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Purification and Properties of *Methanobacterium thermoautotrophicum* DNA Photolyase*

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We have purified DNA photolyase from the autotrophic anaerobic archaebacterium *Methanobacterium thermoautotrophicum* to near homogeneity by a two-column affinity chromatography. The purified enzyme has an M_r = 60,000 and shows near UV absorption peak at 440 nm and a fluorescence emission maximum at 462 nm indicating that it contains 8-hydroxy-5-deazaflavin (coenzyme F420) as an intrinsic chromophore. The photolyase binds with high specificity to thymine dimer in DNA with an equilibrium binding constant, $K_A = 1.4 \times 10^9$ M^{-1}, and a dissociation rate constant, $k_{off} = 1.4 \times 10^{-4}$ s^{-1} ($t_{1/2}$ = 43 min). Despite 6-fold higher affinity compared to the folate-containing *Escherichia coli* photolyase the two enzymes apparently contact the same phosphates around the thymine dimer: the phosphate immediately 5′ and the three phosphates immediately 3′ to the dimer on the damaged strand and the phosphate across from the dimer in the minor groove on the complementary strand. The absolute action spectrum of the *Methanobacterium* photolyase in the 400–500-nm region closely matches the absorption of the enzyme-bound F420. The quantum yield (ϕ) over this region is constant and is approximately 0.2. The value is measurably smaller than the quantum yields reported for other DNA photolyases.

DNA photolyases (EC 4.1.99.3) convert light energy into chemical energy to repair pyrimidine dimers introduced into DNA by far UV (200–300 nm) radiation. The enzymes bind to DNA in a light-independent step but there is an absolute requirement for light (300–500 nm) for catalysis. All photolyases purified and characterized to date seem to fall into two classes. The folate class photolyases as exemplified by the *Escherchia coli* and *Saccharomyces cerevisiae* enzymes, contain two tightly bound chromophores, $FADH_2$ and 5,10-methenyltetrahydrofolate, while the deazaflavin class enzymes, represented by *Scendesmus acutus* and *Streptomyces griseus* photolyases contain the 8-hydroxy-5-deazaflavin coenzyme (F420) and $FADH_2$ (Eker *et al.*, 1981, 1988; Sancar and Sancar, 1984; Johnson *et al.*, 1988; for a review, see Sancar and Sancar, 1988). The folate class enzymes have their absorption and action spectra maxima at around 380 nm and the deazaflavin class enzymes have their maxima at around 440 nm. Characterization of photolyases from both eucaryotes (*S. cerevisiae* and *S. acutus*) and procaryotes (*E. coli*, *S. griseus*, and *Anacystis nidulans*) indicate that members of both kingdoms may have either the folate class or the deazaflavin class enzymes and furthermore, the three photolyase genes that have been sequenced show approximately 40% homology regardless of the class or origin (Yasui *et al.*, 1988).

Archaebacteria have recently been classified as a separate kingdom from eubacteria and eucaryotes (Woese, 1987). Therefore it is of interest to determine the physical and photochemical properties of photolyases from this kingdom and compare them with those of photolyases from pro- and eucaryotes. Previous *in vivo* studies (Kiener *et al.*, 1983) showed that the methanogenic archaebacterium *Methanobacterium thermoautotrophicum* is photoreactivable with a relative action spectrum maximum in the range of 422–433 nm consistent with a F420 chromophore. Indeed these bacteria contain large quantities of this compound which acts as a cofactor in the enzyme system that generates methane by hydride transfer from H_2 to CO_2 (for a review, see Walsh, 1986). In this article we described the purification, spectral, photochemical, and DNA-binding properties of *M. thermoautotrophicum* photolyase.

MATERIALS AND METHODS

Chemicals—Heparin-agarose (Type I), calf thymus DNA, Sepharose CL-2B-300, dimethyl sulfate, and ethylnitrosourea were purchased from Sigma, and 5 M CNBr in acetonitrile was obtained from Aldrich. [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from DuPont-New England Nuclear.

Substrates—UV-irradiated pBR322 containing 5–6 pyrimidine dimers/molecule and a terminally labeled 48-mer duplex containing a centrally located thymine dimer were used as substrates. These substrates were prepared as described by Sancar *et al.* (1984) and Husain *et al.* (1987, 1988), respectively.

UV Single Strand DNA-Agarose—For affinity chromatography of photolyase UV-irradiated single strand DNA-agarose was prepared according to the method of Arndt-Jovin *et al.* (1975). Sonicated calf thymus DNA (3.6 mg/ml) was irradiated with UV from a germicidal lamp for 1 h at a distance of 10 cm prior to coupling. One-half volume of heat-denatured DNA solution was added to 1 volume of cyanogen bromide-activated Sepharose CL-2B-300. Following coupling, the Sepharose was washed with 0.1 M NaOH and then with distilled water.

Assays for Photolyase—The transformation assay (Sancar *et al.*, 1984) was used to locate photolyase during chromatography. The irradiated pBR322 DNA was mixed with column fractions in 50 μl of reaction buffer containing 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol. The mixture was exposed to photoreactivating light and mixed with competent cells. The increase in transformation efficiency with photoreactivating light was taken to be a measure of photolyase activity. The gel retardation assay was used with pure enzyme to study the dark reaction (binding) as well as to obtain the photolytic cross-section ($\epsilon\phi$) of the photochemical reaction. This assay was conducted as described by Husain and

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TABLE I

Purification of M. thermoautotrophicum DNA photolyase

Purification step	Volume	Total protein	Tet^R colonies/μg of protein	Total transformats	Yield	Purification factor
	ml	*mg*			%	
Cell-free extract	100	1200	160	1.9×10^8	100	1
Heparin-agarose	80	20	8300	1.6×10^8	84	52
UV single-stranded DNA-agarose	0.5	0.05	1.1×10^6	5×10^7	26	6250

