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Photoinactivation of Detergent-Solubilized Plasma Membrane ATPase from *Rosa damascena*

ACTION SPECTRA

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ABSTRACT

The photochemistry of vesicular and detergent-solubilized preparations of plasma membrane-associated ATPase was investigated in *Rosa damascena*. The cholate-solubilized ATPase activity fractionated into two peaks on a Sephadex G-150 column with simple, but different ultraviolet (UV) sensitivities. The larger enzyme was UV sensitive; the smaller enzyme was relatively insensitive. The activity of both ATPase fractions depended on environment: both were inactive in cholate, relatively inactive in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, and active in phosphatidylglycerol and phosphatidylserine. The UV sensitivities of both fractions also depended on their environment. For the UV sensitive fraction, the action spectrum differed in the 300 to 400 nanometers range when the fraction was irradiated with and without lipids. For the resistant fraction, UV sensitivity at 290 nanometers differed (up to 6-fold) in different lipids. The resistant fraction solubilized in octylglucoside had an action spectrum very different from that in cholate or in lipid vesicles. The absorption spectra of the different preparations reflected the action spectra. For both UV sensitive and insensitive fractions, the action spectra for photoinactivation had peaks at 290 nanometers, suggesting that the chromophores were tryptophanyl residues. The loss of ATPase activity was strictly correlated with the loss of fluorescence from tryptophan in the partially purified enzymes. Cs^+ protected the UV sensitive activity but not the insensitive one. We propose a model which explains the difference in UV sensitivities based on the positions of the tryptophan residues in the two proteins.

The inactivation of a Mg^{2+}-requiring, K^+-stimulated ATPase activity associated with the plasma membrane of suspension cultured rose cells by UV light has biphasic kinetics (8). The complex curves can be resolved formally into two single-hit component curves, possibly representing two enzymes or two states of one enzyme with different UV sensitivities. Action spectra have been obtained by plotting the slopes of the single-hit components of the photoinhibition curves at different wavelengths from 240 to 405 nm. All spectra show a peak at 290 nm. These spectra resemble the spectrum for the photodestruction of tryptophan (2), suggesting that these aromatic amino acid residues in the protein may be the chromophores. It has been suggested that integral membrane proteins like the plant plasma membrane ATPase (5) may be sensitive to UV due to the role of hydrophobic amino acids, such as tryptophan, in maintaining the protein correctly oriented in the fluid membrane (17).

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The solubilization of the ATPase with the detergent cholate removes all but 1% of the phospholipids and results in a total loss of ATPase activity (9). The subsequent addition of lipids restores the activity. Fractionation of the cholate preparation on a Sephadex G-150 column results in 80% to 90% of the total ATPase activity being recovered in two distinct, equal peaks. This solubilization procedure allows many questions concerning the photobiology of this system to be addressed. Do the two ATPase activities from the Sephadex column have different UV sensitivities? Is the normal lipid environment responsible for UV sensitivity? Are specific phospholipids necessary for ATPase activity? In this paper we have measured the UV inactivation kinetics of the two fractions of ATPase and tested the effects of the hydrophobic environment on their activities and UV sensitivities.

MATERIALS AND METHODS

Plant materials used and procedures for detergent solubilization and gel filtration, as well as ATPase and protein assays used, were described previously (9).

Polar Lipid Dispersion. PC,[2] PE, and PI from soybean, PG from egg lecithin, and PS from bovine brain, all 99.9% purity, were obtained from Sigma Chemical Company. A mixture of lipids (approximately 50% PC from soybean) was also obtained from Sigma. The lipids were dispersed as described before (9).

UV Irradiation. Radiation at all wavelengths, except 254 nm, was obtained from an Oriel high pressure Hg lamp and a Jobin Yvon monochrometer. Vesicles were irrradiated in a quartz spectrophotometer cuvette at room temperature while being stirred. The temperature in the cuvette was monitored and did not change with irradiation. Measurements have been made both in a dark room and in normal laboratory light with no detectable difference between the two. Irradiation at 254 nm was with two 15-w low pressure Hg vapor lamps. The light output was measured at all wavelengths with a silicon photodiode calibrated with potassium ferrioxalate actinometry (10). Morrowitz (11) correction factors for each wavelength were applied as described by Robb *et al.* (13).

