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Membrane damage caused by irradiation of fluorescent concanavalin A

(erythrocyte membranes/fluorescein/membrane protein diffusion)

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ABSTRACT **Visible light irradiation of fluoresceinated concanavalin A (f-Con A) bound to the outside of resealed erythrocyte membranes caused crosslinking of as much as 50% of the membrane proteins. Crosslinking was absent in controls in which equivalent amounts of f-Con A were added to the membranes but prevented from binding by the presence of 10 mM α-methylmannoside. The photodamage was not accompanied by a change in the membrane permeability barrier or membrane shape. Although fluorescein bleaching accompanies the formation of protein aggregates, the amount of aggregated protein is not simply a function of the number of fluoresceins bleached. The percentage of aggregated protein decreases when the same dose of light is given in a shorter time. Although certain antioxidants and free-radical scavengers had no detected effect on the crosslinking, reducing agents such as cysteamine and reduced glutathione either blocked or reversed the protein crosslinking. The mechanism of photoinduced oxidation and the implications of these results for fluorescence studies of cell membranes are discussed.**

In recent years, new fluorescence techniques have been developed for the study of molecular mobility in microscopically small volumes. Fluorescence correlation spectroscopy (1–3) and fluorescence photobleaching recovery experiments (3–6) have used the fluorescence excited by a focused laser beam to characterize dynamics in model membranes and the plasma membranes of single living cells. Other fluorescence techniques, such as nonradiative energy transfer and emission anisotropy measurements originally applied to the study of molecules in bulk solution, are also being extended to the subcellular level. Such *in situ* measurements at high spatial resolution can yield exciting new information, but they must be performed and interpreted with caution. The experiments reported here were designed to investigate the extent to which the relatively high incident light intensities needed in these experiments to extract adequate signals from microscopically small volumes may open the way to significant dye-sensitized photochemical damage.

As a model system for quantitative analysis, we have chosen resealed erythrocyte membranes labeled with fluoresceinated concanavalin A (f-Con A). Our principal assay for photochemical damage is the examination of changes in the banding patterns of membrane proteins in sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels. This approach has been used to examine membranes treated with photosensitizing reagents (7–10). We have found that, in this system, intense laser irradiation of dye located at the outer surface of the membrane in the presence of molecular oxygen can lead to extensive oxidative crosslinking of membrane proteins (11). The magnitude of the effect, however, varies with irradiation time, intensity, and dye concentration in ways that have unexpected practical implications.

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EXPERIMENTAL

Preparation of Resealed Erythrocyte Membranes. Tris ghosts were prepared from washed erythrocytes in 140 mM NaCl/20 mM Tris·HCl, pH 7.4, by hypotonic lysis (1:40 dilution of cells in 10 mM Tris·HCl, pH 7.4) followed by centrifugation at 25,000 × *g* for 10 min. The pellet was resuspended in 10 mM Tris and the process was repeated twice more to yield white erythrocyte membranes. Tris ghosts were resealed by incubation at 37°C for 20 min in 146 mM KCl/20 mM Hepes/1 mM Mg^{2+}/1 mM ATP at pH 7.0. After incubation, the membranes were layered on a 10% sucrose solution in 145 mM NaCl/5 mM phosphate, pH 7.4 (P_i/NaCl) and centrifuged at 14,000 × *g* for 20 min. Sealed membranes that floated on the sucrose layer were aspirated, diluted 1:10 with P_i/NaCl, pelleted at 25,000 × *g* for 10 min, and resuspended with an equal volume of P_i/NaCl.

In some experiments, [^{14}C]inulin was added to the resealing solution. Nonpelletable counts were used later as a measure of membrane leakage.

f-Con A Labeling of Resealed Membranes. Aliquots of resealed membrane suspensions, prepared by the above procedure, were incubated with 50–500 μg of f-Con A (fluorescein isothiocyanate-treated concanavalin A from Miles Yeda) per ml. After incubation for 20 min on ice, the samples were diluted with 5 vol of P_i/NaCl and centrifuged at 25,000 × *g* for 10 min. The pellet was then resuspended with 2 vol of P_i/NaCl in preparation for laser irradiation.

The concentration of bound f-Con A was determined by solubilizing labeled membranes with 1% $NaDodSO_4$ and measuring the fluorescence relative to similarly treated solutions of known concentrations of f-Con A.

