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Chromophore-assisted laser inactivation of proteins is mediated by the photogeneration of free radicals

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ABSTRACT **Chromophore-assisted laser inactivation (CALI) is a technique that selectively inactivates proteins of interest to elucidate their *in vivo* functions. This method has application to a wide array of biological questions and an understanding of its mechanism is required for its judicious application. We report here that CALI is not mediated by photoinduced thermal denaturation but by photogenerated free radicals. Thermal diffusion calculations suggest that the temperature changes resulting from CALI are too small to cause thermal denaturation, and Arrhenius plots of CALI are inconsistent with a photothermal mechanism. CALI shows an energy dose reciprocity above a threshold and can be inhibited by free-radical quenchers, thus demonstrating a photochemical mechanism of protein inactivation. The type of quenchers that are effective in inhibiting CALI indicates that the active species is a hydrogen abstractor which is not derived from molecular oxygen. We suggest that the active free-radical species is the hydroxyl radical and its very short lifetime explains the spatial specificity of CALI such that half-maximal damage is effected within 15 Å from the dye moiety and no significant damage occurs at 34 Å. The data are consistent with free-radical formation resulting from a sequential two-photon process.**

Chromophore-assisted laser inactivation (CALI) of protein function (1) targets laser energy to damage single protein species by binding the protein with a specific antibody that has been labeled with the chromophore malachite green. The bound dye is excited by 620-nm light, a wavelength not significantly absorbed by most cellular components, and damage is localized to within 60 Å of the chromophore (2). CALI has been used to show the *in vivo* roles of several proteins in insect neural development (3–5).

Further characterization and understanding of CALI are required for its optimization and judicious application in biological research. A dependence of CALI on the distance from the dye, the laser power, the number of pulses, and the concentration of dye-labeled ligand has been shown (1, 2). Although thermal denaturation was postulated as the molecular mechanism of CALI, the precise nature of the laser-mediated damage to the protein function has not been established. This study was undertaken to ascertain the mechanism of protein damage by CALI.

The likely sources of photoinduced damage through the absorption of light energy by a chromophore at the radiant flux used for CALI are photothermal and photochemical (6). Photothermal damage would occur through highly localized denaturation of the antibody–antigen complex caused by the rapid laser excitation and the relaxation of the bound dye (5 ps) through vibrational modes to generate heat. For CALI to be based on a photothermal mechanism, the temperature increase at the site of the protein of interest after laser treatment must be sufficiently high to result in thermal denaturation. Photochemical damage would occur through the generation of free radicals by the relaxation of the excited chromophore. If the mechanism of CALI was photochemical, its effectiveness would depend on the total energy delivered and not on the peak power of the laser (7) and it should be possible to inhibit CALI by using free-radical quenchers.

We report here that theoretical and experimental evidence do not support a photothermal model for CALI. Instead, our data demonstrate that CALI is mediated by photoinduced free radicals. We suggest that the mediating free radical is the hydroxyl radical and that its high reactivity and very short lifetime may explain the spatial specificity of CALI.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma unless otherwise specified. Anti-β-galactosidase was obtained from Cappel. Malachite green isothiocyanate was purchased from Molecular Probes, whereas free malachite green (oxalate salt) and cycloocta-1,3,5,7-tetraene (COT) were purchased from Fisher Scientific.

Malachite Green Labeling of Proteins. Anti-β-galactosidase was labeled with malachite green isothiocyanate to a ratio of 6 dye molecules per antibody molecule by the method of Jay (1). The ratio of labeling was determined by measuring the optical density at 620 nm (molar absorptivity = 150,000 $M^{-1}\cdot cm^{-1}$) for a sample of known protein concentration. Free malachite green was intercalated into erythrocyte ghost membranes as described (2).

