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Ultraviolet-Induced Photodegradation of Cucumber (*Cucumis sativus* L.) Microsomal and Soluble Protein Tryptophanyl Residues in Vitro[1]

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The in vitro effects of ultraviolet B (280–320 nm) radiation on microsomal membrane proteins and partially purified ribulose bisphosphate carboxylase (Rubisco) from cucumber (*Cucumis sativus* L.) was investigated by measuring the direct photolytic reduction of tryptophan fluorescence and the formation of fluorescent photooxidation products. Exposure of microsomes and Rubisco to monochromatic 300-nm radiation resulted in the loss of intrinsic tryptophan fluorescence and the production of blue-emitting fluorophores. The major product of tryptophan photolysis was tentatively identified as *N*-formylkynurenine (N-FK). Even though the rates of tryptophan photodegradation and N-FK formation were similar, the amount of blue fluorescence produced was significantly higher in the microsomes relative to Rubisco. Studies with various free radical scavengers and other modifiers indicated that tryptophan photodegradation requires oxygen and that the subsequent formation of N-FK may involve reactive oxygen species. The optimum wavelengths for loss of typtophan fluorescence were 290 nm for the microsomes and 280 nm for Rubisco. The temperature dependence of tryptophan fluorescence and rate of tryptophan photodegradation indicated an alteration in the cucumber microsomal membranes at about 24°C, which influenced protein structure and tryptophan photosensitivity.

One of the potential consequences of stratospheric ozone depletion, which may result from the continued release of chloro-fluorocarbons, is an increase in the intensity of UV-B radiation at the earth's surface (Teramura, 1983; Frederick and Lubin, 1988). Although mechanisms that may reduce UV-B-induced damage to crop plants have been identified (e.g. changes in leaf morphology and epidermal flavonoid content) (Teramura, 1983), these UV-B-induced plant modifications may not be sufficient to prevent decreases in crop yield by enhanced UV-B radiation.

Membrane lipid unsaturated acyl chains (Klamen and Tuveson, 1982; Dearden et al., 1985; Chamberlain and Moss, 1987), sterols (Girotti, 1985), and the sulfhydryl groups (Murphy, 1984; Moan and Vistnes, 1986) and aromatic amino acids (Walrant and Santus, 1974; Caldwell, 1987) of membrane proteins may be susceptible to direct UV-B-induced photooxidation or indirect degradative processes mediated by endogenous photosensitizers, free radicals, and other reactive compounds produced during UV-B irradiation. Murphy (1983) has proposed that some of the phytotoxic effects of UV radiation may result from UV-induced membrane damage. UV radiation can modify processes associated with plant plasma membranes (e.g. K^+-activated, Mg^{2+}-dependent ATPase activity, ion permeability, etc.) (Murphy, 1983). The inactivation of the plasma membrane-bound ATPase by UV irradiation was optimal at 290 nm (Imbrie and Murphy, 1982). Considering the similar UV-B sensitivities of the activity of a microbial membrane enzyme activity and nutrient transport (Robb and Peak, 1979), the results of Imbrie and Murphy (1982) suggest that UV-B radiation can directly modify plant plasma membrane function.

Cucumber varieties with differing UV-B sensitivities have been identified (Krizek, 1981). Therefore, we investigated the effects of UV-B radiation on the microsomal membranes and Rubisco of cucumber leaves. Although Rubisco was utilized primarily to permit comparisons between soluble and membrane proteins, exposure of pea and soybean plants to UV-B radiation decreased the amount of Rubisco and altered its catalytic properties (Vu et al., 1984). Because Trp is a primary protein chromophore in the near UV and UV-induced tryptophan photoproducts may act as photosensitizers (Walrant and Santus, 1974), in vitro changes in the intrinsic protein Trp fluorescence of cucumber microsomal membranes were measured under a variety of conditions. The results indicate that concepts derived from similar studies with isolated proteins may be applicable to more complex membrane systems.

