Photoreactivating Enzyme from the Green Alga *Scenedesmus acutus*. Evidence for the Presence of Two Different Flavin Chromophores

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ABSTRACT: Photoreactivating enzyme was isolated from the green alga *Scenedesmus acutus* and purified 71 000-fold with 16% overall yield. Maximal enzymatic activity was found at 0.07 M NaCl and pH 7.3. A molecular weight of 56 000 was estimated from sodium dodecyl sulfate gel electrophoresis. The absorption spectrum of the purified enzyme shows besides protein absorption (maximum 272 nm) an additional band in the visible region (maximum 437 nm, shoulder 425 nm). The in vitro action spectrum for photoreactivation shows two regions of high photoreactivating activity, viz., in the visible (maximum 440 nm) and the UV-B (maximum 290 nm) regions. The long-wavelength bands (400–500 nm) of the action and absorption spectra are almost completely similar, indicating the presence of an intrinsic chromophore. Fluorescence spectra of denatured photoreactivating enzyme suggested an 8-hydroxy-5-deazaisoalloxazine structure for the chromophore, which was confirmed by reaction with a specific oxidoreductase. This is the first time that the occurrence of an 8-hydroxy-5-deazaisoalloxazine is demonstrated in eukaryotic cells. The absorption spectrum of denatured photoreactivating enzyme indicated the possible presence of a second "hidden" flavin chromophore. Fluorescence measurements identified this chromophore as flavin adenine dinucleotide, which was confirmed by reconstitution with apo D-amino-acid oxidase. Photoreactivating enzyme appears to be the first example of a naturally occurring enzyme containing both a normal flavin and a 5-deazaflavin prosthetic group.

Photoreactivating enzyme (PRE)[1] or photolyase (EC 4.1.99.3) catalyzes the removal of cyclobutane-type pyrimidine dimers in UV-irradiated DNA by splitting them into the constituent pyrimidines. The presence of visible or near-UV light is an absolute requirement in this repair mechanism. This implies that the PRE–UV-irradiated DNA complex must contain a chromophore that absorbs light in this wavelength region. Two concepts have been formulated for the PRE chromophore, viz., the induced and the intrinsic chromophore. An induced chromophore is not present as such in the enzyme, but a new absorption band arises on formation of the complex between PRE and UV-DNA. The (nonflavo-) PRE from *Escherichia coli* is believed to be an example of an induced chromophore (Wun et al., 1977). An intrinsic chromophore is an integral part of the enzyme: its spectral properties do not, or only to a limited extent, change on complexation with UV-DNA. At present, a few examples are known. A reduced FAD chromophore was reported for PRE from *Saccharomyces cerevisiae* (Iwatsuki et al., 1980) and *E. coli* (Sancar & Sancar, 1984; Jorns et al., 1984), while *Streptomyces griseus* PRE contains an 8-hydroxy-5-deazaisoalloxazine chromophore (Eker et al., 1981).

Most green organisms are partly or completely dependent on light energy for growth and maintenance and often live in environments with intense solar radiation. Enzymatic photoreactivation is very efficient for these organisms since visible or near-UV light, the only energy source required for this type of repair, is readily available from sunlight. It can therefore be expected that green organisms possess an active photorepair system. In fact, the very first reports on photoreactivation concerned plants (Hertel, 1904; Hausser & von Oehmcke, 1933) and algae (Whitaker, 1942). No link seems to exist however between photosynthesis and photoreactivation.

The occurrence of photoreactivation is well-known in various green eukaryotic organisms, and a few action spectra have been determined. However, some of these spectra are of rather poor resolution and were measured in vivo, entailing the risk of spectral distortion, for instance, through screening by cellular compounds. Moreover, up to now PRE was not extensively purified from green eukaryotes. As a consequence, no definite conclusions concerning the structure of the involved chromophore were reached.

The present study comprises the isolation, purification, and characterization of photoreactivating enzyme from the green alga *Scenedesmus acutus*. This alga was selected because an axenic isolate was available, it can easily be grown in large quantities, and it has a reasonably high photoreactivating activity compared to, for instance, some *Chlorella* strains we tested. *Scenedesmus* cell extracts also exhibit only moderate nuclease activity compared to cell extracts from higher plants. This prevented disturbances in enzyme assay and enzyme purification.

Materials and Methods

Materials. Red–Sepharose was prepared by coupling Procion Red HE-3B to Sepharose CL-6B (Pharmacia) according to Baird et al. (1976). The preparation of single-stranded DNA–agarose has been described before (Eker & Fichtinger-Schepman, 1975). Heparin–Sepharose CL-6B was obtained from Pharmacia, Cellex-P was from Bio-Rad, and porous silica beads were from Serva (Spherosil type D, 100–200 μm, pore size 40–80 nm, and Servachrom Si200, 50–100 μm, 20-nm pore size) or Merck (Fractosil 500 and 1000, 63–125 μm, mean pore size 60 and 115 nm, respec-

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[1] Abbreviations: PRE, photoreactivating enzyme; SVPD, snake venom phosphodiesterase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; SDS, sodium dodecyl sulfate.

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tively). Snake venom phosphodiesterase was obtained from Sigma (type II from *Crotalus adamanteus*) and hog kidney D-amino-acid oxidase from Boehringer. The apoenzyme of the latter was prepared according to Massey and Curti (1966). NADPH: 8-hydroxy-5-deazaisoalloxazine oxidoreductase was isolated from *S. griseus* (Eker & Hessels, 1983). SF420, 8-hydroxy-N^{10}-[phosphorityllactylpoly(glutamyl)glutamic acidyl]-5-deazaisoalloxazine, was also isolated from *S. griseus* cells, following the procedure of Eker et al. (1980). Solutions of FAD-Na_2 (Boehringer) and FMN-Na (NBC) were freshly prepared just before use.

Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on 10% polyacrylamide gels according to Laemmli (1970) or on polyacrylamide gradient gel slabs [Pharmacia PAA 4/30 with electrode buffer containing 40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM sodium acetate, 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.2% SDS, pH 8.4]. Molecular weight standards were obtained from Pharmacia.

