THE INCISION AND STRAND REJOINING STEP IN THE EXCISION REPAIR OF 5,6-DIHYDROXY-DIHYDROTHYMINE BY CRUDE E. COLI EXTRACTS

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SUMMARY

Strand resealing in the in vitro excision repair of 5,6-dihydroxy-dihydrothymine in osmium tetroxide oxidized polyd(A-T) by crude E. coli extracts is accomplished by polynucleotide ligase. Osmium tetroxide oxidized polyd(A-T) serves as a chemically well defined model substrate containing damage of the kind introduced into DNA by ionizing radiation. In the first incision step of excision repair approximately one endonucleolytic nick is introduced into the polymer by extracts of E. coli endoI$^-$ and E. coli endoI$^-$ uvrA6$^-$ per ring damaged thymine residue removed.

INTRODUCTION

Ring saturation products of the 5,6-dihydroxy-dihydrothymine type (t') represent a major class of lesions formed by γ-rays in the DNA in the living cell (1,2). Excision repair of such products has been demonstrated in bacteria (3) and mammalian cells (4) and is also accomplished from an exogenous, damaged DNA substrate by crude extracts of E. coli (5) and by isolated mammalian nuclei (6). Gamma-irradiated DNA contains a variety of lesions in addition to thymine damage of the 5,6-dihydroxy-dihydrothymine type including damage to the other bases, apyrimidinic and apurinic sites as well as strand breakage. For the study of the damage recognition-and incision step of excision repair, however, a chemically well defined DNA substrate is required. Osmium tetroxide (OsO_4) oxidized polyd(A-T) represents such a substrate since it contains mostly 5,6-dihydroxy-dihydrothymine and a few apyrimidinic sites, but no adenine damage or strand breakage (5,7). With the help of this substrate we have studied the incision and strand-rejoining steps of γ-ray excision repair. It was found that an endonucleolytic break is introduced on average at each ring damaged thymine residue by crude E. coli extracts and that polynucleotide ligase is responsible for the last resealing step.

EXPERIMENTAL PROCEDURES:

Crude extracts of the following E. coli strains were prepared according to methods reported previously (5): E. coli endoI$^-$ (MRW from Dr. R. Boyce),

E. coli endoI⁻ uvrA6 (RB 1001 from Dr. R. Boyce) and E. coli endoI⁻ lig 4 (1200 from Dr. R. McMacken). The latter strain has been obtained by Gellert and Bullock (8) and has been shown to contain a temperature sensitive polynucleotide ligase. The procedures for the selective oxidation of thymine in polyd(A-T)-thymine-methyl[^{3}H] (obtained from Miles Laboratories, Elkhart, Ind.) with osmium tetroxide to 5,6-dihydroxy-dihydrothymine (t') and for the determination of these products by the alkali-acid degradation assay have also been described (5,7). In the present study oxidized polyd(A-T) was used containing 0.7 to 1.6% of t' or approximately 3.5 to 8% total thymine ring destruction.

In a typical experiment 200 µl of freshly prepared E. coli extract was added to OsO_4-oxidized polyd(A-T)-thymine-methyl[^{3}H] (approximately 1 µg of polymer and 5-10 x 10^4 cpm) in 350 µl of 0.05 M phosphate-buffer (pH7) containing 1mM dithiothreitol. In part of the experiments the solution contained additionally the polynucleotide ligase inhibitor nicotinamide mononucleotide (NMN, 2mM) (9). The samples were incubated at 37° (unless

TABLE

Breaks Introduced Into OsO_4-oxidized Polyd(A-T) by Extracts of E. coli endoI⁻ and E. coli endoI⁻ uvrA6 (a)

Strain	Incubation Time (minutes)	Single Strand Breaks per 10^6 daltons	
		expected (b)	observed (c)
E. coli endoI-	7	8	7
	17	20	21
E. coli endoI⁻ uvrA6	7	8	5
	17	20	14

a. Incubation at 37° in the presence of 2mM NMN as described under "Experimental Procedures". Total thymine ring destruction in osmium tetroxide oxidized polyd(A-T) was 3.5% and was calculated from the value for t' by multiplication with a factor of 5 (see Reference 5).

b. The number of breaks expected was calculated from the t'-excision data obtained in the absence of NMN. The t'-excision curves were comparable to those reported in Reference 5.

c. The number of breaks observed was derived from the sedimentation data as described under "Experimental Procedures".

stated otherwise) and 50 µl aliquots were removed for analysis by alkaline sucrose gradient sedimentation. The remaining portions of the samples were treated with 2.5 ml of cold 7% TCA and the t' content of the precipitable material was determined by the alkali-acid degradation assay.