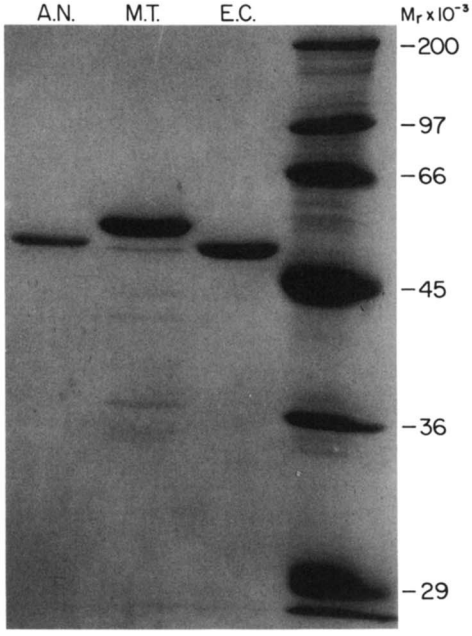

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of photolyase from various bacteria.** *A.N.*, 1.2 μg of *A. nidulans* photolyase; *M.T.*, 2.5 μg of *M. thermoautotrophicum* photolyase; *E.C.*, 2 μg of *E. coli* photolyase. The last lane contains M_r standards whose molecular weights are given on the right margin. The 10% polyacrylamide gel was stained with Coomassie Blue.

Sancar (1987) with the exception that in photolysis cross-section measurements, the enzyme/substrate mixture was exposed to photoreactivating light of proper wavelength before loading onto the gel. The fluence rates were (in ergs mm^{-2} s^{-2}): 30.8 at 405 nm, 30.3 at 420 nm, 16.8 at 433 nm, 63.8 at 450 nm, and 287.3 at 480 nm.

Spectral Measurements—Absorption spectra were recorded on a Hewlett-Packard diode array spectrophotometer. Fluorescence spectra were measured with a Perkin-Elmer Lambda 5 spectrofluorometer. Monochromatic photoreactivation was conducted with a Quantacount monochromator-actinometer from Photon Technology International (South Brunswick, NJ). The quantocount was calibrated with potassium ferrioxalate actinometry of Calvert and Pitts (1966).

Enzymatic and Chemical Footprinting—We probed the photolyase contact sites on a 48-mer duplex (Husain *et al.*, 1988) containing a single thymine dimer with DNase I and dimethyl sulfate protection as well as ethyl phosphotriester and dimethyl sulfate interference as described previously (Husain *et al.*, 1987), except 1 nM substrate was incubated with 18–36 nM photolyase and $E \cdot S$ complexes were separated by gel retardation for the interference studies.

Growth of M. thermoautotrophicum—*M. thermoautotrophicum* Marburg (DSM 2133) was grown in a 20-liter fermenter in the minimal medium described by Schoenheit *et al.* (1980). The cells were grown to stationary phase, harvested in a continuous flow centrifuge, frozen in liquid nitrogen, and stored at −80 °C.

Buffers—The core photolyase buffer (PL buffer) contained 50 mM Tris, pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol (v/v). The other buffers were derived from this by inclusion of the appropriate NaCl concentrations. These buffers are indicated with the prefix indicating the molarity of the sodium chloride, *e.g.* 1 M PL buffer is photolyase core buffer containing 1 M NaCl.

Purification of Photolyase—All steps of enzyme purification were performed at 4 °C except for the UV DNA affinity chromatography which was done at room temperature. One volume of the *M. thermoautotrophicum* cells was resuspended in 3 volumes of 1 M PL buffer and the bacteria were lysed in a French Press. The cell debris was removed by centrifugation at 100,000 × *g* for 2 h. The cell-free extract was dialyzed overnight against 20 volumes of PL buffer (no NaCl) and stored at −20 °C until further use. The cell-free extract was thawed at 4 °C and 50 ml of extract (600 mg of protein) was loaded onto a 100-ml heparin-agarose column (2.5 cm diameter) equilibrated with 0.1 M PL buffer. The column was washed with 200 ml of 0.1 M PL buffer and developed with a 500-ml linear gradient of 0.1 M PL buffer to 0.75 M PL buffer at a rate of 1 ml/min. Photolyase eluted at NaCl concentration of about 0.37 M. Fractions from this step were stored at −20 °C for 2 weeks without loss of activity. The active fractions of two heparin-agarose columns were combined (80 ml, 20 mg of protein) and loaded onto a 1.5-ml UV single-stranded DNA-agarose column at a flow rate of 20 ml/h. The column was equilibrated with 0.6 M PL buffer and covered with a yellow plastic foil ($A_{380-440} > 3$) to prevent photorepair of pyrimidine dimers during loadings of the enzyme. The column was washed with 15 ml of 0.6 M PL buffer. Then, the yellow plastic filter was removed and the column was illuminated with "black light" at a distance of 10 cm while the elution buffer of 1.5 M PL buffer was being applied. Active fractions eluting from the DNA-agarose column were combined and concentrated by ultrafiltration in a Centricon tube (30 kDa cutoff, Amicon) and the buffer was exchanged for 0.1 M PL buffer. The enzyme was stored at −80 °C.

Other Methods—Protein concentrations of crude enzyme preparations were determined by the method of Bradford (1976). The concentration of the pure enzyme was measured spectrophotometrically assuming an extinction coefficient $\epsilon_{433} = 40{,}000$ M^{-1} cm^{-1} for the enzyme that contains the F420 cofactor at 1:1 stoichiometry. The NH_2-terminal amino acid sequence of purified photolyase was determined on a gas-phase sequencing machine at the Microchemistry facility of the Biological Laboratories, Harvard University, Cambridge, MA.