Assay of Enzymatic Activity. Photoreactivating activity was measured with a *Haemophilus influenzae* transformation assay [for experimental details see Piessens and Eker (1975)], using blue fluorescent lamps (Philips TLADK 30W/03). Photoreactivating enzyme was routinely diluted in 40 mM NaCl, 10 mM potassium phosphate, and 5 mM 2-mercaptoethanol, pH 7.0 (buffer A), containing 1 mg/mL bovine serum albumin. Enzyme activity is expressed as $(DF)(N_L - N_D)/N_D$, in which DF is the enzyme dilution factor and N_L and N_D are the number of transformants scored for illuminated or dark-incubated samples, respectively. In order to establish the influence of ionic strength or pH on enzyme activity, time courses of photoreactivation were determined under different conditions. The relative photoreactivating activity was obtained from the slope of plots of $\ln [(N_Z/N_D)^{1/2} - 1] - \ln [(N_Z/N_L)^{1/2} - 1]$ as a function of photoreactivation time (Eker et al., 1986), in which N_Z is the number of transformants found with untreated transforming DNA.

Protein concentrations were determined with the Coomassie Brilliant Blue method (Bradford, 1976).

Growth of Alga. S. acutus strain 141, an axenic isolate, was used for mass cultivation. The alga was grown in a medium containing 0.5 g of KH_2PO_4, 0.5 g of $K_2HPO_4 \cdot 3H_2O$, 1 g of $NaNO_3$, 150 mg of $MgSO_4 \cdot 7H_2O$, and 20 mg of $CaCl_2 \cdot 6H_2O$ per liter, 5 mL of a solution containing 1 g of $FeNH_4(SO_4)_2 \cdot 12H_2O$ and 0.8 g of Na_2EDTA per liter, and 5 mL of a spore element solution containing 573 mg of H_3BO_3, 362 mg of $MnCl_2 \cdot 4H_2O$, 44 mg of $ZnSO_4 \cdot 7H_2O$, 16 mg of $CuSO_4 \cdot 5H_2O$, 16 mg of $Co(NO_3)_2 \cdot 6H_2O$, 4 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 2 mg of NH_4VO_3 per liter. The final pH was 6.6. Ten-liter inoculates were grown at 26 °C in 12-L cylindrical bottles bubbled with 10 mL/min CO_2 and 75 mL/min air and illuminated with two circular 32-W white fluorescent lamps. After 4 days this culture was used to inoculate a 200-L fermentor (130 × 20 × 82 cm, constructed of 1 cm thick glass plates in a steel frame) illuminated with twenty 36-W white fluorescent lamps. The temperature was kept at 26 °C. For stirring, air was bubbled at a rate of 2 L/min. Depending on the growth phase, the flow of CO_2 was raised from 40 to 140 mL/min in order to maintain the pH between 6.7 and 7.0. After 4 days a cell density of about 230 (Klett-Summerson colorimeter with 640 filter) was reached and the cells were harvested in a Sorvall TZ-28 zonal rotor, yielding 600–800 g of wet cells. After the cells were washed with 0.1 M NaCl and 10 mM potassium phosphate, pH 7.0 (buffer B), they were resuspended in buffer B with 15% glycerol, frozen quickly, and stored at −20 °C.

Purification of Photoreactivating Enzyme. About 600 g of frozen cells, total volume 1400 mL, was rapidly thawed and disintegrated by sonication (Eker & Fichtinger-Schepman, 1975). Cell debris was removed by centrifugation at 25000*g*. The pellet was resuspended in half its volume of buffer B and passed once more through the sonic oscillator. After centrifugation, the supernatants were combined (about 1600 mL) and applied to a column (7.6 × 9.5 cm) containing porous silica beads (180 g of Spherosil, flow rate 500 mL/h). The column was washed with buffer B and eluted with buffer B containing 0.4 M NaCl and 6% poly(ethylene glycol) 6000. The eluate (500 mL) was diluted with an equal volume of 10 mM potassium phosphate and 5 mM 2-mercaptoethanol, pH 7.0 (buffer C), and loaded immediately to a Red–Sepharose column (4.4 × 9 cm, flow rate 100 mL/h). The column was washed with buffer D (which is buffer B containing 5 mM 2-mercaptoethanol) and eluted with buffer D containing 2 M NaCl. The eluate (220 mL) was dialyzed during 5 h against 5 L of buffer A. After addition of $^1/_3$rd volume of glycerol, the solution was frozen quickly and kept at −80 °C until further use.

The eluates of three purification runs described above were combined (1155 mL) and applied to a column containing single-stranded calf thymus DNA entrapped in agarose beads (1.6 × 21 cm, flow rate 38 mL/h). The column was washed with buffer D and eluted with buffer D containing 2.4 M NaCl. As only 50–60% of the enzymatic activity was bound to the column, this procedure was repeated twice. The combined eluates (275 mL) were dialyzed against buffer A, diluted with an equal volume of buffer C, and applied to a heparin–Sepharose column (1.6 × 7.5 cm, flow rate 33 mL/h). The column was washed with buffer A and eluted with a linear gradient of 75 mL of buffer A and 75 mL of buffer A containing 0.6 M NaCl. Photoreactivating enzyme eluted at 0.29 M NaCl. Active fractions were combined (45 mL) and dialyzed against buffer A. The dialyzed fractions were diluted with an equal volume of buffer C, adjusted to pH 6.0, and applied to a Cellex-P column (1 × 5 cm, flow rate 25 mL/h) which was previously equilibrated with buffer E (same as buffer A but pH 6.0). The column was washed with buffer E and eluted with a linear gradient of 20 mL of buffer E and 20 mL of buffer E containing 0.6 M NaCl. The enzymatic activity eluted at about 0.3 M NaCl. After addition of $^1/_3$rd volume of glycerol, the active fractions were frozen quickly and stored at −80 °C. All purification steps were performed at 4 °C.

Denatured PRE was prepared by heating purified PRE at 70 °C for 5 min, followed by centrifugation and/or filtration through a membrane filter (Millex-HA, Millipore) to remove denatured protein.

