Scarpa, A., and Azzone, G. F. (1970), *Eur. J. Biochem. 12,* 328-335.
Scarpa, A., and Graziotti, P. (1973), *J. Gen. Physiol. 62,* 756-772.
Schlögel, R. (1954), *Z. Phys. Chem. (Frankfurt am Main) 1,* 305-341.
Selwyn, M. H., Dawson, A. P., and Dunnett, S. J. (1970), *FEBS Lett. 10,* 1-5.
Sordahl, L. A. (1974), *Arch. Biochem. Biophys. 167,* 10-115.
Stucki, J. W., and Ineichen, A. (1974), *Eur. J. Biochem. 48,* 365-375.
Vasington, F. D., Gazzotti, P., Tiozzo, R., and Carafoli, E. (1972), *Biochim. Biophys. Acta 256,* 43-54.
Vinogradov, A., and Scarpa, A. (1973), *J. Biol. Chem. 248,* 5527-5531.

Mutations in *Escherichia coli* Altering an Apurinic Endonuclease, Endonuclease II, and Exonuclease III and Their Effect on in Vivo Sensitivity to Methylmethanesulfonate†

D. M. Kirtikar, G. R. Cathcart, J. G. White, I. Ukstins, and D. A. Goldthwait*

ABSTRACT: The levels of endonuclease II, an apurinic endonuclease, and exonuclease III in the parent strain (AB 1157) of *Escherichia coli* and in various mutants were determined by chromatography on DEAE-cellulose. AB 3027 and NH 5016 lacked endonuclease II and exonuclease III. BW 2001 lacked the apurinic endonuclease and exonuclease III while BW 2007, BW 9093, and BW 9059 lacked only exonuclease III. Deletion mutants BW 9101 and BW 9109 lacked all three enzymes. The latter mutants locate the genes for the two endonucleases in the region of exonuclease III (*xth*) of 38.2 min (White et al., 1976). All of the mutants which were sensitive to methylmethanesulfonate in vivo lacked exonuclease III, but not all mutants lacking exonuclease III were MMS sensitive. The deletion mutants and NH 5016 were the exceptions.

Repair of DNA damage in *E. coli* can occur by excision mechanisms involving endonucleases or *N*-glycosidases. Thymine dimers are excised by an endonuclease which recognizes this lesion and other chemical adducts (Grossman et al., 1975). Endonuclease II recognizes alkylated and aralkylated purine bases such as 3-methyladenine, *O*-6-methylguanine (Kirtikar and Goldthwait, 1974), or the dimethylbenz[*a*]anthracene derivatives of adenine and guanine formed by reaction of DNA with 7-bromomethyl-12-methylbenz[*a*]anthracene (Kirtikar et al., 1975a) as well as lesions in DNA produced by γ irradiation (Kirtikar et al., 1975b). Endonuclease II, purified 12 000-fold, has both phosphodiesterase and *N*-glycosidase activities (Kirtikar et al., 1976a). Many alkylated bases of DNA, especially purines, show increased lability of the glycosidic bond and depurinate spontaneously leaving depurinated sites. An endonuclease from *E. coli* which recognizes apurinic sites has been purified approximately 10 000-fold to homogeneity by Verly and Rassart (1976). An enzyme with a similar molecular weight and chromatographic properties has also been purified to homogeneity in this laboratory (Kirtikar et al., 1976a). It recognizes depurinated (apurinic) sites as well as depurinated sites which have been reduced with $NaBH_4$ (Hadi and Goldthwait, 1971). Endonuclease II and the apurinic endonuclease have been separated from each other (Kirtikar et al., 1976a) and from exonuclease III, an enzyme which degrades double-stranded DNA in a 3′ to 5′ direction and which can remove a terminal 3′-phosphate by its 3′ phosphatase activity (Richardson and Kornberg, 1964).

Although there are several enzymes which recognize apurinic sites in DNA, as will be discussed, we shall refer in this paper to the enzyme activity isolated in peak I from DEAE[1]-cellulose as apurinic endonuclease and also as endonuclease VI.

One mutant, AB 3027, isolated in the laboratory of Dr. Howard-Flanders, was reported to be lacking DNA polymerase I and the apurinic endonuclease (Ljungquist et al., 1976). In Dr. Weiss's laboratory, a mutant, BW 2001, was isolated and at the time was thought to be lacking exonuclease III and endonuclease II, the latter defined as an activity on heavily alkylated DNA (Yajko and Weiss, 1975). With a chromatographic procedure developed in this laboratory which could distinguish between the two endonucleases and exonuclease III (Kirtikar et al., 1976a) an examination of these mutants and their derivatives was instigated. An attempt has also been made to relate the specific enzyme complement of each mutant to its in vivo sensitivity to methylmethanesulfonate.

Methods

The parent strain for all the mutants used for this study was *E. coli* AB 1157 (Howard-Flanders and Theriot, 1965) des-

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received April 1, 1977; revised manuscript received August 25, 1977.* This research was supported by grants from the National Institutes of Health (CA-11322 and CA-18747) and by a contract with ERDA (11-1) 2725. D. A. Goldthwait is the recipient of a National Institutes of Health Research Career Award Fellowship (K-6-GM-21444).

