Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5454–5458, August 1988
Biochemistry

Selective destruction of protein function by chromophore-assisted laser inactivation

(malachite green/localized heating/protein denaturation)

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Communicated by David H. Hubel, April 7, 1988

ABSTRACT **Chromophore-assisted laser inactivation of protein function has been achieved. After a protein binds a specific ligand or antibody conjugated with malachite green (C.I. 42000), it is selectively inactivated by laser irradiation at a wavelength of light absorbed by the dye but not significantly absorbed by cellular components. Ligand-bound proteins in solution and on the surfaces of cells can be denatured without other proteins in the same samples being affected. Chromophore-assisted laser inactivation can be used to study cell surface phenomena by inactivating the functions of single proteins on living cells, a molecular extension of cellular laser ablation. It has an advantage over genetics and the use of specific inhibitors in that the protein function of a single cell within the organism can be inactivated by focusing the laser beam.**

The ultimate understanding of biology lies in knowing the summed interactions of functional proteins that are manifested in the living organism. This is primarily addressed by genetic methods, which require the use of organisms for which mutants are isolated easily and the availability of a suitable screen for the function of interest. One of the main biochemical approaches to understanding functional interactions is the use of specific inhibitors, but such inhibitors are not available for all proteins of interest. There are ligands, such as antibodies or receptor agonists, that bind to specific proteins, but these in general do not block function. A means of converting any specific protein ligand into a reagent that can inactivate protein function would extend the breadth of questions of function that can be investigated.

Miller and Selverston (1) developed a procedure to kill specific cells by injecting them with a fluorescent vital dye and then irradiating them with high-intensity laser light of a wavelength that corresponds to the absorption maximum of the fluorophore but that is not absorbed by cellular components. During this process electrons in the fluorophore are excited and relax to ground state, releasing energy in several forms, including heat. This is effective in killing the target cell, probably by local heating effects, while all other cells remain unaffected. However, there is no analogous procedure that could inactivate the function of a single protein without affecting the rest of the cell.

I report here the chromophore-assisted laser inactivation (CALI) of protein function. Fig. 1 shows the outline for this procedure. Energy is targeted to the protein of interest by conjugating the chromophore to a probe specific to that protein. Such probes could be antibodies, receptor agonists, or enzyme substrates. The sample is irradiated with short pulses of high-intensity laser light at the wavelength of the absorption maximum of the chromophore, which is not significantly absorbed by cellular components. The only energy received by the sample is due to the excitation and relaxation of the chromophore; the remainder of the light energy is transmitted. The vast majority of the absorbed energy is released in the form of heat. This causes a large temperature increase close to the chromophore and denatures proteins in the immediate vicinity. The steepness of the thermal gradient with respect to distance suggests that other cellular components will not be affected, thus maintaining an otherwise viable cell.

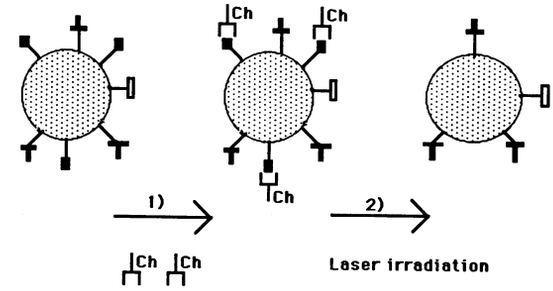

FIG. 1. CALI. In step 1, cells with a diverse array of surface proteins are incubated with a chromophore (Ch)-conjugated ligand specific for one of these proteins. In step 2, these cells are subjected to laser irradiation at a wavelength that is absorbed by the chromophore but not by cellular components. This results in the selective inactivation of the ligand-bound protein without denaturing other proteins in the cell.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma unless otherwise stipulated.