CALI. CALI was performed as described (1). Samples were incubated for at least 1 hr, aliquoted into 96-prong plates (Nunc, Intermed), and subjected to 620-nm laser light under various conditions. A Quanta Ray DCR 3 Nd–yttrium/aluminum garnet (YAG) laser was used to drive a Quanta Ray PDL 2 dye laser containing the fluorescent laser dye DCM, (2-{2-[4-(dimethylamino)phenyl]ethenyl}-6-methyl-4*H*-pyran-4-ylidene)propanedinitrite, to generate a 620-nm pulsed laser beam with a pulse width of 8.5 ns at a frequency of 10 Hz. One of the lasers used was generously provided by the Laser Research Center of the Massachusetts Institute of Technology. The beam was focused with an interjected convex lens and directed vertically with a right-angle prism by total internal reflection to obtain a spot diameter on the samples of 2 mm. Samples were subjected to 5 min of laser pulses of 10 mJ per pulse and an irradiance of 37 MW/cm^2. The 2.5-ns pulse was generated with the same laser and also had a pulse power of 10 mJ per pulse. Use of 1-ps (1.5 mJ per pulse) and 100-fs (1 mJ per pulse) pulsed dye lasers was generously provided by Eric Mazur and Cheng Zai Lu (Department of Physics, Harvard University).

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Abbreviations: BSA, bovine serum albumin; CALI, chromophore-assisted laser inactivation; COT, cycloocta-1,3,5,7-tetraene; D_2O, ^2H_2O.

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Enzyme Assays. Erythrocyte ghosts were prepared as outlined by Dodge *et al.* (8). Microassays of β-galactosidase and alkaline phosphatase were performed in 96-well plates in which the generation of colored product was measured colorimetrically with a microplate reader (Bio-Tek, Winooski, VT). β-Galactosidase was assayed by a method adapted from Wallenfels (9) and alkaline phosphatase was assayed by a method adapted from Bessey *et al.* (10). Acetylcholinesterase was assayed radiometrically by the method of Johnson and Russell (11). SDS/PAGE was done by the method of Laemmli (12).

RESULTS AND DISCUSSION

We used CALI against enzymes as an easy model system to study its mechanism. It has been shown that CALI inactivates 93% of β-galactosidase activity (2) and 80% of alkaline phosphatase and 87% of acetylcholinesterase activity (1) while inactivating <5% of a second enzyme present in the same sample. In all experiments reported here, a second enzyme not associated with dye-labeled antibody was included and not inactivated nonspecifically (<5%).

We calculated the amount of temperature increase at the site of the protein of interest after laser treatment. By solving the heat diffusion equation for a homogeneous and isotropic solid, the predicted temperature increase at the site of the protein of interest was calculated to be too small to cause thermal denaturation. This calculation was done by taking the solution for a continuous point source that releases heat at a prescribed rate per unit time (13). We treated the absorbing dye molecule as a continuous point source because the thermal decay time (0.7 ns) is short compared with the pulse width of the laser (8.5 ns). By assuming that the system has the thermal properties of water and that each single dye molecule absorbs and releases 10^{-16} J per laser pulse (based on parameters for 50% inactivation: optical density = 0.58, irradiance = 37 MW/cm^2), we calculated the temperature increase achieved during a laser pulse at a distance 40 Å from the dye (radius of an antibody molecule). The temperature increase contributed by each dye is ≈0.35°C, which is unlikely to evoke thermal denaturation of proteins. This value is much smaller than originally calculated (1) because the previous model calculations were based on a 10-fold-higher peak power and did not take into account the very rapid heat dissipation. A related calculation determined the temperature change for a 40-Å sphere when a single aqueous malachite green molecule excited by a 620-nm photon (45 kcal/mol; 1 kcal = 4187 J) relaxes to its ground state via internal conversion. The isotropic temperature change is 0.28°C per photon absorbed, a value consistent with the earlier calculation that did not consider the irradiance or the optical density.

The dependence of CALI on the ambient temperature is also not consistent with thermal denaturation. A thermally based mechanism would show a dependence on the ambient temperature that obeys the Arrhenius equation, as all thermal processes do. We measured the efficacy of CALI for a particular subsaturating dose of laser light at various ambient temperatures between 4°C and 40°C to investigate the temperature dependence of CALI. Fig. 1 shows a comparison between the Arrhenius plots of thermal denaturation and CALI of the enzyme β-galactosidase. Although there was a slight increase of efficacy of CALI with increasing ambient temperature, the data were inconsistent with CALI being a completely thermal process (14). A purely thermal process should have a linear Arrhenius relationship with the slope related to the activation energy of thermal denaturation by the value $1/(T + T'/T)$, where T' is the temperature change evoked by a single laser pulse. The value for T' based on the above calculations is quite small and predicts that the slopes