MATERIALS AND METHODS

Plant Material and Membrane Isolation

Cucumber (*Cucumis sativus* L. cv Poinsett 76) seeds were germinated and grown in Jiffy Mix[2] (Ball Jiffy, Chicago, IL) under a 16-h photoperiod (23/17°C). When the first true leaf was about 2 cm wide (11–13 d), the leaves were removed, washed in chilled deionized water, and suspended at a concentration of 3 mL fresh weight in a homogenization solution

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Abbreviations: D_2O, deuterium oxide; N-FK, *N*-formylkynurenine.

[2] Mention of a trademark or a proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

containing 0.25 M sorbitol, 5% (w/v) soluble PVP, 2.5 mM EDTA, 2.5 mM EGTA, 250 μM PMSF, and 10 μg/mL leupeptin in 50 mM Hepes-KOH buffer (pH 7.6). The leaves were vacuum infiltrated with homogenization buffer and then ground by hand (mortar and pestle) at 4°C under reduced light. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 12,000*g* for 15 min. The crude microsomes were collected from the supernatant by centrifugation at 100,000*g* over a 65% (w/v) Suc cushion. The microsomes were collected from the cushion interface and diluted with 10% (w/v) Suc, 1 mM EDTA, 250 μM PMSF, and 10 μg/mL leupeptin in 25 mM Hepes-KOH buffer (pH 7.6). The microsomal membranes were layered over 34% (w/v) Suc, 1 mM EDTA, 250 μM PMSF, and 10 μg/mL leupeptin in 25 mM Hepes-KOH buffer (pH 7.6) and centrifuged at 100,000*g* for 60 min. The membranes that collected at the 10/34% Suc interface were removed, diluted with 10% (w/v) glycerol and 1 mM EDTA in 10 mM potassium phosphate buffer (pH 7), and centrifuged over a 65% Suc cushion. The concentrated membranes were resuspended in the same phosphate buffer to give approximately 2.5 mg membrane protein/mL and used immediately. Membrane protein concentration was determined by the method of Wang and Smith (1975).

Partial Purification of Rubisco

Rubisco was partially purified from leaves of cucumber by the methods of Hall and Tolbert (1978), except that the homogenization solution also contained 10 μg/mL leupeptin and 1% (w/v) casein to reduce endoprotease activity (Rosichan and Huffaker, 1984). The pellet from the $MgCl_2$ precipitation step was resuspended in 10% (w/v) glycerol, 1 mM EDTA, and 10 μg/mL leupeptin in 10 mM potassium phosphate buffer (pH 7) to give approximately 2.5 mg protein/mL and was used immediately. Protein concentration was determined by the method of Wang and Smith (1975).

UV Radiation Treatment Conditions

Unless otherwise noted in the figure and table legends, all UV treatments were performed with membrane or Rubisco diluted with 10 mM potassium phosphate buffer (pH 7) to give 50 μg protein/mL. The samples were stirred in a 1-cm quartz fluorimeter cuvette at 25°C in a modified Aminco SPF-500 fluorimeter optical unit. The light source consisted of a 300-W, UV-enhanced xenon lamp with a model PS-300 power supply (ILC Technology, Sunnyvale, CA). Unweighted light intensities at the cuvette front surface were measured with an Optronic Laboratories (Orlando, FL) model 752 spectroradiometer. Standard illumination conditions consisted of 300-nm (10-nm bandpass) light adjusted to give 200 mW m^{-2} UV-B radiation intensity. The intensity was controlled by applying an appropriate voltage to the light modulation input of the lamp power supply.

Fluorescence Measurements

All fluorescence spectra were obtained with an Aminco SPF-500 corrected spectra fluorimeter, modified as previously described (Caldwell, 1987), and with a ILC Technology model PS-300 power supply. The spectra were corrected for inner filter effects, scattering of the membrane samples, and the Raman band of H_2O (Caldwell, 1987). Time-dependent changes in fluorescence at fixed wavelengths during UV radiation treatments were obtained with this fluorimeter or the fluorimeter optical unit described above equipped with a Hamamatsu R928 photomultiplier, an Ortec (Oak Ridge, TN) model 428 detector-bias power supply, and an Oriel (Stratford, CN) model 70710 current to voltage converter/amplifier module. The time-dependent changes in fluorescence (60-min treatments) were analyzed with the curve-fitting routine of Sigma Plot 4.0 (Jandel Scientific, Corte Madera, CA). Semilogarithmic plots of the change in fluorescence as a function of incubation time were linear with correlation coefficients greater than 0.95, indicating pseudo-first-order kinetics.

