Photoreactivation of Pyrimidine Dimers in the DNA of Normal and Xeroderma Pigmentosum Cells†

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ABSTRACT: Photoproducts formed in the DNA of human cells irradiated with ultraviolet light (uv) were identified as cyclobutyl pyrimidine dimers by their chromatographic mobility, reversibility to monomers upon short wavelength uv irradiation, and comparison of the kinetics of this monomerization with that of authentic cis-syn thymine-thymine dimers prepared by irradiation of thymine in ice. The level of cellular photoreactivation of these dimers reflects the level of photoreactivating enzyme measured in cell extracts. Action spectra for cellular dimer photoreactivation in the xeroderma pigmentosum line XP12BE agree in range (300 nm to at least 577 nm) and maximum (near 400 nm) with that for photoreactivation by purified human photoreactivating enzyme. Normal human cells can also photoreactivate dimers in their DNA. The action spectrum for the cellular monomerization of dimers is similar to that for photoreactivation by the photoreactivating enzyme in extracts of normal human fibroblasts.

The photoreactivating enzyme repairs ultraviolet light induced damage in DNA by monomerizing pyrimidine dimers in a light-dependent reaction (Rupert, 1962). The enzyme binds to the dimer-containing DNA, probably at the site of a dimer [the name cyclobutadipyrimidine has been proposed for the dimer by Madden et al., 1973 and Cohn et al., 1974]. On absorption of a photon in the range 300–600 nm, the enzyme catalyzes the photolysis of the cyclobutane ring, producing two monomer pyrimidines and restoring biological activity to the DNA (Setlow and Setlow, 1963). The specific and exclusive action of the enzyme on pyrimidine dimers (Setlow and Setlow, 1963) make it of potential importance as an analytical tool: if ultraviolet light induced biological damage can be prevented in a true photoenzymatic reaction, pyrimidine dimers were a major contributor to the production of that damage. In human cells the evaluation of the role of pyrimidine dimers in the induction of skin cancer by ultraviolet light (Epstein, 1971) is a case of special interest.

Since a photoreactivating enzyme has only recently been isolated from mammalian cells (Sutherland, 1974; Sutherland et al., 1974), its role in cellular repair phenomena is not yet clear. Sutherland et al. (1975) presented data showing that the cells of the xeroderma pigmentosum line XP12BE (Robbins et al., 1974) could photoreactivate dimers in its DNA. Since previous data on cellular photoreactivation have been contradictory (see Sutherland, 1974, for a discussion), we have examined this cellular photoreactivation phenomenon further.

We show here that the DNA photoproduct from uv-irradiated human cells which can be removed from the DNA by photoreactivation is the cis-syn cyclobutyl pyrimidine dimer, that the extent of cellular photoreactivation of dimers reflects the level of photoreactivating enzyme measured in cell extracts, and that the action spectrum for photoreactivation by the xeroderma line XP12BE is similar in range and maximum to that measured for purified human photoreactivating enzyme (Sutherland et al., 1974; Sutherland and Sutherland, 1975). We also find that normal human cells can photoreactivate dimers in their DNA, and that the action spectrum for dimer photoreactivation by these cells is similar to that measured for in vitro photoreactivation by the photoreactivating enzyme in extracts of these cells.

Experimental Procedures

Cells. The xeroderma line XP12BE (Jay Tim, CRL 1223) was obtained from the American Type Culture Collection; the xeroderma line Sally G was the gift of Dr. James Regan, Oak Ridge National Laboratory. A human embryonic skin and muscle line (HESM) was purchased from Flow Laboratories. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (Flow Laboratories) and contained penicillin (100 units/ml) and streptomycin (20 μg/ml).

Cells were grown routinely in T75 flasks (Falcon Laboratories). For radioactive labeling of the DNA, cells were seeded from one T75 flask into small culture dishes (35 mm diameter). After 12 hr growth, radioactive medium [Dulbecco's modified Eagle's medium containing 6% dialyzed fetal calf serum and 5 μCi/ml of [^{3}H]thymidine (Schwarz/Mann)] was added and the cells were grown for 24 hr. The labeling medium was removed and the cells were washed two times with 1 ml of 0.15 *M* NaCl; finally 1 ml of 0.15 *M* NaCl was added to the dish.