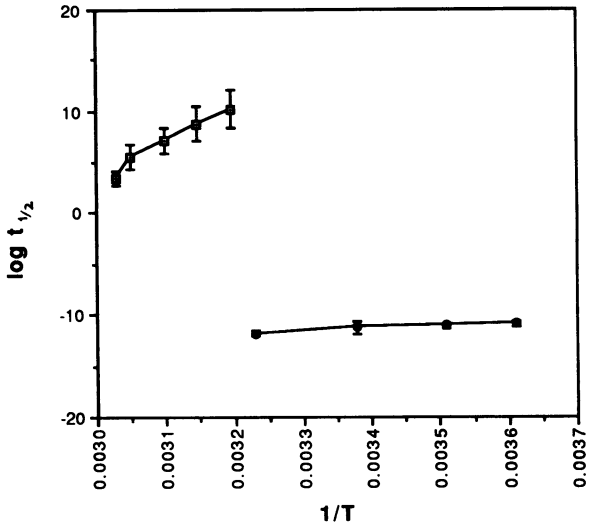

FIG. 1. Comparison of Arrhenius plots of CALI (□) and thermal denaturation (●) of β-galactosidase. The natural logarithm of the time taken for 50% inactivation of the enzyme (in seconds), log $t_{1/2}$, is plotted as a function of the inverse temperature (in kelvins), $1/T$. The $t_{1/2}$ for thermal denaturation was determined by incubating β-galactosidase in Tris-buffered saline at various ambient temperatures from 40°C to 80°C and measuring the remaining enzyme activities after various times. The $t_{1/2}$ for CALI at ambient temperatures from 4°C to 40°C was determined by multiplying the number of laser pulses required for 50% inactivation by the pulse width of the laser (8.5 ns). The slopes of the two plots are very different, which is inconsistent with a photothermal mechanism for CALI. Data are the averaged results of two independent experiments with duplicate data points; the errors bars show the standard deviation between these experiments.

of the Arrhenius plots for CALI and thermal denaturation should be similar. The Arrhenius slopes for CALI and thermal denaturation differ by more than 2 orders of magnitude (Fig. 1), suggesting that CALI is not a photothermal process.

Is there evidence that CALI has a photochemical mechanism? Indig *et al.* (15) have shown that for malachite green bound to bovine serum albumin (BSA), 2.6% of the absorbed energy is not released rapidly as heat and have suggested that this energy may be used to generate photochemical species. Also, laser treatment of malachite green-labeled BSA induces aggregation of the dye-labeled protein. Photoinduced aggregation of dye-labeled proteins has been reported to be a result of free-radical generation (16). Laser irradiation of malachite green-labeled BSA (Fig. 2, lane 2) resulted in a diminution of the band of BSA compared with an unirradiated sample (lane 1), and this diminution was prevented by preincubation with 1% SDS (lane 3). This suggests that the disappearance of BSA is due to aggregation of the protein so that the protein does not enter the gel; this conclusion is supported by amino-terminal analysis indicating that peptide bonds are not broken during CALI (data not shown).

Photoinduced aggregation is not a possible mechanism of CALI. Although the dye-labeled antibody aggregates, the antigen does not and photoinduced aggregation is unlikely to play a role in the inactivation of β-galactosidase by CALI. Samples of dye-labeled anti-β-galactosidase were incubated with β-galactosidase and alkaline phosphatase. In samples subjected to laser irradiation which inactivated β-galactosidase, the dye-labeled antibody disappeared from the gel (Fig. 2, lane 5), but β-galactosidase did not. When laser irradiation was done with antibody that was not dye labeled, there was no effect on the enzymes or the antibody (lane 8).

A photochemical mechanism for CALI is further suggested by the reciprocity between time of exposure and power level.