Absorption Spectra. Absorption spectra were obtained using a Varian Techtron, UV-VIS spectrophotometer, model 635.

Intrinsic Fluorescence. Steady state fluorescence was determined in a Perkin-Elmer PMF 44-A fluorescence spectrophotometer. The sample was contained in a 2 × 10 mm fluorescence

[2] Abbreviations: PC, L-α-phosphatidylcholine; BHT, butylated hydroxy toluene; OG, octylglucoside, *n*-octyl-β-D-glucopyranoside; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidyl-DL-glycerol; PI, L-α-phosphatidylinositol; PS, L-α-phosphatidyl-L-serine; UVC, wavelengths shorter than 280 nm.

cuvette, with a 20-nm split width fluorescence excitation beam and 4-nm slit width for the emission beam. Wavelengths for excitation and emission were scanned from 230 to 310 nm between the light source and the sample chamber and from 310 to 410 nm between the sample chamber and the detector. The spectroscopic measurements were carried out either in distilled H_2O or in 1 M NaCl, CsCl, or $CsNO_3$, and the pH was adjusted to 6.5 with HCl.

RESULTS

UV Inactivation of Enzyme Activity. The two cholate-solubilized ATPase activity peaks from the Sephadex G-150 column (9) had simple but different UV sensitivities (Fig. 1). The ATPase activity in fractions 5 to 8 (127 kD protein) was very sensitive to inactivation by UV light (37% inhibition occurred at 270 J m^{-2}). The ATPase activity in fractions 14 to 17 (82 kD protein) was relatively insensitive to inactivation by UV (37% inhibition at 3940 J m^{-2}).

The ATPase activity that was solubilized with 30 nM octyl glucoside was relatively insensitive to inactivation by UV. It had a linear inactivation curve with a slope slightly more negative than that of the cholate-solubilized fractions 14 to 17 (37% inhibition at 3640 J m^{-2}). When the octyl glucoside preparation was incubated in 1% cholate, then applied to the column of Sephadex G-150, the activity was recovered in fractions 14 to 17 (9) and had the same inactivation kinetics as the lighter enzyme initially solubilized with cholate (37% inhibition at 3890 J m^{-2}).

The ATPase activities of both fractions depended on their environment. Both activities were inactive in cholate, relatively inactive in PE, PC, and PI, and active in PG and PS (Table I).

The UV sensitivities of both fractions also depended on their environment. For the UV sensitive fraction, the action spectrum differed in the 320 to 405 nm region when the enzyme was irradiated with and without lipids present (Fig. 2). There was a high shoulder of action in this region that was present only when the enzyme was associated with lipids. However, for all spectra the peak of inactivation remained at 290 nm. At 290 nm, the UV sensitivity of the UV sensitive enzyme was independent of the kind of lipid present (Fig. 3).

For the UV resistant fraction, the sensitivity at 290 nm differed in different lipids (Fig. 3). The ATPase activity was almost 6 times more sensitive to UV inactivation in PS than in PI. The UV sensitivity was not tested in PE and PC because the activity was so low.

The resistant fraction, solubilized in octylglucoside, had an action spectrum very different from that of the same fraction when it was solublized in cholate or incorporated in lipid vesicles (Fig. 4). For the octylglucoside-solubilized ATPase activity, the action spectrum had a maximum in the UVC region. There was a shoulder at 290 nm and much lower activity from 320 to 400 nm. When the octylglucoside-solubilized preparation was incubated in 1% cholate and then applied to and eluted from the Sephadex column, the action spectrum once again had a peak of 290 nm. When the ATPase activity was irradiated without lipids present, the activity from 320 to 400 nm was lower than when it was irradiated in the presence of lipids.

UV Absorption. The absorption spectra of the various ATPase preparations closely resembled the action spectra (Fig. 5). There was a marked difference in absorbance between the cholate-solubilized preparations in the absence and in the presence of added lipid, whether the lipid added was the mixed lipid from the crude soybean preparation or pure PS. The absorption spectra of the vesicle preparation and the cholate-solubilized plus lipid preparation were very similar. The absorption spectrum of the octylglucoside-solubilized preparation, like the action spectrum, was different from the other spectra, with lower absorbance at

