Spatial specificity of chromophore assisted laser inactivation of protein function

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ABSTRACT Chromophore assisted laser inactivation (CALI) is a new technique that selectively inactivates proteins of interest to elucidate their in vivo functions. This method has application to a wide array of biological questions. An understanding of aspects of the mechanism of CALI is required for its judicious application. A critical concern for CALI is its spatial specificity because nonspecific inactivation of neighboring unbound proteins by CALI is a possibility. We show here that CALI is very dependent on the distance between the chromophore and the protein such that there is no significant effect beyond 60 Å. CALI using antibodies can inactivate other proteins through a complex but its efficacy decreases approximately fourfold for each intervening protein. These data imply that CALI is spatially specific and damage to neighboring proteins is unlikely.

INTRODUCTION

A technique for elucidating the in vivo functions of proteins by their selective laser destruction was recently developed (Jay, 1988). Chromophore assisted laser inactivation of protein function (CALI) effectively inactivates single protein functions on the surfaces of cells or in solution without significantly affecting other proteins in the sample. Laser energy is targeted to single protein molecules by binding the protein with a specific antibody that has been labeled with malachite green. Malachite green is a chromophore that efficiently absorbs 620 nm light, a wavelength not significantly absorbed by cells, and it relaxes to ground state with a relaxation time of ~5 ps. Laser irradiation effectively inactivates the protein that is bound with a malachite green-labeled antibody. This technique is the molecular analogue of cellular laser ablation (Miller and Selverston, 1979). CALI has been tested with enzymes in solution and on the surfaces of cells (Jay, 1988).

Recently CALI was used to show that fasciclin I plays a role in axon adhesion (Jay and Keshishian, 1990) and fasciclin II functions in axonogenesis (Booth et al., 1991) during grasshopper limb bud neurodevelopment. These reports demonstrated that CALI is useful in determining the in vivo function of particular proteins during complex cellular phenomena. CALI may have application in addressing many questions of functional interactions in biology.

A further characterization and understanding of CALI is required for its optimization and judicious application in biological research. A dependence of CALI on the laser power, number of pulses, and the concentration of dye-labeled ligand was established (Jay, 1988) but the nature of the molecular interactions during CALI has not been ascertained. This study was undertaken to further understand the important variables in optimizing CALI and investigate the possibility of nonspecific damage.

The experiments reported here show that CALI is dependent on the number of dye molecules per antibody. CALI can inactivate proteins through a complex but its efficacy decreases with distance, such that proteins that are not directly attached are only slightly affected and those that are two proteins away are not significantly inactivated. We have used dye intercalated into membranes or detergent micelles to inactivate acetylcholinesterase, a membrane protein. The energy requirements and comparable distance of efficacy suggest that this inactivation is similar to CALI. As such, laser inactivation of protein function is independent of the means of localizing the dye to the protein of interest and not dependent on protein–protein interactions. We used this system to define precisely the dependence of laser inactivation on the average distance of the dye from the protein of interest. These results imply that the inactivation of proteins by CALI is quite specific and that nearest neighbor proteins are not significantly affected.

MATERIALS AND METHODS

Alkaline phosphatase, β galactosidase, acetylcholinesterase, bovine serum albumin, and protein A were obtained from Sigma Chemical Co. (St. Louis, MO), anti-β galactosidase and anti-protein A were obtained from Cappell Antibodies (Durham, NC). Malachite green isothiocyanate was obtained from Molecular Probes Inc. (Eugene,

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OR), while the free dye, malachite green (oxalate salt) was purchased from Fisher Scientific Co. (Pittsburgh PA).

Erythrocyte ghosts were prepared as outlined in Dodge et al. (1963). Microassays of β galactosidase and alkaline phosphatase were performed in 96-well plates in which the generation of colored product was measured colorimetrically using a Microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). β galactosidase was assayed by the method of Wallenfels (1962) and alkaline phosphatase was assayed by the method of Bessey et al. (1946). Acetylcholinesterase was assayed radiometrically by the method of Johnson and Russell (1975).

Proteins were labeled with malachite green isothiocyanate by the method of Jay (1988). Incubation times and number of additions of malachite green isothiocyanate in DMSO were varied to alter the ratio of labeling. The ratio of labeling was determined by measuring the optical density at 620 nm (molar absorptivity = 150,000 M^{-1} cm^{-1}) for a sample of known protein concentration.