Reconstitution Experiments. Apo D-amino-acid oxidase was reconstituted with different flavins. The activity of the reconstituted enzyme was measured by monitoring the conversion of D-phenylglycine into benzoylformic acid at 250 nm (Fonda & Anderson, 1967). The reaction mixture contained 50 mM sodium pyrophosphate, pH 8.5, 31 μg of apo D-amino-acid oxidase, and 33 μM D-phenylglycine in a final volume of 2 mL. The cuvette was stirred and thermostated at 25 °C.

Spectral Measurements. Absorption spectra were recorded on a Beckman UV 5260 or Pye-Unicam SP7-500 spectrophotometer. Fluorescence spectra were measured with a home-built spectrofluorometer (Eker et al., 1981). The spectrometers were equipped with a microcomputer for ac-

Table I: Purification of *Scenedesmus acutus* Photoreactivating Enzyme

purification step	volume (mL)	protein (mg)	sp act. (units/mg)	purification (x-fold)	yield (%)
crude extract[a]	4950	70365	149	1	100
porous silica chromatography[a]	2053	35418	288	2	98
Red–Sepharose chromatography[a]	648	2116	4970	32	97
single-stranded DNA–agarose chromatography[b]	210	33	232200	1560	74
heparin–Sepharose chromatography	18	2.52	2420000	16200	58
cellulose phosphate chromatography	13	0.16	10625000	71000	16

[a] Combined results of three separate runs using 1830 g of wet cells in total. [b] Combined results of three consecutive runs (see Materials and Methods).

quisition of data as well as handling and storage of spectra. The sample cuvettes were thermostated at 10 °C. All fluorescence spectra are fully corrected.

For measurement of the action spectrum, a high-intensity monochromatic irradiation apparatus was used, consisting of a 1600-W Xe arc, quartz condensing assembly, grating monochromator (4.1-nm band-pass), and a large-area rhodamine B photon counter [for details see Eker et al. (1986)]. The photon flux was both adapted and corrected for absorption of the sample (Morowitz, 1950). Mixtures containing 1.75 mL of PRE diluted 200-fold in buffer D containing 1 mg/mL bovine serum albumin and 1.75 mL of UV-irradiated transforming DNA were placed in a stirred quartz cuvette (1 × 1 cm) thermostated at 30 °C. After 5 min of dark equilibration, illumination was started. Time courses of photoreactivation were measured by withdrawing samples at regular time intervals, which were immediately frozen in CO_2/acetone and stored at −20 °C until the transformation assay was completed. Photoreactivating activities were obtained from the slopes of kinetic plots as described under Assay of Enzymatic Activity. For wavelengths shorter than 310 nm a correction was made for the inactivation of transforming DNA (Eker et al., 1986).

All irradiation experiments were performed in the dim light of a sodium lamp.

Results

Purification of PRE. Scenedesmus acutus PRE was purified to apparant homogeneity following the purification scheme summarized in Table I. Chromatography on porous silica beads (Spherosil) was used as a replacement of ammonium sulfate fractionation, since it is more reproducible and easier to handle, especially when large volumes are involved. Although the achieved purification is not very large, one gets rid of nearly all green-colored material present in the crude extract, which can persistently adhere to some of the chromatographic materials used later on in the purification scheme. Other types of porous silica beads like Servachrom Si200 and Fractosil 500 or 1000 also gave satisfactory results.

Some nuclease activity was present in the porous silica eluate, which interfered with DNA–agarose chromatography planned as the next purification step. Dye–matrix chromatography was inserted therefore to reduce the nuclease content. It was found that PRE binds to both Blue– and Red–Sepharose. The elution from Blue–Sepharose with high salt concentrations was somewhat retarded; hence Red–Sepharose was used for routine purifications.

The crucial step in most PRE purification protocols is affinity chromatography on UV-irradiated DNA–cellulose, but we could not obtain reproducible results with *Scenedesmus* PRE. Since it is known that PREs also have affinity for unirradiated DNA (Eker, 1985), single-stranded DNA–agarose was used instead. As the binding capacity of this material appeared to be rather low, this step was repeated twice, resulting in a 50-fold purification for the combined eluates.

In the next purification step heparin–Sepharose, which is known to bind enzymes acting on nucleic acids (Farooqui, 1980), was used. Elution was effectively accomplished with a salt gradient, yielding a 10-fold purification.

At pH 7 PRE was only slightly bound to either DEAE-cellulose or cellulose phosphate. The binding could be strengthened by raising the pH in the case of DEAE-cellulose or lowering the pH for cellulose phosphate. At pH 6 the binding to cellulose phosphate was sufficiently strong for use as a final purification step.

The successive application of five chromatographic purification steps resulted in a 71000-fold purification with 16% overall yield. The extensive purification needed to obtain pure PRE reflects the low PRE content in *Scenedesmus* cells, a property shared with other species (Eker, 1983).

Characterization of PRE. Incubation of purified enzyme with chymotrypsin or proteinase A, or heating enzyme solutions at 50 °C, rapidly destroyed photoreactivating activity, indicating the proteinous nature of the photoreactivating agent. The influence of the pH on PRE activity is shown in Figure 1A. An optimum was found at pH 7.3. From the binding behavior of PRE to ion exchangers it can be estimated that the isoelectric point is approximately 7. The influence of ionic strength on the activity of PRE is shown in Figure 1B. Maximal activity was found at 0.07 M NaCl.

The molecular weight was determined with SDS–polyacrylamide gel electrophoresis. Single bands were found in 10% gels and in gradient gels (Figure 2), yielding molecular weights of 55000 and 57000, respectively. A somewhat lower molecular weight was found by gel filtration under nondenaturing conditions, pointing to a single protein chain in native PRE.

Experiments with diluted purified PRE showed that the presence of serum albumin and 2-mercaptoethanol is important in order to retain enzymatic activity. This might indicate the presence of one or more essential SH groups.