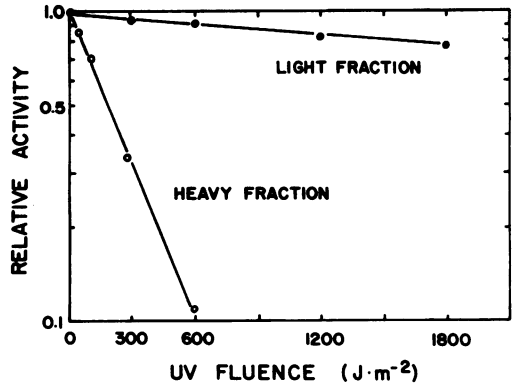

FIG. 1. The UV inactivation of the two cholate-solubilized, fractionated ATPase activities. Samples from G-150 Sephadex were irradiated at 290 nm after they were combined with crude soybean lipids.

Table I. *Acidic Phospholipids Are Essential for Recovery of ATPase Activity after Solubilization with Cholate*

Vesicles were isolated on discontinuous sucrose gradients at the 34/45% interface. The vesicles were incubated at room temperature for 20 min and centrifuged at 100,000*g* for 60 min for the 'cholate' preparation. The cleared cholate solution was layered on a Sephadex G-150 column and eluted by gravity flow at 4°C. Lipids, either as a mixed preparation, (50%) phosphatidylcholine, from soybean or as pure (99.9%) phospholipids were dispersed with a 15-s pulse from the sonicator in a solution containing 2 mM histidine, 0.1 mM EDTA, 2 mM Hepes (pH 6.7) to give a final phosphorus concentration of 10 μM. All phospholipid preparations contained 50 μg BHT/ml. The lipid solution was added to the fractions from the column to give 10 μmol lipid phosphorus/μg protein. The specific activity of the vesicle preparation was 26.2 μmol Pi/mg·h assayed with 50 mM KCl.

Preparation	ATPase Specific Activity	
	Fractions 5–8	Fractions 14–17
	% stimulation	
Vesicle	100	100
Cholate	3.8	3.8
Cholate + mixed lipid	211.9	207.4
Cholate + PE	4.1	7.4
Cholate + PC	8.8	11.4
Cholate + PI	5.7	16.3
Cholate + PG	152.5	173.0
Cholate + PS	224.0	233.6

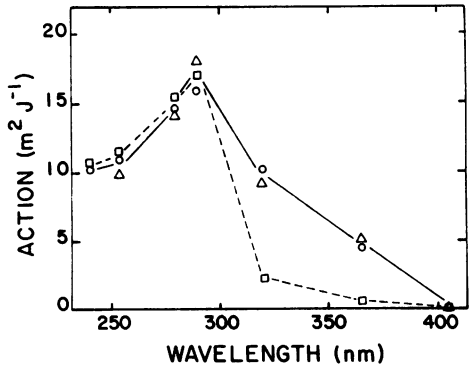

FIG. 2. Action spectra for the inactivation of the UV sensitive ATPase irradiated in native lipid vesicles (○), solubilized in the detergent cholate (□), and solubilized in cholate with PS added (△). The spectrum for native vesicles was calculated by analysis of biphasic inactivation curves (8). The activity of enzyme irradiated in cholate was measured after adding PS.

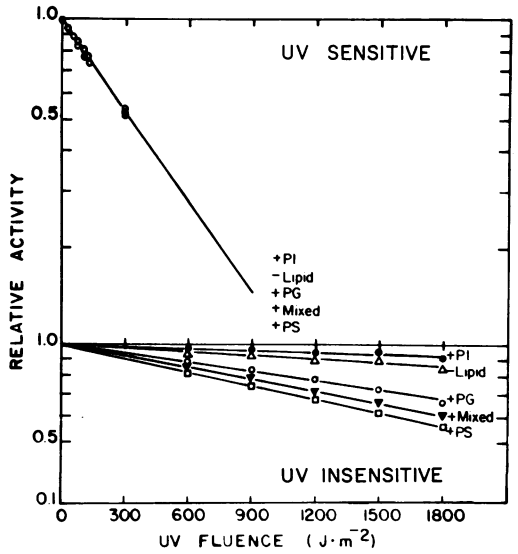

FIG. 3. The effect of lipids on UV sensitivity of the two ATPase activities. Enzymes were irradiated at 290 nm after the lipids were added. Top panel, UV sensitive ATPase; bottom panel, UV insensitive ATPase.