The sedimentation analysis was carried out using 5.2 ml 5-20% alkaline sucrose gradients. Centrifugation was at 19° in a Beckman L2-65B ultracentrifuge in a SW-50.1 rotor at 45,000 rpm for 12 hours. After centrifugation the bottom of each cellulose nitrate tube was punctured and 10 drop fractions were collected into scintillation vials. The samples were counted after addition of 6 ml Aquasol. The number of single strand breaks introduced relative to an unincubated sample of OsO_4-oxidized polyd(A-T) was calculated using the method of Veatch and Okada (10).

RESULTS AND DISCUSSION:

Incubation of OsO_4-oxidized polyd(A-T)-thymine-methyl[^{3}H] with extracts of *E. coli* endoI$^-$ or *E. coli* endoI$^-$ *uvr*A6$^-$ leads to the selective removal of approximately 50% of t' within 30 min. Acid solubilization of thymine label during the same time period is only 15 to 20% for both strains (see Figure 2 and Reference 5). It is evident from the gradient profiles shown on the left side of Figure 1 that the average molecular weight of the polymer is only slightly decreased during the incubation with the *E. coli* endoI$^-$ *uvr*A6 (not shown). It follows that the strand breaks which are introduced by the excision repair process stay open only for a very short time period before they are being rejoined. As shown on the right half of Figure 1, substantial breakdown of the polymer is observed, however, in the presence

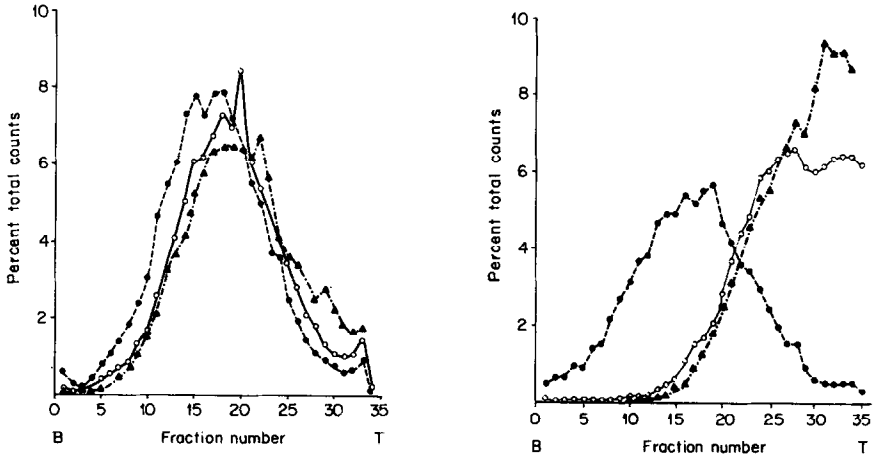

Figure 1: Alkaline sucrose gradient sedimentation profiles of OsO_4-oxidized polyd(A-T) incubated with extracts of *E. coli* endoI$^-$. For experimental conditions see under "Experimental Procedures". Left half: no NMN added; right half: in the presence of 2mM NMN. ●, 0 min. incubation; ○, 12 min. incubation; ▲, 34 min. incubation.

of 2mM nicotinamide mononucleotide (NMN) which inhibits the action of polynucleotide ligase. It is concluded that polynucleotide ligase is responsible for the strand resealing step in the excision repair of 5,6-dihydroxy-dihydrothymine by crude E. coli extracts. This conclusion is further supported by experiments with extracts of the temperature sensitive mutant E. coli endoI⁻ lig 4. As shown in the left half of Figure 2, substantial fragmentation of the polymer is only observed if the incubation with the extract is carried out at the nonpermissive temperature of 42° where polynucleotide ligase is inactive. The right half of Figure 2 gives the t'-excision and polymer degradation data for incubation at the permissive temperature of 30°. The corresponding data for extracts of E. coli endoI⁻ and E. coli endoI⁻ uvrA6 are included as dashed curves for comparison and are taken from work reported in Reference 5. The fact that t'-excision is considerably faster for the ligase mutant may be due to a partial deficiency of the ligase function even at 30° (11) which renders premature strand resealing before product excision

