DNA Photoreactivating Enzyme from the Cyanobacterium Anacystis nidulans*

(Received for publication, July 24, 1989)

Andries P. M. Eker, Patricia Kooiman, Johanna K. C. Hessels, and Akira Yasui

From the Biochemical and Biophysical Laboratory, Delft University of Technology, 2628 BC Delft, The Netherlands and §The Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980, Japan

Photoreactivating enzyme, which specifically monomerizes pyrimidine dimers in UV-irradiated DNA, was purified 21,000-fold from the cyanobacterium Anacystis nidulans to apparent homogeneity with 41% overall yield. The enzyme consists of a single protein chain with 53,000 molecular weight. Maximal activity was found at pH 6.2 and 0.1 M NaCl. Purified photoreactivating enzyme exhibits a marked absorption spectrum with a main band in the blue region (maximum 437 nm), a protein band (maximum 266 nm), and a low intensity band above 500 nm. The molar extinction coefficient of native enzyme was estimated 53,000 at 437 nm. The action spectrum for photoreactivation shows maximal activity at 440 nm and correlates closely with the 437-nm absorption band. The enzyme contains two different intrinsic chromophores in equimolar amounts, which were identified as 7,8-didehydromethyl-8-hydroxy-5-deazariboflavin (FO) and (reduced) FAD. The low intensity absorption band of native photoreactivating enzyme exhibits a shoulder at 498 and maxima at 588 and 634 nm. This band is attributed to a neutral FAD semiquinone radical which accounts for the major part of the FAD present in dark equilibrated enzyme. Preillumination at 585 nm bleaches the semiquinone spectrum due to formation of fully reduced FAD, but exposure to air in the dark restores the spectrum completely. On preillumination at 437 nm the disappearance of FAD semiquinone is more rapid, indicating that the photoreduction is sensitized by the 8-hydroxy-5-deazafavin chromophore. The 8-hydroxy-5-deazafavin and possibly also the reduced FAD chromophore appear to act as a primary photon acceptor in the photoreactivation process.

Cyanobacteria are phototrophic prokaryotes capable of oxygenic photosynthesis. They are mainly found in fresh and marine surface waters. In that habitat the UV component of sunlight will induce lesions in DNA and eventually damage cell. It is expected therefore that cyanobacteria will possess repair systems to reverse the detrimental effects of UV radiation.

Werbin and co-workers (1, 2) described the occurrence of photoreactivation in cyanobacteria. In this repair process pyrimidine dimers in UV-irradiated DNA are split into the constituent pyrimidines by photoreactivating enzyme (pho-...
Determination of Photoreactivating Activity—Photoreactivating activity was determined with the Haemophilus influenzae transformation assay (for details see Ref. 26), using illumination with blue fluorescent lamps (Philips TLADK 30W/83, 7.5 cm, 225 mm) for 30 min at 30°C. PRE was routinely diluted in buffer A (40 mM NaCl, 10 mM potassium phosphate, 5 mM 2-mercaptoethanol, pH 7.0) containing 1 mg/ml bovine serum albumin. Photoreactivating activity is expressed as (DF)(N₀ - Nₐ)/N₀, where N₀ and Nₐ are the number of transformants obtained after photoreactivation or dark incubation, respectively, and DF is the enzyme dilution factor.

Time courses of photoreactivation were determined in order to study the influence of pH and ionic strength. The relative PRE activity was obtained from plots of $\ln(N₀/Nₐ) = 1$ as a function of photoreactivation time (27), where $N_2$ is the number of transformants obtained with untreated transforming DNA.

Protein concentrations were determined with the Coomassie Brilliant Blue method (28).