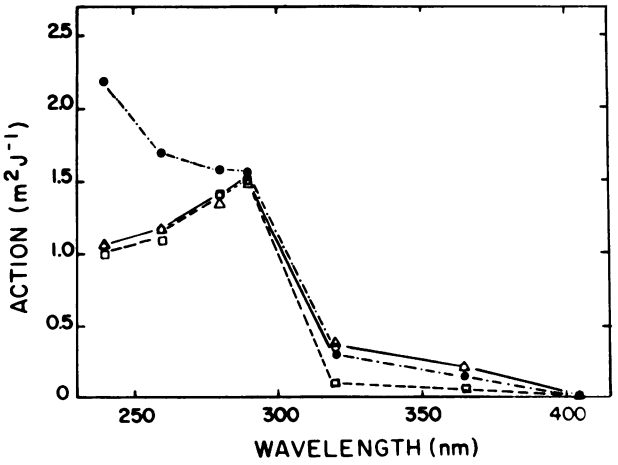

FIG. 4. Action spectra for the inactivation of the UV insensitive ATPase irradiated in native lipid vesicles (○), solubilized in cholate and fractionated in G-150 (□), solubilized in cholate, fractionated, and reincorporated into mixed lipids (△), and solubilized in octylglucoside (●).

wavelengths above 270 nm but a higher absorbance below 270 nm. The PS, the crude lipid preparation from soybean, and sodium cholate did not absorb over the wavelengths tested. The octylglucoside (30 mM) did absorb below 320 nm.

Fluorescence. Peak fluorescence of the ATPase was obtained with an excitation wavelength of 290 nm. The fluorescence spectra of the ATPase preparations were relatively featureless (Fig. 6), as they are in many other proteins (1). The band maximum at 330 nm indicated that the fluorescence was dominated by tryptophans. In confirmation, both Cs^+ and NO^-_3, effective quenchers of tryptophan fluorescence (3), reduced the fluorescence of ATPase relative to that in NaCl. The fluorescence of the UV sensitive enzyme was more sensitive to quenching by Cs^+ than was the fluorescence of the insensitive enzyme.

The effect of inactivating fluences of UV on the relative fluorescence of the two fractions of ATPase was measured. Each fraction of ATPase was irradiated with UV (290 nm) to yield a remaining activity of 10% to 100% for the sensitive enzyme and 80% to 100% for the insensitive enzyme. In both cases there was a linear relationship between loss of ATPase activity and loss of

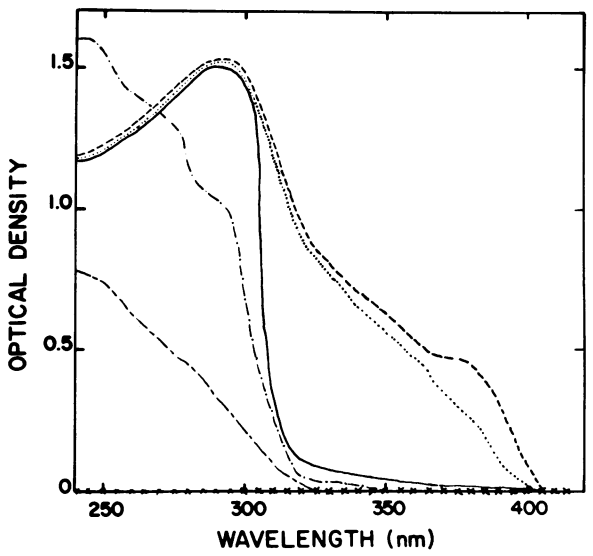

FIG. 5. Absorption spectra for detergents, phospholipids, and ATPase preparations. Cholate (xxx); octylglucoside (—); phosphatidylcholine from the crude soybean preparation (xxx); phosphatidylserine (xxx); ATPase in vesicles (—); ATPase solubilized in 1% cholate and centrifuged for 1 h at 100,000*g* ——); ATPase solubilized in cholate and centrifuged with lipids added back (···); ATPase solubilized in 30 mM octylglucoside (-·-). All preparations were in 1 mM Tris-Mes, pH 6.5, and protein concentrations standardized for comparison.