CALI was performed as described by Jay (1988). Samples were incubated for at least 1 h and aliquoted into 96-prong plates (Nunc, Roskilde, Denmark) and subjected to 620 nm laser light under varying conditions. A Quanta Ray DCR 3 Nd:YAG laser (Spectra Physics Inc., Mountain View, CA) was used to drive a Quanta Ray PDL 2 dye laser containing the fluorescent laser dye DCM (Exciton Chemical Co., Dayton, OH) to generate a 620-nm pulsed laser beam with a pulse width of 8.5 ns at a frequency of 10 Hz. 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}.

RESULTS AND DISCUSSION

Fig. 1 shows efficient inactivation of the enzyme β galactosidase in the presence of malachite green-labeled anti-β galactosidase antibody without the inactivation of alkaline phosphatase in the same solution. This inactivation was dependent on the amount of laser light (number of laser pulses). An inactivation of 95% was obtained after 10 min of laser pulsing. We generally used 5 min of laser irradiation which is sufficient to provide 80% inactivation of β galactosidase.

We had previously shown that CALI was dependent on the number of laser pulses, the power per pulse, and the concentration of the dye-labeled antibody using dye-labeled streptavidin with biotinylated enzymes (Jay, 1988). We also demonstrated that a dye-labeled monoclonal antibody bound to a single epitope on the protein was sufficient to inactivate acetylcholinesterase (Jay, 1988). Fig. 2 shows that CALI is also dependent on the number of dye molecules present per antibody. Anti-β galactosidase was labeled to different ratios of dye to antibody, and used for CALI with samples of β galactosidase subjected to the same amount of laser irradiation. An increase in the dye molecules per antibody increased the efficacy of CALI up to a ratio of 7 dye molecules per antibody. These data suggest that CALI can be optimized by localizing more dye molecules near the protein of interest. How does this compare with the efficacy of

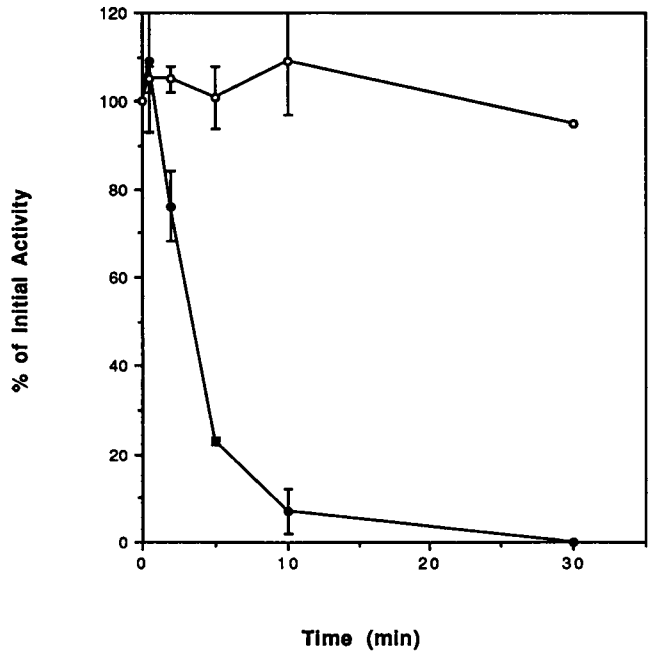

FIGURE 1 Chromophore assisted laser inactivation of β galactosidase. Samples containing 5.5 μg/ml β galactosidase, 3.1 μg/ml alkaline phosphatase, 100 μg/ml bovine serum albumin, 100 μg/ml malachite green-labeled anti-β galactosidase (ratio of labeling = 6.5) in Tris buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) were subjected to increasing times of laser irradiation. Aliquots were assayed for the two enzyme activities and the percentage of initial activity was plotted against the irradiation time. The laser pulse was 8.5 ns of 10 mJ per pulse at a frequency of 10 Hz. The β galactosidase activity is shown by the filled circles, while the concurrent alkaline phosphatase activity of the same sample is shown by the clear circles. The points are based on sample duplicates and the average error is ± 10%.

CALI for labeled streptavidin and biotinylated enzymes used earlier (Jay, 1988)? Higher levels of inactivation were achieved using dye-labeled streptavidin (80% for molecules labeled to a dye ratio of 2). Streptavidin is a smaller protein than an antibody (molecular weight = 70,000) and it binds to biotin as a multimer. As such, the results may not be directly comparable.