Irradiation of Cells. Cells in small culture dishes were exposed to 254-nm radiation from a 15-W low-pressure mercury arc. Exposure rates were measured with a Jagger meter (Jagger, 1961) which had been calibrated using a Hewlett Packard thermopile. Photoreactivation experiments were carried out in two ways. (1) Immediately after irradiation at 254 nm, the dishes containing the cells were placed in a Plexiglas box and exposed to broad spectrum

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white light from a 60-W frosted bulb at 20 cm distance. (2) For action spectrum measurements, the cells were irradiated at 254 nm as described above, then scraped into the overlying saline and transferred to a Pyrex tube which was inserted into the sample chamber of a high intensity monochromator.

Dimer Monomerization. Dimers were monomerized with 248-nm radiation from a high intensity monochromator (Johns and Rauth, 1965a,b). Samples were stirred during irradiation and, in the case of the solution of synthetic dimers, absorbance at 264 nm was monitored with a Zeiss spectrophotometer which is an integral part of the irradiation facility (Johns, 1967).

Action spectra were also measured with radiation from the high intensity monochromator Second-order uv radiation was removed by a 3 in. diameter 0.25 in. thick Pyrex plate between the mercury arc and the entrance slit and, in the case of the 577-nm line, a yellow Corning filter was placed in front of the sample. Samples were placed in 6-mm diameter Pyrex test tubes which were suspended in a 2 in. diameter cylindrical irradiation chamber, the inner walls of which were covered with barium sulfate high reflectance paint (Middleton and Sanders, 1953). The monochromatic beam entered through a 0.25 × 1.25 in. slit in the side of the irradiation chamber and was reflected diffusely from the walls of the chamber, thus reducing focusing effects caused by the test tube. Fluences of the radiation entering the irradiation chamber were adjusted by varying the ganged entrance and exit slits of the monochromator to produce equal numbers of photons during the period of irradiation for each of the different wavelengths. Fluences were measured with the photoelectric cell which is an integral part of the facility (Johns, 1967). The photocell was calibrated with a Hewlett Packard thermopile.

Dimer Analysis. After uv and/or photoreactivating light treatment, cells were killed by the addition of an equal volume of 20% trichloroacetic acid, mixed, and placed on ice. The cell suspensions were centrifuged for 15 min at 15 000 rpm in the SS34 rotor of the Sorvall RC2B centrifuge. The tubes were drained briefly to remove the last traces of the saline solution, 0.15 ml of 98% formic acid was added to each tube and the tubes were sealed. Samples were hydrolyzed at 175°C for 35–40 min, opened, dried, and chromatographed on Whatman No. 1 paper or cellulose thin layers (Brinkman) in 1-butanol–acetic acid–water (40:6:15, v/v). The chromatograms were cut into 1-cm (for paper) or 0.5-cm (for thin-layer) strips, eluted with 0.5 ml water, and counted in a dioxane-based scintillation fluid (containing 120 g of naphthalene and 3.0 g of 2,5-bis (5′-*tert*-butyl-2-benzoazoyl)thiophene per l.) in a LKB scintillation counter.

Cis-Syn Thymine-Thymine Dimers. Thymine dimers were prepared by irradiating a frozen aqueous thymine solution according to the technique of Beukers and Berends (1960). The irradiated solution was thawed and filtered to collect crystals. The crystals were dried at 60°C and extracted in absolute ethanol to remove unreacted thymine. The identity of the dimers was confirmed by three criteria. First, upon 254-nm irradiation in solution the dimer was converted to a product which had an absorption maximum at about 264 nm and a chromatographic mobility (in butanol–acetic acid–water) characteristic of thymine (Setlow and Setlow, 1962). Nuclear magnetic resonance (NMR) spectroscopy indicated a shift in the signal of the vinyl proton of thymine from δ 3.5 to 1.85 after dimerization. The identity of the dimer product as the cis-syn type (also formed in uv-irradiated DNA) (Weinblum and Johns, 1966) was established by infrared analysis of KBr pellet of the product: the spectrum was identical with that obtained for the cis-syn thymine dimer by Weinblum and Johns (1966).