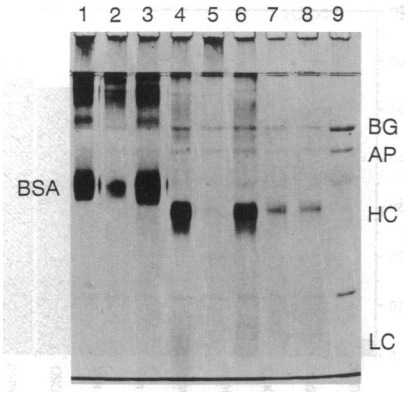

FIG. 2. CALI causes the aggregation of malachite green-labeled proteins, and the aggregation can be prevented by the addition of 1% SDS. Samples of malachite green-labeled BSA (MGBSA) or anti-β-galactosidase (MGaBG) were incubated with alkaline phosphatase and β-galactosidase so that the dye-labeled protein was present in a 10-fold excess. Samples were subjected to 5 min of 620-nm laser irradiation and electrophoresed in an SDS/8% polyacrylamide gel. The proteins were visualized by silver staining. The higher bands in lanes 1–3 are due primarily to multimers of dye-labeled BSA, which tends to aggregate slightly even without laser irradiation. Lane 1, MGBSA, no laser; lane 2, MGBSA plus laser irradiation; lane 3, MGBSA preincubated with 1% SDS plus laser irradiation; lane 4, MGaBG, no laser; lane 5, MGaBG plus laser; lane 6, MGaBG preincubated with 1% SDS plus laser; lane 7, unlabeled aBG, no laser; lane h, unlabeled aBG plus laser; lane I, markers for β-galactosidase (BG), alkaline phosphatase (AP), and heavy chain (HC) and light chain (LC) of the anti-β-galactosidase.

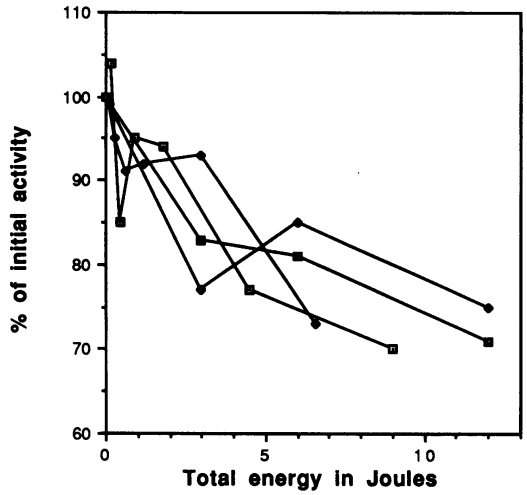

FIG. 3. CALI shows an energy dose reciprocity above a threshold. CALI directed against β-galactosidase was performed at two pulse-width settings with a Nd–YAG-driven dye laser (2.5 ns and 8.5 ns) as well as a 1-ps and 100-fs pulsed laser. Percent inactivation of β-galactosidase was measured for samples exposed to various numbers of laser pulses. Alkaline phosphatase was included in the samples as a control for nonspecific damage, and the activity of this enzyme was unaffected by any of the lasers used (data not shown). Data are plotted as percent of initial activity of β-galactosidase as a function of light energy delivered. The graphs are basically identical over a range of peak power of 4 orders of magnitude. This demonstrates that CALI shows an energy dose reciprocity over the range of peak powers used in this experiment. The data are derived from three different lasers. Each point is the average of duplicates with a standard deviation of 10% or less; error bars are not included because of the number of overlapping graphs presented. The percent inactivation was limited to 30% because of the length of time required to achieve this amount of inactivation with the 100-fs and 1-ps lasers. Data obtained for longer times for the two nanosecond lasers showed a small but significantly higher efficacy for the 8.5-ns laser compared with the 2.5-ns laser. ◆, 100-fs laser; ⊡, 1-ps laser; ◆, 2.5-ns laser; ■, 8.5-ns laser.

When we applied the same total laser energy using peak powers over 4 orders of magnitude, constant energy dose reciprocity applied for CALI. We used dye lasers with four different pulse widths (2.5 and 8.5 ns, 1 ps, and 100 fs) and compared the inactivation of β-galactosidase achieved. Fig. 3 shows the dependence of the percent initial activity on the total energy; the graphs are superimposable for all four pulse widths. At lower power, however, CALI was not effective using a continuous wave laser (2-mW He/Ne laser) or a pulsed laser whose peak power density was lower by a factor of 500 (1). This suggests that constant energy dose reciprocity holds true for CALI only beyond a threshold for photon flux. For the 100-fs and 1-ps lasers, the pulse widths are short with respect to the relaxation time of the dye, and chromophore excitation is saturated under these conditions. Interpretation of the meaning of reciprocity plots for these conditions is problematic. The data suggest that the mechanism of CALI involves events that can occur in times short with respect to the relaxation time of the dye. Although each dye molecule could only undergo a single excitation from ground state per laser pulse, secondary events (such as the sequential absorbance of a second photon by the excited dye) may occur at higher frequency than seen at lower radiant flux.

