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Photoprotection by Topical DNA Repair Enzymes: Molecular Correlates of Clinical Studies

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ABSTRACT

A new approach to photoprotection is to repair DNA damage after UV exposure. This can be accomplished by delivery of a DNA repair enzyme with specificity to UV-induced cyclobutane pyrimidine dimers into skin by means of specially engineered liposomes. Treatment of DNA-repair-deficient xeroderma pigmentosum patients or skin cancer patients with T4N5 liposome lotion containing such DNA repair liposomes increases the removal of DNA damage in the first few hours after treatment. In these studies, a DNA repair effect was observed in some patients treated with heat-inactivated enzyme. Unexpectedly, it was discovered that the heat-inactivated T4 endonuclease V enzyme refolds and recovers enzymatic activity. These studies demonstrate that measurements of molecular changes induced by biological drugs are useful adjuvants to clinical studies.

INTRODUCTION

Bacteriophage T4 endonuclease V is a 16 500 molecular weight polypeptide with activity against UV-irradiated double-stranded DNA (1). The enzyme acts on cyclobutane pyrimidine dimers (CPD)† by releasing the 5′ dimerized base with its glycosylase activity and then incising the DNA backbone at the resultant apyrimidinic site by catalyzing a β elimination reaction (2). Because the DNA repair enzyme has specificity for CPD and was among the first to be purified and its gene cloned, it has also gained some importance as a research tool and a potential therapeutic agent. The enzyme is routinely used to analyze repair of CPD in DNA (3) and was first introduced into excision-repair-deficient xeroderma pigmentosum (XP) cells to increase removal of CPD almost 25 years ago (4). Now the enzyme, encapsulated in a liposomal delivery vehicle, is being tested in clinical trials with XP patients to increase DNA repair *in vivo* and prevent skin cancer (5).

The folding of the T4 endonuclease V polypeptide chain has been deduced by X-ray crystallography (6), and the DNA binding region and active site of the protein have been determined by site-directed mutagenesis (7,8). The enzyme binds to DNA and rotates the undamaged base opposite the CPD out of the DNA helix, in a "base-flipping" reaction, in order to bring the active site amino terminus of the protein into contact with the CPD (9). This cooperation among several domains of the polypeptide chain emphasizes the importance of proper protein folding in the activity of the endonuclease.

The T4 endonuclease V is an early gene product synthesized within 5 min of phage T4 infection of *Escherichia coli* (10). The native promoter is too strong for productive expression in *E. coli,* and the structural gene has been cloned under the *tac* promoter so that its expression can be controlled (11). Even so, induction of T4 endonuclease V is toxic to the host cells (11). The yield of active enzyme in such fermentation is a critical element in the manufacture of enzyme for therapeutic use. Such synthesized protein may be trapped in inclusion bodies that are frequently formed in host cells expressing high concentrations of recombinant proteins (12) or may become inactive due to thermal denaturation during manufacturing and storage. Therefore, the proper folding of the enzyme is of both theoretical and practical interest.

We report here on clinical studies of *in situ* DNA repair and *in vitro* studies on refolding of the heat-denatured enzyme.

MATERIALS AND METHODS

T4 endonuclease V and heat-inactivated enzyme liposomes. The T4 endonuclease V was purified from *E. coli* overexpressing the *den*V gene by gel-filtration and single-stranded DNA affinity chromatography (13). The preparations used produced a single 16 500 Da band by polyacrylamide gel electrophoresis (PAGE), and the identity of

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†*Abbreviations:* BSA, bovine serum albumin; CPD, cyclobutane pyrimidine dimer; EDTA, ethylenediaminetetraacetic acid; MOD, mean optical density; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; XP, xeroderma pigmentosum.

the band as T4 endonuclease V was confirmed by western blots using a polyclonal antibody against T4 endonuclease V (14). Liposomes were prepared by dissolving the heat-inactivated enzyme in 25 m*M* octylglucopyranoside along with 10 m*M* phospholipid mixture (phosphatidyl choline, phosphatidylethanolamine, oleic acid and cholestryl hemisuccinate, 2:2:1:5 molar ratio) followed by overnight dialysis and purification by AcA54 gel filtration (15).