Malachite Green Labeling of Ligands. Malachite green isothiocyanate was custom-synthesized by Molecular Probes (Eugene, OR). Proteins were labeled with this reagent by the following method. Aliquots (10 μl) of a 10-mg/ml solution of malachite green isothiocyanate in dimethyl sulfoxide were added to 200 μg of protein dissolved in 200 μl of 500 mM $NaHCO_3$ (pH 9.8) at 5-min intervals until a reagent/protein molar ratio of 100 was attained. After 1 hr of incubation at room temperature with agitation, free label was separated from the labeled protein by gel filtration. The free label at this pH was slowly converted to the colorless leukoform of the dye by hydroxylation at the central carbon of the triarylmethane. The ratio of labeling was determined by measuring the optical density at 620 nm of a solution of known protein concentration and calculating the dye concentration by using a molar absorptivity $\varepsilon = 150{,}000\ M^{-1}\cdot cm^{-1}$.

CALI. A DCM circulated dye laser (Cooper Lasersonics, Santa Clara, CA) was pumped by a pulsed (10 Hz, 10-nsec pulse width) neodymium/yttrium–aluminum garnet laser (Quanta Ray DCR 3, Spectra Physics, Santa Clara, CA) to generate a 620-nm laser beam. This beam was focused to a

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Abbreviation: CALI, chromophore-assisted laser inactivation.

spot size of 2 mm with an optical lens and directed vertically with a 45-degree mirror or prism to the microtiter well tray below containing the samples. A transferable solid-phase 96-prong plate (Nunc) was used as a microwell tray, with wells of 2.5 mm internal diameter to conform more closely to the laser beam width. The difference between the well diameter and the beam width was in part compensated for by the reflection of light back into the sample by the well's wall.

Enzyme Assays. Alkaline phosphatase was assayed by the method of Bessey *et al.* (2) and β-galactosidase was assayed by the method of Wallenfels (3). Acetylcholinesterase was assayed by a variation of the method of Johnson and Russell (4). Aliquots were incubated for 5 min with 50 μM [^{3}H]acetylcholine (2×10^8 cpm)/100 mM sodium phosphate, pH 7.0, in a total volume of 100 μl. The reactions were terminated by adding 100 μl of 10% (wt/vol) trichloroacetic acid/150 mM sodium chloride. The radioactivity in the samples was measured by scintillation counting after the addition of 3 ml of toluene containing 5% (vol/vol) ScintiVerse (Fisher) and 10% (vol/vol) 1-butanol.

Anion exchange was assayed by a variation of the method of Jay (5). Aliquots were incubated for 45 min with 10^7 cpm of [^{32}P]phosphate in 1 mM sodium phosphate/75 mM sodium citrate, pH 6.4. Cells were separated from free label by sedimentation through oil in 400-μl microcentrifuge tubes. The tubes were frozen on dry ice, the bottoms were cut off, and the [^{32}P]phosphate taken up by the cells was measured by scintillation counting. Data are expressed as a percentage of values for samples without laser irradiation.

RESULTS

For CALI to work, the following criteria must be met. (*i*) The chromophore must have an absorption maximum at a wavelength not significantly absorbed by cellular components, and it must efficiently absorb light and release heat on a fast time scale without photobleaching. (*ii*) The thermal gradient generated by chromophore energy release must be sufficient to denature a cell protein while other cell components are unaffected. (*iii*) The inactivation must be selective and efficient.

N-[4-[[4-(Dimethylamino)phenyl]phenylmethylene]-2,5-cyclohexadien-1-ylidene]-*N*-methylmethanaminium chloride (malachite green, C.I. 42000) (6) is the chromophore of choice for several reasons. Its absorption maximum is at a wavelength (620 nm) not absorbed by cells. It has a very high molar absorptivity (ε = 150,000 $M^{-1} \cdot cm^{-1}$) and thus efficiently absorbs light. The chromophore relaxes from the excited state largely through torsional degrees of freedom and thus has an extremely fast relaxation time (≈5 psec). It does not photobleach, since it does not readily undergo photochemical reaction. In a short pulse of light this chromophore can undergo excitation and relaxation many times. Fig. 2 shows the structure of an isothiocyanate derivative of this dye that reacts covalently with free amino groups. It should be noted that the spectroscopic properties of the dye may be substantially different when the dye is coupled to a protein.

