) is similar and can be repaired by similar or the same enzyme system.

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