Caged Protein Conjugates and Light-Directed Generation of Protein Activity: Preparation, Photoactivation, and Spectroscopic Characterization of Caged G-Actin Conjugates†

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ABSTRACT: A simple method is described to prepare caged (inactive) protein complexes using the amino group-directed photo-deprotection group [(nitroveratryl)oxy]chlorocarbamate (NVOC-Cl). In this study, I show how the polymerization activity of G-actin in physiological salt solution is lost upon conjugation of essential lysine residues of G-actin with NVOC-Cl. Reaction conditions were optimized to prepare caged G-actin in high yield, and the conjugate was characterized by biochemical and absorption spectroscopic methods. Upon excitation of caged G-actin in physiological salt solutions with near-ultraviolet light, an efficient photo-deprotection reaction occurs via photoisomerization of the (nitrophenyl)ethyl group of NVOC, which results in cleavage of the carbamate linkage between the protection reagent and G-actin. A standard irradiation condition was then defined which leads to photoactivation of F-actin from caged G-actin with a yield of more than 90%. Photoactivated F-actin was characterized according to its sedimentation behavior, electron microscopic analysis, and sliding velocity on heavy meromyosin determined with the in vitro motility assay. The results of these assays were similar to those obtained from unmodified F-actin. I also report the preparation of caged G-actin conjugated at cysteine 374 with tetramethylrhodamine iodoacetamide and caged fluorescein maleimide. These caged G-actin conjugates can be used to generate fluorescent, polymerization competent G-actin following near-ultraviolet irradiation. Given the widespread applications of caged substrates and ligands in cell biology, the simple method described herein to prepare and photoactivate caged protein conjugates is expected to advance investigations on the regulation of protein activity in living cells.

Light-based deprotection of masked functional groups of certain biomolecules (caged compounds) has been realized as a simple, noninvasive technique to generate concentration jumps of substrates, ligands, and fluorescent proteins (McCray & Trentham, 1989; Theriot & Mitchison, 1991), and caged cross-linking reagents have been described to photogenerate protein activity (Senter et al., 1985; Marriott et al., 1992). A caged protein is defined as a protein whose activity is lost or modified upon conjugation with a photo-deprotection reagent; irradiation of the caged protein with near-ultraviolet light removes the protection group with the subsequent generation of protein activity. Caged proteins should provide an opportunity to manipulate the activity of specific proteins in complex environments; my particular interest in those proteins is to study regulation of the G-actin monomer pool and the assembly of F-actin filaments in living cells. Photoactivation of microinjected caged G-actin or caged G-actin binding proteins should allow defined concentration jumps of polymerization competent G-actin to be made at defined locations within the cell; thereafter, imaging the relaxation kinetics of the G-actin concentration jump may yield important information on the G-actin-sequestering capacity and dynamics of filament assembly. As part of this strategy, this report describes the preparation and properties of a caged G-actin monomer prepared by conjugation of G-actin with NVOC–Cl,[1] a well-known photo-deprotection reagent of amino groups which forms a photolabile carbamate linkage (Amit et al., 1974; Fodor et al., 1991). The photo-deprotection reaction mechanism is thought to proceed via an aci-nitro intermediate which is attacked by a proton to yield the free amine, carbon dioxide, and nitrosoacetophenone (Figure 1). A photo-deprotection condition is described which efficiently removes NVOC groups from caged G-actin, resulting in a high yield of polymerization competent G-actin. The preparation, photoactivation, and spectroscopic properties of TMR-labeled caged G-actin and caged fluorescein-labeled G-actin proteins are also described; these fluorescent conjugates of caged G-actin should be useful for subsequent intracellular studies outlined above.

MATERIALS AND METHODS

NVOC-Cl, phalloidin, and TMR-phalloidin were purchased from Fluka. NVOC-Cl was stored under dry silica gel at 4 °C in the dark. TMR-IA was purchased from Molecular Probes Inc. (Eugene, OR). All other reagents, unless otherwise noted, were purchased from Sigma Chemical Co. and were of the highest quality grade available. Operations involving caged reagents and caged protein conjugates were performed under either red light or dimmed room light. G-actin was prepared from rabbit muscle according to the method of Spudich and Watt (1971) and used within 1 week. HMM

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[1] Abbreviations: ATP, adenosine triphosphate, sodium salt; NVOC-Cl, [(nitroveratryl)oxy]chlorocarbamate; TMR, tetramethylrhodamine; TMR-IA, tetramethylrhodamine iodoacetamide; FTIC, 5,6-fluorescein isothiocyanate; Prodan, 6-proprionyl-2-(dimethylamino)naphthalene.