I have calculated the temperature change in a 100-Å (10-nm) sphere around a single malachite green molecule in an aqueous environment. The energy input was determined from Beer's law (7) and was converted to a temperature change by using the specific heat of water. At a laser power output of 200 $MW \cdot cm^{-2}$ (above which nonspecific cellular damage could result from nonlinear absorption) a 10-nsec pulse should cause a temperature change of 130°C. This is well above the critical denaturation temperatures shown for equilibrium heating of proteins (data not shown) and near those estimated for 10-nsec heating times (8). Since the temperature gradient decreases with the cube of the distance, the temperature rise experienced by sites 400 Å away would be ≈2°C. The thermal dissipation occurs on the nanosecond time scale, which suggests that intense heating near the chromophore could be effected without substantial heating of the rest of the cell. The simplifying assumptions make this model calculation quite approximate. Although the quantitative results should be interpreted with this in mind, the calculation is indicative of the feasibility of CALI.

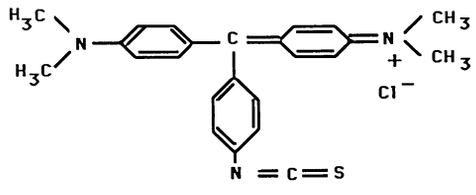

FIG. 2. Structure of malachite green isothiocyanate.

The efficiency and selectivity of inactivation in solution were tested by using two enzymes for which there were convenient specific probes. Malachite green-labeled streptavidin was added to a mixture of a biotinylated enzyme and a second, unbiotinylated enzyme, and the solution was irradiated with 620-nm laser light. The complementary experiment with the biotin moiety on the second enzyme demonstrated that inactivation was not due to a selective sensitivity of one enzyme over the other. Fig. 3 shows the effects observed with the enzymes alkaline phosphatase and β-galactosidase. Irradiation for 5 min at 80-mW power inactivated 75% and 85% of the initial activity for biotinylated alkaline phosphatase and β-galactosidase, respectively, whereas the unbiotinylated species showed only a 27% loss for alkaline phosphatase and a 9% loss for β-galactosidase. Thus, a selective inactivation of protein function was effected by irradiating malachite green-streptavidin bound to a biotin moiety of a target protein.

Although the sample was irradiated for 5 min, the laser pulses at a frequency of 10 Hz, so that there is between each 10-nsec pulse a rest period of ≈100 msec, a very long time with respect to thermal dissipation. The average peak power

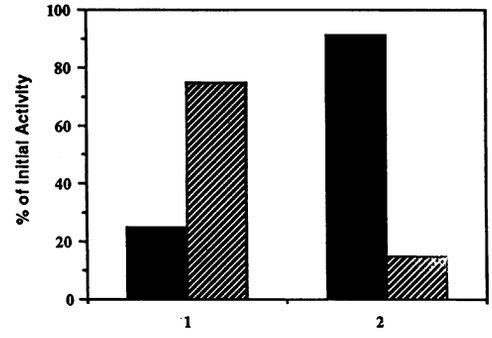

FIG. 3. CALI of biotinylated enzymes. Samples containing two enzymes, one with a biotin moiety attached, were combined with malachite green-streptavidin (labeled to a dye/protein molar ratio of 2.0) in phosphate-buffered saline (5 mM sodium phosphate, pH 8.0/145 mM NaCl) in a total volume of 20 μl and incubated for 1 hr before irradiation. Samples were irradiated for 5 min at a laser power output of 80 mW. Aliquots (5 μl) were taken to assay activity of alkaline phosphatase (2) and β-galactosidase (3). The data are expressed as a percentage of the control activity (measured in samples that were not irradiated) and are derived from the average of duplicate samples. All the data presented are representative of experiments performed on 3–5 separate occasions, and the standard errors varied from 1 to 10% of the mean. Bars 1: 50 ng of biotinylated alkaline phosphatase, 1.25 μg of β-galactosidase, 2.5 μg of malachite green-streptavidin. Bars 2: 50 ng of alkaline phosphatase, 1.25 μg of β-galactosidase, 2.5 μg of malachite green-streptavidin. Black bars represent alkaline phosphatase activity; hatched bars represent β-galactosidase activity.

during a pulse was 0.8 MW with a beam width of 2 mm, for a power density of 25 MW·cm^{-2}, which is one-eighth the power density used in the initial calculations although the total energy input per molecule is much greater than that used in the calculation. If the observed inactivation resulted solely from thermal effects, the data suggest that the energy input can be parceled into discrete pulses with their effect being cumulative. It should be noted that other photoinduced effects that may contribute to the inactivation have not been ruled out.