The data presented thus far suggest that the mechanism of CALI is photochemical. We examined the effect of free-radical quenchers on CALI to directly test this possibility. We employed two systems: (*i*) acetylcholinesterase in human erythrocyte ghosts with free malachite green intercalated in the membrane and (*ii*) β-galactosidase with malachite green-labeled antibody. The first system allowed us to use lipid-soluble quenchers that would also intercalate in the membrane, whereas CALI of β-galactosidase was used to test only the water-soluble quenchers.

There was a significant quenching effect on laser treatment of acetylcholinesterase resulting from pretreatment with sodium azide, butylated hydroxytoluene, and vitamin E but only a slight effect by mannitol (Fig. 4). These reagents share a capacity to quench hydrogen-abstracting free radicals. The ability of both water-soluble and lipophilic reagents to inhibit the protein damage suggests that the free radical species involved in CALI is not limited to the membrane. The amount of inactivation of acetylcholinesterase was not significantly affected when H_2O was replaced by D_2O, which extends the lifetime of singlet oxygen, suggesting that singlet oxygen is not involved (see below). These data show that laser damage of acetylcholinesterase by malachite green intercalated into a membrane is caused by hydrogen-abstracting free-radical species generated by laser excitation of malachite green.

Despite the close agreement of both power requirements and the distance dependence for these processes (2), one cannot rule out the possibility that the mechanism of inactivation is different when the dye is intercalated in a membrane than when the dye is bound to an antibody. Moreover, quenchers are likely to concentrate on the surface of the membrane, depending on the lipophilicity; hence accurate determination of concentrations for effective quenching is problematic. To circumvent these concerns, we added quenchers to samples of β-galactosidase for CALI using dye-labeled antibodies and found that antibody-mediated CALI was also inhibited by free-radical quenchers. Azide at 10 mM was not effective, while 40% and 85% of the inactivation was inhibited at 100 mM and 200 mM azide, respectively; 100 mM mannitol had only a slight effect (12%) (Fig. 5). These results are similar to those shown in Fig. 4 suggesting that antibody-mediated CALI and damage mediated by light excitation of free dye intercalated into membrane show similar sensitivities to different free-radical quenchers.

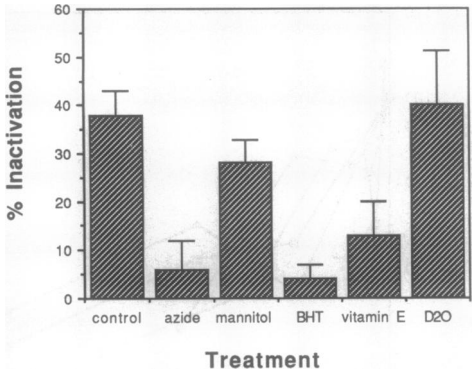

FIG. 4. Effect of free-radical quenchers on CALI using free dye in erythrocyte ghosts. Free malachite green was added to erythrocyte ghosts so that 38% inactivation of the enzyme was achieved by laser irradiation. In similar samples, free radical quenchers were added or in one case H_2O was replaced by ^2H_2O (D_2O). The samples were subjected to CALI and samples were assayed for acetylcholinesterase and β-galactosidase. β-Galactosidase was added as a control enzyme not associated with the erythrocyte ghosts, and its activity was unaffected by laser treatment (data not shown). Data are expressed as the percent inactivation of acetylcholinesterase achieved by laser irradiation and are the average of four independent experiments; error bars represent the standard deviation between experiments. Control, laser irradiation without added quencher; azide, 10 mM sodium azide; mannitol, 10 mM mannitol; BHT, 10 mM butylated hydroxytoluene; vitamin E, 10 mM vitamin E; and D2O, a sample in which H_2O is replaced by D_2O.