Can CALI work in a complex in which the dye molecule is present not on the antibody but on another protein that binds to the antibody? We could not rule out the possibility that the perturbation of axon adhesion in the grasshopper pioneer neurons was due to the inactivation of a protein bound to fasciclin I in a complex (Jay and Keshishian, 1990). We addressed this question by comparing the efficacy of CALI against β galactosidase when malachite green was bound to the following proteins: (*a*) to the anti-β galactosidase directly, (*b*) to protein A which binds to the Fc domain of the antibody, or (*c*) to an antibody that recognizes protein A such that

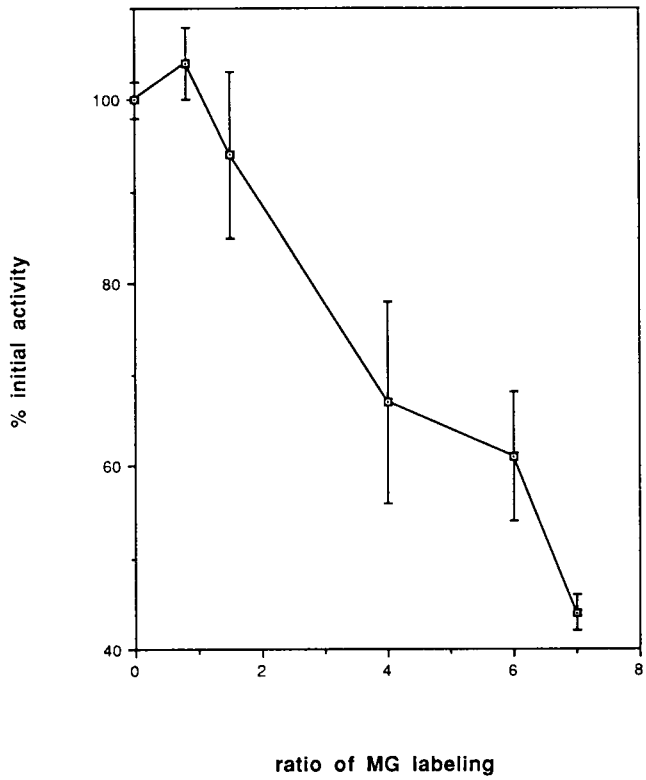

FIGURE 2 The dependence of CALI on the number of chromophores per antibody. Dye labeled anti-β galactosidase with varying ratios of labeling were made by changing the time of incubation before desalting and by increasing the concentration of the labeling reagent. The ratio of dye labeling was calculated by dividing the dye concentration by the protein concentration. The dye concentration was determined by measuring the optical density at 620 nm of a solution of the desalted dye labeled antibody and dividing by the molar absorptivity (150,000 M^{-1} cm^{-1} in a 1 cm wide cuvette). The protein concentration was determined by dividing the mg/ml by the molecular weight of IgG (150,000). These differing samples of dye labeled antibody were used for CALI under standard conditions and β galactosidase activity was measured to ascertain the percentage inactivation achieved. The percentage of initial activity was plotted against the ratio of malachite green labeling.

