Progress Report and Renewal Application

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"In Vitro Enzymatic Studies on the Nature and Repair of x-ray Induced Lesions in DNA".

Principal Investigator: Susan S. Wallace, Ph.D.

MASTER

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PROGRESS REPORT

Progress Report

Period: 9/1/78 to 5/31/79

I Purification and Properties of Enzyme Probes for X-ray-Induced DNA Lesions.

A. Escherichia coli x-ray endonuclease

The E. coli x-ray endonuclease has been purified about 5,000 fold by DNA agarose affinity chromatography, hydroxylapatite and phosphocellulose ion exchange chromatography. Purified fractions after DNA agarose chromatography recognize alkali-stable but not alkali-labile damages. The alkali-labile damage-specific activity that was present in less purified fractions was removed by DNA agarose. The enzyme also nicks osmium tetroxide-treated DNA and purified fractions do not exhibit any activity on unirradiated DNA either in the presence or absence of magnesium. Purified E. coli x-ray endonuclease does not require magnesium, has a broad pH optimum and is fully active in the presence of 10 mM EDTA. After DNA agarose chromatography, the enzyme is useful as a reagent for probing the number of alkali-stable x-ray-induced damages in DNA as described in the next section.

B. Saccharomyces cerevisiae endonuclease E

Extracts of Saccharomyces cerevisiae were chromatographed through DNA agarose affinity columns and screened for endonuclease activity against x-irradiated PM2 DNA substrate (14). Three activities were observed against x-irradiated DNA and one of the activities was investigated further and found to nick alkali labile lesions in x-irradiated DNA as well as apurinic DNA. This activity has been named endonuclease E in conjunction with the present terminology for naming yeast endonucleases. Endonuclease E does not require magnesium, is partially inhibited by EDTA and is apparently an apurinic endonuclease. Endonuclease E also incises PM2 DNA that has been heavily irradiated with ultraviolet light.

The ability of both the E. coli x-ray endonuclease and the yeast endonuclease E to bind very tightly to DNA agarose makes them useful as reagents because this one step leads to substantial purification.

II. Use of Enzyme Probes

The dose response of the induction of single strand breaks and E. coli x-ray endonuclease-sensitive sites has been determined for PM2 supercoiled DNA irradiated in Tris-EDTA buffer plus 50 mM KI. Small volumes (50-100 μ l) of DNA samples are irradiated in air at room temperature with a Picker x-ray source having a beryllium window. When operated at 60 kVp and 2.5 mA, the dose-rate is 27.6 krads/min. As can be seen in Figure 1, Fraction III (DNA agarose fraction) of the enzyme induces about 1.8 breaks for every single strand break produced by x-rays. Cruder fractions of the x-ray endonuclease have been shown to recognize both alkali-labile and alkali-stable base damages (1,2), however the number of endonuclease-sensitive sites depicted here includes primarily the latter class of damages. This appears to be the case since subsequent incubation in glycine buffer and analysis on alkaline sucrose gradients generates a new dose-response curve accounting for an additional 1.3 lesions per single strand break which is about 75% of the total number of alkali-labile lesions present in PM2 DNA irradiated under these conditions (3). Thus, DNA agarose chromatography appears to have removed an activity which in cruder preparations incised at alkali-labile lesions. At least one specific base damage has been shown to be recognized by the E. coli x-ray endonuclease, since it incises osmium tetroxide-induced damages in PM2 DNA (1). These damages correspond to the 5,6 dihydroxy-dihydrothymine product produced by ionizing radiation. The number of alkali-stable damages recognized by Fraction III and more purified fractions of x-ray endonuclease are fewer than originally reported (1,2) for partially purified fractions. It is possible that chromatography on DNA

agarose also removes a glycosylase activity, that in combination with the apurinic activity of the less purified fractions gave rise to an incision. Although fewer alkali-stable damages are recognized by Fraction III than by partially purified preparations, Fraction III is much more useful as a reagent since 85 to 90% of the sites recognized are alkali-stable, and in addition non-specific EDTA-sensitive nucleases have been removed.

Figure 2 shows the neutral sucrose gradient analysis of single-strand breaks and Saccharomyces cerevisiae endonuclease E- (Fraction IV, DNA agarose fraction) induced breaks in PM2 supercoiled DNA x-irradiated as above. Here the number of endonuclease E-sensitive sites is about 70% of the potential alkali-labile lesions (1.7 per single-strand break) (3) determined by alkaline sucrose sedimentation analysis of PM2 DNA after 16 hours incubation in glycine buffer. Thus, the number of nicks introduced by endonuclease E is greater than the number of alkali-labile lesions measured on alkali gradients immediately after x-irradiation (0.6 per single-strand break) (3) but fewer than the total potential number of alkali-labile lesions. Since the incubation conditions alone convert some of the slow-converting class of alkali-labile lesions to the fast-converting class, thus providing additional abasic substrate, endonuclease E could be valuable as a probe for abasic lesions formed under a variety of irradiation and post-irradiation conditions.