Laser Irradiation. For relatively long time (15 sec to 100 min), low-intensity exposures, samples of membrane suspensions (≈25 μl) were drawn into 1.4-mm (inside diameter) capillaries (fluid length = 1.4 cm) and aligned coaxially with an unfocused incident laser beam (λ = 488 nm, Argon/Krypton ion laser, Control Laser Corp., Orlando, FL). A microscope coverslip was placed perpendicular to the optical axis, in contact with the column of fluid, to serve as a planar optical entrance window. Total laser powers of 7.0–700 mW were used in a mixed mode operation, with roughly half the power in each case falling within the bore of the capillary tube. Fluorescence intensity during irradiation was collected from the back 50% of the sample and monitored with a silicon photodiode shielded by a colored-glass barrier filter to block the scattered laser light.

For higher power densities, the incident laser beam was focused to a 300-μm-diameter spot by the ×10 objective of a fluorescence microscope vertical illuminator. The cell sus-

Abbreviations: f-Con A, fluoresceinated concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate; P_i/NaCl, 5 mM phosphate, pH 7.4/145 mM NaCl.

pension, in a 300-μm (inside diameter) capillary mounted on a translational stage perpendicular to the incident beam, was translated through the beam at controlled velocities (30–300 μm/sec).

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to Fairbanks *et al.* (12) with 3.35% acrylamide gels. Samples were solubilized with 1% $NaDodSO_4$ either at 37°C for 30 min or in boiling water with 5% 2-mercaptoethanol for 2 min.

RESULTS

Protein Aggregation. When resealed erythrocyte membranes (0.7–1.0 mg of protein per ml) labeled with f-Con A were irradiated with laser light, subsequent analysis of membrane proteins by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the appearance of protein aggregates with apparent molecular weights of over 3×10^5 (see Figs. 1 and 2). Irradiated controls of unlabeled membranes or membranes labeled with unlabeled Con A had less than 10% aggregation. Sample heating does not appear to be an important factor in the crosslinking. Irradiation of samples in ice resulted in the normal amount of crosslinking and incubation at 50°C for 5 min resulted in no crosslinking. Examination of Coomassie blue staining patterns of the $NaDodSO_4$ gels of irradiated samples showed that the primary crosslinked protein was spectrin. Under conditions in which about 50% of the total protein appeared as high molecular weight aggregates, 75% of the spectrin but only 30% of band 3 was lost. Qualitatively similar effects were obtained when tetramethylrhodamine isothiocyanate-labeled Con A was used and when fluorescein was attached directly to the membrane by the reaction of intact cells with fluorescein isothiocyanate or fluorescein mercuric acetate. Laser irradiation of labeled membranes produced no detectable change in membrane shape.

Localization of the Photosensitizer. A number of control experiments were performed to demonstrate that the dye molecules mediating the formation of protein aggregates were indeed bound to lectin molecules specifically attached at the outer surface of resealed membranes. Membranes labeled with f-Con A were washed after incubations with and without the addition of 10 mM α-methylmannoside. The fluorescence excited by laser irradiation of the sample incubated with α-methylmannoside was less than 20% of the control level.

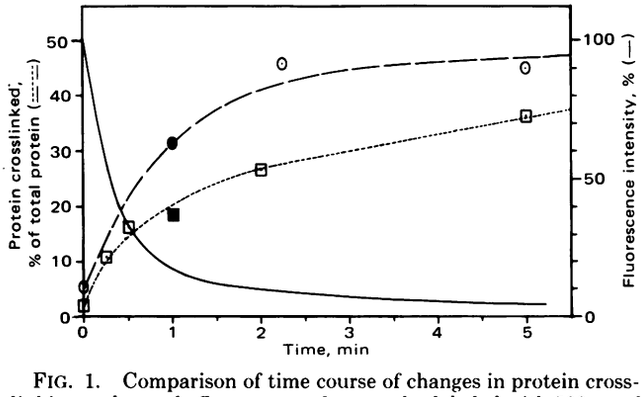

FIG. 1. Comparison of time course of changes in protein crosslinking and sample fluorescence for samples labeled with 500 μg of f-Con A per ml and irradiated with 15 W/cm^2 at 488 nm. The percentage protein crosslinked is calculated as the percent of Coomassie blue staining greater than spectrin in apparent molecular weight either with (⊡) or without (⊙) 5% 2-mercaptoethanol present during the solubilization of membrane proteins by $NaDodSO_4$. See *Discussion* for explanation of solid symbols.