The equilibrium binding constant of photolyase to *cis-syn* thymine dimer and the dissociation rate constant were determined by the gel retardation method described previously (Husain and Sancar, 1987).

RESULTS

Purification of Photolyase—M. thermoautotrophicum DNA photolyase was purified 6200-fold with a 26% yield to approximately 95% homogeneity following the two-step procedure summarized in Table I. The DNA affinity resin used for purification gave a high recovery of photolyase and minimized the amounts of contaminating proteins. Other factors that affect recovery are column size and exposure to light during elution. It is important to use the smallest possible amount of affinity resin in the column since oversized columns lead to a decreased yield of photolyase. Similarly, if the affinity column remained covered with the yellow plastic foil during elution with the 1.5 M PL buffer, the recovery was only about 10% of that obtained after elution off the illuminated resin. The same protocol has been used to purify the *A. nidulans* DNA photolyase from a *Streptomyces coelicolor* strain carrying the *A. nidulans* photolyase gene[1] and a slightly modified procedure to purify the *E. coli* photolyase from an overproducing strain (Sancar *et al.*, 1984).

[1] A. Kiener, J. Piret, and A. Yasui, unpublished results.

TABLE II

NH_2-terminal sequence of M. thermoautotrophicum DNA photolyase

Asterisk denotes residue homologous to a patch in *E. coli* photolyase extending from residue 431 through 449.

Residue	1									10					15
Sequence	Met	Ile	His	Asp	Glu	Arg	Ile	Arg	Ser	Leu	Asn	Thr	Glu	Lys	Pro
	|	|			●		*			|			*	
Yield (pmol)	101	63					41			33					
Residue	16				20					25			28		
Sequence	Ala	Arg	Asp	Gly	Lys	Tyr	Val	Ile	Tyr	Trp	Met	Gln	Ala		
	|			*	*		*						|		
Yield (pmol)	23						85						7.1		

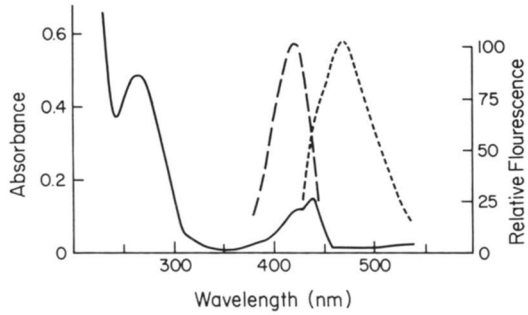

FIG. 2. **Optical spectra of *M. thermoautotrophicum* photolyase.** ——, absorption spectrum; – – –, fluorescence excitation (λ emission = 462 nm); and · · ·, fluorescence emission (λ excitation = 420 nm) spectra.

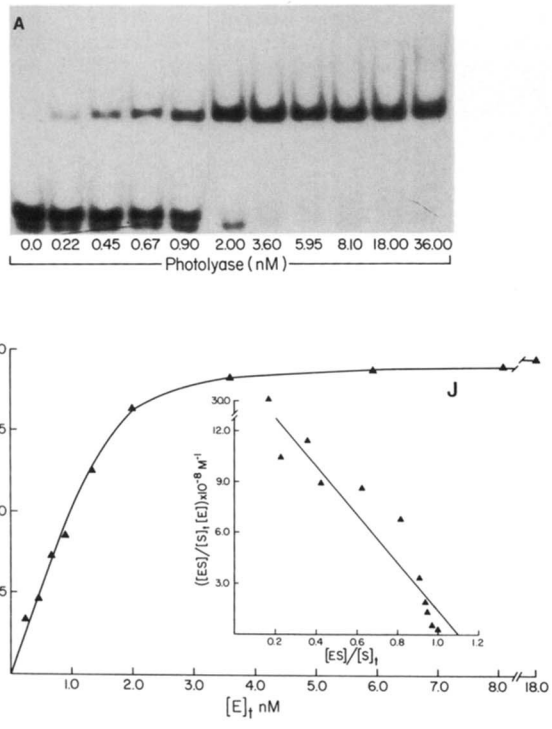

FIG. 3. **Equilibrium binding of photolyase to DNA.** The defined substrate (1 nM) was mixed with increasing concentrations of photolyase and the $E \cdot S$ complexes were separated from free DNA by the "gel retardation" method and quantitated. *A*, a typical run of a gel retardation experiment with the indicated concentrations of photolyase. *B*, analysis of data from gel retardation. The averages from three gel retardation experiments are plotted as a saturation curve and replotted in the *inset* in the form of Eadie-Scatchard plot to obtain the binding constant and stoichiometry.

In Fig. 1 photolyases purified by this two-column procedure are compared on a 10% sodium dodecyl sulfate-polyacrylamide gel. The *E. coli* and *A. nidulans* photolyases which have calculated M_r values of 53,994 (Sancar *et al.*, 1984) and 54,475 (Yasui *et al.*, 1988), respectively, exhibit apparent molecular weights of 50,000 relative to the standards used in this gel. Relative to these standards the *M. thermoautotrophicum* photolyase has M_r = 55,000. However, the molecular weight of this enzyme is probably closer to 60,000 based on its mobility relative to the two other photolyases. Indeed the NH_2-terminal amino acid sequence of the purified enzyme revealed that the archaebacterial photolyase, like the eucaryotic (yeast) photolyase, has an NH_2-terminal tail (Sancar, 1985; Yasui *et al.*, 1988) that is non-homologous to the eubacterial photolyases.