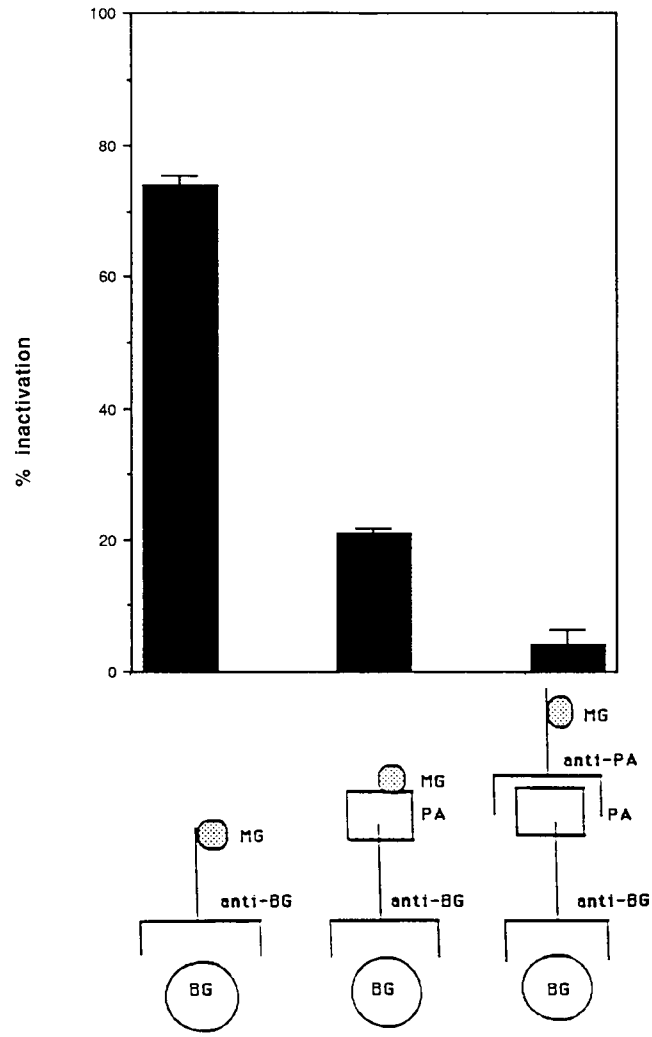

FIGURE 3 The efficacy of CALI in a complex. CALI against β galactosidase was done as in Fig. 1. In samples where the dye was present on anti-β galactosidase directly, the final concentration of 44 μg/ml. Protein A was added to 44 μg/ml (2-fold molar excess) and anti-protein A was added to 176 μg/ml (2-fold molar excess). Reagents were all dye labeled to a ratio of greater than 6. MG, malachite green; BG, β galactosidase; anti-BG, anti-β galactosidase; PA, protein A; anti-PA, anti-protein A. Data are expressed as the percentage inactivation compared with similar samples without laser irradiation. Alkaline phosphatase in the same samples were assayed and were not affected by laser light.

the dye-labeled ligand was separated through two intervening proteins from β galactosidase. These results are shown in Fig. 3. Under conditions where CALI, using dye-labeled anti-β galactosidase, inactivated 74% of the initial activity, only 21% inactivation occurred when the dye label was present on protein A bound to the first antibody. Moreover, when the dye label was present on the anti-protein A, the inactivation was 4%, which was indistinguishable from controls. The efficacy of CALI decreases four- to fivefold for each intervening protein between the malachite green and the protein of interest. Thus, it is unlikely that we would inactivate secondary proteins in complexes, though we cannot rule out a small amount of inactivation. The geometry of protein complexes on cell surfaces may be different than the linear array of this model system such that inactivation of auxiliary proteins in a membrane complex by CALI may be possible.

A parallel study using biotinylated anti-β galactosi-

dase and dye-labeled streptavidin showed that CALI resulted in 17% inactivation when the dye was on the streptavidin compared with 65% inactivation when the dye was directly bound to anti-β galactosidase (data not shown). Previous studies showed that malachite green-labeled streptavidin was effective in CALI against biotinylated enzymes (Jay, 1988).

These results of CALI in a complex, and the dependence of number of dye molecules per antibody, suggested that the proximity of dye to the protein of interest is important for CALI to work. A more direct measure of the distance dependence of CALI was studied in the following manner. We had previously shown that acetylcholinesterase could be inactivated by CALI using dye-labeled anti-acetylcholinesterase (Jay, 1988). Acetylcholinesterase is a membrane protein that imbeds a small hydrophobic tail into the outer leaflet of the red cell membrane or into micelles in detergent-solubilized samples of this protein. The structure of malachite green is a planar hydrophobic molecule with a delocalized positive charge that adheres to micelles or the outside of red cell membranes. The dye is localized or tethered near to the acetylcholinesterase without an interceding protein such as an antibody. If the agent of inactivation is transferred solely through the medium, then the proximity of the dye to the enzyme is the important parameter in inactivation of the enzyme. Moreover, by varying the number of dye molecules present per micelle or ghost, we observed the dependence of CALI on the average distance between the dye molecules and the enzyme. This experiment could not be done with soluble enzymes in solution because the concentration of dye required to obtain close separation distances would be so high that the laser light would be completely absorbed in the first few microns of the sample.

Fig. 4 shows the result on the efficacy of CALI of increasing the number of dye molecules per micelle in a Triton X-100-solubilized sample of purified acetylcholinesterase. This shows that 0.7 malachite green molecules per Triton X-100 micelle are required to effect a 50% inactivation of acetylcholinesterase. The diameter of a Triton X-100 micelle is 25 Å; this defines an average separation of 12.5 Å between dye and protein for 50% inactivation. Alkaline phosphatase and β galactosidase were included in the same samples and do not insert into micelles or membranes. They were not affected by laser light (data not shown) and serve as negative controls against nonspecific damage.