Thus, under neutral conditions a combination of two enzyme probes can differentiate between abasic alkali-labile and alkali-stable lesions introduced into DNA by ionizing radiation. The data depicted in Figures 1 and 2 were derived using the DNA agarose fractions of both enzymes under conditions of enzyme saturation which is essential if the enzymes are to be used to quantify the number of potential enzyme-sensitive damages. The advantages of this system include the ease of

purification of the supercoiled DNA used as substrate as well as the enzymes used for quantification of damages. The latter occurs because both enzymes bind extremely tightly to DNA agarose columns and thus a single step allows for a high purification to a point where they are useful as reagents. In addition, the assay itself is very sensitive allowing for the measurement of a single nick. The disadvantages include the fact that enzyme-induced breaks are always measured in the background of x-ray-induced single strand breaks. Also, the E coli x-ray endonuclease has not been well characterized with respect to particular base damages recognized and neither enzyme necessarily represents a unique activity. However, as presented here, the system is an extremely useful model system for in vitro studies since enzymatic probes can be combined with physical probes to give good quantification at high sensitivity levels.

III. Use of Physical, Chemical and Enzymatic Probes to Quantify X-ray-Induced Lesions in Viruses and Cells

A. Viral Studies

As a model system for the quantification of DNA damages induced by ionizing radiation, we have chosen PM2 bacteriophage. This system has a number of advantages. The one of primary interest is that the DNA exists in the phage head as a supercoiled circular DNA molecule. Thus, strand breaks, alkali-labile lesions and enzyme sensitive sites can be readily quantified by the technique of sucrose gradient sedimentation. As described above, we have quantified the number of alkali-labile, alkali stable and enzyme sensitive sites in PM2 DNA irradiated in vitro under several radical scavenging conditions. We are presently examining these same parameters in the PM2 bacteriophage in an attempt to relate a particular damage to the lethal event. We have thus far determined the number of single strand breaks, fast-converting alkali labile lesions and slow converting alkali-labile lesions in PM2 bacteriophage irradiated in 10X citrate broth, 50 mM KI and

0.1 M histidine. All three of these agents are about equally protective with respect to the number of alkali labile lesions, however, 10X citrate broth is more effective in protecting against single strand breaks. Thus, for both KI and histidine there exists approximately 1.1 alkali labile lesions for every single strand break for which about 0.4 of these are fast converting alkali labile lesions. For 10X citrate broth there exists approximately 1.8 alkali labile lesions per single strand break of which 0.4 are fast converting. There appears to be no significant difference among these three protective agents when examined for their effects on strand breaks and alkali labile lesions in PM2 DNA irradiated in vitro. We are presently preparing to measure the number of alkali stable x-ray endonuclease sensitive sites in PM2 bacteriophage DNA irradiated under these three protective conditions. In the case of PM2 bacteriophage irradiated in 10X citrate broth, there are about 1.7 times as many lethal events as single strand breaks and about 3 times as many lethal events as alkali labile lesions. Thus, it appears that damages other than or in addition to these damages are responsible for the lethal event in PM2 bacteriophage.

B. Cellular Studies

For the quantification of x-ray-induced lesion in cells, Saccharomyces cerevisiae will be used as a model system. Yeast have been chosen because they are a simple eukaryote and amenable to both biochemistry and genetics. As a point of departure for these studies, this laboratory is presently measuring the number of 5,6 dihydroxy-dihydrothymine damages (T') produced by ionizing radiation in yeast DNA as determined by the method of Hariharan and Cerutti (5). To date, the columns have been set up and T' damages have been measured in x-irradiated PM2 bacteriophage DNA. These studies will be related to the above described PM2 studies and used as a basis for quantifying these same lesions in yeast DNA. We

are presently isolating yeast petite mutants which are deficient in mitochondria so that incorporation of TMP will be exclusively confined to nuclear DNA. For these studies mutants able to take up TMP are being employed. When T' damages have been quantified in yeast DNA, enzymatic probes will be employed.

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Figure Legends

- FIG. 1. Dose response of x-irradiated PM2 DNA analyzed on neutral sucrose gradients (0); analyzed on neutral sucrose gradients after incubation with saturating amounts of Fraction III E. coli x-ray endonuclease (Δ); analyzed on alkali gradients after incubation with saturating amounts of E. coli x-ray endonuclease and subsequent incubation for 16 hours in glycine buffer ($\square$).
- FIG. 2. Dose response of x-irradiated PM2 DNA analyzed on neutral sucrose gradients (0); analyzed on neutral sucrose gradients after incubation with saturating amounts of Fraction IV of S. cerevisiae endonuclease E (Δ); analyzed on alkali gradients after 16 hours incubation in glycine buffer (---).

Figure 1

Figure 2

Personnel:

Faculty

P.I., S. Wallace (20%)

Non-Faculty

Res. Associate, R. Melamede (100%)

Technician, S. Pope (33 1/3%)

Graduate Students, E. Moran, B. Zerler

Publications:

Wallace, S.S., Katcher, H.L. and Armel, P.R. The measurement of x-ray damage in supercoiled DNA using enzyme probes,. in "Techniques in DNA Repair", E.C. Friedberg and P. Hanawalt, Eds.

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