Activity assay. Plasmid pMJR1560 (0.2 mg/mL) was irradiated with 40 J/m^2 of UVC from a Westinghouse T15G germicidal lamp (predominantly 254 nm) and mixed with an equal amount of unirradiated pSV2neo plasmid DNA. Each assay mix included 200 ng of the plasmid DNA mix and 200 ng of enzyme in 20 μL of Endo buffer (20 m*M* $NaHPO_4/NaH_2PO_4$, pH 6.5, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* β-mercaptoethanol) incubated for 60 min at 37°C. The reaction was terminated by extraction with an equal volume of phenol, mixed with 2 μL of gel loading dye and loaded on a neutral 0.8% ultrapure agarose (Gibco/BRL) gel in a Gibco/BRL H5 Midigel apparatus for electrophoresis at 50 V. The gel was then stained with 1 μg/mL ethidium bromide, destained with 1 m*M* $MgSO_4$, viewed with a 313 nm transilluminator and the fluorescence image captured by a Photometrics® (Tucson, AZ) Star I digital camera and the fluorescence intensity of each band quantified by Quantiscan® (Ferguson, MO) image analysis software. The fraction of total UV-irradiated plasmid remaining supercoiled was calculated for each reaction. In this assay, the relaxation of the UV-irradiated pMJR1560 plasmid represents UV endonuclease activity. The relaxation of the pSV2neo plasmid, a control for nonspecific nuclease activity, was negligible. The fraction of total UV-plasmid fluorescence in the supercoiled band is reported.

Denaturation and refolding. For heat inactivation experiments, the enzyme at 1 mg/mL was either heated in a water bath at 60°C for 60 min or boiled in 1% sodium dodecyl sulfate (SDS) for 2 min. For refolding, the inactivated enzyme preparations were then diluted between 1:50 (8 μL) and 1:200 (2 μL) into 400 μL of either standard buffer (50 m*M* Tris, pH 8, 0.1 mg/mL bovine serum albumin [BSA], 1 m*M* ethylenediaminetetraacetic acid [EDTA]) or refolding buffer (50 m*M* Tris, pH 8, 0.1 mg/mL BSA, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 0.1 mg/mL lauryl maltose) and incubated at 25°C for 90 min.

Clinical studies in skin cancer patients. Fifteen human subjects with a prior history of skin cancer were recruited under a protocol approved by the Ethical Committee of the Karl Franzens University, Graz, Austria. Six patients had basal cell carcinoma, five had squamous cell carcinoma and four had both. They were exposed to two minimal erythema doses of UV radiation on sites of their buttock skin. Liposomes containing either active T4 endonuclease V or heat-inactivated (60 min boiling) enzyme were applied in a hydrogel lotion at 1 μg/mL to different sites immediately and at 2, 4 and 5 h after UV irradiation, and 4 mm biopsies were taken at 6 h from unirradiated, irradiated and irradiated and treated sites. The formalin-fixed biopsies were sectioned and stained with TDM-1 antibody specific for thymine–thymine CPD (16). The amount of specific antibody staining was visualized by immunohistochemical microscopy, quantified by digitized image analysis and expressed in terms of integrated mean optical density (MOD) per cell, which is proportional to UV dose (17). This same method was used in the study of DNA repair in XP patients treated with T4N5 liposome lotion (5).

RESULTS

In vivo DNA repair by T4 endonuclease V

Skin sites of the 11 XP patients treated with T4N5 liposomes removed on average 20% of CPD in 6 h while untreated sites removed only about 6% (Fig. 1). Although we observed that 9 of 11 XP patients showed reduction in CPD at the treated site compared to the untreated site ($P < 0.05$), we were surprised to find that in 6 of those 11 patients there was also some reduction in CPD at the site of treatment with heat-inactivated enzyme in liposomes compared to the untreated site, although it was not statistically significant (5).

The repair of DNA in skin cancer patients was also increased by treatment with T4N5 liposome lotion (Fig. 1). In contrast to patients with XP, these repair-proficient patients with a prior history of skin cancer removed on average 10% of CPDs in 6 h. The T4N5 liposomes enhanced repair to a level similar to that found in XP patients or nearly 20% in 6 h. The observation that repair was not further enhanced in these patients compared to the XP patients suggests that the repair process is saturated at this dose of T4N5 liposome lotion, and that a downstream step in repair has become rate limiting. In this study, too, we observed that the sites treated with liposomes containing heat-inactivated enzyme had slightly greater, but not statistically significantly greater, repair of CPD than the untreated site (12% repair *vs* 10% repair, data not shown).