Spectral Properties. The absorption spectrum of the final enzyme preparation (Figure 3) shows a protein band with a maximum at 272 nm and a slight inflection at 289 nm, which may point to the presence of tryptophan residues. There is also a characteristic absorption band in the visible region with a maximum at 437 nm and a shoulder at 425 nm. This absorption spectrum is comparable to that of *S. griseus* PRE (Eker et al., 1986), indicating the presence of an 8-hydroxy-5-deazaisoalloxazine chromophore.

The action spectrum for photoreactivation was measured by using the purified enzyme preparation. One of the conditions for correct measurement of an action spectrum is the validity of the reciprocal correlation between irradiation time and photon flux. We found that this condition was satisfied for photon fluxes up to 10 neinstein/s. In the double logarithmic plot of photoreactivating activity as a function of

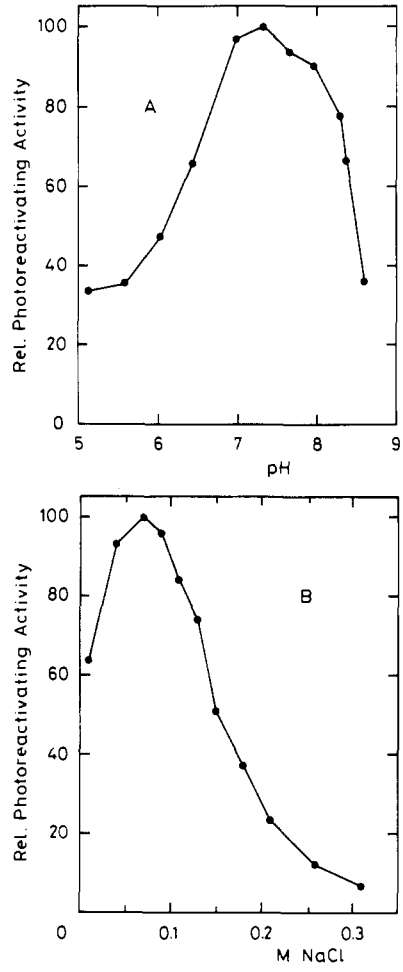

FIGURE 1: Influence of pH (panel A) and of ionic strength (panel B) on the enzymatic activity of *S. acutus* PRE.

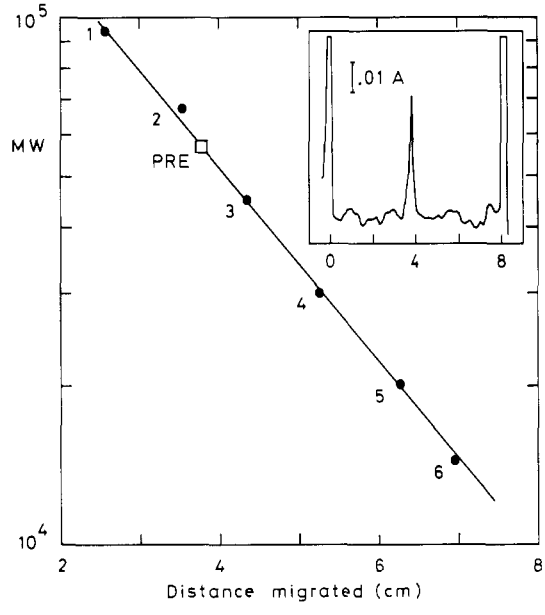

FIGURE 2: Molecular weight determination of *S. acutus* PRE by SDS–polyacrylamide gradient (4–30%) gel electrophoresis. The molecular weight standards are phosphorylase B (1), bovine serum albumin (2), ovalbumin (3), carbonic anhydrase (4), soybean trypsin inhibitor (5), and α-lactalbumin (6). A molecular weight of 57 000 was found. Inset: Scan (577 nm) of a gel with PRE, dyed with Coomassie Brilliant Blue G-250.

photon flux a straight line was found with slope 1.08 (not shown). This indicates that the photochemical reaction catalyzed by *Scenedesmus* PRE is a monophoton process. The

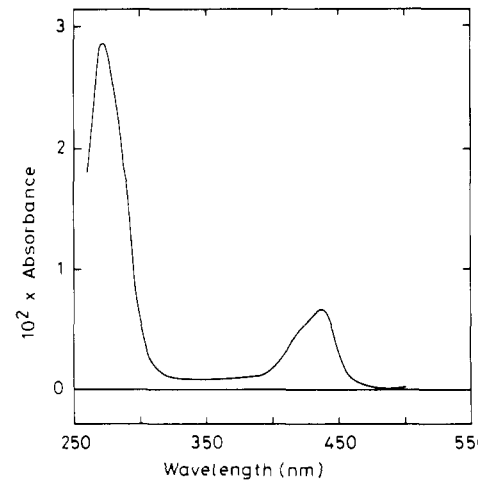

FIGURE 3: Absorption spectrum of purified *S. acutus* PRE (8 μg/mL) in 0.3 M NaCl, 10 mM potassium phosphate, and 5 mM 2-mercaptoethanol, pH 7.

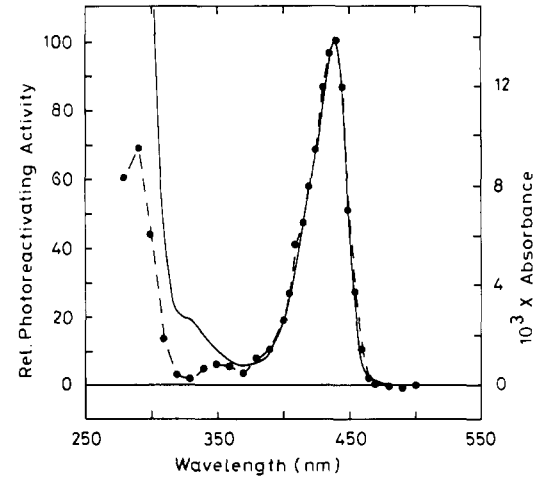

FIGURE 4: Action spectrum for photoreactivation (●--●). Photoreactivating activities were obtained from the slopes of time courses of photoreactivation with constant photon flux of 2.29 neinstein/s at different wavelengths. The photoreactivating activity at 440 nm (maximum) was arbitrarily set at 100. For comparison, the absorption spectrum of *S. acutus* PRE is also shown (—).

action spectrum itself is shown in Figure 4. The 380–500-nm region (maximum at 440 nm) is almost completely congruent with the long-wavelength absorption spectrum of PRE (maximum at 437 nm), establishing the intrinsic nature of the chromophore. A second region of high photoreactivating activity was found below 320 nm, which may be attributed to either the PRE chromophore or the protein part (Eker et al., 1986). In between, there is a region of low activity with a maximum around 350 nm.