Continuous-wave laser irradiation is not needed, or useful, since the heat flux would soon reach a steady state and lead to nonspecific damage at sites away from the chromophore. Inactivation was not observed when samples were subjected to the same total energy with a continuous-wave laser (2-mW HeNe laser) or with a pulsed laser whose peak power density was lower by a factor of 500 (0.05 MW·cm^{-2}). These data argue that the peak power density is the important parameter for inactivation.

To optimize the specificity of the inactivation, the time of irradiation (number of pulses), the power output of the laser, and the concentration of malachite green-streptavidin were varied. The inactivation increased with time of irradiation (Fig. 4*A*), amount of malachite green-labeled ligand (Fig. 4*B*), and power output of the laser (Fig. 4*C*). The data in Fig. 4*B* show that CALI is dependent on the saturable binding of the dye-labeled ligand. From the threshold values for the time of irradiation (300 pulses) and the power output (50 mW) from Fig. 4*A*, it can be calculated that the total energy input required per molecule for inactivation is 30-fold higher than that used in the temperature-change calculation cited earlier and could add a maximum of 9×10^6 kcal (3.77×10^{10} J) per mol of bound protein to the sample. This value is quite high, suggesting that the energy transfer is somewhat inefficient. Thermal diffusion during the laser pulse would also decrease the amount of energy actually transferred to the protein. This energy input causes a generalized heating of the bulk solution of <0.001°C per pulse, implying that generalized heating is not likely to be a concern.

At the best values for these parameters, selective inactivation of >80% of biotinylated alkaline phosphatase activity occurred without a significant loss of unbiotinylated enzyme activity. Inactivation plateaued at this value because the ratio of labeling, for both biotin and malachite green with respect to protein, was ≈2:1; hence, the Poisson distribution predicts that 10% of each protein will have no label attached. Since 10% of the enzyme molecules lack biotin and 10% of the streptavidin molecules lack malachite green, only 80% of the enzyme molecules should be inactivated. Higher levels of inactivation can be achieved with dye-labeled ligands that bind directly to the protein of interest. CALI done with dye-labeled antibody to β-galactosidase caused a 93% inactivation of β-galactosidase without affecting alkaline phosphatase in the same solution (data not shown).

Fig. 5 shows controls that test the specificity of CALI. There was no significant inactivation of alkaline phosphatase activity by laser irradiation when biotin was not conjugated to the enzyme (Fig. 5, bar 1). Without laser irradiation, malachite green-streptavidin binding alone did not inhibit the activity of biotinylated alkaline phosphatase (bar 2), nor was there any loss of activity without malachite green-streptavidin or laser irradiation (bar 3). Laser irradiation of a sample of biotinylated alkaline phosphatase with only soluble malachite green present did not result in inactivation (bar 4), and the presence of excess biotin in solution, which would prevent the binding of streptavidin to the biotinylated enzyme, also prevented the loss of activity when the sample was irradiated (bar 5). These last two data imply that the binding of the dye-conjugated probe to the enzyme is essential for the laser inactivation of protein function. The use of