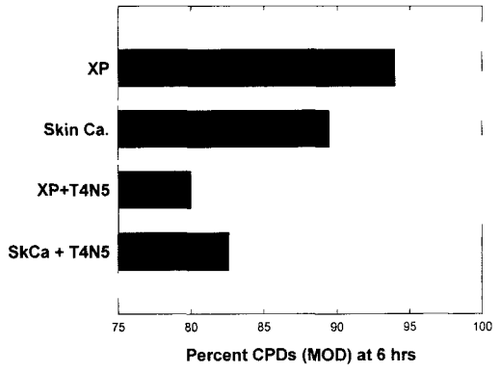

Figure 1. Repair of CPD in XP and skin cancer patients treated with T4N5 liposome lotion. Eleven XP and 15 skin cancer patients were UV irradiated and treated with T4N5 liposome lotion (1 μg/mL) or left untreated, as described in the Materials and Methods. After 6 h the sites were biopsied and analyzed for remaining CPD by immunohistochemistry. The percent of initial MOD is reported for treated and untreated control groups.

We investigated whether this repair at sites treated with heat-inactivated enzyme could be due to refolding of the denatured protein.

In vitro studies of T4 endonuclease activity

The enzyme activity of T4 endonuclease V was assayed by its ability to relax specifically UV-irradiated supercoiled plasmid DNA without acting on unirradiated supercoiled plasmid DNA in the same reaction mixture. As is shown in Fig. 2A, active enzyme relaxed a UV-irradiated smaller plasmid without incision of a larger unirradiated plasmid in the same reaction mix (lane 1). However, boiling the enzyme for 2 min immediately prior to adding to the plasmid mixture completely eliminated this enzyme activity (lane 2). The loss of enzyme activity is dependent on temperature, as shown in panel B. At 37°C the $T_{½}$ is about 60 days, at 25°C it is about 90 days, and refrigerated at 10°C the enzyme activity has a $T_{½}$ of about 180 days.

Recovery of activity from inactive T4 endonuclease V

Enzyme activity was recovered from detergent-denatured protein under refolding conditions (Fig. 3). Lane 1 shows that the active enzyme almost completely incises the UV-irradiated plasmid, compared to untreated plasmid mix (lane 2). When the T4 endonuclease V was boiled for 2 min in

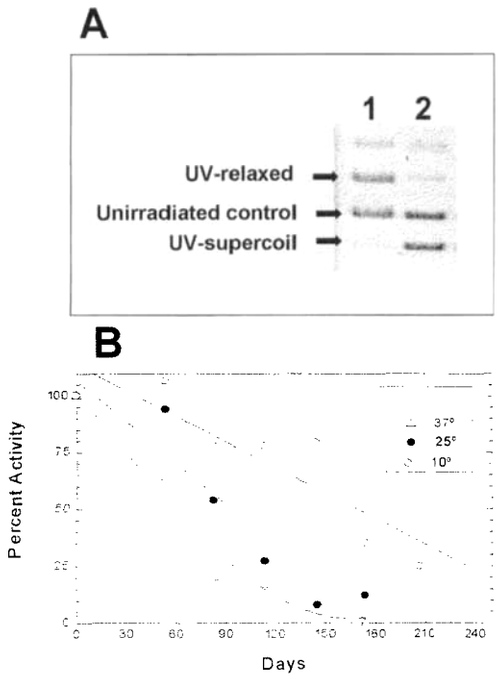

Figure 2. Stability of T4 endonuclease V at different temperatures. A: The activity assay measures the conversion of supercoiled UV-irradiated plasmid into the relaxed form by T4 endonuclease V, while unirradiated control plasmid is unchanged (lane 1). Heating the enzyme at 60°C for 60 min inactivates the enzyme activity (lane 2). B: Three preparations of purified T4 endonuclease at 0.2 mg/mL reaction buffer were sealed in Eppendorf tubes at refrigeration, 10°C (○), room temperature, 25°C (●) or incubated in a bacterial plate incubator, 37°C (△). Samples were periodically assayed for enzymatic activity and the values compared to the activity at the start of the experiment. The results are presented as the average percentage of activity remaining, and the lines are drawn by first (○,●) or second (△) order linear regression.