Presence of an 8-Hydroxy-5-deazaisoalloxazine Chromophore. Native *Scenedesmus* PRE is hardly fluorescent, but after release of the chromophore by heat denaturation a relatively strong fluorescence was observed (Figure 5). Maxima were found at 469 nm in the emission spectrum and 420, 293, and 242 nm in the excitation spectrum. This fluorescence spectrum is very characteristic for 8-hydroxy-5-deazaisoalloxazines, as can be seen by comparison with the fluorescence spectrum of compound SF420, an 8-hydroxy-5-deazaflavin isolated from *S. griseus* (Eker et al., 1980).

Further evidence was obtained by using a NADPH:8-hydroxy-5-deazaisoalloxazine oxidoreductase which catalyzes the reaction:

NADPH + 8-OH-5-deazaisoalloxazine ⇌
NADP$^+$ + 8-OH-5-deazaisoalloxazine-H_2

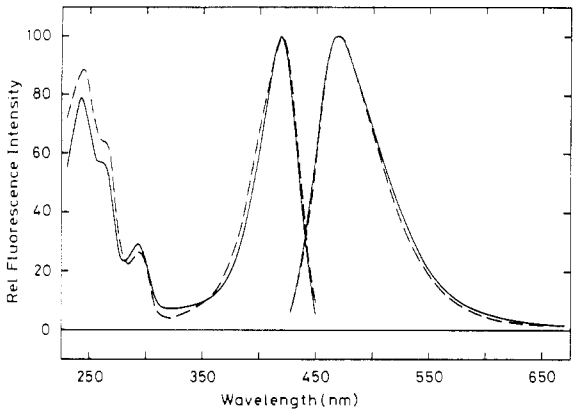

FIGURE 5: Fluorescence excitation (λ_{em} 462 nm) and emission (λ_{ex} 420 nm) spectra of heat-denatured *S. acutus* PRE (—) and SF420 (---) in 40 mM NaCl and 10 mM potassium phosphate, pH 7.

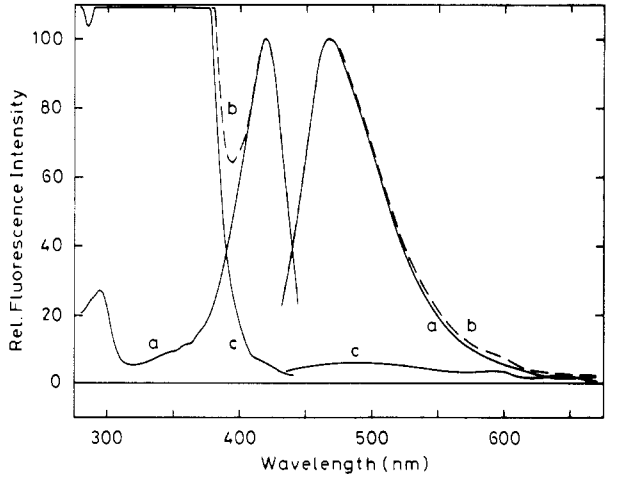

FIGURE 6: Identification of the PRE chromophore by means of reaction with a specific oxidoreductase. Fluorescence excitation (λ_{em} 462 nm) and emission (λ_{ex} 420 nm) spectra are shown of denatured *S. acutus* PRE (curve a), after addition of NADPH (curve b), and after addition of NADPH:8-hydroxy-5-deazaisoalloxazine oxidoreductase (curve c). In the latter case the chromophore is almost instantaneously reduced to the nonfluorescent dihydro form. The pH was 6. The large peak (280–390 nm) in excitation spectra b and c is due to the added NADPH.

This enzyme has an absolute requirement for both the 5-deazaisoalloxazine structure and the presence of an 8-hydroxy substituent in the flavin substrate (Eker & Hessels, 1983). *Scenedesmus* PRE was heat denatured, and the pH was brought to 6, close to the optimal pH for enzymatic reduction. A small amount of solid NADPH was added, which did not appreciably influence the fluorescence spectrum above 400 nm (compare curves a and b in Figure 6). Addition of the oxidoreductase resulted in an almost instantaneous decrease of fluorescence intensity (Figure 6, curve c) which can be attributed to reduction of the chromophore, since reduced 8-hydroxy-5-deazaisoalloxazine shows, in contrast to the oxidized form, no fluorescence on excitation at 420 nm. This confirms the presence of both the 5-deazaisoalloxazine structure and the 8-hydroxy substituent in the PRE chromophore.

Fluorescence spectra and enzymatic reduction together establish the 8-hydroxy-5-deazaisoalloxazine structure of the PRE chromophore.

Evidence for the Presence of a Second Chromophore. Although the absorption spectrum of native PRE has its maximum at 437 nm, an absorption maximum at 420 nm was found after release of the chromophore by denaturation (Figure 7, curve a), in agreement with the fluorescence excitation

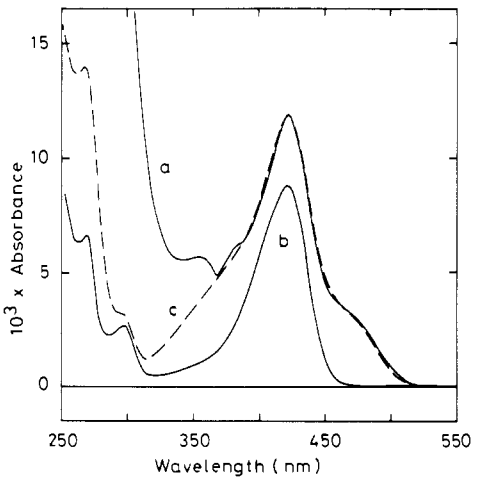

FIGURE 7: Comparison of absorption spectra of denatured *S. acutus* PRE (19 μg/mL, curve a) and SF420 (curve b). A much closer fit is found with the absorption spectrum of an equimolar mixture of SF420 and FAD (curve c).

spectrum shown in Figure 5. Comparison with the absorption spectrum of compound SF420 (Figure 7, curve b) indicates some additional absorption on the flanks of the 420-nm absorption band, pointing to the presence of another absorbing species. The absorption spectrum of denatured PRE was compared with that of SF420/FAD mixtures of different composition, yielding a good fit in the 370–500-nm region with an equimolar mixture (Figure 7, curve c). Below 370 nm an increasing discrepancy was observed due to scatter and end-absorption of the remaining protein.