Absorbance Measurements

Absorbance measurements were performed with a Beckman model DU-8 spectrophotometer. The absorbance of the samples during UV treatment was monitored with a Hamamatsu UV-enhanced photodiode in the photovoltaic mode.

RESULTS

Fluorescence Spectra

Exposure of both cucumber microsomal membranes and Rubisco to UV-B radiation (300 nm, 200 mW m^{-2}) resulted in the loss of intrinsic protein fluorescence measured with excitation at 295 nm (1 nm bandpass) (Fig. 1). The emission maxima for the membrane proteins (333 nm) and Rubisco (343 nm) indicated Trp fluorescence, with the tryptophanyl residues of the membrane proteins being in more hydrophobic environments than those of Rubisco (Teale and Weber, 1957). Unlike treatment of Rubisco, UV-B treatment of the microsomal membranes resulted in a blue shift of the emission maxima (Fig. 1A), suggesting that tryptophanyl residues in relatively hydrophilic environments were more susceptible to photodegradation.

The loss of intrinsic protein fluorescence was accompanied by increased fluorescence intensity at wavelengths above 400 nm (Fig. 1). With excitation at 320 nm, the increase in blue fluorescence became more apparent (Fig. 1, insets). Even though the amount of UV-B-induced reduction in Rubisco Trp fluorescence was similar to that of the microsomal membranes (Fig. 1), the increase in blue fluorescence was significantly less with Rubisco compared to the microsomal membranes. Furthermore, the fluorescence emission maxima for Rubisco (320 nm excitation) was red shifted relative to those of the microsomal membranes (Fig. 1, insets).

Even though the microsomal fractions had no visible green coloration, the absorbance of the microsomes at wavelengths above 350 nm suggested the presence of Chl (data not shown). One hour of exposure of both microsomes and Rubisco to UV-B radiation (300 nm, 10 nm bandpass) increased the absorbance of both the microsomes and Rubisco at wavelengths between 275 and 290 nm (Fig. 2). There was

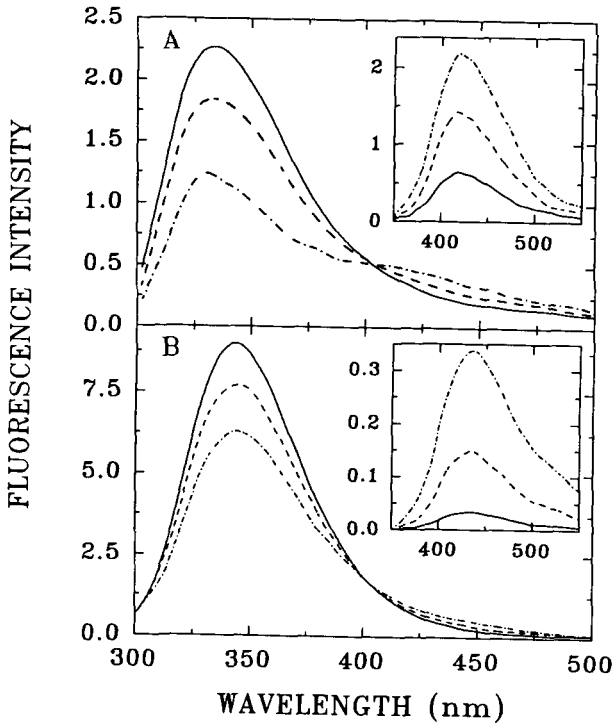

Figure 1. UV-B (300 nm)-induced changes in the fluorescence emission spectra of cucumber microsomal membranes (A) and Rubisco (B). Protein samples were treated with 200 mW m^{-2} UV-B radiation for 0 (—), 30 (- -), and 60 min (-·-). The intrinsic fluorescence emission spectra were then measured with excitation at 295 nm (1-nm bandpass) and emission with a 5-nm bandpass. The formation of blue-fluorescing photoproducts was measured with excitation at 325 nm (1-nm bandpass) and emission with a 5 nm bandpass (insets).