NH_2-terminal Sequence—The NH_2-terminal sequence of the methanogen photolyase was determined by gas-phase sequencing method using 100 pmol of photolyase. The sequence of the 28 NH_2-terminal amino acids is shown in Table II. The sequence shows no homology to the NH_2-terminal sequences of the two procaryotic (*E. coli* and *A. nidulans*) and one eucaryotic (*S. cerevisiae*) photolyases that have been sequenced. However, the region spanning Glu^5-Val^{22} is 39% homologous to the region Glu^{431}-Val^{448} of *E. coli* photolyase.

Spectral Evidence for 8-OH-5-Deazaflavin Chromophore—The near UV absorption and fluorescence spectra of the methanogen photolyase are shown in Fig. 2. The enzyme has an absorption maximum at 434 nm and fluorescence excitation and emission maxima at 420 and 462 nm, respectively. As these spectroscopic properties are typical of photolyases containing an F420 cofactor (Eker *et al.*, 1988), we conclude that the *M. thermoautotrophicum* photolyase has an 8-OH-5-deazaflavin cofactor. Assuming an extinction coefficient of 40,000 M^{-1} cm^{-1} (Walsh, 1986) there is a stoichiometric amount of cofactor bound per enzyme molecule. Since our supply of purified photolyase has to date been limited, we have not yet been able to analyze for the presence of a stoichiometrically bound flavin adenine dinucleotide cofactor which is present in all photolyases (as $FADH_2$ or $FADH^{\cdot}$) reported in the literature (Sancar and Sancar, 1988).

Binding of Photolyase to a Thymine Dimer—We investigated the kinetics and thermodynamics of DNA-photolyase binding using a gel retardation assay (Husain and Sancar, 1987) and a 48-mer duplex with a centrally located thymine dimer as substrate (Husain *et al.*, 1988). Fig. 3*A* shows the titration of the substrate with photolyase, and in Fig. 3*B* we have plotted the data from *A* and two other experiments conducted under identical conditions. Photolyase-substrate complexes run as a sharp band in contrast to those formed with the *E. coli* photolyase which "streak" throughout the lanes. From the Eadie-Scatchard plot in the *inset* of Fig. 3*B* we obtain an equilibrium binding constant $K_A = 1.4 \times 10^9$ M^{-1} and a stoichiometry of 1.1 photolyase molecule per thymine

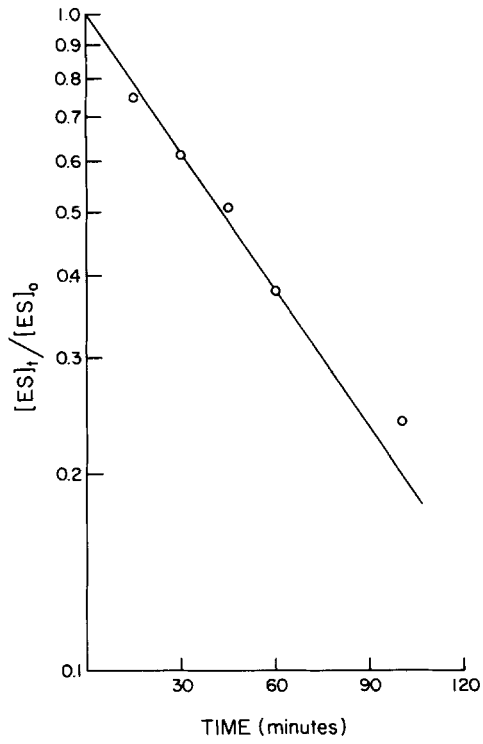

FIG. 4. **Dissociation of photolyase from thymine dimer.** The ^{32}P-labeled 48-mer (1 nM) was incubated with 1 nM photolyase to equilibrium, competing DNA in the form of UV-irridated pBR322 was added to 36-fold molar excess (pyrimidine dimers) and at the indicated time points the photolyase-radiolabeled DNA complexes were quantitated by the gel retardation assay and plotted as a fraction of the initial concentration.

dimer. Thus the methanogen photolyase, like other photolyases, binds as a monomer; however, it has 5–6-fold higher affinity to thymine dimer than *E. coli* photolyase (Husain and Sancar, 1987). The dissociation rate constant K_2 was also determined by gel retardation using the competing cold substrate method. The results of such an experiment are shown in Fig. 4. About 70–80% of the complexes dissociate with a rate constant $k_2 = 1.4 \times 10^{-4}$ s^{-1}; the remaining complexes dissociate considerably slower as is the case with the *E. coli* photolyase. However, in contrast to the *E. coli* enzyme (where $k_{off} = 4.4 \times 10^{-2}$ s^{-1}) the main off-rate is exceedingly slow, corresponding to a $t_{1/2}$ of about 43 min. In fact, the association rate constant calculated from $K_A = k_1/k_2$ or $k_1 = K_A \cdot k_2 = 1.4 \times 10^9 \times 1.4 \times 10^{-4} = 2 \times 10^5$ M^{-1} s^{-1} is about 10–100-fold slower than the on-rate for a diffusion-controlled reaction. The k_{cat} for *M. thermoautotrophicum* photolyase is 9 min^{-1} under saturating light (data not shown) which is comparable to the corrected value of 25 min^{-1} for *E. coli* photolyases[2] perhaps indicating that the dissociation of the enzyme from repaired DNA is also slower than that of *E. coli*.

DNA Contacts of Photolyase—The fact that *E. coli* and *M. thermoautotrophicum* photolyases with two different photoharvesting cofactors, bind to the same substrate with different kinetic and thermodynamic parameters raised the intriguing possibility that the structural determinants for binding by the two enzymes were different. To investigate this possibility we conducted enzymatic and chemical footprintings on both enzymes using the uniquely modified substrate, the thymine dimer-containing 48-mer.