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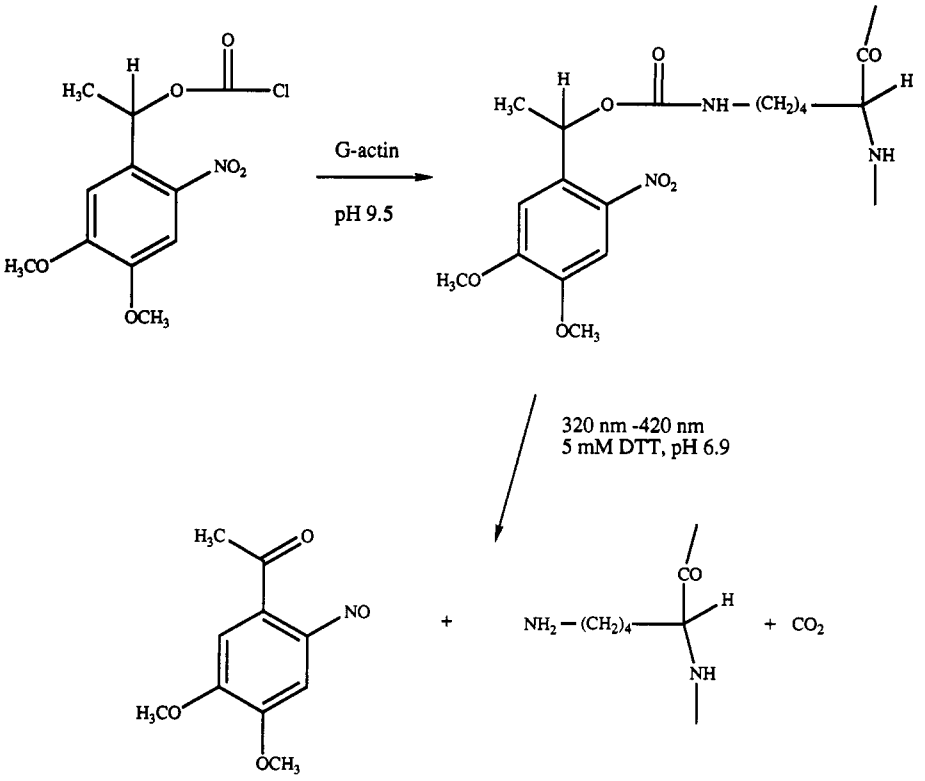

FIGURE 1: Proposed reaction mechanism of [(nitrophenyl)ethyl]carbamate photo-deprotection reaction.

was prepared according to Kinosita et al. (1991). The in vitro motility assay demonstrating F-actin sliding on HMM was performed essentially according to Kinosita et al. (1991).

Preparation of NVOC-Labeled Poly(lysine). Poly(lysine) (2 mg), average molecular weight of 10 000, was dissolved in 2 mL of 0.1 M Na_2CO_3, pH 9.5; 100 μL of a 10 mM solution of NVOC-Cl in dry acetone was added to the poly(lysine) in the dark and the reaction mixture left for 30 min at 20 °C. The conjugate was centrifuged at 10000*g* for 20 min and the supernatent dialyzed overnight against 3 L of 10 mM PIPES, pH 6.9.

Preparation of Caged G-Actin. A freshly prepared 0.2 M NVOC-Cl solution (50 μL) in dry acetone was added in rapid succession to 1.95 mL of sodium carbonate-based G-buffer (2 mM Na_2CO_3, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM DTT, pH 9.5) and 6 mL of a 36 μM G-actin in sodium carbonate-based G-buffer, in the dark at room temperature for 30 min. The addition of NVOC-Cl in this manner reduced the amount of denatured protein normally encountered upon addition of acetone directly to the protein solution. In order to establish an optimum labeling condition, a range of NVOC-Cl concentrations was tried from 25 μM to 2.5 mM. After the 30 min reaction time, the sample was centrifuged at 5000*g* for 10 min to remove insoluble material and dialyzed against 2 mM PIPES-based G-buffer, pH 6.9, at 4 °C. The NVOC-labeled G-actin was then treated with 2 mM magnesium chloride and 0.1 M KCl to initiate polymerization and, after 90 min at 200 °C, centrifuged at 100000*g* for 60 min at 4 °C. The pellet, which contained polymerizable actin, was discarded. The supernatent, containing caged G-actin, was collected and the NVOC/G-actin labeling ratio determined by absorption spectroscopy (see also Results section); the concentration of unlabeled G-actin was determined by absorption at 290 nm against an identical G-buffer using an extinction coefficient of 0.63 mL/mg cm. The concentration of NVOC was calculated from its absorption at 350 nm, where the extinction coefficient is 5000 M^{-1} cm^{-1}. Consistent yields of caged G-actin were obtained using the above protocol with a final NVOC-Cl concentration of 1.0 mM, this method will be subsequently referred to as the standard labeling condition.