1% SDS and then incubated with Standard assay buffer for 90 min at 25°C no activity was detected (lane 4). However, when the SDS-denatured enzyme was subsequently diluted into refolding buffer and incubated for 90 min at 25°C, almost complete activity was recovered (lane 3). The relaxed form of the UV-irradiated plasmid shows aberrantly greater migration in this lane of the gel, which may be due to the high concentration of detergents (both SDS and lauryl malt-

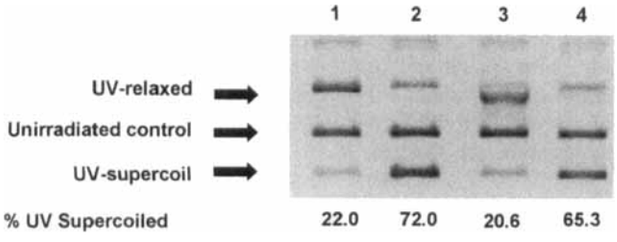

Figure 3. Refolding of detergent-plus-heat denatured T4 endonuclease V in refolding buffer. Lanes 1 and 2: Positive control of 200 ng T4 endonuclease V and negative control of no enzyme, respectively. The T4 endonuclease V (200 ng) denatured by boiling for 2 min in 1% SDS was inactive (lane 4). Activity was restored by incubation in refolding buffer for 90 min at 25°C (lane 3). The percentage of supercoiled UV-irradiated plasmid remaining is shown at the bottom of each lane.

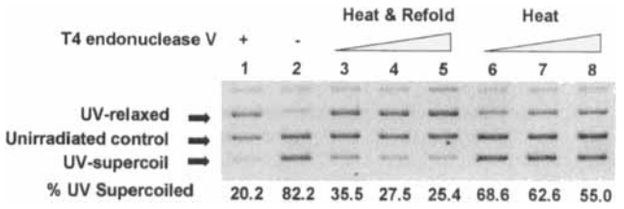

Figure 4. Refolding of heat-inactivated T4 endonuclease V in refolding buffer. Lanes 1 and 2: Positive control of 200 ng T4 endonuclease V and negative control of no enzyme, respectively. Lanes 3, 4 and 5 show 50, 100 and 200 ng, respectively, of heat-inactivated T4 endonuclease V incubated with refolding buffer at 25°C for 90 min prior to assay. Lanes 6, 7 and 8 show 50, 100 and 200 ng, respectively, of heat-inactivated T4 endonuclease V incubated only in standard buffer at 25°C for 90 min prior to assay. The percentage of supercoiled UV-irradiated plasmid remaining is shown at the bottom of each lane.

ose). Many researchers have recovered enzyme activity from proteins extracted from SDS-PAGE gels by gentle removal of the detergent under renaturing conditions.

Surprisingly, we found that enzyme denatured by heating alone, without linearization by SDS, could also be renatured (Fig. 4). Initially, the enzyme was active (compare enzyme in lane 1 with no enzyme in lane 2). Heating the enzyme preparation for 60 min at 60°C, followed by incubation in standard reaction buffer for 90 min at 25°C resulted in a small and concentration-dependent increase in incision activity represented by a decline in the percentage of supercoiled UV-plasmid DNA (lanes 6–8). Heat-denatured enzyme incubated in refolding buffer for 90 min at 25°C recovered virtually all the incision activity (lanes 3–5), and the fraction of UV-supercoiled DNA declined with increasing protein concentrations.

Heat-inactivated T4 endonuclease V has been used as a control in animal and human studies with active T4 endonuclease V encapsulated in liposomes (T4N5 liposomes) (5,18). In order to test the ability of the encapsulated heat-inactivated enzyme to refold, heat-inactivated enzyme was encapsulated in liposomes. Due to the encapsulation step, the enzyme concentration in these reactions was reduced by 10-fold compared to earlier experiments. As shown in Fig. 5, these liposomes, encapsulating 20 ng of protein, were ei-

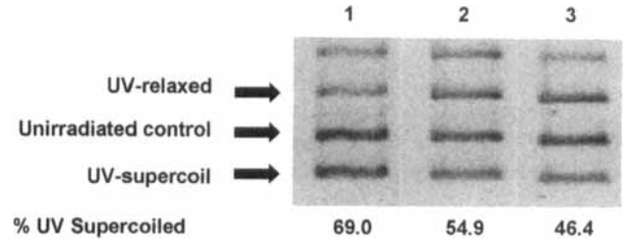

Figure 5. Refolding of heat-inactivated T4 endonuclease V in liposomes. The T4 endonuclease V heat inactivated by 60°C for 60 min was encapsulated in liposomes and 20 ng of encapsulated enzyme showed no activity (lane 1). Incubation of 20 ng of liposome-encapsulated enzyme in standard buffer with 0.1% Triton X-100 at 25°C for 90 min prior to assay recovered some activity (lane 2), while incubation in refolding buffer and 0.1% Triton X-100 prior to assay recovered more activity (lane 3). The percentage of supercoiled UV-irradiated plasmid remaining is shown at the bottom of each lane.