It is conceivable therefore that a second chromophore with a "normal" flavin structure is present in *Scenedesmus* PRE.

Identity of the Second Chromophore. On excitation at 420 nm (the absorption maximum of the released 8-hydroxy-5-deazaisoalloxazine chromophore), no significant difference was found between the fluorescence emission spectra of denatured PRE and SF420 (Figure 5). On the other hand, when the excitation wavelength is chosen at 450 nm, a divergence was observed in the 470–600-nm region (Figure 8A). The difference spectrum (Figure 8B) obtained by subtracting the SF420 spectrum from the denatured PRE spectrum has a maximum at approximately 525 nm. It resembles the fluorescence emission spectrum of a flavin as shown in Figure 8C. The addition of a small amount of snake venom phosphodiesterase (SVPD) induced a substantial enhancement of the fluorescence of denatured PRE (Figure 8A). Again, the difference spectrum (Figure 8B) compares well with the result obtained for FAD (Figure 8C). For the ratio of fluorescence intensities with and without phosphodiesterase a value of 7.8 was obtained for denatured PRE, compared to 8.2 for FAD. The increase of fluorescence intensity must be attributed to the release of FMN from FAD on incubation with phosphodiesterase, as this abolishes the fluorescence quenching due to stacking of adenine and isoalloxazine rings in FAD (Miles & Urry, 1968; Spencer & Weber, 1972). Phosphodiesterase had no influence on the fluorescence of FMN or riboflavin.

In another experiment the 8-hydroxy-5-deazaisoalloxazine chromophore in denatured PRE was first reduced with NADPH and NADPH:8-hydroxy-5-deazaisoalloxazine oxidoreductase, whereupon the fluorescence emission was measured at pH 6 with excitation at 445 nm (Figure 9A), yielding a flavin-type emission spectrum. When the pH was lowered to 2.6, the fluorescence intensity increased by a factor 3.5. For FAD a 4.9-fold increase of fluorescence intensity was obtained on changing the pH (Figure 9B). This increase of fluorescence

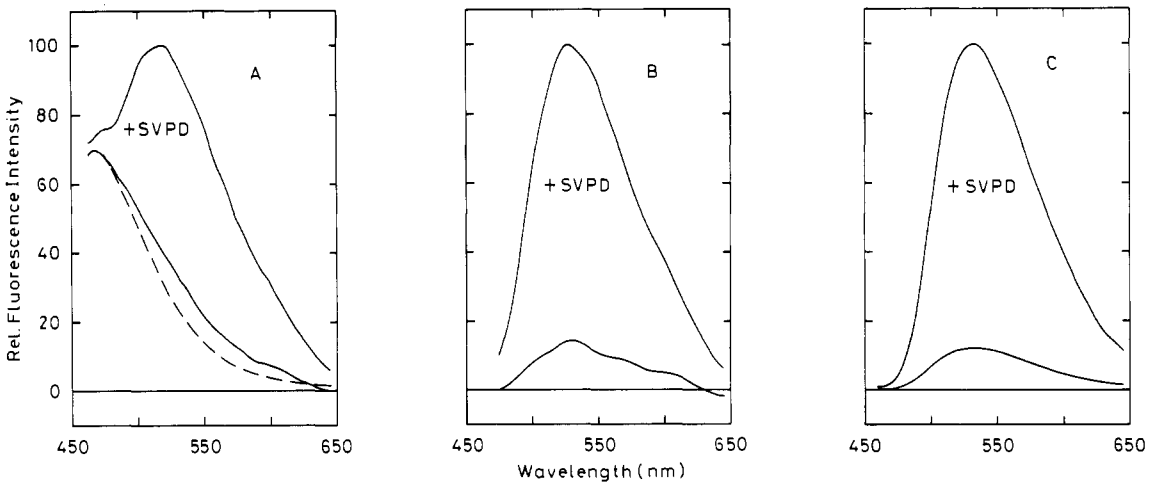

FIGURE 8: Fluorescence emission spectra of denatured *S. acutus* PRE (excitation at 450 nm). In panel A the fluorescence emission of denatured PRE (—) and SF420 (---) is compared. Also shown is the fluorescence emission of denatured PRE after addition of snake venom phosphodiesterase (SVPD). Phosphodiesterase had no appreciable influence on the fluorescence of SF420. In panel B difference spectra are shown, obtained by subtracting the SF420 spectrum from the denatured PRE spectra shown in panel A. Panel C shows fluorescence emission spectra of FAD before and after addition of SVPD. The spectra in panels B and C are normalized, retaining relative intensities.

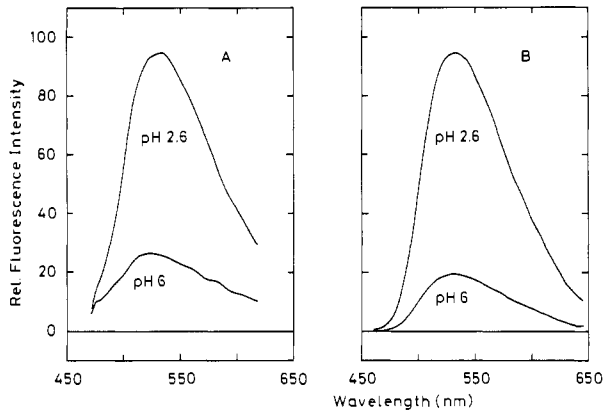

FIGURE 9: Influence of pH on the fluorescence emission of denatured *S. acutus* PRE. Small amounts of NADPH and NADPH:8-hydroxy-5-deazaisoalloxazine oxidoreductase were added to denatured PRE in order to reduce the 8-hydroxy-5-deazaisoalloxazine chromophore (compare Figure 6). Subsequently, fluorescence spectra were measured at pH 6 and 2.6 (panel A). Fluorescence spectra of FAD under the same conditions are shown in panel B. The excitation wavelength was 445 nm.