Preparation of TMR-Labeled Caged G-Actin and Caged Fluorescein-Labeled Caged G-Actin. G-actin at a concentration of 100 μM was dialyzed against 2 mM Tris-based G-buffer, pH 8.0, in the absence of DTT, clarified by centrifugation, and treated with a 2.0 mol excess of TMR-IA or caged fluorescein maleimide (a gift from Do-Jindo Chemical Co., Japan) overnight at 4 °C. Free dye was removed from each conjugate by dialysis against three rapid changes of 2 mM sodium carbonate-based G-buffer, pH 9.5, containing 2 mM mercaptoethanol and then each conjugate centrifuged at 100000*g* for 120 min. Each supernatent was adjusted to approximately 25 μM and then NVOC-Cl added to 1.0 mM. After a 30 min reaction time at 20 °C, the double labeled conjugate was centrifuged at 10000*g* for 20 min and then dialyzed against 2 mm PIPES-containing G-buffer, pH 6.9, and centrifuged at 100000*g* for 60 min. $MgCl_2$ and KCl were added to the supernatent to a final concentration of 2 mM and 0.1 M, respectively. The conjugates were stored in the dark on ice.

Photoactivation of Caged Actin. Prior to photoactivation, caged G-actin in PIPES-containing F-buffer, pH 6.9, was centrifuged at 100000*g* for 60 min. The protein concentration of the supernatent fraction was quantitated by absorption spectrophotometry and DTT added to a final concentration of 5 mM. For macrovolume investigations (>100 μL), the output of a 100 W high-pressure mercury arc lamp (Carl Zeiss, Oberkocken) was directed through the sideport of an SLM-Aminco AB2 spectrophotometer; the excitation wavelength was selected using an interference filter with a broad transmission wavelength between 320 and 400 nm (Dr. Rapp Optoelecktronic, Hamburg). The intense heat generated by the arc lamp was filtered out with two glass copper sulfate filters and a distilled water filter contained in a quartz cuvette. The near-ultraviolet light beam was focused onto the sample

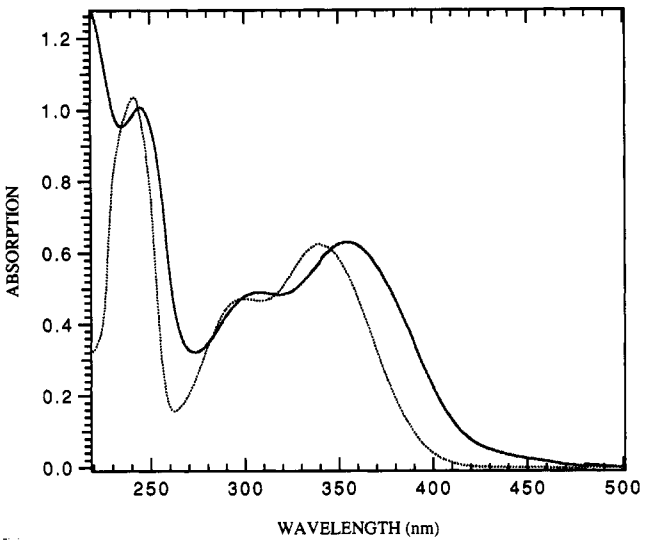

FIGURE 2: Absorption spectra of NVOC-labeled poly(lysine) at approximately 120 μM in 10 mM PIPES, pH 6.9 (solid line), and NVOC-Cl dissolved in 5% aminoethanolamine at approximately 120 μM in ethanol (dotted line).

contained in a semi-microquartz cuvette if the volume was less than 200 μL or else defocused to obtain a uniform excitation for larger sample volumes.

RESULTS

Spectroscopic Properties of NVOC-Labeled Poly(lysine). NVOC-Cl-labeled poly(lysine) was used as a model system to define the near-ultraviolet and ultraviolet absorption characteristics of NVOC-labeled protein conjugates. The absorption spectrum of NVOC-labeled poly(lysine) is shown in Figure 2 (solid line). The low-energy band is centered at 350 nm with an overlapping ultraviolet band centered at 305 nm and a deep ultraviolet band centered at 250 nm. The spectrum of NVOC-labeled poly(lysine) is red-shifted with respect to that of NVOC-Cl dissolved in 5% aminoethanolamine in ethanol (Figure 2, dotted line), suggesting the NVOC group interacts with poly(lysine). Using an extinction coefficient of NVOC at 350 nm as 5000 M^{-1} cm^{-1}, the extinction coefficient of NVOC at 290 nm was calculated to be 3400 M^{-1} cm^{-1}.