[1] Abbreviations used: UV, ultraviolet; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMS, methylmethanesulfonate; apurinic endonuclease is used to designate the enzyme which recognizes depurinated or depurinated reduced sites in DNA and which is present in peak I. This enzyme has the same properties as that isolated by Verly and Rassart (1975) and has been designated endonuclease VI.

ignated wild type. AB 3027 and NH 5016 were obtained from Dr. Paul Howard-Flanders. Dr. Bernard Weiss provided strains BW 2001, BW 2007, BW 2030, BW 2021, BW 9101, BW 9109, BW 9093, and BW 9059 (Milcarek and Weiss, 1972; Yajko and Weiss, 1975).

Cells were grown to late log phase in EM-9 media (Kushner et al., 1971) supplemented with 20 μg/mL of L-arginine, L-histidine, L-proline, L-leucine, and L-threonine in a 10-L fermentor. Strains AB 1157, BW 2001, BW 2007, BW 2021, and BW 9059 were grown at 42 °C. Strains AB 1157, AB 3027, NH 5016, BW 2030, BW 9101, and BW 9109 were grown at 37 °C and BW 9093 was grown at 25 °C. The cells were harvested using a Sharples centrifuge. The yield of cells was between 25 and 48 g, depending on the strain and temperature of growth. The cells were washed with buffer A (0.05 M Tris-HCl, 0.1 mM EDTA, pH 8.0) and frozen at −20 °C.

Cells were disrupted in buffer A by homogenization with glass beads or by sonication. No differences in any of the enzyme activities were observed when the two procedures were compared. The cell homogenate was then centrifuged at 13 200g for 45 min to remove unbroken cells and debris. The supernatant fraction was centrifuged at 94 000g for 3 h. The clear supernatant fraction was then adjusted to a nucleic acid concentration of 3 mg/mL and streptomycin sulfate (in 0.05 M Tris-HCl, pH 8.0, buffer B) was added to a final concentration of 0.8%. A precipitate was removed by centrifugation at 16 300g for 45 min. The clear supernatant fraction was brought to 40% $(NH_4)_2SO_4$ saturation and centrifuged, and the resulting supernatant fraction was adjusted to 70% $(NH_4)_2SO_4$ saturation. After centrifugation at 16 300g for 1 h, the pellet was suspended in 20 mL of buffer C (0.05 M Tris-HCl, pH 8.0, 0.1 mM dithiothreitol, 20% glycerol) and dialyzed against 100 volumes of the same buffer with three changes over 24 h. This material was designated fraction III (Hadi et al., 1973).

Fraction III was applied to a 2.5-cm diameter × 50 cm column of DEAE-cellulose (DE-52, Whatman) equilibrated with buffer C. The column was washed with 1 column volume of 0.03 M NaCl in buffer C (250 mL) and then with 1 L of 0.1 M NaCl in buffer C at a flow rate of 1.3 mL/min. Ten milliliter fractions were collected starting with the 0.1 M NaCl wash. A linear gradient of 0.1 M to 0.5 M NaCl in buffer C (total volume of 1600 mL) was then applied. Every fourth fraction was assayed for endonuclease activity on depurinated–reduced DNA or alkylated DNA. Exonuclease III 3′-phosphatase was assayed only in fractions 1–100. Exonuclease III, apurinic endonuclease, and endonuclease II were eluted as described in the Results section. An endonuclease activity for both depurinated DNA and alkylated DNA (peak II) was eluted at 0.16 M NaCl. Fractions containing enzyme activity were pooled and concentrated first on a small DEAE column and then by concentration dialysis. The specific activity of each enzyme was determined in the pooled, concentrated fraction.

The assays for the two endonucleases as well as the definitions of their units have been described (Kirtikar et al., 1976a). These assays employ DNA entrapped in a polyacrylamide gel (Melgar and Goldthwait, 1968) which is either treated with methylmethanesulfonate (MMS) at a molar ratio of MMS to nucleotide of 500/1 or depurinated and then reduced with $NaBH_4$ (Kirtikar et al., 1976a). Assays were done on the day of preparation of the substrates. Incubation mixtures contained 20 nmol of DNA substrate, 0.05 M Tris-HCl pH 8.0, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M dithiothreitol, and 1.5 mg of bovine serum albumin in a volume of 1.5 mL. Incubations were for 30 min at 37 °C. The assay was linear with increasing enzyme concentration. The 8-hydroxyquinoline does not inhibit the enzyme activity at various stages of purification. Mg^{2+} gives only a twofold stimulation with both endonucleases II and VI, but was not used for these endonuclease assays because of the possibility of other nucleases dependent upon divalent metals. The assays have been used to purify extensively endonuclease II and VI. Highly purified endonuclease II and VI make approximately four single strand breaks per one double strand break in their respective substrates, free in solution. This suggests that the sites are not random. The authors recognize that the DNA gel procedure is not the ideal assay but its convenience and rapidity allow easy detection of these enzymes.

The 3′-phosphatase activity of exonuclease III was measured under the conditions described by Richardson and Kornberg (1964), and only fractions 1–100 were analyzed. Exonuclease III is found between fractions 30 and 70.

In vivo sensitivity to methylmethanesulfonate of the wild type strain and of each mutant was tested. Cells were grown at the desired temperature (25, 37, or 42 °C) in 10 mL of L-broth (Ljungquist et al., 1976), then harvested and resuspended in 0.05 M KPO_4, 0.1 M NaCl, pH 7.3 to a density of 3×10^7 CFU/mL. A 1-mL aliquot was removed at zero time for serial dilution with cold buffer and plating. MMS was added to a 15-mL culture at a final concentration of 0.05 M and the culture was incubated in a gyrotory water bath at the desired temperature. One-milliliter aliquots were removed at various times, immediately diluted with cold buffer, and subsequently serially diluted, plated on L–agar (L-broth + 1.6% agar) and incubated overnight at 25, 37, or 42 °C for determination of survivors. Data are expressed as N_t/N_0, colonies surviving at time t divided by colonies surviving at zero time.