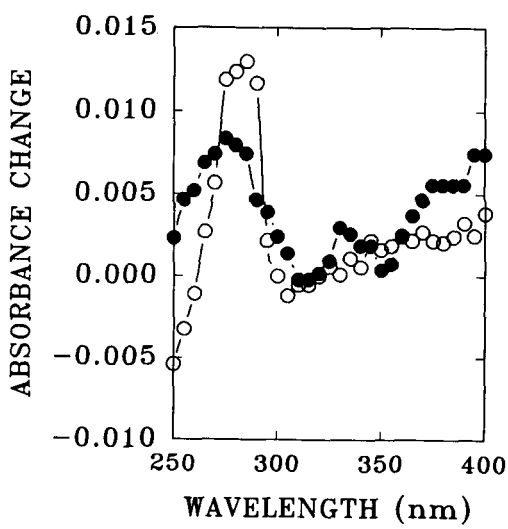

Figure 2. Effects of UV-B (300 nm) on the absorbance of aqueous solutions of cucumber microsomal membranes (●) and Rubisco (○). The change in absorbance was obtained by subtracting the absorbance of untreated samples from that of samples treated with 200 mW m^{-2} UV-B radiation for 60 min.

a slight increase in microsome absorbance at about 325 nm and above 350 nm (Fig. 2).

Effect of UV-B Intensity on the Rate of Trp Photodegradation

As shown in Figure 3, the intensity of the UV treatment (300 nm, 10 nm bandpass) influenced the rate of Trp photodegradation in both microsomal proteins and Rubisco. The change in rate as a function of intensity was linear from 0 to 300 mW m^{-2}, indicating that the processes associated with Trp photodegradation were not saturated under the standard illumination conditions (200 mW m^{-2}). The tryptophanyl residues of the microsomal membrane proteins were more sensitive to UV-B-induced photodegradation than those of Rubisco.

Effect of Free Radical and Reactive Oxygen Modifiers on the Rate of Trp Photodegradation

The formation of the blue fluorescent product was inhibited by 20 mM hydrazine hydrate and anaerobic conditions during illumination (Table I). The effects of different reactive oxygen quenchers on both the rates of Trp photodegradation and blue fluorescence production suggested the involvement of singlet oxygen ($^1O_2$), hydroxyl radical (·OH), and superoxide anion (O_2^-) (Table I). Although the rate of Trp fluorescence reduction by UV-B radiation was not influenced by

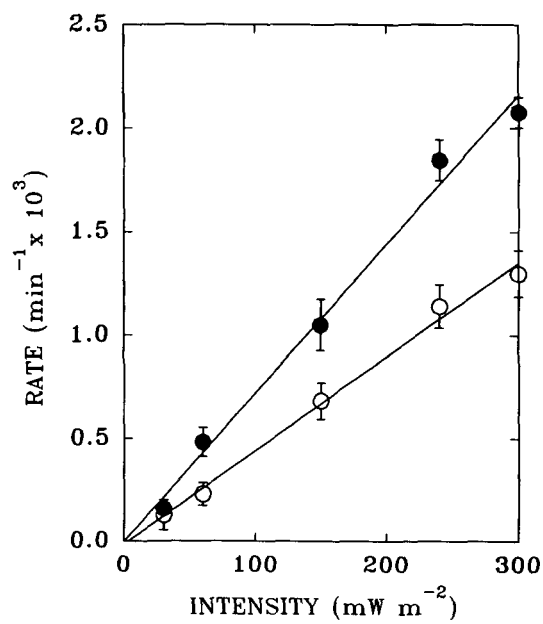

Figure 3. Effect of UV-B (300 nm) treatment intensity on the rate of protein Trp photodegradation. Cucumber microsomal membranes (●) and Rubisco (○) were treated with UV-B at 300 nm for 60 min. At regular intervals during this time period, the fluorescence emission of the membranes (335 nm) and Rubisco (345 nm), both a with 5-nm emission bandpass, was measured using 295-nm excitation (1-nm bandpass). The pseudo-first-order reaction rates were then computed.