Is this method of protein inactivation comparable to CALI? The requirement of laser energy and irradiation time is the same as that found for CALI using malachite green-labeled anti-acetylcholinesterase. This suggests that the mechanisms for the inactivation from interca-

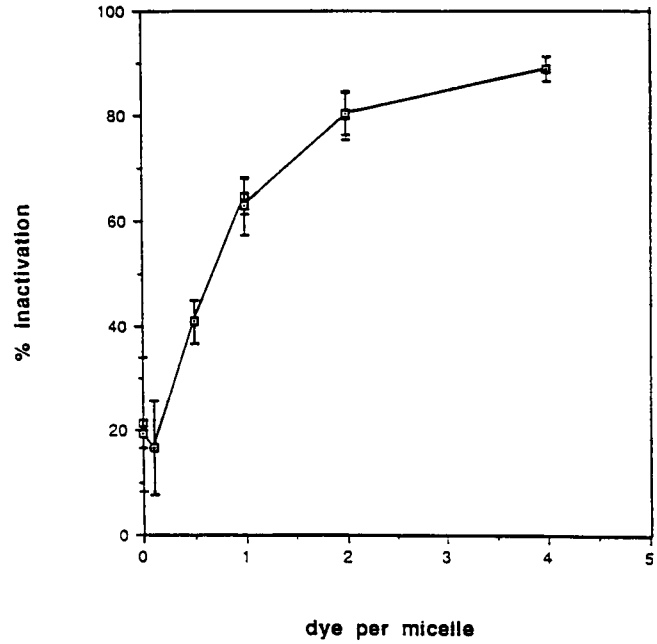

FIGURE 4 CALI of acetylcholinesterase using free dye in micelles. Acetylcholinesterase was dissolved in 0.6 mM Triton X-100 (final micelle concentration = 4.3 μM) in distilled water to a final concentration of 3.3 μM. Samples included either alkaline phosphatase or β galactosidase at the concentrations used in Fig. 1 and these enzymes served as controls for soluble enzymes not associated with micelles. CALI was done on detergent solubilized samples of acetylcholinesterase. Free malachite green was added which adsorbed onto the micelles. The dye to micelle ratio was determined by dividing the number of dye molecules added by the number of detergent micelles. The number of micelles is calculated by subtracting the concentration of the critical micellar concentration (0.21 mM in saline) from the concentration of Triton X-100 and dividing by the aggregation number (140). Varying aliquots of a stock solution of 100 mg/liter malachite green in water was added to samples of the acetylcholinesterase solution and the number of effective dye molecules in micelles was determined from the optical density at 620 nm (molar absorptivity = 150,000 $M^{-1}cm^{-1}$). This was necessary because there is an equilibrium between the dye and its hydroxylated-leuco (colorless) form that is dependent on the pH of the solvent. The number of effective dye molecules per micelle was determined by dividing the dye concentration by the micelle concentration. Samples (in duplicate) were subjected to 5 min of laser irradiation and assayed for the enzyme activities. There was no effect on the soluble control enzyme and the data presented are the percentage inactivation of acetylcholinesterase as a function of the number of malachite green molecules per micelle.

lated dye and dye-labeled antibody are likely to be similar. We cannot completely rule out the possibility that the mechanisms of these two phenomena are different.

These data also demonstrate that no interceding protein is required for laser inactivation of protein function mediated by malachite green. One model for

the mechanism of inactivation requires protein–protein contacts that misarrange after the absorbed laser energy is dissipated. These data suggest that these protein interactions are not necessary.

We were unable to test the efficacy of CALI at longer separation distances using detergent micelles due to their small size. To determine longer distances for which CALI is effective, we used acetylcholinesterase present in erythrocyte membrane ghosts which are much larger than detergent micelles. With this system, we titrated the distance required for CALI by increasing the number of dye molecules bound per ghost and calculating the average distance between the enzyme and the nearest dye molecules.

Fig. 5 shows the results of an experiment with laser irradiation of red blood cell ghosts with increasing quantities of free malachite green so that the distance of free dye to the acetylcholinesterase is varied. We assume that the dye will distribute randomly in the plane of the membrane such that they are equidistant from each other. The distance between acetylcholinesterase and the nearest 4 dye molecules is calculated by dividing the surface area per ghost (145 μm^2) by the number of dye molecules per ghost and determining the radius. The results show a linear dependence of CALI efficacy and distance of dye molecules to enzyme. Assays of β galactosidase in the same solution showed no inactivation.