Cultivation of Cells—A. nidulans strain 1402–1 SAUG (synonym Synecococcus FCC3001 (29)) was grown in a mineral medium modified from Kraz and Myers (30), containing/liter 0.5 g of KH₂PO₄, 0.5 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 1 g of NaNO₃, 165 mg of NaCl, 10 mg of MgCl₂·6H₂O, 10 mg of FeCl₂·4H₂O and 1 ml of a solution of spore elements containing/liter 2.86 g of HB₈₀, 1.81 g of MnCl₂·5H₂O, 222 mg of ZnCl₂, 26 mg of NaN₃, and 0.79 mg of CuSO₄·5H₂O. The total pH was 6.4. Inoculats (4 liters) were grown in stirred 5-liter flasks illuminated with three circular 32-watt white fluorescent lamps. The cell suspension was flushed with air (250 ml/min) and CO₂ injection was regulated between 50 and 100 ml/min in order to keep pH at 7.0 ± 0.2. After 5 days a culture density of about 300 (Klett Summerson model 6), was reached, and cells were harvested by centrifugation in a Sorvall T28-B (3,000 rpm, 15 min), yielding 250–300 g of wet cells. Cells were washed with buffer B (0.1 M NaCl, 10 mM potassium phosphate, pH 7.0), suspended in an equal volume of buffer B and half a volume of glycerol, frozen quickly, and stored at -80°C. This protocol was repeated several times using approximately 2 kg of wet cells in total.

Three or four batches of partially purified enzyme were rapidly thawed, centrifuged to remove a fine precipitate, and loaded overnight in the dark on a UV-DNA-cellulose column (4.4 X 10 cm, containing 180 g of spherosil-type D, 100–200 mesh). The column was washed with buffer B and eluted with buffer B containing 0.1 M NaCl and 6% poly (ethylene glycol) 6000. The eluate (750 ml) was diluted with an equal volume of buffer C (10 mM NaCl, 10 mM potassium phosphate, 5 mM 2-mercaptoethanol, pH 7.0) and loaded overnight in the dark on a UV-DNA-cellulose column (4.4 X 10 cm, containing 50 g of UV-DNA-cellulose, flow rate 96 ml/h). The column was washed with buffer D (which is buffer B containing 5 mM 2-mercaptoethanol) and eluted with buffer D containing 1.9 M NaCl (flow rate 170 ml/h). The eluate (227 ml) was dialyzed for 5 h against 5 liters of buffer A. After addition of ½ volume of glycerol the solution was frozen quickly and stored at -80°C. This protocol was repeated several times using approximately 2 kg of wet cells in total.

For three or four batches of partially purified enzyme were rapidly thawed, centrifuged to remove a fine precipitate, and loaded overnight in a heparin-Sepharose column (Pharmacia LKB Biotechnology Inc., 2.3 X 10 cm, flow rate 61 ml/h). The column was washed with buffer B and eluted with a 400-ml linear gradient 0-0.9 M NaCl in buffer D. Active fractions were combined and dialyzed overnight against 5 liters of buffer A. The dialyzed eluates of 2 heparin-Sepharose columns were combined and dialuted with an equal volume of buffer E (10 mM potassium phosphate, 5 mM 2-mercaptoethanol, pH 7.0). After centrifugation this solution was loaded overnight on a DEAE-cellulose column (Whatman DE52, 1.6 X 5 cm, flow rate 19 ml/h). The column was washed with buffer E containing 0.7 M NaCl and 0.1 M NaCl (flow rate 70 ml/min), and eluted with buffer E containing 0.7 M NaCl and 0.3 M NaCl in buffer A. The last purification step was repeated with pooled active fractions using a DEAE-Sepharose CL-6B column (Pharmacia LKB Biotechnology Inc., 1 X 5 cm, flow rate 35 ml/h). Active fractions were rapidly frozen after addition of 1/3 volume of glycerol and stored at -80°C. All purification steps were carried out at 4°C.

Denatured PRE was prepared by heating at 65°C for 5 min, followed by centrifugation and/or filtration through a membrane filter (Millipore Milllex GV4) to remove denatured protein.

Spectral Measurements—Fluorescence spectra were measured with a home-built apparatus (18): the spectra are fully corrected. Absorption spectra were recorded with a Beckman UV 5290 or Pye-Unicam SP7–500 spectrophotometer. Action spectra were obtained from time courses of photoreactivation (see before) measured in samples illuminated in a high intensity monochromatic irradiation apparatus, which is standard equipped with a large area rhodamine B photon counter (for details see Refs. 20, 27). Since the absorbance of rhodamine B in ethylene glycol decreases rapidly with wavelength increase, a flow-through cell (Kipp pyranometer) (Kipp CM10 without transparent hemisphere) with a μV meter (Keithly 150B) was used instead for wavelengths greater than 575 nm. Appropriate cut-off filters (Schott GG400, 420, and 495) were used to reduce straylight. Action spectra were measured with a constant photon flux of 5.29 or 22.9 einstein/s at fixed pmt sensitivity and irradiation time was constant. The photon flux was corrected for absorption of the sample according to Morewitz (31), while a correction was made for the inactivation of transforming DNA when illuminated with wavelengths below 310 nm (27).