Results

Apurinic Endonuclease, Endonuclease II, and Exonuclease III in Wild Type E. coli. The presence or absence of any of these three enzymes can be determined by DEAE chromatography of a 40–70% $(NH_4)_2SO_4$ precipitate of a cell extract (Kirtikar et al., 1976a). The elution profile of apurinic endonuclease, endonuclease II, and exonuclease III present in wild type *E. coli* is shown in Figure 1A. Apurinic endonuclease and exonuclease III were eluted together in peak I with 0.1 N NaCl. The peak of activity of exonuclease III consistently preceded the peak of activity of the apurinic acid endonuclease. Endonuclease II (peak III) was eluted during the linear NaCl gradient at approximately 0.25 N NaCl. Peak II included material which was active on both MMS treated DNA and depurinated reduced DNA. This is currently under investigation.

Mutants Lacking Exonuclease III and Endonuclease II. The strain AB 3027 was isolated by Dr. Paul Howard-Flanders (Ljungquist et al., 1976). This was derived by treatment of AB 1157 with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and selected by its inability to grow in the presence of 1.2 mM MMS. When this strain was examined by DEAE-cellulose chromatography, the results, shown in Figure 1B, were obtained. AB 3027 lacks both exonuclease III and endonuclease II.

This strain, AB 3027, has also been shown to lack DNA polymerase I (Ljungquist et al., 1976). In order to obtain a strain with a normal DNA polymerase I, *met* E^- (very close to pol I) was transduced into AB 3027 and transductants were selected for their ability to grow on 1.2 mM MMS (Ljungquist et al., 1976). The resulting strain, NH 5016, had normal levels of DNA polymerase I. The DEAE elution profile of NH 5016

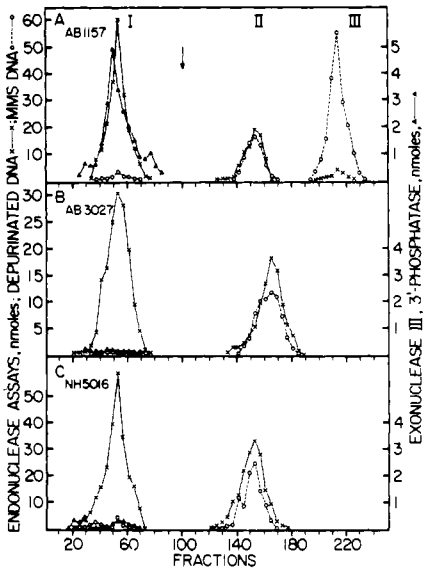

FIGURE 1: DEAE-cellulose column profile of AB 1157, AB 3027, and NH 5016. In all profiles, endonuclease levels were tested in every fourth fraction. Exonuclease III was tested in every fourth fraction only in the region of fractions 1-100. Where no values are shown, the level of enzyme activity is insignificant. Fractions 1-100 were eluted with 0.1 N NaCl and fractions 100-260 were eluted with a linear 0.1-0.5 N NaCl gradient (arrow) as described in Methods. AB 1157 was grown at 42 °C, AB 3027 at 37 °C, and NH 5016 at 37 °C. The activities are presented in nmol per mL of fraction in Figures 1-4. (X-X) Apurinic endonuclease; (O- - -O) endonuclease II; (▲-▲) exonuclease III.

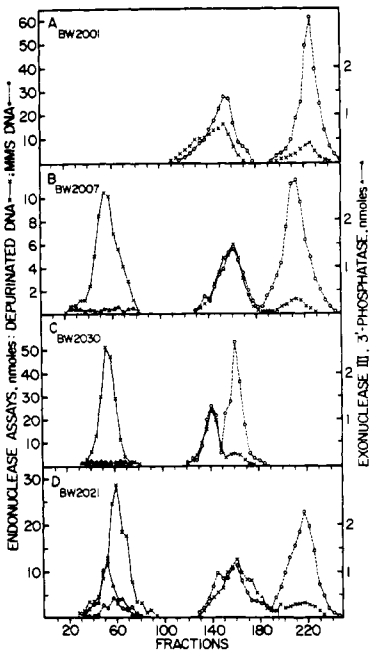

FIGURE 2: DEAE-cellulose column profile of BW 2001, BW 2007, BW 2021, and BW 2030. BW 2001, BW 2007, and BW 2021 were grown at 42 °C; BW 2030 was grown at 37 °C. The profile for strain BW 2030 shows peak II and III close together. Between fractions 135 and 150 the collector malfunctioned and tubes overflowed. Sodium chloride molarities for fractions were as follows: 120, 0.1; 130, 0.115; 140, 0.16; 150, 0.23; 160, 0.27; 170, 0.295; 180, 0.35. If the gradient was linear, peak III would be displaced to the right by at least eight fractions.

is shown in Figure 1C. Exonuclease III and endonuclease II were both absent as in AB 3027.