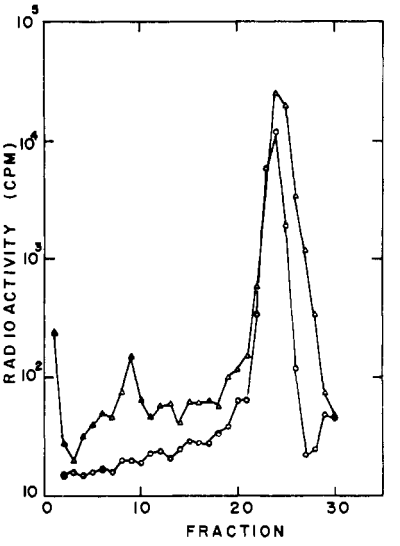

FIGURE 1: Radioactivity profile of chromatogram of a hydrolysate of HESM cells. Cells were labeled with [^{3}H]thymidine, exposed to ultraviolet light (△), or given no uv treatment (○), killed by the addition of trichloroacetic acid, and subjected to formic acid hydrolysis. The samples were dried, chromatographed in 1-butanol–acetic acid–water (40:6:15) on Whatman No. 1 paper, and counted. Fractions 8–10 of the chromatogram of the irradiated DNA were eluted and pooled.

Photoreactivating Enzyme. Cell extracts were prepared by washing a T75 flask of cells twice with 0.15 *M* NaCl (60 ml), scraping them into 2 ml of 0.15 *M* NaCl, centrifuging at 5000 rpm for 10 min in the SE12 rotor of the RC2B centrifuge, rinsing with 2.0 ml of 0.15 *M* NaCl, centrifuging as before, and resuspending in buffer E (10 m*M* Tris (pH 7.0), 0.1 m*M* dithiothreitol, and 0.1 m*M* EDTA). Cells were sonicated for 45 sec in a Kontes sonicator at a power setting of 6–7. Photoreactivating enzyme activity was measured by the method of Sutherland and Chamberlin (1973); units of enzyme activity are given in pmol per mg hr. Protein concentrations were determined by the Lowry method (Lowry et al., 1951).

Results

Ultraviolet irradiation of DNA in intact eucaryotic cells can result in a complex array of photoproducts (see, for example, Sutherland et al., 1968). Since the only known substrate for the photoreactivating enzyme is the pyrimidine dimer, it is important to measure accurately DNA dimer content; if photoproducts other than dimers appeared in the dimer region of the chromatogram, their disappearance might be mistaken for true photoreactivation. We have thus examined the identity of the photoproduct appearing in the dimer region of chromatograms of hydrolysates of uv-irradiated [^{3}H]thymidine-labeled human cells.

A radioactivity profile of such a chromatogram is shown in Figure 1; a profile of a chromatogram of unirradiated DNA is also shown in Figure 1. The photoproduct appearing in fractions 8–10 was eluted from the chromatogram and aliquots were given increasing exposures to 248-nm radiation from a high intensity monochromator. Profiles of chromatograms of the initial (no radiation), an interme-

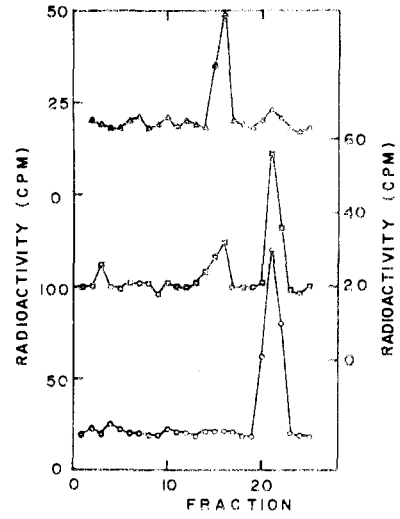

FIGURE 2: Conversion of "dimer" to thymine by 248-nm irradiation. Pooled "dimer" peak from the chromatogram of uv-irradiated HESM DNA (see Figure 1) was rechromatographed on Brinkman cellulose thin layers on 1-butanol–acetic acid–water (40:6:15) without exposure to light (top), after 4-min exposure to 248 nm (middle; right axis), or after 16-min exposure (bottom). The 248-nm radiation was delivered at a rate of 2×10^{16} photons/sec.