Table I. *Effect of reactive oxygen modifiers and protective enzymes on the rates of microsomal membrane protein and Rubisco Trp photodegradation and N-FK production*

Membrane (M) or Rubisco (R) was treated with UV-B radiation (300 nm, 200 mW m^{-2}) for 60 min. At 5-min intervals, the Trp fluorescence intensity at 335 nm (5-nm bandpass) for the membranes and 345 nm (5-nm bandpass) for Rubisco was measured with excitation at 295 nm (1-nm bandpass). The blue fluorescence of the same sample was then measured with excitation at 320 nm (1-nm bandpass) and emission at 415 nm (20-nm bandpass). The pseudo-first-order reaction rates were then computed. The effects of the various additives are presented as a percentage of the reaction rate obtained without additives.

Sample	Addition	Concentration	Rate	
			Tryptophan	N-FK
			% of control	
M	NaN_3	20 mM	82.6	77.3
M	NaFormate	20 mM	97.3	78.9
M	D-Mannitol	20 mM	95.4	82.5
M	Hydrazine	20 mM	87.7	46.4
M	EDTA	2 mM	101.4	97.8
M	Catalase	10 μg/mL	98.7	80.8
M	SOD	10 μg/mL	94.3	88.2
M	D_2O		92.3	33.7
M	N_2		99.4	31.7
R	NaN_3	20 mM	76.9	75.8
R	NaFormate	20 mM	98.5	82.3
R	D-Mannitol	20 mM	95.5	84.1
R	Hydrazine	20 mM	55.3	18.5
R	EDTA	2 mM	98.6	102.4
R	Catalase	10 μg/mL	97.6	93.4
R	SOD	10 μg/mL	87.6	81.7
R	D_2O		72.5	66.7
R	N_2		98.4	21.2

anaerobic conditions, the formation of the blue-fluorescing product required oxygen and was reduced by azide, suggesting the involvement of $^1O_2$ (Table I). However, the effects of D_2O, a promoter of $^1O_2$-dependent processes, were not consistent with this interpretation (Table I).

Wavelength Dependence of Protein Trp Photodegradation

Samples of both microsomal membranes and Rubisco were illuminated at different wavelengths (10 nm bandpass), and at regular intervals, the Trp fluorescence was measured with 295-nm excitation (1-nm bandpass) and emission at 335 or 345 nm (10-nm bandpass) for the microsomes and Rubisco, respectively. The rates were corrected for the relatively small changes in fluorescence caused by the brief exposure to 295-nm light. The tryptophanyl residues of the microsomal membrane proteins were optimally photodegraded at 290 nm with a second optimum at 390 nm (Fig. 4A). There was significant photodegradation at all wavelengths between 250 and 400 nm. The Trp photodegradation at wavelengths above 350 nm may have resulted from absorbance by Chl or other pigments. In contrast, the optimal wavelength for Rubisco Trp fluorescence reduction was 280 nm, with the rate of photodegradation being significantly lower than that of the membrane proteins at wavelengths above 350 nm (Fig. 4C).

Effect of Temperature on Trp Photodegradation

With increasing sample temperature, the intensity of the Trp fluorescence declined (Fig. 5). The reduction in Rubisco intrinsic fluorescence as a function of sample temperature was linear from 6 to 60°C. However, the temperature-induced reduction of microsomal membrane protein fluorescence was biphasic, with two linear segments that intersect at about 24°C (Fig. 5).