The highest concentrations of dye had an average enzyme to dye distance of 19 Å, a distance far less than the diameter of an antibody molecule and this resulted in 85% inactivation of acetylcholinesterase. This value corresponded to the inactivation seen with acetylcholinesterase incorporated in a Triton X-100 micelle in which four dye molecules had been inserted on average. The diameter of the Triton X-100 micelle is 25 Å, very close to the value obtained using the ghosts. The inactivation leveled off at distances of greater than 60 Å.

The percentage of inactivation seemed to reach a minimum value at around 25%. This was also observed for acetylcholinesterase in Triton X-100 micelles (20%) in which a nonzero inactivation was observed after laser irradiation despite the absence of malachite green. This was not observed for other enzymes such as alkaline phosphatase or β galactosidase (Jay, 1988). The reason for this is unclear, though one possibility is that the acetylcholinesterase preparation and the membrane ghosts are both slightly colored, suggesting that they have hemoglobin present which absorbs 620 nm light (molar absorptivity = 3,750 $M^{-1} cm^{-1}$).

A comparison of these results with those obtained by antibody directed CALI (Figs. 1 and 2) was revealing. The experimental setup of the ghosts effectively placed 4 dye molecules within the average distance plotted in Fig. 5. We compared this with the inactivation of β galactosidase using anti-β galactosidase labeled with 4 dye molecules per antibody in Fig. 2 which is 35%. An IgG antibody molecule is 85 Å long (Sarma et al., 1971). The location of the dye moieties on the antibody are not known. Though most of the mass of the antibody is closer than the midpoint of the length (43 Å) we have

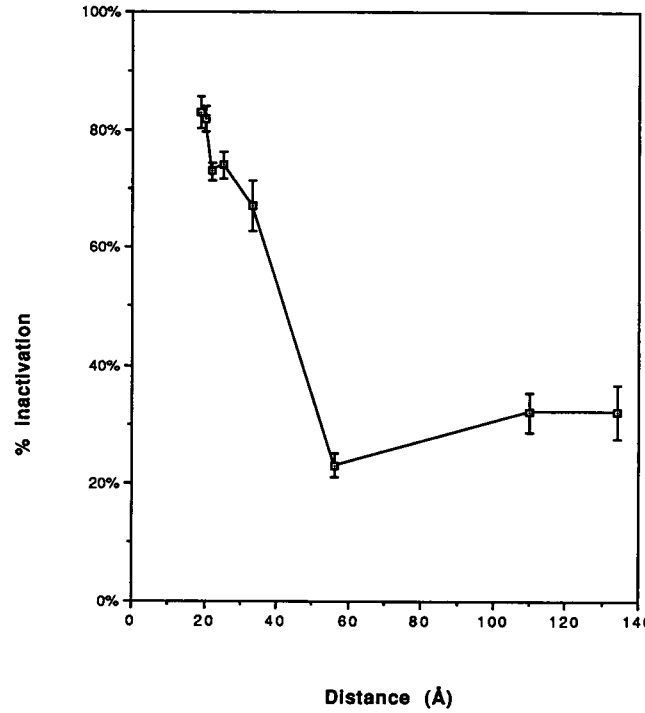

FIGURE 5 CALI of acetylcholinesterase using free dye in erythrocyte ghosts. Samples of human erythrocyte ghosts (100 μg in 1 ml of 75 mM sodium citrate, pH 5.5) were incubated with varying amounts of 2 mM stock solution of malachite green. The optical densities at 620 nm of these samples were measured, the samples centrifuged, and the optical densities of the supernatants were measured. The number of dye molecules incorporated was determined by subtraction of supernatant from the initial solution and dividing by the molar absorptivity and multiplying by the volume (0.001 liters). The pellet was washed once and appeared slightly blue colored and was resuspended in 1 ml of the sodium citrate solution containing 5.5 μg/ml of β galactosidase, a soluble enzyme not associated with the ghosts. The number of ghosts was determined by the amount of protein added (1.7×10^9 ghosts per mg ghost protein). The distance between dye molecules and the acetylcholinesterase was the radius surrounding each dye molecule in the ghost determined by dividing the surface area of a ghost by the number of dye molecules incorporated and determining the radius assuming a circle. Duplicate samples were subjected to 5 min of laser irradiation and aliquots were assayed for acetylcholinesterase and β galactosidase. There was no laser inactivation observed for the control enzyme, β galactosidase. The data are presented as the percentage inactivation of acetylcholinesterase as a function of distance from malachite green. Note that the plane of the membrane is described by a repeating unit of one enzyme surrounded by four equidistant dye molecules.