Gel Electrophoresis—SDS gel electrophoresis was done in polyacrylamide gel slabs (Pharmacia 4/30) and in 10% polyacrylamide gel slabs according to Weber and Osborn (32). Electrophoresis under nondenaturing conditions was done using 8% polyacrylamide gel rods (6 mm diameter) in an imidazole-barbitol system, pH 7.1. (22). When electrophoresis was finished, gel rods were either stained with Coomassie Brilliant Blue or sliced. Gel slices were crushed and extracted overnight with buffer D. Enzyme activity in the supernatants was determined by the transformation assay.

Amino Acid Sequence Determination—Proteins were purified on SDS-gel electrophoresis prior to N-terminal amino acid sequence determination with an Applied Biosystems 470A protein sequencer.

RESULTS

Purification of Photoreactivating Enzyme—Photoreactivating enzyme was isolated from A. nidulans cells and purified 21,000-fold with 41% overall yield by consecutive column chromatography on porous silica beads, UV-DNA-cellulose, heparin-Sepharose, DEAE-cellulose, and DEAE-Sepharose (Table I). The final preparation was apparently homogeneous as judged from SDS-electrophoresis (not shown). Moreover, gel electrophoresis under nondenaturing conditions revealed a single protein band after Coomassie Brilliant Blue staining. This band coincided with the photoreactivating activity profile obtained from an identical gel which was sliced, extracted, and assayed instead of stained (Fig. 1), indicating that the protein band in question is photoreactivating enzyme indeed.

Properties of Photoreactivating Enzyme—the molecular weight of A. nidulans PRE was estimated to be 49,000 by gel filtration for native enzyme and 58,000 for denatured protein by SDS-polyacrylamide gradient gel electrophoresis. This indicates that PRE consists of a single protein chain. The influence of pH on enzymatic activity gave a rather broad profile with a plateau ranging from pH 5.2 to 7.9 (80% activity). Maximal activity was found at pH 6.2 and 0.1 M NaCl, while there was hardly any activity above 0.3 M NaCl (not shown).

Purified PRE was used to determine the N-terminal amino acid sequence:
TABLE I

Purification of A. nidulans photoreactivating enzyme

The amount of PRE causing \((N_1 - N_0)/N_0 = 1\) in the transformation assay under conditions mentioned under "Materials and Methods" is defined as one unit.

| Purification step                  | Volume | Protein | Total activity | Specific activity | Purification | Yield |
|-----------------------------------|--------|---------|----------------|-------------------|--------------|-------|
|                                   | ml     | mg      | megounits      | units/mg          | x fold      | %     |
| Crude extract*                    | 10,520 | 240,700 | 152            | 0.6               | 1           | 100   |
| Porous silica chromatography*     | 10,512 | 144,683 | 142            | 3.7               | 6           | 93    |
| UV-DNA-cellulose chromatography*  | 1,587  | <500    | <76            | 143.5             | 144         | 30    |
| Heparin-Sepharose chromatography* | 68     | 25.4    | 68             | 2,700             | 4,280       | 45    |
| DEAE-cellulose chromatography     | 24     | 7.4     | 58             | 7,800             | 12,400      | 38    |
| DEAE-Sepharose chromatography     | 10     | 4.7     | 63             | 13,400            | 21,200      | 41    |

* Combined results of seven separate runs using 2,090 g of wet cells in total.

b Combined results of two separate runs.

Fig. 1. Gel electrophoresis of A. nidulans PRE in an imidazole-barbital, pH 7.1, buffer system under non-denaturing conditions. One gel rod was stained with Coomassie Brilliant Blue G-250 and scanned at 546 nm. Another rod was sliced, extracted, and assayed for photoreactivating activity.

For AA-23 an Ala signal was obtained, which could be due however to the two preceding Ala residues. For AA-25 both a Trp and an Ala signal were found.