The quenching data for azide may be explained by the spatial specificity of CALI. The intermolecular distance between the dye and an azide molecule is 34 Å at 10 mM, 16 Å at 100 mM, and 12.5 Å at 200 mM azide. These distances are within the range for which CALI is effective (2). Therefore, free radicals generated during CALI could react with the protein before significant quenching occurred at 10 mM but not at 100 mM azide. By interpolating between these data, a half-maximal quenching distance for azide is 15 Å, suggesting that the free radical involved is short-lived in the environment used for CALI.

Possible free-radical candidates consistent with the quenching data include hydroxyl radicals and singlet oxygen, both of which belong to the class of oxygen-derived radicals and are common in biological systems (17). Since all four quenchers can scavenge both hydroxyl radicals and singlet oxygen, two experimental tests were run to distinguish between them. First, D_2O was substituted for H_2O in one sample to differentiate between hydroxyl radicals and singlet oxygen. The lifetime of singlet oxygen is longer in D_2O than in H_2O by a factor of 10–15, but substitution of D_2O for H_2O has no effect on hydroxyl radicals (17); replacing H_2O with D_2O should significantly enhance the damaging effect of CALI if singlet oxygen were the mediating species and should have no effect if hydroxyl radicals caused protein inactivation. Substitution of H_2O for D_2O had no effect on CALI against acetylcholinesterase (Fig. 4) or β-galactosidase (Fig. 5). Second, molecular oxygen was removed by bubbling nitrogen gas in the sample thus removing the source of singlet oxygen production; this had no effect on CALI of β-galactosidase (Fig. 5). Protein inactivation in response to laser light in the absence of molecular oxygen shows that singlet oxygen is not involved in the mechanism of CALI.

Differential quenching achieved by azide compared with mannitol is also consistent with the damaging species in CALI being a hydroxyl radical. Azide has a 9-fold faster reaction rate than mannitol in the quenching of hydroxyl radicals (18); hence, azide is more effective at inhibiting CALI at the concentrations employed. There was no significant quenching observed at 10 mM sodium azide, whereas

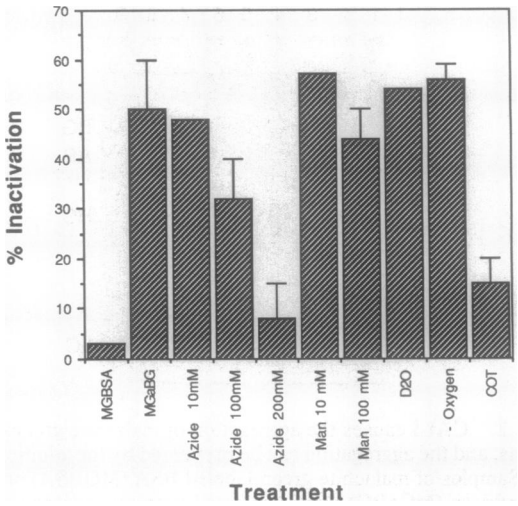

FIG. 5. CALI using antibodies is inhibited by free-radical quenchers. CALI was done by adding a 10-fold molar excess of malachite green-labeled anti-β-galactosidase (MGaBG) to a solution of β-galactosidase (10 μg/ml) and alkaline phosphatase (10 μg/ml) and subjecting the mixture to 5 min of laser irradiation (*Materials and Methods*). Alkaline phosphatase was added to each sample as a control for nonspecific damage. Samples were mixed with a series of free-radical quenchers to compare their effects on the efficacy of CALI. After the 5-min laser treatment, the activities of alkaline phosphatase and β-galactosidase were assayed. Data are presented as the percent inactivation of β-galactosidase in the presence of various quenchers. Alkaline phosphatase activity was unaffected in all samples (data not shown). Data are based on duplicate assays and averaged for three similar experiments; error bars represent the standard deviation between experiments. MGBSA, laser irradiation of a sample incubated with dye-labeled BSA instead of antibody; MGaBG, CALI of β-galactosidase; Azide, CALI of β-galactosidase in the presence of 10, 100, and 200 mM sodium azide; Man, CALI of β-galactosidase in the presence of 10 and 100 mM mannitol; D2O, CALI of β-galactosidase in a sample in which H_2O has been replaced with D_2O; − Oxygen, CALI of β-galactosidase in a sample in which oxygen has been replaced by nitrogen; COT, CALI of β-galactosidase in the presence of 10 mM COT.

singlet oxygen-dependent crosslinking of proteins is 20% quenched by azide at concentrations as low as 3 mM (16).