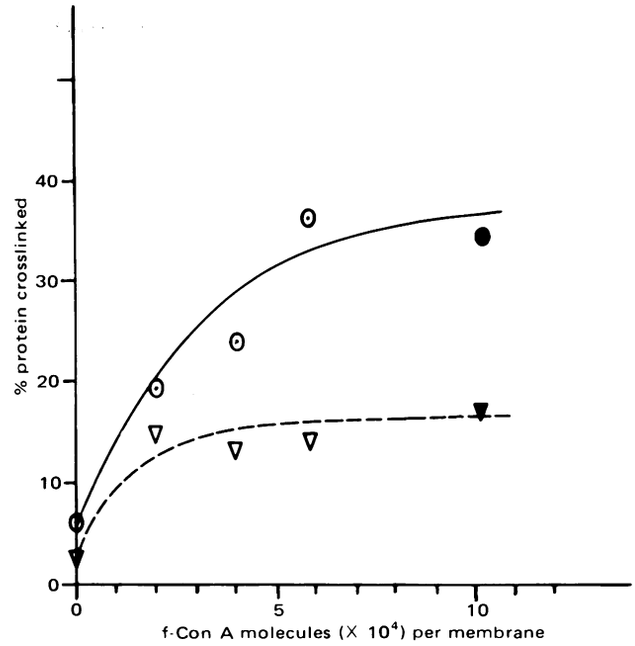

FIG. 2. Plot of percentage of protein crosslinked after 1-min irradiations of 15 W/cm^2 at 488 nm versus amount of fluorescent Con A bound to membranes. Membrane concentration was estimated from the protein concentration. Protein crosslinking was determined with (▽) and without (⊙) 5% 2-mercaptoethanol present during the solubilization of the membrane proteins by $NaDodSO_4$. There are 2 fluoresceins per Con A in this preparation. The percentage of dye photobleached was unaffected by Con A concentration. See *Discussion* for explanation of solid symbols.

Protein crosslinking was correspondingly diminished. Crosslinking was totally absent in controls in which equivalent amounts of f-Con A were added to the membrane suspension but prevented from binding by the presence of 10 mM α-methylmannoside. It was shown, finally, that the membrane barrier was not broken even during the laser irradiation of labeled membranes. Less than 5% of [^{14}C]inulin resealed within the membranes escaped from the cells during the 5-min irradiation at 15 W/cm^2.

Time, Concentration, and Dose Dependence. The time course of the crosslinking (Fig. 1) is consistent with the view that intact fluorescein, and not its photobleached product, acts as the photosensitizing agent. The extent of crosslinking, however, does not appear to be limited by the availability of intact dye. Reducing the level of f-Con A labeling below 1×10^5 molecules per membrane does not cause corresponding decreases in the extent of crosslinking generated during 1-min irradiations (see Fig. 2).

The data of Figs. 1 and 2 were taken at an average laser intensity of $\approx$15 W/cm^2. We found, however, that changing the laser intensity at which a given irradiation dose is administered changes the extent of crosslinking. The three groups of data designated 1X, 2X, and 4X in Fig. 3 correspond to three different total irradiation doses (225, 450, and 900 J/cm^2, respectively). Each dose was applied at three different power densities (15, 1.5, and 0.15 W/cm^2) with irradiation times varied inversely in the appropriate way. It is seen in every case that the photodamage *decreases* when a given dose is applied in a shorter time with a higher intensity. Concurrently, the amount of bleaching decreases, but to a much smaller extent. Experiments performed by using the microscope scanning system described above show that the same trend exists at power densities between 45 and 450 W/cm^2.

Inhibitors of Photodamage. The addition of 5% 2-mercaptoethanol disaggregated a significant fraction of the crosslinked material (Figs. 1 and 2), which indicated that oxidative crosslinks were being formed. Several compounds known to act as free radical scavengers or singlet oxygen quenchers were tested as possible inhibitors of protein crosslinking. Histidine (100 mM), α-tocopherol (0.8 μl in 20 μl of ethanol solution per ml of cell suspension), and butylated hydroxytoluene (5 mM) had little or no effect. Cysteamine (50 mM) and reduced glutathione (2.0–20 mM), however, eliminated practically all evidence of photo-induced protein crosslinking (see Fig. 4). These compounds are also reducing agents and may have acted, to some extent, after the irradiation to reverse oxidative crosslinks. In experiments in which excess iodoacetimide was added with 1% $NaDodSO_4$ immediately after the irradiation to react with free glutathione, however, the inhibition of crosslinking by glutathione was not affected. Finally, as expected, reducing the concentration of O_2 in the sample prior to laser irradiation decreased the extent of protein crosslinking (Fig. 4).