From DNase I footprints of both enzymes on the dimer containing and the complementary strand, respectively (data not shown), both enzymes essentially protect a 11–16-base pair region around the dimer from DNase I attack, and therefore no conclusion can be made as to the probable cause of the differential affinities of the two enzymes.

Higher resolution footprints were obtained by conducting ethylation interference and methylation interference and protection experiments. The results of ethylation interference are shown in Fig. 5. The *E. coli* enzyme contacts the phosphodiester bond immediately 5′ and the three phosphodiester bonds immediately 3′ to the dimer, as reported previously (Husain *et al.*, 1987). We also see that a contact 5′ phosphodiester bonds 3′ to the cross-dimer AA on the complementary strand. This relatively weak contact was missed in the previous report because the enzyme-substrate complexes were separated from unbound substrate by nitrocellulose filtration where the efficiency of $E \cdot S$ complex retention is only 30%. We find that separation of complexes on a 5% polyacrylamide gel is more satisfactory for interference studies. Surprisingly, we find that the *M. thermoautotrophicum* photolyase makes exactly the same phosphate contacts as the *E. coli* enzyme.

Finally, the comparative bindings of the two enzymes were probed by methylation protection and interference experiments which reveal groove contacts. The results of methylation protection experiments are shown in Fig. 6. Methylation of the G immediately 5′ to the dimer is partially blocked by both photolyases while the *M. thermoautotrophicum* enzyme but not *E. coli* photolyase protects strongly the second and fourth G 3′ to the dimer. In addition, the G 5 nucleotides 3′ to the dimer (G31) becomes hypersensitive to alkylation in the presence of the methanogen enzyme. Repair by either enzyme changes the methylation pattern of the DNA by rendering the G immediately 3′ to the dimer hypersensitive to methylation. This is perhaps due to the fact that the 48-mer is bent into the major groove at the dimer, making the groove less accessible to dimethyl sulfate; upon repair the DNA returns to normal B conformation (Husain *et al.*, 1987). There is no strong effect of photolyases on the methylation pattern of the complementary strand except that the A across from the 5′ T of the dimer becomes hypersensitive to methylation in the presence of *E. coli* photolyase, whereas there is a slight inhibition of methylation of this A with *M. thermoautotrophicum* photolyase. Thus it appears that the *M. thermoautotrophicum* enzyme is more deeply embedded into the major groove around the dimer compared to the *E. coli* enzyme. This conclusion is further supported by the methylation interference experiments shown in Fig. 7. The methylation of the second G 3′ to the dimer interferes strongly with binding of the *M. thermoautotrophicum* enzyme but only marginally with the binding of the *E. coli* photolyase. The methylation of the first G 3′ to the dimer, in contrast, increases the affinity of the *E. coli* photolyase but has no effect on binding of the *M. thermoautotrophicum* enzyme.

The summary of the footprinting data is given in Fig. 8. Qualitatively the contacts made by the two enzymes are remarkably similar. However, quantitatively the contacts of the methanogen enzyme in the major groove, especially 3′ to the dimer, is more intimate and this may explain the 5–6-fold higher binding constant and slower dissociation of the enzyme.

The Absolute Action Spectrum of M. thermoautotrophicum Photolyase—The absolute action spectra of both folate and deazaflavin class enzymes have been reported and for both classes of enzymes quantum yields of about 1.0 have been proposed (Sancar *et al.*, 1987; Eker *et al.*, 1986). We therefore determined the absolute action spectrum of the pure *M.*

[2] Y. F. Li and A. Sancar, unpublished data.