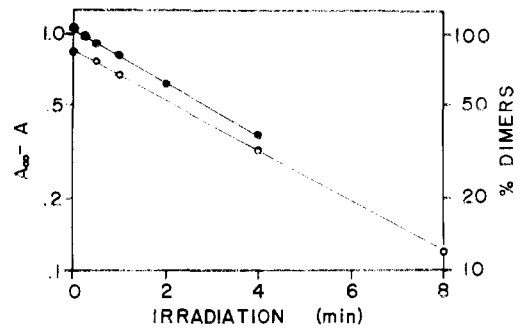

FIGURE 3: Kinetics of the conversion of the radioactive labeled photoproducts obtained as shown in Figure 1 and the monomerization of authentic pyrimidine dimers plotted as a function of time of exposure to 248-nm radiation. The incident fluence was 2×10^{16} photons/sec. The disappearance of the authentic dimers was measured by plotting $A_\infty - A$ (○) where A was the absorbance observed after a given fluence and A_∞ was the limiting absorbance achieved after extensive exposure. The percent unconverted radioactive photoproduct (●) was measured chromatographically. The two lines were determined by the method of least squares.

diate (4 min of 248-nm radiation), and the final (16 min) aliquots are shown in Figure 2, top, middle, and bottom, respectively.

We have also compared the kinetics of monomerization of authentic thymine–thymine dimers with that of the photoproduct from uv-irradiated human cells. Figure 3 shows that the human DNA photoproduct is converted from a product with an R_f of 0.27 [cf. R_f of 0.3 for thymine–thymine dimers (Setlow and Carrier, 1966)] to one of R_f 0.58 [R_f of thymine = 0.6 (Setlow and Carrier, 1966)] with the same kinetics as the conversion of thymine dimer to monomeric thymine. We thus conclude that the photoproduct from uv-irradiated human DNA—which has an R_f of about 0.27 in butanol–acetic acid–water, is converted to a product of R_f of thymine, and is monomerized with kinetics similar to that of authentic thymine dimer—is indeed a cyclobutyl pyrimidine dimer.

Levels of Dimer Photoreactivation and Photoreactivating Enzyme. If the apparent dimer photoreactivation we observe is due to true enzymatic photoreactivation, the amount of photoreactivation observed in a fixed time should depend directly on the level of photoreactivating enzyme measured in cell extracts. In order to facilitate comparison, we have chosen two cell lines with very low excision capacity (<5% of normal) and varying levels of photoreactivating enzyme. Table I shows the level of photoreactivating enzyme observed in cell extracts for the lines XP12BE and Sally G as well as the fraction of dimers photoreactivated by the cells in 5- or 30-min exposure to white photoreactivating light. The data indicate that the line XP12BE, with about 40% of photoreactivating enzyme activity, photoreactivates almost 60% of its cellular dimers in 5 min, and about 70% in 30 min. The line Sally G, however, with only about one-third as much enzyme activity, removed almost none of its dimers in a 5-min exposure to photoreactivating light, and only about 15–20% in 30-min exposure. Thus the rate of cellular photoreactivation of dimers directly reflects the level of photoreactivating enzyme measured in cell extracts.

Table I

Cell Line	Excision Repair (%)	Photoreactivating Enzyme Activity[a] (%)		Fraction of Cellular Dimers Photoreactivated[b] in 5 min	30 min
XP12BE	<2[d]	36–40		0.58	0.72
Sally G	<5[e]	10–17[c]	Expt I	0.04[c]	0.22
			Expt II	0.01[c]	0.15

[a] Photoreactivating enzyme levels were compared to those in normal cells (HESM), taken as 100%. These cells contained photoreactivating enzyme at a specific activity of 600–625 pmol per mg per hr. [b] Fraction of dimers photoreactivated was calculated by dividing the dimer content of cells exposed to photoreactivating light by the value for cells kept in the dark for the same period. [c] Photoreactivating enzyme levels below 20% and dimer photoreactivation measurements below 0.10 are subject to uncertainty. [d] Robbins et al. (1974). [e] J. Regan, personal communication.