The rate of protein Trp photodegradation was sensitive to sample temperature. When presented as an Arrhenius plot, temperature dependence of the rate of Rubisco Trp photodegradation was linear from about 4 to 55°C (Fig. 6). Above 55°C, the rate declined, suggesting the onset of thermal denaturation of Rubisco. The Arrhenius plot of microsomal membrane protein Trp photodegradation was triphasic, with an increase in apparent activation energy above about 24°C and thermal denaturation above 40°C (Fig. 6).

DISCUSSION

Molecular mechanisms for the biological effects of UV-B radiation have been investigated extensively in animal and

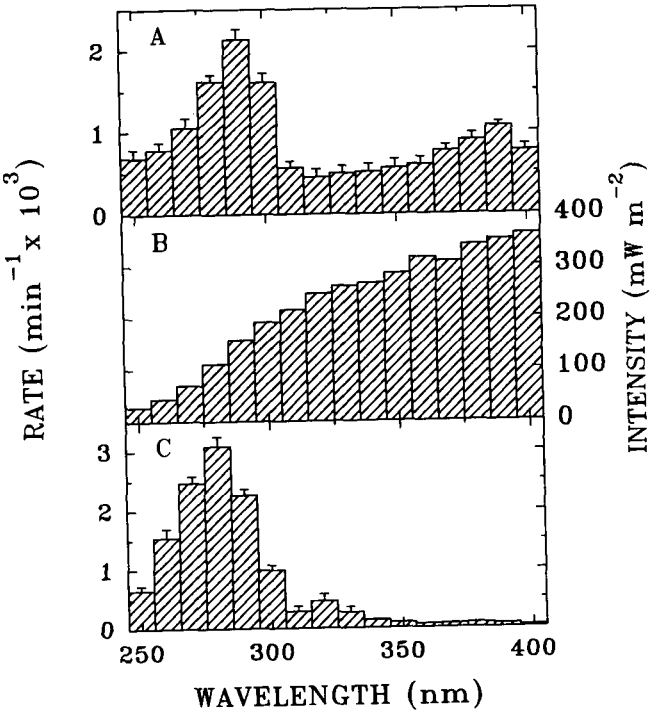

Figure 4. Influence of treatment wavelength on the rate of Trp fluorescence loss. Cucumber microsomal membranes (A) and Rubisco (C) were treated with a variety of wavelengths (10-nm bandpass) at the intensities shown in B. Over a 60-min period, the fluorescence intensities at 335 nm (5 nm bandpass) for the membranes and 345 nm (5-nm bandpass) for Rubisco were measured with excitation at 295 nm (1-nm bandpass). The pseudo-first-order reaction rates were then computed.

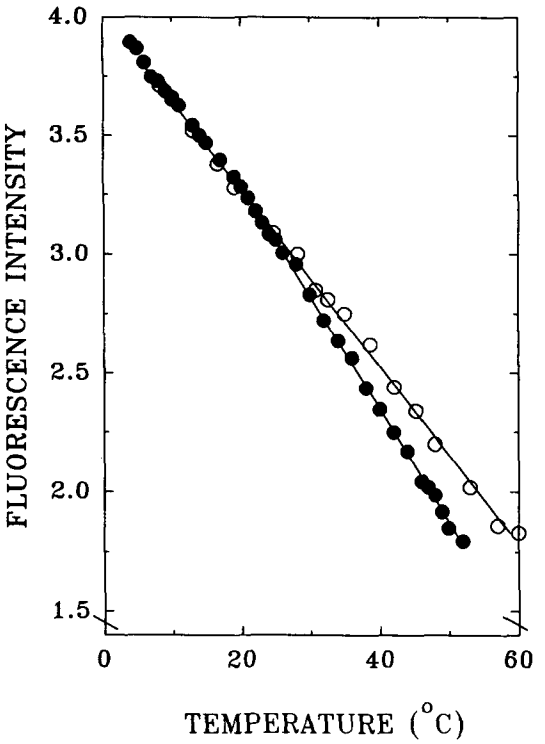

Figure 5. Temperature dependence of the intrinsic fluorescence of cucumber microsomal membranes and Rubisco. The fluorescence intensities at 335 nm (5-nm bandpass) for the membranes (●) and 345 nm (5-nm bandpass) for Rubisco (○) were measured, with excitation at 295 nm (1-nm bandpass) as a function of sample temperature. An ascending temperature gradient (1°C/min) was applied to the samples, and the fluorescence was determined at regular intervals. The excitation shutter of the fluorimeter was closed between measurements. The fluorescence intensity of Rubisco at 4°C was normalized to the fluorescence of the membranes at that temperature.