A Mutant Lacking Exonuclease III and Apurinic Endonuclease, and Its Revertants. Strain BW 2001 was isolated by Yajko and Weiss (1975) by treating AB 1157 with MNNG and selecting a clone, the extract of which had a decreased ability to release heavily alkylated DNA from a gel. It was found to be deficient in exonuclease III and in an endonucleolytic activity on heavily alkylated DNA. The DEAE elution profile of this mutant is shown in Figure 2A. Exonuclease III is missing as well as the apurinic endonuclease, but endonuclease II is present in normal levels.

The strain BW 2001 grows less rapidly than the parent strain. A spontaneous revertant, BW 2007, was selected by Yajko and Weiss (1975) because of its more rapid growth and therefore larger colony size. The DEAE elution profile, (Figure 2B), shows that this mutant is lacking exonuclease III but has now a normal level of the apurinic endonuclease.

Another derivative of BW 2001 was constructed by the transduction of *phe*S and *pps* markers into BW 2001, followed by the transduction of these markers plus *xth*A11 into AB 1157. The DEAE elution profile of this mutant, BW 2030, is shown in Figure 2C. It is apparent that the apurinic endonuclease has returned to normal but exonuclease III is still absent. Peaks II and III are close together because of malfunction of the collector as explained in the legend.

Another revertant of BW 2001 was isolated by Yajko and Weiss (1975) because of its MMS resistance and was called BW 2021. The profile of this mutant is shown in Figure 2D where the level of the apurinic endonuclease is normal and the exonuclease III level approaches normal.

Mutants Lacking Apurinic Acid Endonuclease, Endonuclease II, and Exonuclease III. A deletion mutant BW 9101 was defined by Yajko and Weiss (1975) in which the deletion included the *pnc*A gene and extended into or through the exonuclease III (*xth* gene). It was of interest to note that in the

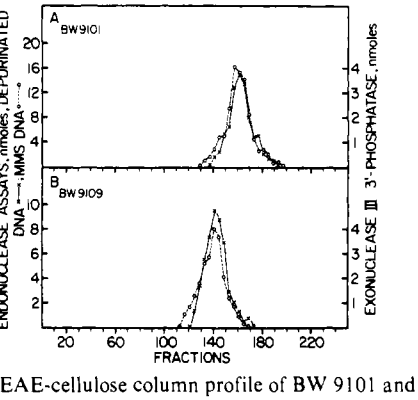

FIGURE 3: DEAE-cellulose column profile of BW 9101 and BW 9109. Both strains were grown at 37 °C.

DEAE elution profile (Figure 3A) all three enzymes, apurinic endonuclease, endonuclease II, and exonuclease III, were absent. This suggests that the genes for all of these enzymes lie in the region of the *xth* gene located by White et al. (1976) at 38.2 min on the revised map. Also of interest is the presence of the unaltered activities of peak II indicating that the genetic locus for this material must be at a site other than the deletion. The previous suggestion (Kirtikar et al., 1976b), based on preliminary evidence, that this activity might be due to a dimer formed of one subunit of apurinic endonuclease and one subunit of endonuclease II is incorrect.

The deletion mutant BW 9109 was obtained by Dr. Weiss by transduction of the deletion mutant, BW 9101, with the *pnc*A marker into AB 1157. The DEAE profile of this mutant (Figure 3B) is similar to that of BW 9109.

Mutants Lacking Exonuclease III. Milcarek and Weiss (1972) isolated a series of mutants of AB 1157 produced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine which showed altered levels of exonuclease III. One of the mutants, BW 9093, had

TABLE I: Enzyme Activities in DEAE Peaks I and III.[a]

Strain	Temp of growth (°C)	Peak I			Peak III	
		Depurinated reduced DNA (μmol/(mg h))	MMS DNA (μmol/(mg h))	3′-Phosphatase (nmol/(mg 30 min))	Depurinated, reduced DNA (μmol/(mg h))	MMS DNA (μmol/(mg h))
AB 1157	37	0.69	0.006	32.2	0.059	0.68
AB 1157	42	0.40	0.009	4.5	0.064	0.44
AB 3027	37	0.54	0.007	0.44	—	—
NH 5016	37	0.57	0.009	0.11	—	—
BW 2001	42	—	—	—	0.047	0.51
BW 2007	42	0.54	0.011	0.37	0.036	0.55
BW 2030	37	0.42	0.008	0.23	0.039	0.49
BW 2021	42	0.48	0.008	13.1	0.021	0.54
BW 9093	25	0.40	0.009	1.3	0.030	0.52
BW 9059	42	0.51	0.008	0.44	0.031	0.53

[a] BW 9101 and 9109 had no apurinic endonuclease or exonuclease III activity in peak I and no endonuclease II activity in peak III. The fractions in each peak were pooled and concentrated prior to measurement of specific activity. Where a blank (—) appears, no enzyme activity could be detected in the column fractions and therefore they were not pooled.

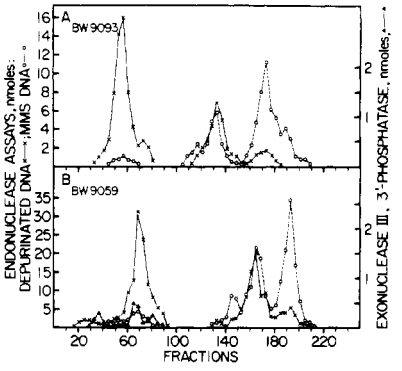

FIGURE 4: DEAE-cellulose column profile of BW 9093 and BW 9059. BW 9093 was grown at 25 °C and BW 9059 at 42 °C.