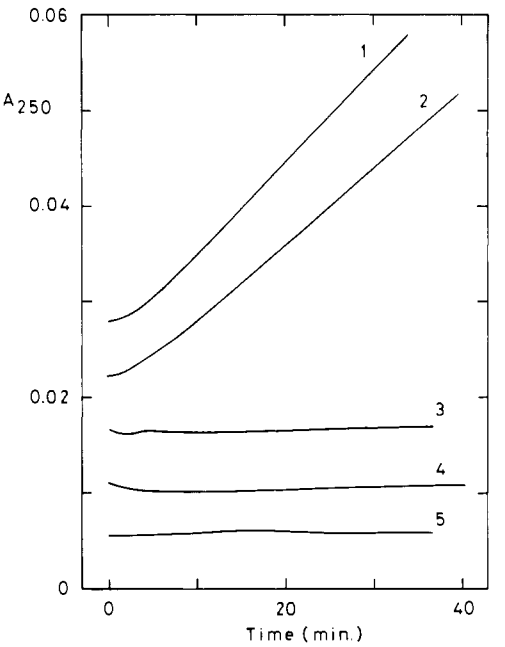

FIGURE 10: Reconstitution experiments with apo D-amino-acid oxidase. The activity of the reconstituted enzyme was measured by recording the conversion of D-phenylglycine into benzoylformic acid at 250 nm. The apoenzyme was reconstituted with denatured *S. acutus* PRE (1), 11.2 pmol of FAD (2), 1.52 nmol of SF420 (3), or 0.97 nmol of FMN (4). The control (no addition) is shown in curve 5. The curves are arbitrarily shifted along the *y* axis.

is also characteristic for FAD (Weber, 1950; Cerletti & Siliprandi, 1958; Tsibris et al., 1965). For FMN a decrease of fluorescence intensity is observed on lowering the pH (Bessey et al., 1949).

Further evidence was obtained from reconstitution experiments with apo D-amino-acid oxidase, which uses FAD as natural coenzyme. Incubation with denatured PRE resulted in an unequivocal restoration of enzymatic activity (Figure 10). No activity was found on incubation with SF420, FMN, or riboflavin, while denatured PRE showed no D-amino-acid oxidase activity.

Both fluorescence measurements and reconstitution experiments establish that the second chromophore in *Scenedesmus* PRE is FAD.

Discussion

Photoreactivation in Green Organisms. The two types of PRE with intrinsic chromophore known at present can be distinguished on the basis of action or absorption spectrum. For the reduced FAD type PRE, a maximum is found at 380 nm, while the 8-hydroxy-5-deazaisoalloxazine type has a maximum at 440 nm (Eker, 1983). From absorption (Figure 3) and action spectra (Figure 4) it is clear that *Scenedesmus* PRE belongs to the 8-hydroxy-5-deazaisoalloxazine type, which was confirmed by identification of the chromophore.

Photoreactivation has been demonstrated in a great variety of green organisms (Halldal & Taube, 1972), but only in a few cases are action spectra available. The in vivo action spectrum of the green alga *Platymonas subcordiformis* (Halldal, 1961) shows maximal activity at 430 nm and resembles that of *Scenedesmus* rather well, while for the desmid alga *Netrium digitus* maximal activity between 400 and 500 nm was reported (Marcenko, 1968). Cell extracts of the red alga *Porphyridium cruentum* exhibit 4.2-fold higher photoreactivating activity with 440-nm light compared to 370 nm (Eker, unpublished results). A concise action spectrum for photoreactivation of spores of the moss *Bryum pseudotri-*

quetrum was reported (Kinugawa & Otsuka, 1972), with maximal activity at 436 nm. Hence, there are indications that an 8-hydroxy-5-deazaisoalloxazine-type PRE is present in these organisms.

For vascular plants the situation is less clear. Extracts of *Zea mays* pollen showed maximal activity at 385 nm (Ikenaga et al., 1974), and the action spectrum resembles that of a reduced FAD PRE. On the other hand, a maximum at 420 nm was found for *Ginkgo biloba* pollen (Klein, 1963), while extracts of *Phaseolus vulgaris* seedlings exhibit maximal activity at 405 nm (Saito & Werbin, 1969). Obviously there is no uniformity.

A remarkable congruency exists between the photoreactivation action spectra of *Scenedesmus* and the prokaryotic cyanobacteria *Anacystis nidulans* (Saito & Werbin, 1970; Eker, 1983) or *Agmenellum quadruplicatum* (Van Baalen & O'Donnell, 1972). PRE from *Anacystis* contains without doubt an 8-hydroxy-5-deazaisoalloxazine chromophore (Eker, 1983; Eker, unpublished results), which explains this similarity. It is therefore tempting to assume that lower planta contain an 8-hydroxy-5-deazaisoalloxazine PRE of prokaryotic origin, introduced during evolution through an endosymbiontical cyanobacterium, while in higher plants a reduced FAD PRE, possibly from nuclear or mitochondrial origin, predominates.