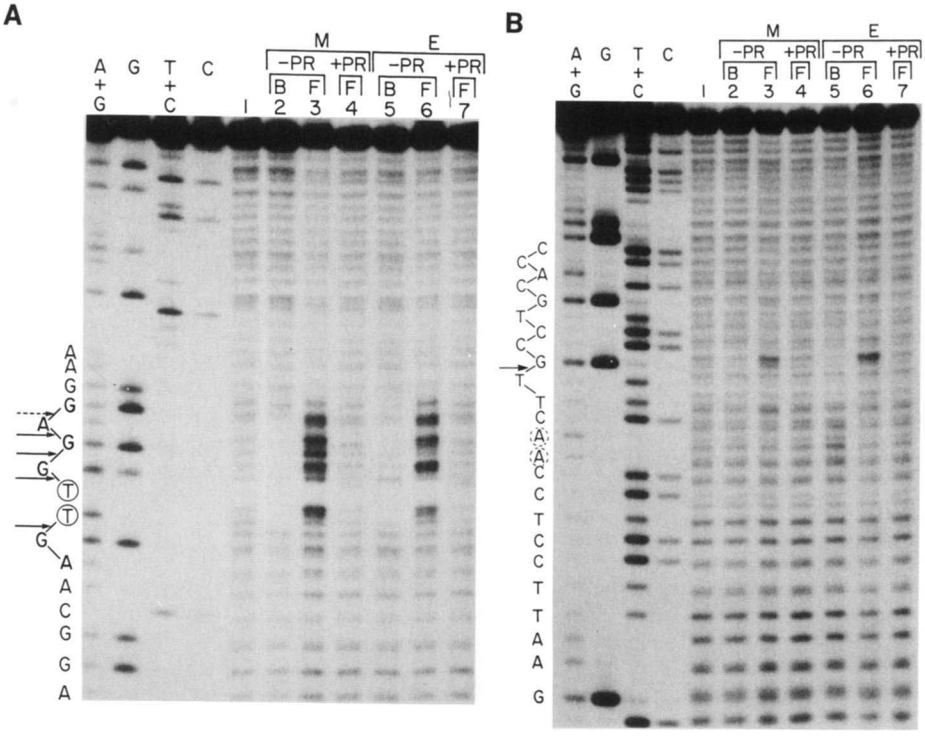

FIG. 5. **Phosphate contacts of *M. thermoautotrophicum* (*M*) and *E. coli* (*E*) photolyases.** Ethylnitrosourea-treated DNA was mixed with photolyase, enzyme bound (*B*) and free (*F*) DNA were separated by the gel retardation method, cleaved by heating with alkali, and samples were analyzed on a 10% sequencing gel. *A*, (top strand), *lane 1* contains ethylated DNA that was heated in alkali, *lanes 2* and *3* contain photolyase-bound and free DNA that was cleaved with alkali. *Lane 4*, contains alkylated DNA that was mixed with *M. thermoautotrophicum* photolyase, photoreactivated (*+PR*), and then cleaved with alkali. *Lanes 5–7* contain DNA that was treated in the same way as in *lanes 2–4* except *E. coli* photolyase was used. The phosphates whose ethylation strongly interfere with binding are indicated by *solid arrows* and the one phosphate whose ethylation weakly interferes with binding is indicated by a *broken arrow*. *B* (bottom strand), the ethylated DNA in *lanes 1–7* was treated in a similar manner as in *lanes 1–7* of the top strand. One phosphate whose ethylation interferes with binding is indicated by an *arrow*.

thermoautotrophicum photolyase in the 400–500-nm region. In order to determine the quantum yield we conducted photoreactivation experiments under enzyme excess condition such that all substrate was bound to the enzyme and therefore the reaction kinetics was simplified to a first order kinetics (k_p = first order rate coefficient) with the light dose (L) as the variable (Rupert, 1962). Under these conditions:

$$\ln \frac{[ES]}{[ES]_0} = -k_p \, L \tag{1}$$

and therefore k_p is obtained from the slope of the enzyme-substrate decay kinetics. k_p is related to the photolytic cross-section ($\epsilon\phi$) by a simple equation (Rupert, 1962).

$$\epsilon\phi = \frac{k_p \times 5.2 \times 10^9}{\lambda} \tag{2}$$

We determined k_p, and therefore $\epsilon\phi$, at wavelengths defining the near UV peak of the *M. thermoautotrophicum* enzyme and superimposed these values on the absorption spectrum of this enzyme (Fig. 9, *top*). The absolute action spectrum has the same shape as the absorption spectrum, thus confirming that the 8-hydroxy-5-deazaflavin responsible for absorption in this region acts as a photosensitizer in dimer photoreversal. However, the magnitude of the photolytic cross-section at all wavelengths is approximately 5-fold lower than the extinction coefficients at the corresponding wavelengths. Thus the quantum yield (ϕ) of the *M. thermoautotrophicum* photolyase is about 0.20 in the near UV region (Fig. 9, *bottom*).

DISCUSSION

Evolutionarily the pyrimidine dimer cleaving DNA photolyase may have been the first DNA repair system to have evolved. The early atmosphere was presumably poor in oxygen and thus early organisms were exposed to high UV flux. It is therefore of interest to characterize repair systems in organisms that even at present maintain their capabilities to thrive under conditions of the early atmosphere, anaerobiosis, high H_2 and CO_2 content. *M. thermoautotrophicum*, which belongs in the "third kingdom" of the biological world, the archaebacteria, is a representative of this group of organisms. In this article we have characterized the photoreactivation system of this bacterium. The following points of interest emerged from our study. First, *M. thermoautotrophicum* appears, in comparative terms, extremely rich in photolyase. This enzyme constitutes 0.016% of total cellular proteins which is nearly 10-fold higher than the amount of photolyase in *E. coli* and yeast. Whether this high level is an archaeological remnant or indicates that the enzyme may have another function in this organism is uncertain. The content of the free 8-hydroxy-5-deazaflavin (coenzyme F420) is very high, up to 100 mg/kg (Walsh, 1986) and F420 is utilized as a low potential, diffusable redox cofactor in these organisms.

The second point of interest of this study is the fact that the *M. thermoautotrophicum* (deazaflavin class) and *E. coli* (folate class) photolyases appear to make essentially the same contacts with a DNA fragment containing a thymine dimer yet bind to it with different affinities and the *M. thermoautotrophicum* enzyme dissociates (in the dark) from the substrate about 30-fold slower. The higher affinity of the *M. thermoautotrophicum* enzyme and the lower dissociation rate are probably due to the fact that this enzyme is buried deeper into the major groove 3′ to the dimer (as revealed by meth-