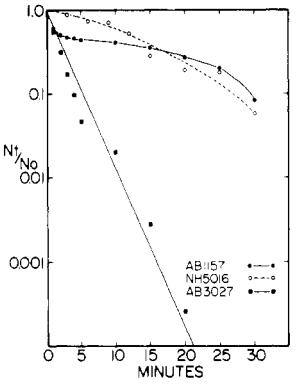

FIGURE 5: In vivo sensitivity to MMS of AB 1157, AB 3027, and NH 5016. Strains were tested at 37 °C.

a thermosensitive exonuclease III which, after a purification of 710-fold, showed an activity at 25 °C which approached that of the parent strain, but at 42 °C was more rapidly inactivated than the wild type enzyme (Milcarek and Weiss, 1972). This strain, grown at 25 °C in our laboratory, was used to prepare fraction III for DEAE column chromatography. The profile, shown in Figure 4A, has no detectable exonuclease III, but normal levels of apurinic endonuclease and endonuclease II.

Another mutant, BW 9059, isolated by Milcarek and Weiss (1972), was obtained by transduction of the *phe*S and *pps* markers into BW 9099. The latter was an exonuclease III mutant which was obtained in a fashion similar to BW 9093 and which had a low level of enzyme even at 25 °C. The DEAE profile of BW 9059, grown at 42 °C, is shown in Figure 4B. Again, as in the other mutant, exonuclease III is absent while the endonuclease levels are normal.

A summary of the specific activities of peaks I and III of the DEAE elution patterns is presented in Table I. Fractions through each entire peak of activity were pooled and concentrated and this material was then tested with the different substrates: depurinated-reduced DNA in the gel, MMS treated DNA in the gel, and [^{32}P]DNA with 3′-phosphomonoesters. Small differences in the numbers are not significant since the number of fractions pooled varied from column to column. Where no figures are given, there was no activity in the column fractions and therefore no fractions were pooled. The level of exonuclease III was measured only in peak I since it was present only there (Kirktikar et al., 1976a). Both endonuclease substrates were used. The activity observed in peak I with the MMS-DNA is approximately 1-2% of that observed with depurinated-reduced DNA and may be due to depurinated sites in the MMS DNA. In peak III, the level of activity found with depurinated-reduced DNA is approximately 5-10% of that observed with MMS-DNA. This ability to recognize depurinated DNA is a property of endonuclease II which has been observed even after purification of the enzyme to homogeneity, and it is also present in peak III of mutant BW 2001, which lacks the apurinic enzyme. Levels of the two endonucleases, when present, were reasonably consistent. The levels of exonuclease III were more variable even when not altered by mutation.

Sensitivity in Vivo of the Mutants to Methylmethanesulfonate. Experiments were then done to determine the degree of sensitivity in vivo of the parent and mutant strains to methylmethanesulfonate. The wild type and mutant strains of *E. coli* were exposed to 0.05 M methylmethanesulfonate for varying times after which they were plated. Survival curves, determined for the wild type, for AB 3027 and for NH 5016 are shown in Figure 5. As noted previously by Ljungquist et al. (1976), AB 3027 was extremely sensitive to MMS. This mutant lacks polymerase I, exonuclease III, and endonuclease II. The mutant NH 5016 with a normal polymerase but deficient in exonuclease III and endonuclease II was slightly more sensitive to MMS than wild type in their experiments, but in our laboratory had approximately the same sensitivity as the wild type strain.

The mutant strain, BW 2001, which lacked the apurinic

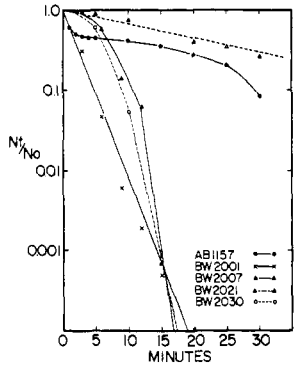

FIGURE 6: In vivo sensitivity to MMS of AB 1157, BW 2001, BW 2007, BW 2021, and BW 2030. Strains were tested at 37 °C.

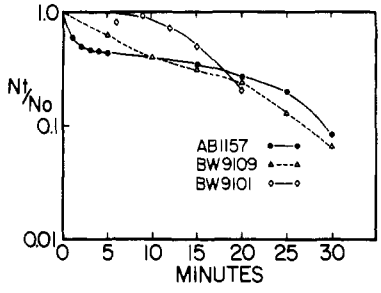

FIGURE 7: In vivo sensitivity to MMS of AB 1157, BW 9101, and BW 9109. Strains were tested at 37 °C.

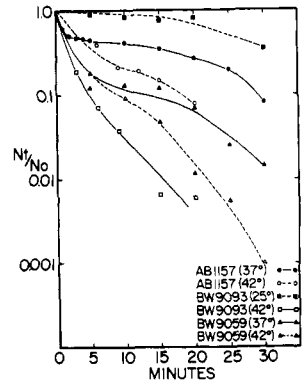

FIGURE 8: In vivo sensitivity to MMS of AB 1157, BW 9093, and BW 9059. AB 1157 was tested at 37 and 42 °C; BW 9093 was tested at 25 and 42 °C and BW 9059 was tested at 37 and 42 °C.

endonuclease as well as exonuclease III, was also extremely sensitive to MMS both in the experiments of Yajko and Weiss (1975) and as shown in Figure 6. This mutant was not as sensitive as AB 3027. The revertant BW 2007 which had regained the apurinic endonuclease, but not the exonuclease III, had approximately the same sensitivity as BW 2001, (Figure 6), while the other revertant BW 2021, with normal levels of both endonucleases and exonuclease III, was slightly more resistant than wild type. The mutant BW 2030, constructed from BW 2001, and lacking only exonuclease III had a sensitivity which was intermediate between BW 2001 and BW 2007 (Figure 6).