8-Hydroxy-5-deazaisoalloxazine in Scenedesmus. It was rather recently that 8-hydroxy-5-deazaisoalloxazines were recognized as naturally occurring compounds (Eirich et al., 1978). At present, two functions are known. They can act as a cofactor in electron-transfer reactions like the reduction of $NADP^+$ by molecular hydrogen in methanogenic bacteria (Tzeng et al., 1975), or the reduction of 5a,11a-dehydrochlortetracycline to chlortetracycline in *Streptomyces* (McCormick & Morton, 1982). Second, 8-hydroxy-5-deazaisoalloxazines are chromophoric coenzymes in DNA-photoreactivating enzymes, where they are involved in the photochemical splitting of pyrimidine dimers (Eker et al., 1981). In the green alga *S. acutus* we found evidence for the second function only. In *Scenedesmus* cell extracts the presence of 8-hydroxy-5-deazaisoalloxazine could not readily be demonstrated, in contrast with methanogens and actinomycetes which contain rather high levels (Eirich et al., 1979; Eker et al., 1980; Naraoka et al., 1984; Daniels et al., 1985). The low 8-hydroxy-5-deazaisoalloxazine level is in agreement with the low PRE content of *Scenedesmus*.

Besides in methanogenic archaebacteria and certain prokaryotes, 8-hydroxy-5-deazaisoalloxazine has now also been demonstrated to be present in eukaryotic cells. Consequently, this type of modified flavin seems to occur in organisms belonging to all three primary kingdoms.

Two Chromophores in Scenedesmus PRE. For *E. coli* PRE some evidence has been given for the presence of a second chromophore in addition to reduced FAD (Jorns et al., 1984), but the structure of this chromophore was not revealed. As appears from the present study, *Scenedesmus* PRE contains two different flavin chromophores. To our knowledge, it is the first example of a naturally occurring enzyme containing both a normal flavin and a 5-deazaflavin.

From the absorption spectrum of denatured PRE (Figure 7) molar ratios of 1.04 for FAD and 0.81 for 8-hydroxy-5-deazaisoalloxazine to PRE were calculated, using molar extinction coefficients of 11 400 and 33 500 (obtained for SF420 at pH 7), respectively. The somewhat low 8-hydroxy-5-deazaisoalloxazine content may be the result of a slow release of chromophore, as has been found for *Streptomyces* PRE (Eker, 1980). A comparable chromophore content was reported for PREs from *S. cerevisiae* (molar ratio 0.74–0.83; Iwatsuki et al., 1980) and *E. coli* (molar ratio 0.69; Sancar & Sancar, 1984). The molar chromophore to protein ratios and the single protein band observed in gel electrophoresis indicate that the presence of FAD is not due to some flavoprotein contamination. The possibility of two PREs with different chromophores is also rejected since copurification through the extensive purification protocol mentioned in Table I, yielding an equimolar mixture of these PREs, is extremely unlikely.

Only a few enzymes are known to contain two different flavins, like NADPH–cytochrome P-450 reductase or NADPH–sulfite reductase, both possessing 1 FAD and 1 FMN per protein monomer [Kamin and Lambeth (1982) and references cited therein]. The presence of different flavins allows here electron transfer from the two-electron donor NADPH through FAD and FMN to a one-electron acceptor like ferriheme or sulfite. A major difference in functioning between PRE and these reductases is that in the latter case ground-state flavins shuttle between different redox states, whereas in PRE at least one optically excited flavin is involved (no pyrimidine dimer splitting is found in the absence of photoreactivating light).

Comparison of the absorption spectra of native PRE and a model system consisting of 8-hydroxy-10-methyl-5-deazaisoalloxazine in an aprotic dipolar solvent (Jacobson & Walsh, 1984; Eker et al., 1986) shows that the 8-hydroxy-5-deazaisoalloxazine chromophore in PRE is in the fully oxidized state. Moreover, the long-wavelength bands (400–500 nm) of absorption and action spectra are highly similar (Figure 4), indicating that oxidized 8-hydroxy-5-deazaisoalloxazine is a primary photon acceptor in PRE. The redox state of FAD is more difficult to estimate, partly because this chromophore is spectrally hidden due to overlap with the strongly absorbing 8-hydroxy-5-deazaisoalloxazine. The similarity in the 400–500-nm region of the action spectrum and the absorption spectra of PRE and model system points to the absence of oxidized FAD. The lack of photoreactivation above 500 nm seems to exclude the semiquinone form as a photon acceptor. An indication for the presence of reduced FAD may be found in the small but significant band around 350 nm in the action spectrum (Figure 4). This band corresponds well with the range of absorption maxima reported for reduced flavoproteins (Ghisla et al., 1974). The low molar extinction coefficient observed for reduced flavins compared to the high value of 44 000 estimated for the 8-hydroxy-5-deazaflavin chromophore in PRE (Eker et al., 1986) accounts for the large difference in photoreactivating activity at 350 nm as compared to 440 nm. In this context, it is interesting that 4a,5-reduced flavins are able to sensitize the photochemical splitting of thymine dimers (Eker & van Buyten, unpublished results).

The question can be raised as to why PRE contains two chromophores. Since enzymatic photoreactivation is strictly dependent on light, the presence of more than one chromophore can be advantageous by extension of the active wavelength region, comparable to accessory pigments in photosynthesis. The reduced FAD-type PRE should be considered then as the archetype of PRE, which acquired during evolution a second chromophore in a limited number of species. Furthermore, the photochemical yield may be increased by an additional chromophore with high molar extinction coefficient like 8-hydroxy-5-deazaisoalloxazine.

Another possibility is that the two PRE chromophores are functionally coupled. In this case, 8-hydroxy-5-deazaisoalloxazine would act as the primary photon acceptor, while its

excited state reacts with FAD, yielding a reactive intermediate that splits the pyrimidine dimer. In addition, it is conceivable that the 8-hydroxy-5-deazaisoalloxazine acts as a photosensitizer to maintain the FAD chromophore in *Scenedesmus* PRE in a reduced state, since 5-deazaflavins can efficiently reduce flavins in flavoproteins (Massey & Hemmerich, 1977).

Finally, although less probable, a pure structural role of the FAD prosthetic group (Cromartie & Walsh, 1976) cannot be excluded at present. Further studies on the mechanism of photoreactivation will be necessary to reach more definite conclusions.

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Registry No. PRE, 37290-70-3; FAD, 146-14-5; 8-hydroxy-5-deazaisoalloxazine, 80547-92-8.

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