Action Spectrum for Photoreactivation by XP12BE Cells. If the dimer disappearance observed in cells is a true photoenzymatic monomerization of pyrimidine dimers, the action spectrum for the dimer monomerization by the cells should agree with that for dimer monomerization by the purified human photoreactivating enzyme. The action spectrum of the human enzyme, with a maximum at about 400 nm, extending from about 300 nm to at least 577 nm (Sutherland et al., 1974; Sutherland and Sutherland, 1975) is shown in the lower part of Figure 4. We have determined the action spectrum for dimer monomerization by the intact cells. The upper part of Figure 4 shows that the maximum is also about 400 nm and that the range of the spectrum extends from about 300 nm to at least 577 nm. The agreement between the action spectra for photoreactivation by the cells and by the purified enzyme indicates that the cellular dimer monomerization is indeed due to the action of the human photoreactivating enzyme.

Photoreactivation by Normal Human Cells. We first examined photoreactivation in human cells with low excision capacity, as photoreactivation can be masked by an efficient excision repair system (see Sutherland, 1975). We have now examined the ability of normal human fibroblasts to photoreactivate dimers in their DNA. A typical time course of photoreactivation is shown in Figure 5. Although the cells removed few of the dimers in the dark in 30 min, in

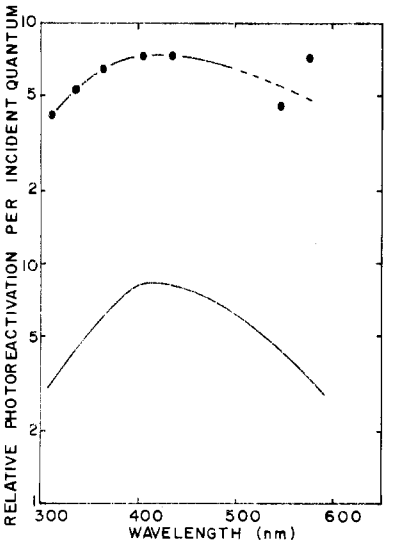

FIGURE 4: Action spectrum for dimer monomerization in XP12BE cells (upper curve) and the action spectrum of the photoreactivating enzyme from human leukocytes (Sutherland et al., 1974) (lower curve). In the cellular photoreactivation experiments the fluence entering the irradiation chamber was 10^{16} photons/sec and the irradiation time was 2 min. During this time, no significant excision of dimers occurred.

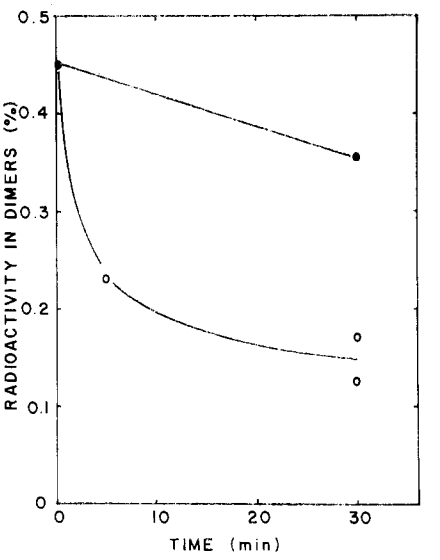

FIGURE 5: Photoreactivation of pyrimidine dimers in the DNA of normal human cells (HESM). Cells were labeled with [^{3}H]thymidine, exposed to 100 J/m^2 of 254-nm radiation, and kept in the dark (●) or exposed to broad spectrum photoreactivating light from a 60-W white incandescent bulb (○). Cells were killed by the addition of ice-cold trichloroacetic acid, hydrolyzed in formic acid, dried, chromatographed in butanol–acetic acid–water, sliced, and counted.

the presence of photoreactivating light about 60% of the dimers disappeared from their DNA. (Some variability was observed in experiments with normal human cells: photoreactivation similar to that shown in Figure 5 was observed in five of six experiments; in one experiment no photoreactivation could be detected with the same cell line under apparently identical conditions.) We have also examined the action spectrum for photoreactivation by the normal human cells. A comparison of the action spectrum for cellular dimer photoreactivation by HESM cells and that of in vitro photoreactivation by extracts of the same cell line is shown in Figure 6. The similarity of the spectra indicates that the enzyme responsible for in vitro photoreactivation is the same as the one which carries out dimer monomerization in the cells.