The deletion mutants BW 9101 and BW 9109 were unusual in that, in spite of a lack of apurinic endonuclease, endonuclease II, and exonuclease III, they were slightly more resistant to MMS than the wild type strain (Figure 7).

The mutants in which there is a defective exonuclease III were then examined (Figure 8). BW 9093 was tested at 25 °C and found to be less sensitive than the wild type, but at 42 °C it was considerably more sensitive. This mutant was shown by Milcarek and Weiss (1972) to have a thermolabile exonuclease III. BW 9059 which at 42 °C did not have exonuclease III was considerably more sensitive than wild type at 42 °C (Figure 8) but at 37 °C it was only slightly more sensitive.

Discussion

A summary of the presence or absence of the enzymes, apurinic endonuclease, endonuclease II, and exonuclease III, in the various mutants is given in Table II. Several mutants BW 9093, BW 2007, and BW 2030 are deficient only in exonuclease III. One mutant, BW 2001, is deficient both in apurinic endonuclease and exonuclease III, while two mutants, AB 3027 and NH 5016, are deficient in endonuclease II and exonuclease III. AB 3027 also lacks DNA polymerase I (Ljungquist et al., 1976). To date there is no mutant defective only in apurinic endonuclease or endonuclease II. These mutants suggest that there are three different genetic loci for the three enzymes. The mutants BW 9101 and 9109, with a deletion of 0.2 min which includes the *pnc*A gene and the exonuclease III (*xth*) gene, are missing not only exonuclease III, but also apurinic endonuclease and endonuclease II. This suggests that the genetic loci for these two endonucleases are also in the region close to the *xth* gene at 38.2 min on the revised map (White et al., 1976). The mutants AB 3027 and BW 2001 are most likely double mutants produced by MNNG. From the data available, it is not possible to order these genes. The presence of normal levels of endonuclease activity for depurinated-reduced and MMS treated DNA in peak II in both deletion mutants indicates that this activity is unrelated to the apurinic endonuclease and to endonuclease II.

TABLE II: The Relationship of Apurinic Endonuclease, Endonuclease II, and Exonuclease III to in Vivo Sensitivity to MMS in the Parent and Mutant Strains.[a]

Strain	Apurinic endonuclease	Endonuclease II	Exonuclease III	MMS sensitivity
AB 1157	+	+	+	1
AB 3027	+	−	−	4
NH 5016	+	−	−	1
BW 2001	−	+	−	4
BW 2007	+	+	−	3
BW 2030	+	+	−	3
BW 2021	+	+	+	0
BW 9101	−	−	−	0
BW 9109	−	−	−	0
BW 9093 25 °C	+	+	±	0
42 °C	ND[b]	ND	ND	3
BW 9059 37 °C	ND	ND	ND	1
42 °C	+	+	−	2

[a] MMS sensitivity is graded on a scale of 0-4 with 1 representing the parent strain, 4 the most sensitive strain, and 0 the least sensitive strains. [b] ND means not done.

The in vivo sensitivity of the various mutants to methylmethanesulfonate is presented in Table II. The first point of interest is that all of the mutants which are sensitive have a deficiency of exonuclease III. Mutant BW 9093 was shown to have a thermosensitive exonuclease III (Milcarek and Weiss, 1972). In this laboratory when BW 9093 cells were grown at 25 °C and fractions from the DEAE column tested at 25 °C, no exonuclease III activity was found. When this mutant was tested for in vivo sensitivity to MMS, it was normal at 25 °C but at 42 °C it had increased sensitivity (Table II). This provides further evidence that a deficiency of exonuclease III is

related to in vivo MMS sensitivity. The thermosensitivities of apurinic endonuclease and endonuclease II from this strain were found to be similar to those of the wild type strain. If either endonuclease was coded for by the same gene as exonuclease III, it would be expected to be as thermosensitive as exonuclease III.

There are two exceptions to the association of in vivo MMS sensitivity and exonuclease III deficiency. The first is mutant NH 5016 which lacks both exonuclease III and endonuclease II yet is not sensitive (Figure 5), while the second is the deletion mutation in the two different backgrounds, BW 9010 and BW 9109, which is also not sensitive in vivo (Figure 7). Ljungquist et al. (1976) noted that NH 5016 was slightly more sensitive to MMS than AB 1157, and they also proposed that the absence of DNA polymerase I in AB 3027 was the reason for its sensitivity.

Sensitivity of bacteriophage T7, treated in vitro with MMS and then used to infect a host, was related not to alkylated bases, but to depurinated sites (Lawley et al., 1969). The production of such sites in *E. coli* in vivo after treatment with MMS could occur by enzymatic or by spontaneous (Lindahl and Nyberg, 1972) loss of alkylated bases. The sensitivity to MMS of strains lacking exonuclease III suggests that this enzyme has a major role in repair. Recently Weiss (1976) and Ljungquist and Lindahl (1977) have presented evidence that exonuclease III acts as an endonuclease at apurinic sites. Furthermore, Ljungquist and Lindahl (1977) have provided evidence that exonuclease III is responsible for the major activity at apurinic sites in *E. coli* extracts. Thus, either the exonucleolytic or the endonucleolytic activity of exonuclease III may be required for efficient repair.