Based on these data, we conclude that the free-radical species responsible for CALI is not derived from molecular oxygen and suggest that the active species is a hydroxyl radical. This radical reacts with many different classes of biological molecules, including amino acids and nucleic acids, and damages the active sites of several enzymes (19). The reaction rates of the hydroxyl radical with a variety of biological substrates are very fast (10^8–10^{10} $M^{-1}\cdot s^{-1}$) (20). In CALI, photogenerated hydroxyl radicals are spatially localized to the protein of interest bound by dye-labeled antibody, and the high reactivity and short lifetime of this species may explain the spatial specificity of CALI (2). Based on the concentration of potential reactive targets in the cell, and the lifetime of the hydroxyl radical, the range of distance over which the hydroxyl radical will effect damage is ≈10 Å. This value is quite small and in good agreement with the half-maximal quenching distance for azide (15 Å). It is interesting to compare this value with that determined for molecular singlet oxygen (21). The lifetime in cells of singlet oxygen in cells is 200 ns compared with 1 ns for the hydroxyl radical. If singlet oxygen were responsible for CALI, the extent of damage would exceed 200 Å from the dye, leading to nonspecific damage of nearest-neighbor proteins, since the calculated average interprotein distance in cells is 80 Å (2).

Addition of the triplet-state quencher COT significantly inhibited CALI (Fig. 5). While this result is consistent with

the involvement of a triplet-state intermediate in the mechanism of CALI, it is known that olefinic compounds such as COT are also capable of quenching free radicals, and COT may function in this capacity. We do not believe that CALI involves a triplet-state intermediate, for the following reasons. The energy of the triplet state derived from malachite green must be less than that of the singlet state (45 kcal/mol). This is insufficient to generate a hydroxyl radical, for which the energy of formation in water at pH 7 is 55 kcal/mol. This value was obtained by the calculation based on the electrical potential required for the single-electron oxidation of liquid water with the concomitant formation of a solvated proton. Furthermore, molecular oxygen should be a good quencher of the triplet state, and we observed no effect resulting from depletion of molecular oxygen (Fig. 5).

The small percentage of the total absorbed energy used to generate free radicals may explain why CALI requires high levels of laser energy and thousands of laser pulses to be effective (1). Once power above this threshold is achieved, then CALI obeys an energy dose reciprocity. The conjugation of the dye to a macromolecule may decrease its ability to relax solely by vibrational energy modes, as suggested by the comparison between the energy transfer achieved by protein-bound malachite green and that achieved by malachite green free in solution (15).

Based on these results and previous work, we suggest the following model for the mechanism of CALI. A sequential two-photon process results in the generation of a hydroxyl radical from water. The energy of formation of the hydroxyl radical is larger than the energy of the first excited singlet state for malachite green. Thus, if the hydroxyl radical is the damaging species for CALI, then malachite green must absorb more than one photon to generate this species during CALI. While the photon flux used for CALI is insufficient to result in a direct two-photon process, it is possible that a sequential two-photon process occurs in which the excited singlet state absorbs a second photon to generate a high-energy species. This model is consistent with the requirement of a threshold of photon flux for CALI, and the high-energy species would have sufficient energy to generate the hydroxyl radical from water. It is also consistent with the observation that lasers with pulse widths shorter than the relaxation time of the dye function in CALI. Although each dye molecule can be excited from ground state only once per laser pulse, the likelihood of a second photon being absorbed by the excited dye is higher due to the greater radiant flux.

A free-radical mechanism for CALI raises the possibility of nonspecific oxidative damage to membrane lipids as well as neighboring proteins. In the grasshopper embryo, CALI does not affect viability or morphology (3). This stated, the rate of free-radical generation may also limit the peak laser power that can be used for CALI. The quenching data for azide suggest that damage is not likely for molecules at distances beyond 15 Å from the dye. In addition, the apparent photogeneration of free radicals is inefficient, so that thousands of laser pulses spread out over a time of 5 min is required for the specific inactivation of a bound protein. This time allows for lateral diffusion of neighboring lipids so that damage to a critical local concentration of lipids in the bilayer is not probable. Thus, nonspecific damage is not likely to be significant.

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