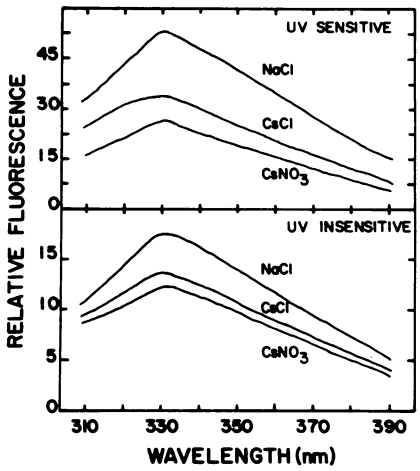

FIG. 6. Fluorescence of the UV sensitive (upper) and UV insensitive (lower) ATPase preparations determined in 1 M NaCl, 1 M CsCl, or 1 M $CsNO_3$, pH 6.5; excitation wavelength 290 nm. Each sample contained 0.02 mg of protein in 2 ml.

tryptophan fluorescence (Fig. 7). However, the degree of relationship differed. For the sensitive enzyme, a loss of 50% fluorescence corresponded to a loss of 55% of activity. For the insensitive enzyme, a loss of 50% fluorescence corresponded to a loss of 10% activity.

The effect of UV on tyrosine fluorescence contrasted to that on tryptophan fluorescence. The emission spectra obtained when the enzyme was excited at 280, 292, and 297 nm were recorded to yield the same fluorescence at 375 nm. This procedure normalizes the spectra to give the same tryptophanyl fluorescence (15). Under these conditions, the difference between the emission spectrum obtained at 280 nm excitation and that obtained at 292 nm excitation represents tyrosyl fluorescence, which has a peak at 303 nm (Fig. 8). There was no difference in the tyrosyl fluorescence of the UV sensitive enzyme after an irradiation at

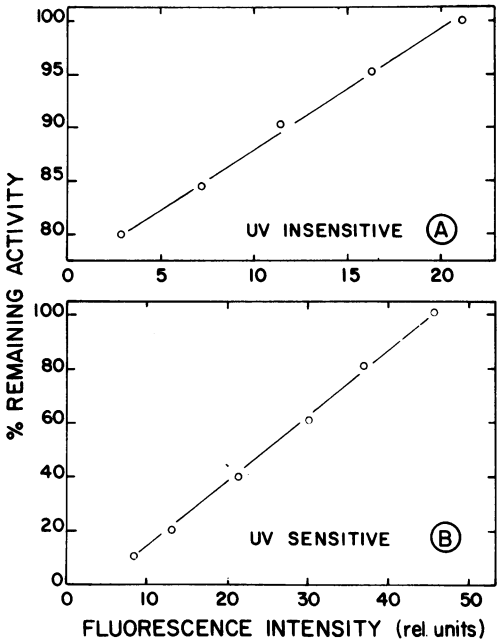

FIG. 7. The relative fluorescence of the UV insensitive (A) and UV sensitive (B) ATPase activities measured after partial inactivation by UV irradiation. Excitation wavelength was 290 nm; emission wavelength was 330 nm.

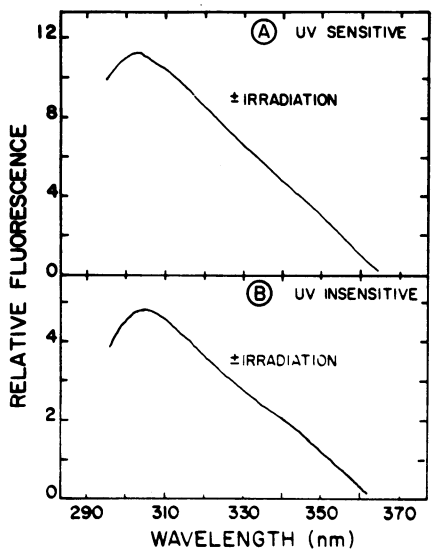

FIG. 8. The difference between ATPase fluorescence excited at 280 and 292 nm. A, UV sensitive ATPase before and after UV (290 nm) irradiation resulting in a 90% loss of activity. B, UV insensitive ATPase before and after UV irradiation resulted in a 20% loss of activity.

290 nm that inactivated 90% of the ATPase activity. After irradiation of the insensitive enzyme that resulted in a 20% loss of activity, there was also no change in the tyrosyl fluorescence.