An explanation for the absence of sensitivity of the deletion mutants BW 9101 and 9109 and NH 5016, all of which lack exonuclease III, is not obvious. Since these mutants also lack endonuclease II, it is possible that the rate at which apurinic sites are generated in these strains is slower, and that these slowly generated sites can be repaired in the absence of exonuclease III by other apurinic enzymes to be discussed below. However, this explanation does not take into account the similar degrees of sensitivity to nitrous acid found in these mutants (Da Rosa et al., 1977). Further knowledge of the enzymes involved and the availability of new mutants will be needed to clarify this confusing picture.

A number of endonucleases have been isolated from *E. coli*. Endonuclease I, an enzyme active on native and denatured DNA, was described originally by Lehman (1962). Endonuclease II is the enzyme activity in peak III (Figure 1A) which has been purified and characterized (Kirtikar et al., 1976a). Endonuclease III, described by Radman (1976), recognizes UV-treated DNA. Endonuclease IV, isolated by Ljungquist (1977), acts on apurinic sites in the presence of 10^{-3} M EDTA. Endonuclease V, isolated by Gates and Linn (1977), acts on double- and single-stranded DNA as well as UV-irradiated, depurinated or OsO_4-treated DNA and DNA containing uracil.

The apurinic endonuclease present in peak I, Figure 1A, and purified (Kirtikar et al., 1976a) is designated endonuclease VI. It can be distinguished from endonuclease IV because its activity is lost in the presence of 10^{-3} M EDTA or 0.5 M NaCl or by heating the enzyme at 45 °C (Kirtikar et al., 1976b). These conditions do not inactivate endonuclease IV. Since endonuclease VI has chromatographic properties and a molecular weight similar to the enzyme purified by Verly and Rassart (1975), it is reasonable to assume that it is the same enzyme.

It should be emphasized that the enzymes which have activity on depurinated DNA include endonuclease IV, V, VI, and exonuclease III, while endonuclease II also has a minimal residual activity on this substrate. Lingquist and Lindahl (1977) have indicated that the endonucleolytic activity of exonuclease III is the major activity on apurinic sites, and they suggest that other enzymes active at apurinic sites play a minor role. This interpretation would support the experimental findings of Yajko and Weiss (1975) and Weiss (1976) who claimed that exonuclease III was endonuclease II, the latter defined as an endonucleolytic activity observed by the release of DNA from a heavily alkylated DNA gel. This treatment results in a significant number of depurinated sites which could then be a substrate for exonuclease III. The role of divalent metal in the phosphatase, endonuclease, and exonuclease activities of this enzyme still remains to be clarified (Kirtikar et al., 1976a).

Acknowledgments

The authors wish to thank Dr. Paul Howard-Flanders and Dr. Bernard Weiss for providing the bacterial strains.

References

Da Rosa, R., Friedberg, E. C., Duncan, B. K., and Warner, H. R. (1977), *Biochemistry 16* (in press).

Gates, F. T., and Linn, S. (1977), *J. Biol. Chem. 252*, 1647–1653.

Grossman, L., Braun, A., Feldberg, R., and Mahler, I. (1975), *Annu. Rev. Biochem. 44*, 19–43.

Hadi, S. M., and Goldthwait, D. A. (1971), *Biochemistry 10*, 4986–4994.

Hadi, S. M., Kirtikar, D. M., and Goldthwait, D. A. (1973), *Biochemistry 12*, 2747.

Howard-Flanders, P., and Theriot, L. (1965), *Genetics 53*, 1137–1150.

Kirtikar, D. M., Cathcart, G. R., and Goldthwait, D. A. (1976a), *Proc. Natl. Acad. Sci. U.S.A. 73*, 4324–4328.

Kirtikar, D. M., Dipple, A., and Goldthwait, D. A. (1975a), *Biochemistry 14*, 5548–5553.

Kirtikar, D. M., and Goldthwait, D. A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2022–2026.

Kirtikar, D. M., Kuebler, J. P., Dipple, A., and Goldthwait, D. A. (1976b), Cancer Enzymology, Schultz, J., and Ahmed, F., Ed., New York, N.Y., Academic Press, pp 139–155.

Kirtikar, D. M., Slaughter, J., and Goldthwait, D. A. (1975b), *Biochemistry 14*, 1235–1244.

Kushner, S. R., Nagaishi, H., Templin, A., and Clark, A. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68*, 824–827.

Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. (1969), *J. Mol. Biol. 39*, 181–198.

Lehman, I. R. (1962), *J. Biol. Chem. 237*, 819–828.

Lindahl, T., and Nyberg, B. (1972), *Biochemistry 11*, 3610–3618.

Ljungquist, S. (1977), *J. Biol. Chem. 252*, 2808–2814.

Ljungquist, S., and Lindahl, T. (1977), *FEBS Lett.* (submitted).

Ljungquist, S., Lindahl, T., and Howard-Flanders, P. (1976), *J. Bacteriol. 126*, 646–653.

Melgar, E., and Goldthwait, D. A. (1968), *J. Biol. Chem. 243*, 4401–4408.

Milcarek, C., and Weiss, B. (1972), *J. Mol. Biol. 68*, 303–318.

Radman, M. (1976), *J. Biol. Chem. 251*, 1438–1445.

Richardson, C. C., and Kornberg, A. (1964), *J. Biol. Chem. 239*, 242–250.