The absorption spectrum of purified PRE is shown in Fig. 2. In addition to a protein band (maximum at 266 nm), a band is present in the visible region (maximum at 437 nm) as well as a low intensity absorption band above 475 nm. At enhanced sensitivity this band is resolved into a shoulder at 498 and two maxima at 588 and 634 nm. The high resolution action spectrum for photoreactivation (Fig. 2) shows a large band in the blue region (maximum at 440 nm) which coincides almost exactly with the 437-nm absorption band, indicating the presence of an intrinsic photochemically active chromophore. A second region of high photoreactivating activity was found in the UV-B region (maximum at 290 nm), likewise in good correspondence with the absorption spectrum. No photoreactivating activity, even at 10-fold enhanced light intensity, was found with wavelengths greater than 500 nm, and the absorption spectrum of 8-OH-10-methyl-5-deazaisoalloxazine in dimethyl sulfoxide contains 10 mM triethylamine and 17.5% H2O (6:4). Shaded area, presumed contribution of the FADH2 chromophore.

The absorption spectrum of purified PRE was recorded at normal (curve A, ---) and 30-fold enhanced (curved B, ---) sensitivity in order to show the FAD semiquinone spectrum. The action spectrum for photoreactivation (pol) is compared with the absorption spectrum (curve C, ---). Also shown is the action spectrum obtained with 10-fold higher light intensity (pol-pol) which shows that photoreactivation is absent at wavelengths greater than 500 nm, and the absorption spectrum of 8-OH-10-methyl-5-deazaisoalloxazine in dimethyl sulfoxide containing 10 mM triethylamine and 17.5% H2O (- - -). Shaded area, presumed contribution of the FADH2 chromophore.

Two Different Chromophores in A. nidulans PRE—The close resemblance of absorption and action spectrum (Fig. 2) points to the presence of an intrinsic chromophore, while the visible absorption spectrum of native PRE is largely similar to that of model compound 8-OH-10-methyl-5-deazaisoalloxazine (I Scheme 1 legend). Denaturation of PRE, which will release noncovalently bound cofactors, induced a relatively strong fluorescence in contrast with native PRE which is almost non-fluorescent. The fluorescence excitation and emission spectra are nearly completely identical with the fluorescence spectra of 7,8-didemethyl-8-OH-5-deazariboflavin (II) (Fig. 3). The 8-OH-5-deazafavin structure was corroborated by reaction with 8-OH-5-deazafavin:NADPH oxidoreductase which reduces the chromophore to the non-fluorescent dihydro form (Fig. 3). Since this oxidoreductase is very specific, requiring the presence of both the 5-deazafavin structure and the 8-OH substituent in its substrate (24), this enzymatical conversion together with the fluorescence measurements establishes the 8-OH-5-deazafavin structure of the PRE chromophore, leaving the structure of the N(10)-side chain unknown.

Some information on the N(10)-substituent can be obtained from the pKc of the 8-OH group. At low ionic strength the pKc increases from 5.91 for 7,8-didemethyl-8-OH-5-deazariboflavin to 6.56 for F+ and 6.59 for SF240, apparently due to the presence of the charged phosphate in the N(10)-side chain. We calculated a value of 5.92 from fluorescence emission spectra of the released A. nidulans PRE chromophore at different pH (Fig. 4), indicating the absence of such a charged group.
During fluorescence measurements a small but significant difference was found between the emission spectra of denatured PRE and 7,8-didemethyl-8-OH-5-deazariboflavin on excitation at 450 nm (Fig. 5, curves A and B). The difference spectrum (curve C) has a maximum at 530 nm and resembles the fluorescence emission spectrum of normal flavins, pointing to the presence of a second flavin chromophore in PRE. In order to differentiate between FAD and riboflavin or FMN, denatured PRE was incubated with snake venom phosphodiesterase (curve D), which converts the released 8-HDF chromophore into the nonfluorescent reduced form. The high fluorescence below 380 nm in curve B is due to the added NADPH.

The structure of the second chromophore was further corroborated by reconstitution experiments with apo-d-aminoc acid oxidase which specifically uses FAD as coenzyme. We found that the enzymatic activity of apo-d-aminoc acid oxidase was restored on incubation with denatured A. nidulans PRE (results not shown).