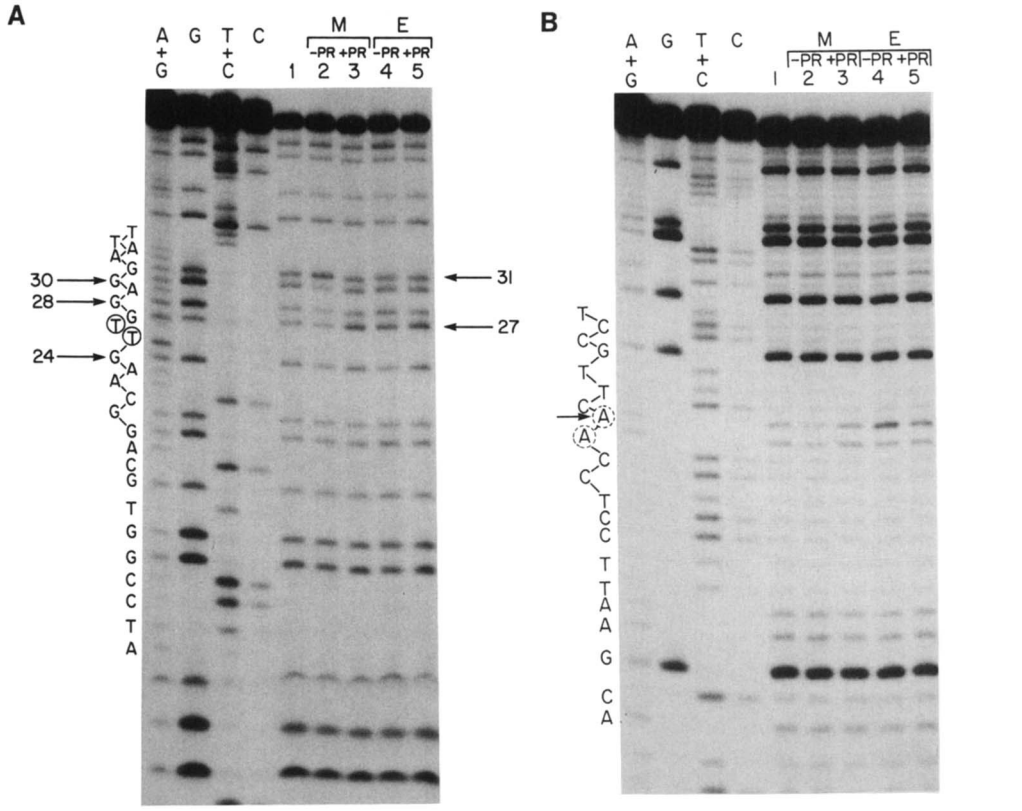

FIG. 6. **Methylation protection by *M. thermoautotrophicum* (*M*) and *E. coli* (*E*) photolyases.** DNA was incubated with excess photolyase, treated with dimethyl sulfate, and then cleaved either by piperidine (G reaction; top strand) or 10% ammonium acetate and piperidine (G>A reaction; bottom strand). *A*, (top strand), *lane 1*, G reaction; *lane 2*, methylation in the presence of *M. thermoautotrophicum* photolyase in the dark (*−PR*); *lane 3*, methylation in the presence of *M. thermoautotrophicum* photolyase after camera flash (*+PR*). *Lanes 4* and *5* are analogous to *lanes 3* and *4* except the reaction mixtures contained *E. coli* photolyase. G24 is protected by both photolyases. In addition, *M. thermoautotrophicum* photolyase also protects G28 and G30. G27 becomes hypersensitive after repair by either photolyase and G31 becomes hypersensitive when the *M. thermoautotrophicum* enzyme is bound to the substrate. *B* (bottom strand), samples in *lanes 1–5* were treated similarly with *lanes 1–5* in the top strand except after methylation, G>A reaction was performed. The *arrow* represents the A whose methylation is either slightly inhibited (*lane 2*, *M. thermoautotrophicum*) or enhanced (*lane 4*, *E. coli*) upon binding of enzyme.

ylation protection and interference experiments) and thus probably displaces 3–4 water molecules from the major groove during binding. We suspect that entropy contribution to binding is the main cause of difference between the binding of the *M. thermoautotrophicum* and *E. coli* photolyases.

Finally, this study has shown that the *M. thermoautotrophicum* photolyase is an efficient photocatalyst but not as efficient as previous work with other DNA photolyases seemed to suggest. The *M. thermoautotrophicum* photolyase has a quantum yield of 0.2 compared to the remarkable quantum yield of 1.0 reported for *E. coli* (Sancar *et al.*, 1987b) and *S. griseus* (Eker *et al.*, 1986) photolyases. We have also determined the quantum yield of *A. nidulans* photolyase and found it to be 0.26.[3] We are therefore inclined to think that photolyases in general are not perfect photocatalysts and that the quantum yields for these various enzymes is in fact less than 1.0. Indeed, a more recent study with the *E. coli* enzyme has found a quantum yield of 0.44 (Payne *et al.*, 1987) suggesting that the value reported earlier was an overestimate. Similarly, the quantum yield cited above for the *S. griseus* enzyme was determined from photoreactivation rates under enzyme turnover condition. Under this experimental setup the repair rate is governed by the combined rates of the dark (complex formation) and light (photolysis) reactions and therefore it is conceivable that the reported value is 2–5-fold higher than the actual value because of underestimate/overestimate of the two-rate coefficients governing the dark reaction. The rate of dissociation from photorepaired DNA by *M. thermoautotrophicum* DNA photolyase is about 1000-fold faster ($k_{cat} \geq 9\ min^{-1}$) than from unrepaired dimer-containing DNA. It is likely that essentially every photolyase-pyrimidine dimer complex lasts long enough to absorb a photon of blue-green light prior to dissociation (about once every 43 min from the *in vitro* data).

In summary, in this report we have partially characterized a purified DNA-photolyase from *M. thermoautotrophicum* and thus photolyases from all three kingdoms, eubacteria (*E. coli* and *S. griseus*), eucaryotes (*S. cerevisiae* and *S. acutus*), and archaebacteria (*M. thermoautotrophicum*) have now been purified and characterized. These enzymes appear to have some common features. (i) All have a strongly absorbing chromophore which is either a folate (*E. coli* and yeast) or a deazaflavin (*S. griseus*, *S. acutus*, *A. nidulans*, and *M. thermoautotrophicum*). (ii) All photolyases analyzed in detail (*E. coli*, yeast, and *S. acutus*) also have a $FADH_2$ chromophore which appears to be at the catalytic center (Sancar *et al.*, 1987b). We suspect that the *M. thermoautotrophicum* photolyase also has an $FADH_2$ cofactor although we have not

[3] A. Kiener, I. Husain, A. Sancar, and C. Walsh, unpublished results.