Verly, W. G., Paquette, Y., and Thibodeau, L. (1973), *Nature*

(*London*), *New Biol. 244*, 67-69.
Verly, W. G., and Rassart, E. (1975), *J. Biol. Chem. 250*, 8214-8219.
Weiss, B. (1976), *J. Biol. Chem. 251*, 1996-1901.
White, B. J., Hochhauser, S. J., Cintron, N. M., and Weiss, B. (1976), *J. Bacteriol. 125*, 1082-1088.
Yajko, D. M., and Weiss, B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72*, 688-692.

Specificity of Photochemical Cross-Linking in Protein–Nucleic Acid Complexes: Identification of the Interacting Residues in RNase–Pyrimidine Nucleotide Complex†

Abraham Havron and Joseph Sperling*

ABSTRACT: This report presents evidence in favor of the supposition that photochemical cross-linking between the partners of nucleoprotein complexes is specific and involves only residues which are in close proximity in the native structure. RNase was covalently linked to its ^{14}C-labeled competitive inhibitor uridine 2′(3′),5′-diphosphate (pUp) by ultraviolet irradiation of the enzyme–inhibitor complex. The amino acid residues which took part in the photochemical reaction were identified as Ser-80, Ile-81, and Thr-82. Ion-exchange chromatography of the irradiated complex afforded modified RNase containing one covalently bound molecule of [^{14}C]pUp (RNase-pUp). A tryptic digest of the performic acid oxidized RNase-pUp gave a single labeled peptide whose amino acid composition was consistent with the sequence Asn-67-Arg-85. Further degradation with thermolysin gave five peptides, only two of which were radioactive. One of these, Thr-76-Arg-85, contained one molecule of the labeled inhibitor and was totally deficient in Ser-80 and Ile-81. In the second, Ile-81-Arg-85, half of Thr-82 was missing and it contained half an equivalent of bound inhibitor. It is suggested that the initial attack of the excited pUp occurred at Ser-80 with subsequent modification of Ile-81. Thr-82 was modified, most probably, by a different mechanism to give a labile, though covalent, addition product. The pyrimidine ring of the nucleotide in the enzyme–inhibitor complex points in the direction of peptide 78–82 which constitutes the bottom of the binding site for the pyrimidine ring (Richards, F. M., and Wyckoff, H. W. (1973), in Atlas of Molecular Structures in Biology, Phillips, D. C., and Richards, F. M., Ed., Oxford, Clarendon Press, p 1). Thus, the identification of Ser-80, Ile-81, and Thr-82 as the residues which cross-linked photochemically to pUp indicates that such cross-linking is specific and involves only neighboring groups in the native structure of the nucleoprotein complex.

The tendency of proteins and nucleic acids to form stable covalent complexes as a result of ultraviolet irradiation has increasingly been used as a probe for studying the structure of native nucleoprotein complexes. Recent examples of problems which were studied by this approach are the interactions between aminoacyl-tRNA synthetases and their cognate tRNAs (Schoemaker et al., 1975; Budzik et al., 1975), ribosomal proteins and ribosomal RNA (Gorelic, 1976), coat protein and RNA in bacteriophage MS2 (Budowsky et al., 1976), and the interactions between DNA and histones in chromatin (Strniste and Rall, 1976; Sperling and Sperling, 1977). These studies are based on the assumption that the UV[1]-induced covalent cross-links are formed between interacting regions on the macromolecules, or between residues which are in close proximity in the native complex. The compliance with this condition would mean that the photochemical cross-linking "freezes" existing contact points in the irradiated protein–nucleic acid complex and, thereby, allows the identification of the interacting regions by chemical means. It is apparent, therefore, that the reliability of the photochemical approach depends on the ability of both purines and pyrimidines to form covalent adducts with a major number of amino acids—without particular preference toward specific ones. At the same time it requires that specific covalent bonds would be formed only between neighboring residues in the native structure.

The photochemical induction of addition products between pyrimidines and amino acids has been reported in several publications (Jellinek and Johns, 1970; Gorelic et al., 1972; Varghese, 1974; Sawada, 1975; Sperling and Havron, 1976). Recently, it has also been shown that stable covalent cross-links occur upon irradiation of complexes of various proteins and purine nucleoside triphosphates or cyclic phosphates (Sperling, 1976; Antonoff et al., 1976). As far as amino acids are concerned it has been shown, mainly by using low molecular weight model compounds, that a large variety of amino acids takes part in cross-linking to uracil and thymine derivatives (Smith, 1969; Schott and Shetlar, 1974; Shetlar et al., 1975).

In our previous work (Sperling and Havron, 1976) we have photochemically linked RNase A to two of its competitive inhibitors—pUp and pCp. We have shown, by three different criteria, that the cross-linking was specific: (a) the denatured enzyme failed to cross-link with the inhibitors; (b) the extent of covalent binding of pUp could be reduced by the addition of increasing amounts of another competitive inhibitor (3′-

†From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel. *Received March 17, 1977.* This work was supported by a grant from the Israel Commission for Basic Research.

[1] Abbreviations used are: pUp, uridine 2′(3′),5′-diphosphate; pCp, cytidine 2′(3′),5′-diphosphate; 3′-UMP, uridine 3′-monophosphate; HVPE, high voltage paper electrophoresis; DBAE-cellulose, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose; UV, ultraviolet; IR, infrared; TLC, thin-layer chromatography.