Finally, the identity of both chromophores was confirmed by thin layer chromatography in four different systems (Table II). The results of fluorescence measurements, specific enzymatic reactions, and thin layer chromatography established unequivocally the structure of the A. nidulans PRE chromophores as 7,8-didemethyl-8-OH-5-deazariboflavin and FAD.

Chromophore Ratio—When A. nidulans PRE was denatured its main visible absorption band shifted from 437 to 418 nm (Fig. 6) due to the release of intrinsic cofactors. The shape of this spectrum obtained by subtracting B from A, as shown in curve C, resembles the fluorescence emission spectrum of flavins. Also shown is the large increase of fluorescence intensity of denatured PRE after addition of snake venom phosphodiesterase (curve D), which is characteristic for FAD. The difference spectrum of D and B (curve E) is identical with the fluorescence emission spectrum of FAD (---).

TABLE II

Identification of PRE chromophores by thin layer chromatography

| Sample                  | A     | B     | C     | D     |
|-------------------------|-------|-------|-------|-------|
| Denatured A. nidulans PRE | 0.75  | 0.10  | 0.75  | 0.65  |
| Denatured A. nidulans PRE | 0.30  | 0.35  | y     | 0.06  |

Reference compounds

| Sample | RF values in system |
|--------|---------------------|
| F420 (IV) | 0.55 ** 0 ** 0 ** |
| F+ (III) | 0.61 0.18 0 0     |
| FO (II)  | 0.78 0.19 0.78 0.65 |
| FAD     | 0.50 y 0.57 y 0 y 0.06 y |

**Excessive trailing, no sharp spots were obtained. TLC systems: A, silica (Merck 5577), n-butanol/acetic acid/water 5:2:3; B, cellulose (Bakerflex IB2), 3% NH4OH; C, silica, chloroform/methanol/ethyl acetate/acetic acid 100:100:40:2; D, silica, acetonitrile/water, 40:10, formic acid until pH 2.5.**
FIG. 6. Absorption spectra of native and denatured *A. nidulans* PRE. The absorption spectrum of native PRE, maximum at 437 nm (curve A), changes on denaturation due to the release of chromophores yielding spectra with maxima at 419 (pH 7.3, curve B) or 375 nm (pH 2.8, curve C). Also shown is the close fit between the absorption spectra of denatured PRE and an equimolar mixture of FO and FAD (---).

Fig. 7. Effect of preillumination on the PRE absorption spectrum. Native *A. nidulans* PRE (---) was irradiated in the presence of 5 mM 2-mercaptoethanol with 585 nm light (57 neinstein/s) at 10°C for 30 min (---). The sample was left in the dark for 2 h (---) to show the recurrence of the flavin radical. Inset: time course of dark recovery at 10°C after preillumination.

The protein concentration was estimated 12 μM assuming Mr = 53,000, indicating that approximately 28% of the PRE molecules lack the chromophores. From Fig. 6 it is also estimated that the molar extinction coefficient of native PRE is 53,000 at 437 nm.

Effect of Preillumination—The long wavelength absorption spectrum of purified PRE (Fig. 2) has a close resemblance with the absorption spectra of neutral (blue) flavin semiquinone radicals in polar solvents (33), suggesting that part of the FAD chromophore is present as semiquinone (FADH'). Assuming a molar extinction coefficient of 4500–5600 for the 580-nm absorption band of flavin semiquinones in flavoproteins (33), it can be calculated that 75–95% of the FAD chromophore is in the semiquinone form in dark equilibrated PRE.

When native PRE was preilluminated at 585 nm in the presence of 2-mercaptoethanol, the semiquinone band bleached whereas there were no large changes in the rest of the absorption spectrum (Fig. 7). On standing in the dark in air the original spectrum was slowly restored (see inset of Fig. 7). Even after several cycles of preillumination and dark recovery the final absorption spectrum was not altered (not shown). Photoreactivating activity did not change signifi-

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**Discussion**

We have purified photoreactivating enzyme from the cyanobacterium *A. nidulans* till apparent homogeneity, enabling the elucidation of the structure of the two intrinsic PRE chromophores as well as the N-terminal amino acid sequence. Meanwhile, this sequence appeared to be sufficiently informative to permit the cloning of the *Anacystis* *phr* gene using synthetic oligonucleotides. This has led to the determination of the complete base sequence of this gene, the first of a 8-HDF type PRE (35).