chosen to use this as the average value for distance between the dye and the protein because labeling of the antibody at the binding face may perturb binding of the antibody. When the four dye molecules are 43 Å away, the inactivation of acetylcholinesterase achieved was 42%, quite close to the value observed using antibody-directed CALI against β galactosidase (35%). This was in reasonably close agreement despite using two different enzymes with varying means of localizing the dye to the enzymes. This correlation strengthens the assertion that the mechanisms of inactivation of these two methods are similar.

These data suggest that the major parameter in the efficiency of CALI is the distance of the dye molecules from the protein of interest. At values greater than 60 Å, the inactivation levels off to that of controls without dye present. This is comparable to the length of an antibody (85 Å). This dependence on distance may explain why CALI does not work for all antibodies (Jay, 1988). The location of dye moieties on the labeled antibody is dependent on the location of free amino groups on the surface of the antibody which is variable between antibodies. If the majority of labeled sites are far away from the antigen binding site, then CALI will not be effective. This also explains the low inactivation achieved when the dye is bound to protein A when CALI was done in a complex in Fig. 4. One prediction from this is that the use of Fab fragments of antibodies labeled with malachite green would optimize the efficacy of CALI.

CONCLUSIONS

The strict dependence of CALI on distance or on intervening proteins in a complex imply that nonspecific damage to proteins that are not directly bound is unlikely. As such, the attribution of *in vivo* function to proteins subjected to CALI can be made with greater confidence. The limit of effective distance for CALI of 60 Å corresponds well with the length of an antibody molecule (85 Å). Thus, a random labeling of sites on an antibody should provide a number of dye moieties in range to be effective in CALI.

Knowledge of the effects of distance on CALI also has bearing on the application of CALI intracellularly. Since cytoplasmic protein concentration is high, inactivation of nearest neighbor proteins or even those bound in a complex due to their proximity is of concern. In a cell with a protein concentration of 300 mg/ml (average molecular weight = 50,000), the average separation is 80 Å. If proteins were motionless, what would be the extent of damage due to proximity of a dye-labeled protein to its nearest neighbor (labeling ratio = 4)?

Fig. 5 shows that CALI is not effective at distances greater that 60 Å, suggesting that nonspecific inactivation by proximity of nearest neighbors is not likely. In addition, because proteins are always in relative motion, and CALI takes place over thousands of laser pulses over several minutes, the actual average distance between nonbinding nearest neighbors would be much greater, such that the probability of nonspecific inactivation is insignificant in practice. This suggests that it will be possible to use CALI directed against intracellular proteins without problems due to nonspecific inactivation. However, the inactivation of proteins in a complex remains a possibility though we have shown here that the efficacy of CALI decreases dramatically with each intervening protein.

In the erythrocyte membrane we asked the same question of distance between nearest neighbor membrane proteins. There are 5×10^6 membrane proteins per red cell which have a surface area of 145 μm^2 (Fairbanks et al., 1971). If we assume that the proteins are randomly distributed, the interprotein distance is 60 Å. This is just at the limit of distance for CALI to work, and lateral diffusion between pulses makes the effective distance much greater. As in the example of cytoplasmic proteins, inactivation due to random proximity of neighboring membrane proteins to dye molecules is unlikely.

Nonspecific inactivation in the absence of dye remains a concern and limits the peak power that can be used such that we conduct CALI in the range of 10–50 megawatts per cm^2, and we have observed nonspecific damage with peak power values that are twofold higher. For this reason, it is necessary to perform control experiments in the absence of dye or using dye-labeled reagents that do not specifically bind to the protein of interest.

Does this level of spatial localization for CALI suggest a mechanism for protein inactivation? Since both photothermal and photochemical mechanisms for CALI would be dependent on the proximity of the dye, the correlation of distance and efficacy does not infer the mechanism of CALI. Knowledge of the spatial specificity of CALI does provide a better basis on which to ascribe the inactivation to only the bound protein. As such, CALI can be more confidently applied to study the in vivo role of proteins of interest in complex cellular phenomena.

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