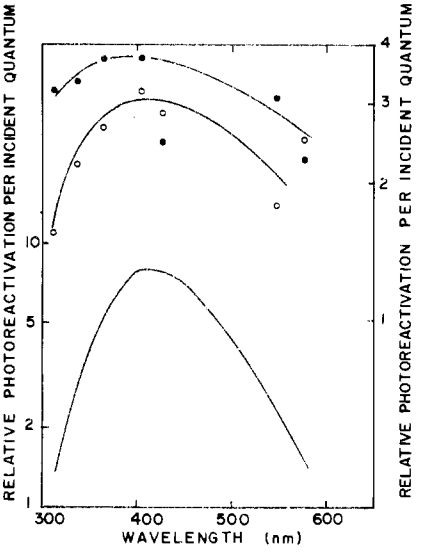

FIGURE 6: Action spectra for dimer monomerization in HESM cells (upper curves) and for photoreactivating enzyme activity in HESM cell extracts (lower curve). Labeled cells were exposed to 100 (○) or 200 (●) J/m^2 of 254-nm radiation, then to photoreactivating light from a high intensity monochromator at a rate of 10^{16} photons/sec for 2 min.

Discussion

Sutherland et al. (1975) presented evidence for dimer monomerization in human cells. Since previous data on photoreactivation in mammalian cells are contradictory (see Sutherland, 1975), we felt it imperative to examine this photoreactivation effect in greater detail. We have examined the identity of the photoproduct from the DNA of uv-irradiated human cells measured by Sutherland et al. (1975) as pyrimidine dimers. The photoproduct has the same R_f in 1-butanol–acetic acid–water on Whatman No. 1 paper as does thymine–thymine cyclobutyl dimer, and is converted to a product with a R_f of thymine by irradiation at 248 nm in solution. The kinetics of this conversion are identical with that for the monomerization of authentic thymine–thymine dimers prepared by the irradiation of thymine in ice. We thus conclude that the photoproduct measured by Sutherland et al. is indeed the cyclobutyl pyrimidine dimer.

We have also tested for correlation of the level of cellular photoreactivation with the photoreactivating enzyme activity measured in cell extracts. The xeroderma line XP12BE, with about 40% of the normal level of photoreactivating enzyme, photoreactivates dimers in its DNA at a much faster rate than the line Sally G which contains only about 15% of the normal enzyme level. Therefore there is a direct correlation between the level of enzyme activity measured in cell extracts and its expression in the cells.

If the photoreactivating enzyme isolated from human cells (Sutherland, 1974; Sutherland et al., 1974) is responsible for the photoreactivation observed in the cells, the action spectrum for photoreactivation by the isolated enzyme should be similar to the action spectrum for cellular photoreactivation. We find that the action spectrum for dimer monomerization by cells of the xeroderma line XP12BE agrees in both range (300–600) and wavelength maximum (405 nm) with that for the isolated human enzyme. We

have also shown (see Figures 5 and 6) that normal human cells can photoreactivate dimers in their DNA, and that the action spectra for photoreactivation of dimers in these cells agree with that measured for in vitro photoreactivation by extracts of these cells.

The demonstration that the photoproduct from uv-irradiated human cells (Sutherland et al., 1974) is a cyclobutyl pyrimidine dimer indicates that its disappearance in a light-dependent enzymatic reaction is indeed photoreactivation. The correlation between photoreactivating enzyme levels and rates of cellular photoreactivation of dimers implies that the enzyme isolated from human cells is responsible for cellular photoreactivation. The correlation of action spectra for photoreactivation of isolated enzyme and of intact cells also indicates that the cellular disappearance of dimers is true enzymatic photoreactivation.

Acknowledgments

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