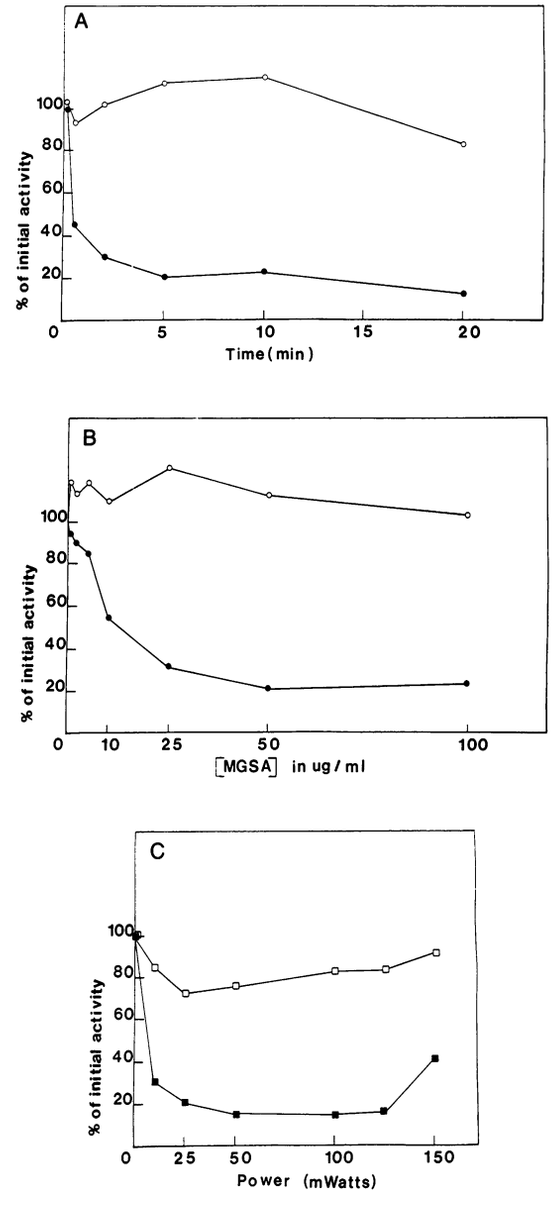

FIG. 4. Optimization of parameters for CALI. Two sets of samples containing 50 ng of biotinylated (filled symbols) or unbiotinylated (open symbols) alkaline phosphatase in a total volume of 20 μl of phosphate-buffered saline (pH 8) were subjected to 620-nm laser irradiation under various conditions. Data are expressed as a percentage of alkaline phosphatase activity of samples without laser irradiation. (*A*) Samples containing 2.5 μg of malachite green-streptavidin were subjected to increasing times of laser irradiation at a power output of 50 mW. (*B*) The concentration of malachite green-streptavidin ([MGSA], expressed in μg/ml instead of μg per 20-μl sample) was varied and samples were subjected to 5 min of laser irradiation at a power output of 50 mW. (*C*) Samples containing 2.5 μg of malachite green-streptavidin were irradiated for 5 min at various laser power outputs (mW).

an alternative ligand, a malachite green-labeled antibody against biotin, was also effective in laser inactivation (bar 6), showing that there is nothing inherently special about streptavidin and protein inactivation. When a different dye moiety, Texas red, was conjugated to streptavidin, it was ineffective in laser inactivation (bar 7). Although the absorption maximum and molar absorptivity of Texas red are comparable to those of malachite green, the relaxation time of the Texas red fluorophore is 3 orders of magnitude greater than that of malachite green. Thus, in a short pulse of light, the energy delivered to the sample would be 3 orders of