Although our results are on the whole in agreement with earlier publications, discrepancies were found concerning molecular weight and spectral properties of the enzyme. Saito and Werbin (5) estimated a molecular weight of 93,000 by gel filtration for *Anacystis* PRE, approximately twice the value we obtained. This points to a dimer molecule, in accordance with the reported tendency for aggregation (5), possibly enhanced by ammonium sulfate precipitation. We performed porous silica chromatography instead: the use of poly (ethylene glycol) for elution apparently suppresses any tendency of aggregation at this stage of purification without afflicting enzyme activity. The low level of PRE activity found between start and major protein band in nondenaturing gel electrophoresis (Fig. 1) may indicate some residual aggregation.

The action spectrum reported by Saito and Werbin (5) is roughly similar to our results (Fig. 2), but they found a maximum at 418 nm in the absorption spectrum of native PRE compared with 437 nm in this study. Also the enzyme was reported to be fluorescent with excitation/emission maxima at 420 and 470 nm, respectively (7), while we found native PRE hardly fluorescent. These spectral properties resemble those of released 8-OH-5-deazaflavin chromophore (see Fig. 3). In retrospect we think that the preparation used in early reports contained a substantial amount of released fluorescent 8-IDF chromophore. We encountered a similar problem with *S. griseus* PRE (21) due to spontaneous release of chromophore, but we found *Anacystis* PRE to be far more stable.

Both spectral data and results of thin layer chromatography identified the PRE chromophores as 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO, II) and FAD, present in equimolar amounts. This classifies *Anacystis* PRE as a 8-HDF type PRE. The action and absorption spectra of *Anacystis* (cyanobacterium) and *Scenedesmus* (green alga) PRE (20) are almost identical. Preliminary experiments indicate that the 8-HDF chromophore in *Scenedesmus* PRE has the same structure (FO) as in *Anacystis* PRE, and a common origin from a pre-endosymbiotic bacterium has been suggested (20). *Anacystis* cells are relatively rich in PRE, compare 5.5 with 0.5 mg/kg wet cells for *Scenedesmus*, in accordance with the fact that cyanobacteria must relay on photoreactivation for the rapid removal of UV-induced DNA damage as efficient

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1. A. P. M. Eker, unpublished results.
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The visible absorption band of Anacystis PRE has a high molar extinction coefficient of 53,000 (437 nm) which compares well with the value of 54,300 (420 nm) reported for P420 (IV) at pH 13.5 (36). The shape of this band (Figs. 2 and 6) indicates deprotonization of the 8-hydroxy group of the 8-HDF chromophore in PRE, whereas the high molar extinction coefficient points to deprotonization of N(6), e.g. due to interaction with charged groups inside the protein. This might be crucial for the ability to act as a photosensitizer in dimer splitting.

8-HDF-type PREs have the reduced FAD chromophore in common with pterin-type PREs from E. coli and S. cerevisiae and the question arises why different chromophores are present in various PREs. In Fig. 8 the absorption spectra of both types of PRE are compared. Besides the higher molar extinction coefficient of Anacystis PRE (compare 53,000 with 28,000), it is questionable whether E. coli and S. cerevisiae PREs could act with a quantum yield of 1 like S. griseus PRE (27) since they are fluorescent and will lose excitation energy. Moreover, a better overlap with the solar radiation spectrum at earth surface is obtained compared with S. cerevisiae PRE.

Therefore, the presence of the 8-HDF chromophore permits a more efficient use of available sunlight compared with the reduced pterin or reduced FAD chromophores. This is the more important since in eutrophic waters, a normal environment for cyanobacteria, the relative contribution of the far blue and near UV region of sunlight is severely diminished. In that habitat the presence of an 8-HDF chromophore will be even more advantageous for these organisms.