microbial systems. The consensus is that there are two potential primary lesions involved in UV-B-induced cellular damage. The first involves the formation of nonthymine dimer DNA photoproducts that interfere with DNA replication and transcription (Rahn, 1979). Second, UV-B radiation can cause direct photochemical modification of proteins, particularly those associated with membranes, which reduces enzyme activity and may sensitize subsequent photochemical reactions (Walrant and Santus, 1974; Valenzeno, 1987).

UV-C and UV-B radiation inhibited the activity of rose petal plasma membrane-bound ATPase and induced an efflux of K^+ from rose cells (Imbrie and Murphy, 1982; Murphy, 1983, 1984). Because the optimal reduction in ATPase activity occurred with UV-B radiation (290 nm) (Imbrie and Murphy, 1982), this modification in enzyme activity may have resulted from the direct photooxidation of protein Trp groups (Walrant and Santus, 1974). Trp photodegradation could directly alter protein structure or modify membrane lipids and proteins through the production of O_2^- and H_2O_2 (Walrant and Santus, 1974; Andley et al., 1984).

The results presented in this report are consistent with in vitro UV-B-induced Trp photooxidation of both membrane proteins and Rubisco. The wavelength dependence (Fig. 4) and oxygen requirement (Table I) support the contention that Trp is a primary UV-B chromophore and the source of the blue-fluorescing photoproduct in these samples. The blue shift of the fluorescence emission maximum of the microsomal membranes exposed to UV-B radiation (Fig. 1A) is consistent with the results of Pigault and Gerard (1984). They demonstrated that the photosensitivity of protein tryptophanyl residues is dependent upon their location within the protein with greater rates of photodegradation for exposed tryptophanyl residues.

There are a variety of potential UV-B-induced photoproducts that have blue fluorescence emissions similar to that shown in Figure 1. Bityrosine has similar fluorescence properties (excitation at 325 nm, emission at 410–420 nm), which have been utilized to evaluate the effects of various reactive oxygen species on proteins (Davies, 1987). Another possible source of the blue fluorescence in the UV-B-treated microsomes is lipid peroxidation products (Dillard and Tappel, 1984; Salmon et al., 1990). However, no thiobarbituric acid-reactive lipid peroxidation products could be detected after the 60-min UV-B treatments (data not shown). This suggests that lipid peroxidation is not the primary cause for the increasing levels of blue fluorescence during UV-B irradiation of the microsomal membranes.