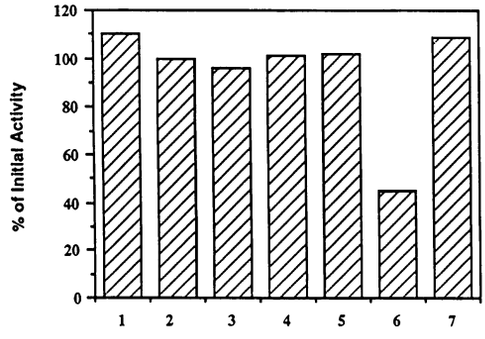

FIG. 5. Controls for CALI. Samples containing 50 ng of biotinylated or unbiotinylated alkaline phosphatase in 20 μl of phosphate-buffered saline were incubated for 1 hr at room temperature. Samples subjected to laser treatment were irradiated for 5 min at a power output of 80 mW. Samples were assayed for alkaline phosphatase activity and data were expressed as a percentage of activity without laser irradiation. Bars: 1, alkaline phosphatase, 2.5 μg of malachite green-streptavidin, laser irradiation; 2, biotinylated alkaline phosphatase, 2.5 μg of malachite green-streptavidin, no laser; 3, biotinylated alkaline phosphatase, no malachite green-streptavidin, no laser; 4, biotinylated alkaline phosphatase, 2.5 μg of free malachite green dye, laser irradiation; 5, biotinylated alkaline phosphatase, 2.5 μg of excess biotin, 2.5 μg of malachite green-streptavidin, laser irradiation; 6, biotinylated alkaline phosphatase, 2.5 μg of malachite green conjugated to anti-biotin antibody, laser irradiation; 7, biotinylated alkaline phosphatase, 2.5 μg of Texas red-streptavidin (Molecular Probes), laser irradiation.

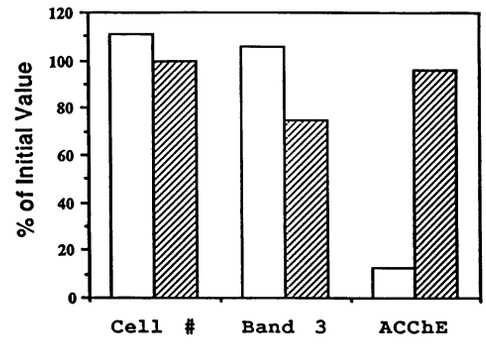

FIG. 6. Inactivation of acetylcholinesterase on human erythrocytes by malachite green conjugated to anti-acetylcholinesterase. A monoclonal antibody against human erythrocyte acetylcholinesterase (a gift from D. Fambrough, Johns Hopkins University, Baltimore) was labeled with malachite green isothiocyanate to a ligand/protein molar ratio of 2:1. Samples of human erythrocytes (4 × 10^6 cells) in a total volume of 20 μl of 1 mM sodium phosphate/75 mM sodium citrate, pH 6.4, were incubated with 10 μg of malachite green-anti-acetylcholinesterase or with buffer alone for 1 hr at room temperature. The samples were laser irradiated at a power output of 75 mW for 5 min. Aliquots (5 μl) were used to determine cell number (by hemacytometer), anion-exchange activity (band 3), and acetylcholinesterase activity (ACChE). Low cell densities were employed in the above samples since hemoglobin has a slight absorbance of 620-nm light. At higher cell densities, there was a significant absorption of the laser light by hemoglobin and decreased inactivation was observed. Open bars, samples with 50 μg of malachite green-anti-acetylcholinesterase; hatched bars, samples with buffer alone.

magnitude lower and would likely be insufficient to generate the thermal gradient required for denaturation.

Laser inactivation of specific surface proteins on whole cells would be useful to dissect cell surface phenomena. However, the selective inactivation of cell surface proteins might be difficult because surface proteins are attached to the membrane and their movement is constrained to two dimensions. Also, the heat-conduction properties of the membrane are not well understood, and the integrity of the membrane may be nonspecifically disrupted by laser irradiation. I have used acetylcholinesterase to demonstrate CALI of protein function on whole cells.

Initially, laser inactivation of acetylcholinesterase in solution was tested. When samples containing dye-conjugated antibody to acetylcholinesterase were subjected to 620-nm laser light for 5 min at 75-mW power, 77% of acetylcholinesterase activity was lost but there was no observable inactivation of alkaline phosphatase in the same solution (data not shown). The antibody against acetylcholinesterase is a monoclonal antibody (5) that most likely binds to a single epitope on the enzyme. Since acetylcholinesterase exists as a homodimer, a single bound malachite green-labeled probe per monomer is sufficient to inactivate the protein's function.