We measured the relative fluorescence of the two ATPase activities in different lipids (Table II). The fluorescence of the UV sensitive enzyme was independent of the type of lipid present. The fluorescence of the insensitive enzyme was dependent on the hydrophobic environment. As with UV inactivation, there was the most fluorescence in the presence of PG and PS, and less fluorescence in PI.

The effect of tryptophan fluorescence quenchers (CsCl and $CsNO_3$) on the inactivation kinetics was tested by irradiating enzymes in the presence of 1 M NaCl, 1 M CsCl, and 1 M $CsNO_3$.

Table II. *The Effect of the Hydrophobic Environment on Tryptophan Fluorescence of the Two ATPase Fractions*

The enzyme preparations were as described in Table I. Relative fluorescence was measured using a Perkin-Elmer PMF 44-A fluorescence spectrophotometer with an excitation wavelength of 290 nm and emission at 330 nm. Neither the cholate nor any of the lipids used absorbed or fluoresced at these wavelengths.

Lipid	Relative Fluorescence of ATPase	
	Fractions 5–8	Fractions 14–17
Cholate + PI	45.0	8.0
Cholate	46.0	10.7
Cholate + PG	48.0	13.7
Cholate + mixed lipid	44.0	14.0
Cholate + PS	46.0	20.7

CsCl and $CsNO_3$ reduced the UV sensitivity of the sensitive enzyme by factors of 1.2 and 1.6, respectively. The fluences giving 37% survival were 200 J m^{-2} in 1 M NaCl, 350 J m^{-2} in 1 M CsCl, and 453 J m^{-2} in 1 M $CsNO_3$. The presence of quenchers had no effect on the slope of inactivation of the insensitive enzyme (data not shown).

DISCUSSION

The two forms of ATPase from the plasma membrane of suspension cultured cells of rose differ in mol wt (estimated as 82 and 127 kD by comparison with BSA on Sephadex G-150; [12]) and UV sensitivity. Since the ATPase activity fractionated into two peaks with simple, but different sensitivities, we conclude that the biphasic inactivation curves of the vesicle preparations (8) represent these two different enzyme fractions.

Since the partially purified ATPase activities retained their sensitivity to UV, we suggest that the proteins themselves are the photoreceptors. The fact that neither the crude lipid preparation nor the purified phospholipid absorbed in this region further supports this. However, we cannot exclude the possibility that proteins that copurify with ATPase may play some part in the inactivation process. When the ATPase activities were solubilized with cholate, the action spectra for the two fractions were very similar to the absorption spectrum of tryptophan (2), with peaks at 290 nm (Ref. 8, Figs. 2 and 4), suggesting that tryptophan may be the sensitive component of the enzyme (12). Evidence obtained with substances that quench tryptophan fluorescence, Cs^+ and NO^-_3, supports this contention. Our data do not exclude the possibility that absorption by tyrosine and other amino acids contribute to inactivation. However, it would be unusual if these contributions were large (7).

The increased action between 320 and 400 nm associated with the presence of lipids, but absent in cholate, could be a novel effect of the environment on tryptophan absorption. While solute-solvent interactions have been shown to influence the excitation wavelength dependence of the fluorescence of indoles, the previously observed red shift of emission has been associated with increasing polarity of the solvent (16). At this point, we cannot rule out the possibility that there is a sensitizer with a peak of absorption in the 300 to 400 nm range, which absorbs light in the presence of lipid but not in cholate. Natural pigments, such as hypericins and psoralens, have been shown to have absorption and action spectra in this region, to be lipid soluble, and to act as photosensitizers of cell membranes (6).

The ATPase activity from both fractions depends on the environment. The cholate treatment removes phospholipids, and ATPase activity was barely detectable in cholate solution. Neutral lipids such as PE and PC did not restore ATPase activity.

However, the acidic phospholipids PG and PS effectively reversed the sodium cholate inhibition of the ATPase activity. These findings are consistent with the interpretation that cholate extracts acidic phospholipids from the membranes, leading to an inactivation of the enzyme activity. From these results it is reasonable to speculate that the ATPase *in situ* is associated with acidic phospholipids and that these lipids are somehow necessary for catalytic activity. Similar acidic phospholipids requirements have been reported for the Na^+,K^+-stimulated Mg-ATPases from a variety of animal cells (4) and the *Neurospora* plasma membrane ATPase (14).