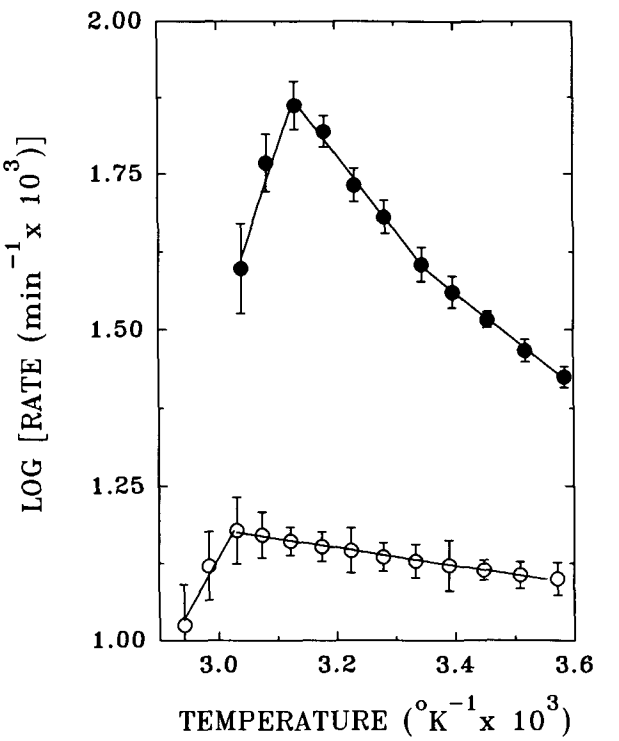

Figure 6. Arrhenius plots of the temperature-dependent changes in the rate of cucumber microsomal membrane protein (●) and Rubisco (○) Trp fluorescence loss by UV-B (300 nm) radiation.

The simultaneous loss of protein Trp fluorescence and the formation of a blue-fluorescing product suggests the photodegradation of Trp to N-FK. The tentative identification of N-FK as the primary blue-fluorescing UV-B photoproduct in both the membrane and Rubisco samples is based primarily on circumstantial evidence. A number of different photoproducts of free Trp have fluorescent properties comparable to that of UV-B-irradiated cucumber microsomes and Rubisco (i.e. anthranilic acid and kynurenic acid) (Tatischeff et al., 1976). However, only N-FK can be produced by photooxidation of Trp in a protein. The fluorescence properties of the photoproduct are similar to those obtained with other protein systems and attributed to N-FK (Walrant et al., 1975; Fujimori, 1981). The addition of hydrazine hydrate to the samples prior to UV-B treatment reduced the formation rate of the blue-fluorescing photoproduct (Table I). Fujimori (1981) observed a similar hydrazine-induced reduction in the production of blue fluorescence in UV-B-treated proteins, which he related to the ability of hydrazine to react with N-FK, forming a hydrazone product.

The reduction in the rate of protein Trp photodegradation by hydrazine (Table I) is also consistent with N-FK being a primary photoproduct of the UV-B treatment, because N-FK can sensitize the photooxidation of Trp. Walrant et al. (1975) have proposed that N-FK can act as an internal photodynamic sensitizer in proteins. The N-FK produced by UV-B illumination may sensitize the system to UV-A radiation, resulting in UV-A-induced production of reactive oxygen species.

The results presented in Table I indicate that, unlike N-FK formation, the UV-B-induced decrease in Trp fluorescence may not require O_2. The reduction in the rate of N-FK formation by mannitol, formate, and azide suggests the involvement of $\cdot OH$ and $^1O_2$ in N-FK production. However, because D_2O, a promoter of $^1O_2$-dependent reactions, reduced the rate of N-FK production, participation of $^1O_2$ in the UV-B-induced photodegradation of the protein tryptophanyl residues is not certain. Reactive oxygen species can directly modify proteins, causing both inter- and intramolecular cross-links and fragmentation that may increase their susceptibility to endogenous proteases (Davies, 1987).

The effects of temperature on the rates of Trp photodegradation (Fig. 6) suggest that cucumber Rubisco is thermally very stable, with no significant change in structure until the temperature exceeds about 55°C. Conversely, the microsomal membranes apparently undergo a conformational change centered near the maximal growth temperature of the plants (23°C) (Figs. 5 and 6) that influences the photosensitivity of protein Trp.

Both membrane and soluble proteins can be modified by UV-B radiation in vitro. Furthermore, the UV-B reactivity of the protein tryptophanyl group may be influenced by membrane structure. However, the relationship of these results to whole plant responses to UV-B radiation is not clear. Vu et al. (1984) observed quantitative and qualitative changes of Rubisco from pea and soybean plants exposed to UV-B radiation. The UV-B treatments also increased the UV-B absorbance of extracted pea pigments, suggesting that modification of the UV-B-filtering ability of the intact leaves may not be sufficient to protect the plant from UV-B-induced damage. Therefore, it seems likely that UV-B exposure can influence the activity of plant enzymes in vivo either by direct photolysis or through the production of reactive oxidants.

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