Acetylcholinesterase is located on the extracellular surface of human erythrocytes (9), and malachite green-labeled anti-acetylcholinesterase binds to this enzyme on whole cells (data not shown). After laser irradiation, erythrocyte samples incubated with malachite green-labeled anti-acetylcholinesterase or with citrate buffer alone were assayed for cell number, anion-exchange activity of band 3 (a transport protein of the erythrocyte membrane), and acetylcholinesterase activity. Fig. 6 shows that CALI can be applied to whole cells. Under conditions in which 87% of the acetylcholinesterase activity was lost, there was no inactivation of anion exchange, and there was no significant difference in the cell number compared to that of nonirradiated samples, implying no cell lysis. Without the dye-labeled antibody, there was no loss of acetylcholinesterase activity or decrease in cell number, but there was a slight decrease in anion-exchange activity. These data show that one surface protein function on living cells can be inactivated by CALI without cell lysis or the inactivation of a second surface-protein function. Although it is not possible to assay all cellular protein functions, the lack of inactivation of a second protein function (that is even more dependent on membrane integrity, since band 3 is an integral membrane transport protein) suggests that other functions are not affected by this treatment. In addition, the morphology of the cells was unaltered after laser irradiation as judged by visual inspection. Hemoglobin, a major constituent of the erythrocyte cytoplasm, has a slight absorbance of 620-nm light (ε = 4000 $M^{-1} \cdot cm^{-1}$ at 630 nm; ref. 10), although much less than that of malachite green. Therefore, erythrocytes represent a worst-case scenario with respect to nonspecific absorbance and damage. No discoloration of the hemoglobin was observed after laser irradiation, implying that hemoglobin was not denatured by this treatment. The absence of cellular damage to erythrocytes suggests that other cells will not suffer nonspecific damage by laser irradiation.

Erythrocytes are a model system for CALI uncomplicated by recovery of function through *de novo* protein synthesis. For cells capable of protein synthesis, it is clear that laser inactivation would lead to only a short-term loss of protein function, with the time of recovery dependent on the rate of protein turnover. Thus, for certain applications the use of inhibitors of protein synthesis would be required together with CALI.

For four different enzymes studied, I observed very different power thresholds for inactivation. Power outputs of >100 mW produced nonspecific inactivation of acetylcholinesterase in the absence of dye-labeled antibody; however, if the power output was <50 mW no inactivation occurred even with dye-conjugated antibody present. This contrasts with the selective inactivation of alkaline phosphatase and β-galactosidase, in which the allowable range of power output varied from 10 mW to 150 mW. The enzyme hexokinase could not be selectively inactivated at power outputs up to 200 mW (at which nonspecific damage occurred), although

the dye-conjugated streptavidin did bind to the biotinylated hexokinase, and the critical denaturation temperature for this enzyme is lower than that of the other enzymes tested (data not shown). Other potential variables include the distance from the chromophore to the site of denaturation and the nature of the interaction between the ligand and the protein of interest. This implies that selective inactivation will have to be separately characterized and optimized for each protein, not an unexpected result due to the vast heterogeneity of protein structures.

DISCUSSION

I have shown that selective inactivation of protein function can be effected by binding a malachite green-labeled probe to the enzyme and subjecting the sample to short pulses of laser light. This inactivation is dependent on the power of the laser pulse, the number of pulses, and the concentration of dye-labeled ligand. Although CALI is generally applicable to proteins, inactivation conditions for each protein must be separately optimized.

Highly selective changes can be produced in living cells by using this procedure, if specific probes that can be conjugated with malachite green isothiocyanate are available. This is tantamount to being able to biochemically introduce short-term phenotypic changes comparable to nonsense mutations. CALI may be thought of as the molecular extension of cellular laser ablation (1) and can be used to investigate the function of cell surface proteins. The laser can be focused onto single cells by using microscope optics, so that proteins on single cells in the organism can be inactivated upon laser irradiation. This would allow for the specific disruption of spatially and temporally unique events that are mediated by protein function in the lifetime of the organism. Moreover, CALI correlates protein function to a specific ligand that binds to that protein. This reagent can then be used to assist in purification of that protein or in cloning the gene that encodes it.

I wish to thank David Hubel for support; Allan Oseroff, Leslie Shinobu, Jim Foley, and Michael Otteson for helpful discussion; Walter Gilbert and Guido Guidotti for critical reading of the manuscript; and the Massachusetts Institute of Technology Laser Biomedical Research Center for the use of the laser. This work was supported by grants from the National Institutes of Health, the Harvard Society of Fellows, and the William H. Milton Fund.

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