DISCUSSION

Mechanisms. These studies have shown that extensive crosslinking of erythrocyte membrane proteins can occur during visual light excitation of fluorescein localized at the membrane surface. The effect requires molecular oxygen and is inhibited or reversed to a considerable extent by reducing agents. All evidence thus points to the operation of a Type II photosensitized oxidation (13, 14), a process characterized by the initial interaction between oxygen and the electronically excited dye triplet state (reached by intersystem crossing from the excited singlet state, the initial product of light absorption). The most probable of the Type II interactions leads to the deexcitation of the dye molecule back to the ground state, with the transfer of the excitation producing an electronically excited singlet state of oxygen. A single dye molecule could go through many such cycles, catalytically producing one molecule of singlet oxygen in each cycle. Reactions initiated by the singlet oxygen, in this scenario, would lead to the subsequent photodamage. Many photosensitizers would thus be expected to induce qualitatively identical effects (15). The chemical nature of membrane photodamage sensitized by protoporphyrin has been investigated by Dubbelman *et al.* (8).

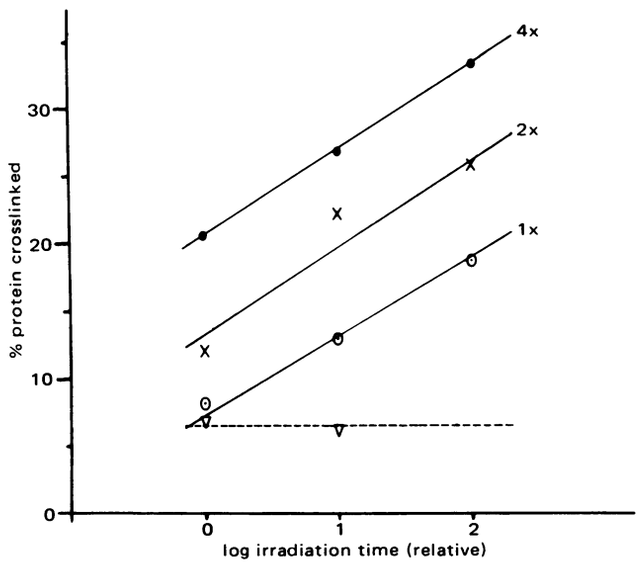

FIG. 3. Percentage of crosslinked protein determined for three different doses of irradiation, 225, 450, and 900 J/cm^2, designated here as 1X(○), 2X(x), and 4X(●), respectively. Each dose was delivered over three different irradiation periods: 0.25, 2.5, and 25 min for 1X; 0.5, 5, and 50 min for 2X; and 1, 10, and 100 min for 4X, with the intensities varied inversely in the appropriate way. The control samples (▽) were irradiated for 1 and 10 min with total doses of 900 J/cm^2.