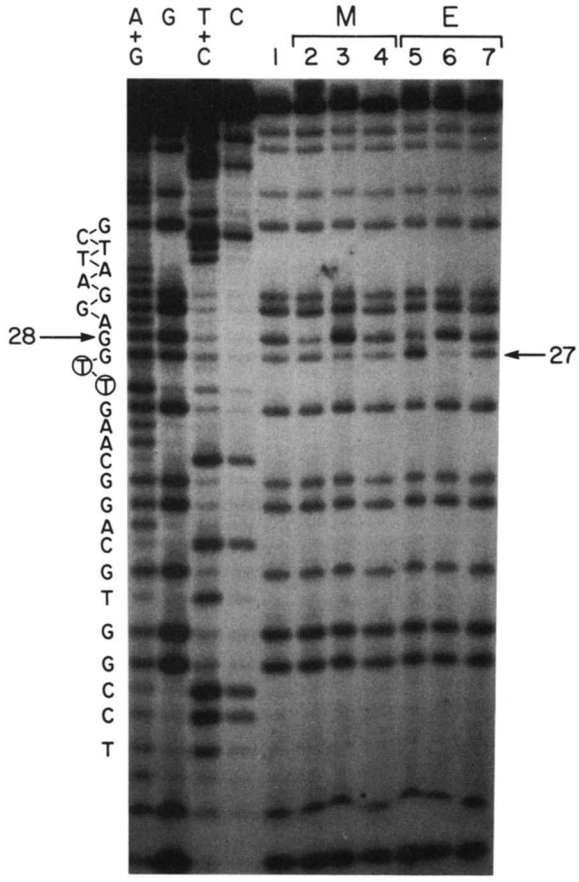

FIG. 7. **Methylation interference.** Methylated DNA was mixed with photolyase (*M*, *M. thermoautotrophicum*; *E*, *E. coli*), free and enzyme bound fractions were separated by gel retardation and cleaved by piperdine. *Lane 1*, Maxam-Gilbert G reaction; *lanes 2–4*, DNA incubated with *M. thermoautotrophicum* photolyase. *Lane 2*, enzyme bound; *lane 3*, free DNA; *lane 4*, free DNA following repair by camera flash. *Lanes 5–7* contain DNA incubated with *E. coli* photolyase. *Lane 5*, enzyme bound; *lane 6*, free DNA; and *lane 7*, photorepaired DNA. Methylation of G27 enhanced binding of *E. coli* photolyase and had no effect on the binding of *M. thermoautotrophicum* photolyase. Methylation of G28 strongly interfered with binding of *M. thermoautotrophicum* photolyase compared to *E. coli* photolyase. In addition, G24 (the G immediately 5′ to the dimer) also partially interfered with the binding of both *M. thermoautotrophicum* and *E. coli* photolyases (not marked in the figure).

FIG. 8. **Summary of footprinting data.** *Top*, *M. thermoautotrophicum* photolyase; *bottom*, *E. coli* photolyase. The DNase I footprints are *bracketed* ▲, and △, phosphates whose ethylation interferes with photolyase binding strongly and weakly, respectively. ○, methylation of these G is inhibited by photolyase binding; ●, methylation of this G interferes with photolyase binding; □, methylation of this G is enhanced by photolyase binding. ■, methylation of this G enhances photolyase binding; ↑, methylation is inhibited, and ↑, methylation is enhanced by binding of photolyase.

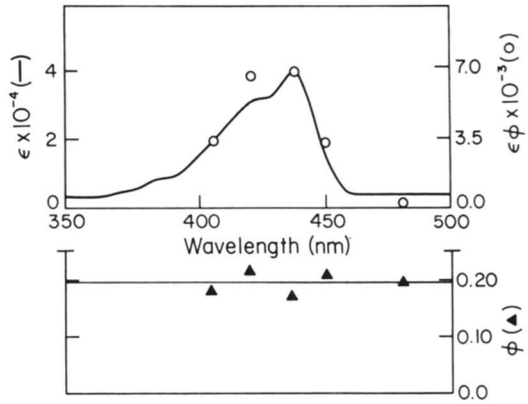

FIG. 9. **Absolute action spectrum of *M. autotrophicum* photolyase.** The near UV absorption spectrum of the enzyme is plotted on absolute scale taking $\epsilon_{440} = 40{,}000\ M^{-1}\ cm^{-1}$. The data points for photolytic cross-sections are superimposed on the absorption spectrum. *Top*, absorption and action spectra; *bottom*, quantum yield as a function of wavelength.

demonstrated it experimentally for lack of sufficient quantities of this enzyme. (iii) Photolyases that have been sequenced so far are 30–40% homologous (*E. coli*, *S. cerevisiae*, and *A. nidulans*) whether they belong to the folate or the deazaflavin class and whether they are of procaryotic or eucaryotic origin. The 28 NH_2 terminal amino acids of the *M. thermoautotrophicum* enzyme did not show significant homology to the three photolyases that have been sequenced. This is not surprising as the NH_2 terminus of the yeast enzyme also is a "tail" without homologous sequence in other photolyases. We expect that when the sequence of the methanogen enzyme becomes available (cloning is in progress) it will show considerable homology to other photolyases. (iv) Photolyases are quite efficient photocatalysts, working with a quantum yield of 0.2–0.4, but they are not perfect photocatalysts and the previously reported quantum yields of 1.0 for both classes of enzymes are likely to be in error. Whether this lower than perfect value is due to inefficient energy transfer from the primary chromophore (folate, deazaflavin) to the cofactor at the photocatalytic center ($FADH_2$), or in the proposed subsequent electron transfers in cyclobutane ring breakage remains to be determined.

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