ther added to the substrate for 90 min at 25°C (lane 1), or dissolved with 0.1% Triton X-100® in standard reaction buffer and incubated for 90 min at 25°C (lane 2), or dissolved with 0.1% Triton X-100 in refolding buffer and incubated for 90 min at 25°C (lane 3). Under these conditions, 69% of the UV-irradiated plasmid remained supercoiled without treatment, while dissolving the liposomes with Triton X-100 alone reduced the supercoiled fraction to about 55%, but dissolving the liposomes in refolding buffer further reduced the supercoiled fraction to 46%. The recovery of enzyme activity from these heat-inactivated liposomes did not result in as much incision activity as in the prior experiments because the amount of enzyme in the liposome assay was reduced 10-fold.

DISCUSSION

Enzyme therapy is a powerful tool to replace missing activity in a temporally and spatially controlled manner. Specially engineered liposomes as described here are able to deliver DNA repair enzymes into skin and stimulate repair of UV-induced DNA damage. In the case of XP patients, the repair enzyme circumvents the missing recognition and incision event. In the case of skin cancer patients, the T4 endonuclease V activity shifts the repair from a nucleotide excision repair to a base excision repair pathway. Animal and human skin explant studies established that the dosage of 1 μg/mL of T4N5 liposomes saturates the repair machinery and greater enzyme concentrations do not increase the rate (15). These studies suggest that treatment of XP patients with T4N5 liposomes at 1 μg/mL restores DNA repair to approximately the same rate as found in T4N5 liposome-treated normal skin, supporting the concept that at this dosage repair is maximal.

An anomalous finding was that repair of CPD was increased in the sites treated with inactive enzyme in liposomes. We hypothesized that the inactive enzyme may regain activity by refolding. An enzyme acquires activity when the polypeptide chain is folded into the proper three-dimensional structure. While this folded conformation is at an energy minimum, it often must be assisted by chaperones and protected from unfolding by heat-shock proteins. Other energy-minimum conformations are possible, and with heating the polypeptide chain can assume alternate conformations and enzyme activity may be lost. Reactivation of heat-inactivated protein after boiling in an ionic detergent like SDS is facilitated by the linearization of the peptide chain by the detergent charge, followed by removal of the detergent and repetition of *de novo* protein folding. However, inactivation by heat alone may convert the enzyme into another folding pattern from which there may be no simple way to recover enzyme activity without a linear intermediate.

The T4 endonuclease V has three α-helices bundled in the central portion of the molecule, running in the energetically favored antiparallel configuration to each other (6). The loops connecting helix 1 with helix 2 (around amino acid 44) and helix 2 with helix 3 (around amino acid 100) are essential to proper folding (6). Deletion of 3 amino acids in either of these loops lowers enzyme activity and drastically reduces the accumulation of intracellular protein, suggesting that these two regions are structurally required for efficient folding and orientation of the α-helices (19). Heating may destroy the activity of T4 endonuclease V by changing the bending of these loops without denaturing the α-helices.

We report here that the T4 endonuclease V protein may refold under mild conditions after heat denaturation. This refolding occurs in the presence of high protein concentration (0.1 mg/mL total protein) and is facilitated by micelle-forming emulsifiers or liposomes. In fact, the scaffolding provided by the liposome membrane and micelles has been exploited in studies of the refolding of denatured proteins (20). It is possible that the anionic charge of the liposome membrane serves as an anchor for one or more of the α-helices and this facilitates the reformation of the turns in the loop regions.

These studies demonstrate the durability of the T4 endonuclease V enzymatic activity even under conditions that inactivate other endonucleases such as restriction enzymes. Heat denaturation of T4 endonuclease V may not be a sufficient guarantee of inactivation for use as clinical controls. The results further suggest that users of T4 endonuclease V for analytical assays may recover activity from stored preparations by incubation in refolding buffer prior to use. In addition, the loss of activity in liposome-encapsulated enzyme during storage may be recovered when the enzyme is released from the liposome.

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