The UV sensitivity of both fractions also depends on the environment. The presence or absence of lipids and the kind of lipid present during irradiation affected the UV sensitivity of the insensitive component. That ATPase was almost 6 times more sensitive to inactivation by 290 nm UV in the presence of PS than it was in PI. The sensitivity of the UV sensitive enzyme to 290 nm UV was independent of the type of lipid present. For both cholate-solubilized enzymes, the presence or absence of lipids affected the action in the 300 to 400 nm range. An increase in the presence of lipids was also seen in the absorption spectra, which closely resembled the action spectra, indicating that the effect of the lipids is on absorption, rather than on the subsequent reactions leading to the inactivation.

Data obtained with octylglucoside support the idea that both ATPase activity and UV sensitivity are affected by the hydrophobic environment. The octylglucoside must have denatured or irreparably inactivated the UV sensitive component, since this component could not be recovered at any point after the detergent was introduced. Dupont and Leonard (5), working with corn root plasma membrane ATPase, reported a similar rapid decay of enzyme activity when it was solubilized with octylglucoside. That UV sensitivity of the insensitive component is affected was shown by the difference in inactivation kinetics and in action spectra when the ATPase was solubilized in octylglucoside and cholate. The spectra diverged sharply in the 240 to 280 nm range, with increased action in the octylglucoside-solubilized ATPase. Here again the absorption spectra for the octylglucoside-solubilized ATPase activity closely resembled the action spectra. This increase in UV sensitivity and action may, however, be related to the absorbance of octylglucoside. While cholate does not absorb in the range tested (240–410 nm), octylgluocoside, or some impurity in the octylglucoside, does absorb UV light. We would suggest some form of energy transfer from the chromophore in the detergent to the protein, resulting in increased action.

The finding that quenchers of tryptophan fluorescence (Cs^+ and NO^-_3) protected the ATPase from inactivation provides further evidence that environment influences UV sensitivity. However, this protection occurred only with the UV sensitive fraction. Cs^+ quenching of tryptophan fluorescence is collisional and so is most effective when the amino acid is located at a site accessible to water. The difference between the two fractions in both the amount of quenching and the amount of protection by Cs^+ suggests that their tryptophans are in different positions. The different effects of lipids on UV sensitivity and relative fluorescence of the two fractions further supports this.

The biological activity of an enzyme is a unique property of certain key amino acid residues and the overall macromolecular conformation as determined by intimate relationship between structure, conformation, and function. Localized alterations at sites not considered as essential can induce changes in the three-dimensional structure leading either to a loss of catalytic ability or a damaged, active enzyme (7).

We propose the following model. Inactivation of both ATPases results from the photodestruction of tryptophan residue(s). In the lighter, relatively insensitive protein, the tryptophanyl residues are associated with the lipids, while in the UV sensitive enzyme the tryptophanyl residues are in another hydrophobic area of the molecule that is more accessible to the solvent. In the insensitive enzyme, the destruction of tryptophan residue(s) could result in partial inactivation by changing the protein/lipid interaction and shifting the protein in the fluid membrane. In the sensitive component, inactivation could be a result of the destruction of tryptophan residue(s) associated with the active site or located adjacent to a key catalytic residue. Since activity would respond differently to the destruction of tryptophans, this model implies that the two ATPase forms represent different enzymes.

An alternative model would suggest that the inactivation of the enzyme does not result from the destruction of tryptophan. Light absorbed by tryptophan would destroy the active site as well as the tryptophans themselves through an indirect photosensitization process. In the UV sensitive fraction, the rates of destruction of tryptophans and active sites would be equal; in the UV insensitive fraction, they would not. This model is consistent with the idea that a common catalytic subunit may be bound to another polypeptide that confers UV sensitivity.

The biphasic inactivation kinetics of the vesicle preparation have been shown to represent two distinct ATPase activities separable not only by UV sensitivity but also by mol wt. That the action spectra for the partially purified ATPase activities are very similar to the spectra for the vesicle preparations suggests that the proteins themselves contain the UV chromophores. Both ATPase activity and UV sensitivity are affected by their environment. There is strong evidence in the action spectra and from the fluorescence data that tryptophan is the sensitive component in the enzyme. Further, we suggest that the difference in UV sensitivity of the two enzymes is a result of different locations of the tryptophanyl residues within the two proteins.

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