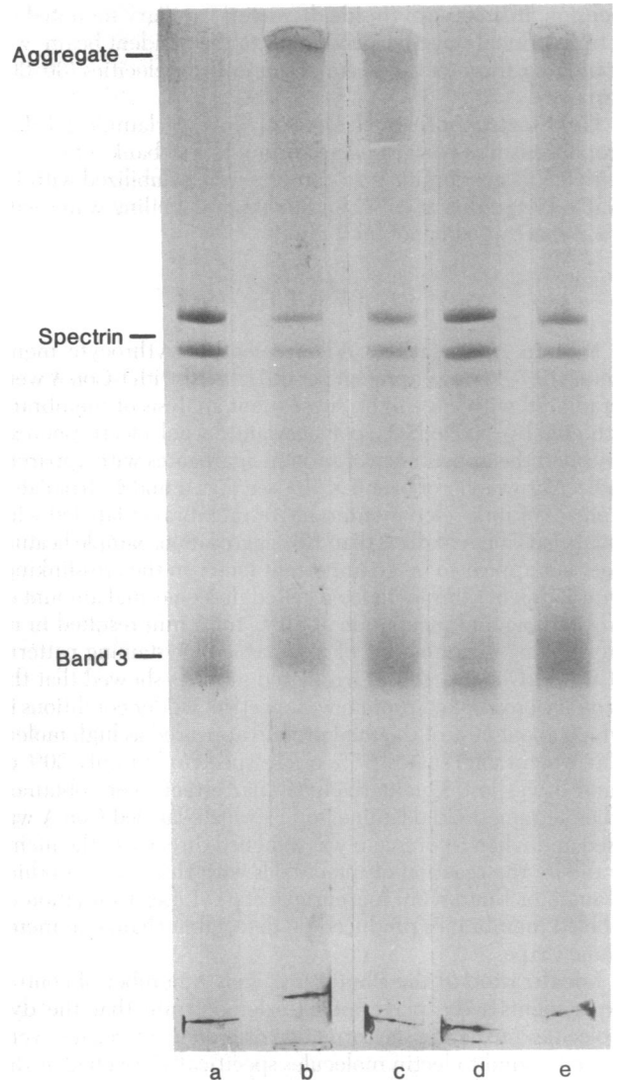

FIG. 4. $NaDodSO_4$/3.25% polyacrylamide gels of erythrocyte membranes treated with 500 μg of f-Con A per ml. Membranes were (a) not irradiated or irradiated for 5 min in the presence of (b) nothing, (c) 2 mM reduced glutathione, (d) 50 mM cysteamine, or (e) after purging the sample for 10 min with N_2. The percentage of fluorescein bleached was (a) 0%, (b) 93%, (c) 92%, (d) 78%, and (e) 89%.

Dye "bleaching," a process utilized in the fluorescence photobleaching recovery technique to produce spatial concentration gradients, most likely occurs by a competing Type II interaction in which the dye triplet state is irreversibly oxidized by the oxygen. For fluorescein, it has been estimated that between 10 (at pH 12) and 100 (at pH 2) singlet oxygen molecules will be formed on average before the dye is oxidized (16).

One might expect that the magnitude of the observed effect, the membrane protein crosslinking, would be a function of the dye concentration and the irradiation time and intensity in a combination that simply reflects the total number of singlet oxygen molecules produced per cell. This, however, appears not to be the case. Fig. 3 shows directly the variation with exposure time for given total irradiation doses. Careful examination of Figs. 1 and 2 reveals a similar effect. The solid data

points, two in each figure, represent the same experiment—a 1-min exposure of a sample labeled with 1×10^5 f-ConA molecules per membrane. Notice that reducing the number of singlet oxygen molecules by a factor of ≈5 produces practically no effect if it is accomplished by reducing the dye concentration (Fig. 2), but leads to a marked decrease in crosslinking if it is done by reducing the exposure time (Fig. 1). Again, a given number of singlet oxygen molecules appear to have a smaller observable effect if they are generated in a shorter time.

This dependence upon exposure time suggests the presence of some intermediate rate-limiting step in protein crosslinking. Perhaps it is related to the permeability barrier presented by the membrane itself; remember that although f-ConA is restricted to the external surface, spectrin, a protein limited to the cytoplasmic aspect of the membrane, is affected preferentially. The inability of soluble singlet oxygen quenchers to prevent crosslinking may be related to the fact that primarily membrane, not solvent, separated fluorescein and spectrin (see ref. 17 for similar observations).

Implications. Membrane protein crosslinking of the type that we have observed could seriously affect the results of fluorescence experiments on single cells. In preliminary experiments (unpublished results) we have observed extensive changes in erythrocyte lipid fatty acid composition as well.

Fluorescence photobleaching recovery experiments which by design require relatively high irradiation doses seem especially susceptible to these effects. If the trend demonstrated in Fig. 3 continues to the much higher intensities and shorter exposure times that are customarily used, the protein crosslinking may be minimal. We could not assess this directly here, because it was not possible to irradiate the large amounts of material needed for conventional gel electrophoresis without going to a considerably expanded beam geometry. Membrane lipid damage, however, may not exhibit a similar time and intensity dependence.

Considerable indirect evidence suggests the absence of damage during photobleaching experiments (18). It must now be recognized, however, that some of the suggested criteria—the maintenance of the integrity of the permeability barrier and the lack of gross morphological changes—do not rule out photodamage of the type reported here.

We have found that cysteamine and reduced glutathione (at physiological levels) act to prevent or reverse protein crosslinking. Similar results have been reported for hydroxylamine (8) and sodium azide (10). Additional work needs to be done to evaluate the effects of these and other reagents, as well as the relative merits of different fluorescent probes. It is thereby to be hoped that photochemical damage can always be kept at acceptable levels.

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