Action and absorption spectra of Anacystis PRE are highly similar to each other and to the absorption spectrum of model compound 8-OH-10-methyl-5-deazaisoalloxazine (Fig. 2). This leaves no doubt that 8,8-didemethyl-8-OH-5-deazariboflavin in the oxidized form acts as a primary photon acceptor in the photoreactivation process. Concerning the FAD chromophore, from the absence of photoreactivation at wavelengths above 500 nm (Fig. 2), it is clear that FAD semiquinone does not act as a photon acceptor in photoreactivation although it represents the major FAD species in dark equilibrated PRE. The same holds for oxidized FAD since an appreciable shoulder in the 450-500 nm region of the action spectrum is expected from superposition of absorption spectra of enzyme-bound 8-HDF and FAD (compare Fig. 6), which is not found. However, a significant difference between action spectrum and absorption spectrum of the model compound is present in the 330-390 nm region (Fig. 2, shaded area) which might be attributed to FADH2. A similar discrepancy has been found for PREs from Streptomyces (27) and Scenedesmus (20). The difference spectrum has a maximum at 350 nm (as far as can be estimated from the 5-nm resolution of the action spectrum), in agreement with the absorption spectrum of FADH2 bound to E. coli PRE (40), while a molar extinction coefficient of 3600 is calculated assuming a quantum yield of 1. This is comparable with the values of 3500-6200 reported for the near UV absorption band of reduced flavins in flavoproteins (37). Although other explanations cannot be completely ruled out, we assume therefore that FAD in the fully reduced form might act as a photon acceptor for photoreactivation but only in the near UV and not in the blue region.

This conclusion is further corroborated by the results of experiments with Anacystis PRE lacking the 8-HDF chromophore, which is still functionally active (38) although to a lower level. A similar conclusion was reached for the pterin-type PREs (39, 40).

From the long wavelength absorption spectrum of dark equilibrated PRE (Figs. 2 and 7), it appears that the major part of FAD is present as semiquinone. However, it is possible that PRE in living cells contains FADH; and the semiquinone is formed, despite the absence of 2-mercaptoethanol, during purification as was found for E. coli PRE (42). A rapid disappearance of the semiquinone was found on irradiation at 437 nm, which can be explained by photo reduction sensitized by the 8-HDF chromophore. This indicates a coupling of both chromophores (Fig. 9), i.e. a geometry which enables an efficient transfer of energy or electrons between the chromophores. It is conceivable that during photoreactivation the chromophores also act as a couple.

No appreciable enhancement of photoreactivating activity (as measured at 425 nm) was found after preillumination. At this wavelength only the 8-HDF and not the FADH2 chromophore will absorb light. The lack of enhancement might be explained by a mechanism in which photoreactivation, at least in the 437 nm band, mainly takes place directly through the 8-HDF chromophore, bypassing FADH2 (Fig. 9). An alternative explanation is a rapid reduction of FADH+ in the initial stage of photoreactivation so that in general these experiments are performed with PRE containing FADH2. The photoreduction of flavins in flavoproteins sensitized by 5-deazaflavins is well known (43).

In conclusion, the findings mentioned before led us to the following model for 8 HDF type PRE (Fig. 9). The 8-HDF (oxidized form) and possibly the FADH2 chromophore can act as a primary photon acceptor in dimer splitting, although in different wavelength regions. Photoreduction of FADH+ is

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**Fig. 9. Schematic model of 8-HDF-type PRE.** Preillumination (----) causes reduction of FAD semiquinone (FADH1) to fully reduced FAD (FADH2) either directly (>500 nm) or sensitized by 8-hydroxy-5-deazaflavin (8-HDF, 437 nm). During photoreactivation (-----) splitting of pyrimidine dimers (TT) in UV-DNA may occur by direct energy or electron transfer mediated by 8-HDF (path 1) or FADH2 (path 2), or indirectly through FADH2 (path 3).
possible either by irradiation of the semiquinone or through sensitization by 8-HDF, indicating that these chromophores are geometrically close enough to permit energy or electron transfer from 8-HDF to FADH'. Further experiments are necessary to refine this model, e.g. to distinguish between direct dimer splitting by 8-HDF or through a pathway with FADH₂ as an intermediary.

Acknowledgment—The skillful technical assistance of A, Wagtman, N. Otting, and M. Snoeren is acknowledged. We are most grateful to Drs. C. T. Walsh and A. Kiener for performing the amino acid sequencing and to Drs. W. H. Laarhoven and G. D. Vogels for their generous gift of 8-OH-5-deazaflavins.

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A P Eker, P Kooiman, J K Hessels and A Yasui

J. Biol. Chem. 1990, 265:8009-8015.

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