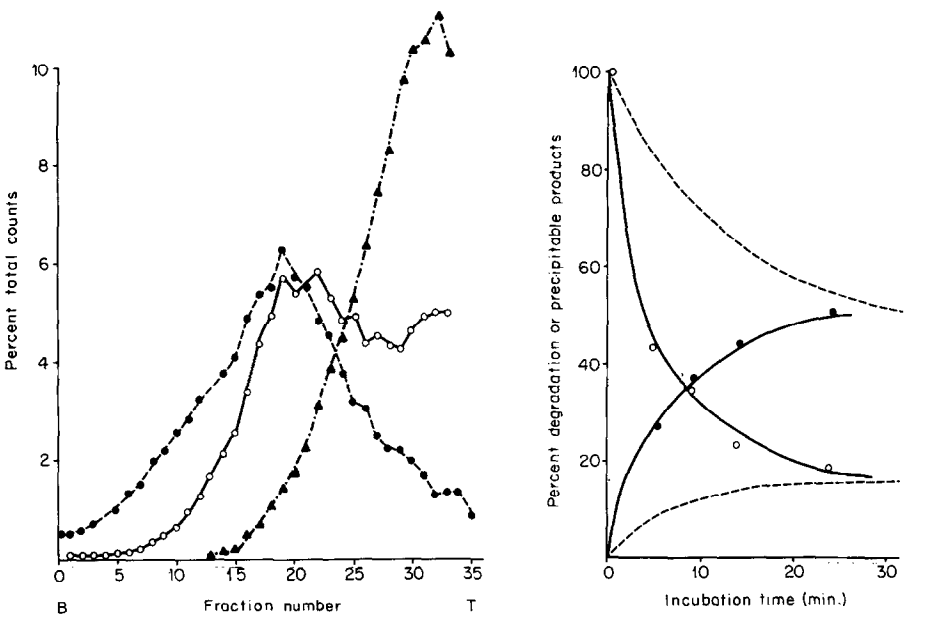

Figure 2: Alkaline sucrose gradient sedimentation profiles and t'-excision curves of OsO_4-oxidized polyd(A-T) incubated with extracts of E. coli endoI⁻ lig 4. For experimental conditions see under "Experimental Procedures". Left half: alkaline sucrose gradient sedimentation profiles. ●, 0 min. incubation; O, 5 min. incubation at 30°; ▲, 5 min. incubation at 42°. Right half: t'-excision and polymer degradation. O, percent t' remaining acid precipitable; ●, percent thymine label rendered acid soluble. The dashed curves give the corresponding data for extracts of E. coli endoI⁻ and E. coli endoI⁻ uvrA6 and are comparable to those reported in Reference 5.

has been accomplished less likely. While product excision could be demonstrated at short incubation times at 42° for extracts of E. coli endoI⁻ lig 4 (not shown) a clear distinction between product excision and unspecific polymer degradation was no longer possible later on.

The correlation of the level of t'-excision to the number of strand breaks introduced and remaining open under conditions of ligase inhibition yields valuable information concerning the endonucleolytic incision step. The data is given in the table. The number of breaks remaining open is calculated from the sedimentation data shown in Figures 1 and 2 according to Veatch and Okada (10); the number of strand breaks expected was derived from excision data in the absence of NMN for E. coli endoI⁻ and E. coli endoI⁻ uvrA6. The excision data under conditions of ligase inhibition could not be used for this purpose since unspecific polymer hydrolysis was substantially increased. It is evident that the values for the expected and observed number of breaks are very close for extracts of E. coli endoI⁻ and it follows that one break is on average introduced for each ring damaged thymine residue. For extracts of E. coli endoI⁻ uvrA6⁻ the observed number of breaks is 3/4 of that expected and it follows that 0.75 breaks are introduced per ring damaged thymine residue. These values could be underestimates if product excision is more efficient under conditions of ligase inhibition under which premature strand rejoining cannot occur. In any case, it is clear that endonucleolytic incision at ring damaged thymine in OsO_4-oxidized Polyd(A-Y) by crude E. coli extracts is quite efficient and complete.

REFERENCES

1. Cerutti, P. (1974) Naturwissenschaften 61, 51-59
2. Swinehart, J. and Cerutti, P., unpublished results
3. Hariharan, P. and Cerutti, P. (1972) J. Mol. Biol. 66, 65-81
4. Mattern, M., Hariahran, P., Dunlap, B. and Cerutti, P. (1973) Nature New Biol. 245, 230-232
5. Hariharan, P. and Cerutti, P. (1974) Proc. Natl. Acad. Sci. 71, 3532-36
6. Hariharan, P., Remsen, J. and Cerutti, P. (1974) in "Molecular Mechanisms for The Repair of DNA" (eds. P. C. Hanawalt and R. B. Setlow) Plenum Press, New York, in the press; see also Rad. Res. 59, 177 (1974)
7. Beer, M., Stern, S., Carmalt, D. and Mohlenrich, K. (1966) Biochemistry 5, 2283-2288
8. Gellert, M. and Bullock, M. L. (1970) Proc. Natl. Acad. Sci. 67, 1580-1587
9. Campbell, J., Fleischman, A. and Richardson, C. (1971) Fed. Proc. 30, 1313
10. Veatch, W. and Okada, S. (1969) Biophys. J. 9, 330-346
11. Gottesman, M., Hicks, M. and Gellert, M. (1973) J. Mol. Biol. 77, 531-547

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