Characterization of Caged G-Actin. The absorption spectrum of an 11.3 μM solution of caged G-actin containing 3.9 NVOC groups/actin monomer in 2 mM PIPES-based G-buffer, pH 6.9, is shown in Figure 3A (solid line). The absorption maximum wavelength of NVOC on G-actin is 350 nm and extends to 420 nm, making it susceptible to photoisomerization even in room light. It is imperative therefore that all operations on caged G-actin are performed under either red light or dimmed room light. The actin concentration in caged G-actin was calculated by first determining the NVOC concentration from its absorption at 350 nm, then calculating the absorption contribution of NVOC at 290 nm, and then subtracting this value from the absorption of caged G-actin conjugate at 290 nm. The labeling ratio of seven different preparations of caged G-actin using the standard labeling condition was between three and five NVOC groups/G-actin monomer with a yield between 30% and 60%. In a control experiment, unlabeled G-actin was found to polymerize to more than 95% in F-buffer (data not shown); 20 μM caged G-actin labeled with three to five NVOC groups polymerizes slowly when stored on ice in PIPES-based F-buffer, reflecting the stability of the carbamate linkage. In any case, prior to

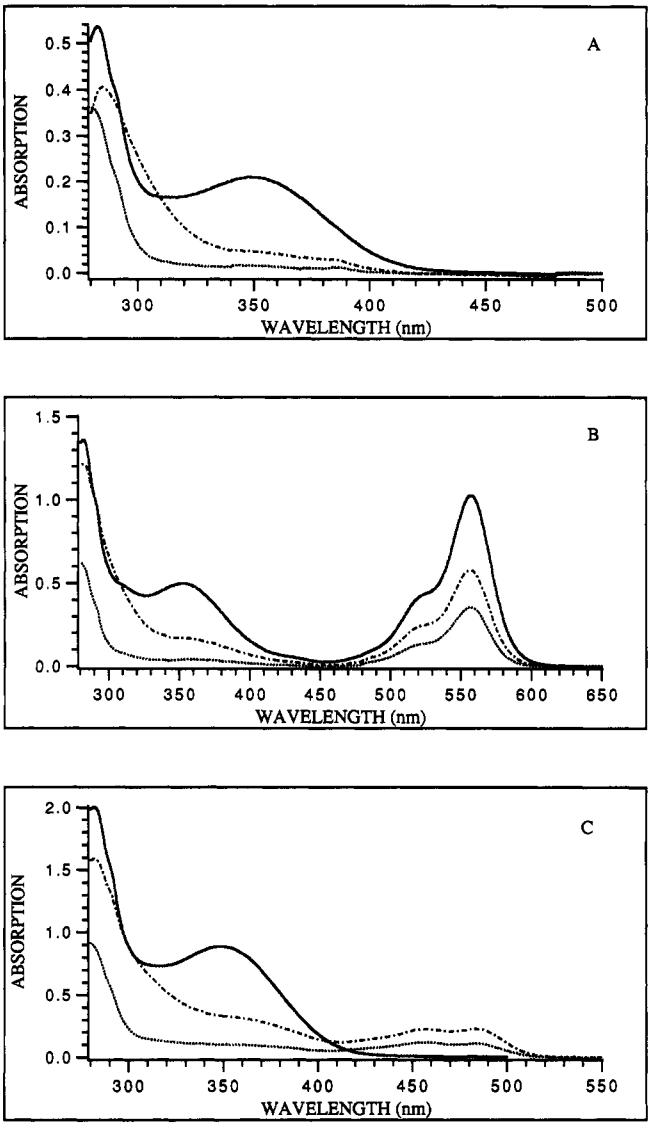

FIGURE 3: Absorption spectra of (A) NVOC actin, (B) TMR-labeled caged G-actin, and (C) caged fluorescein-labeled caged G-actin (solid line, caged; dotted line, decaged pellet; dash–dotted line, decaged supernatent).

photoactivation, caged actin preparations were centrifuged at 100000*g* for 60 min to remove any filamentous actin.

Burtnick (1984) has reported that G-actin labeled at lysine 61 with FITC cannot polymerize in F-buffer, although Miki (1987) found this deficiency could be overcome in the presence of phalloidin, which is believed to stabilize actin–actin contacts close to lysine 61 (Lorenz et al., 1993). When a 1.5 mol equiv excess of phalloidin is added to 8 μM caged G-actin for 90 min at 20 °C, analysis of the supernatant and pellet fractions from the spin-down assay by SDS–PAGE (Figure 4) shows that almost all of the caged G-actin polymerizes, consistent with the idea that NVOC-Cl modifies lysine 61 and/or other residues that lie between subdomains II and IV of G-actin (Lorenz et al, 1993).

Spectroscopic Characterization of TMR-Labeled Caged G-Actin and Caged Fluorescein-Labeled Caged G-Actin. The yield of TMR-labeled caged G-actin was found to be higher if G-actin was first treated with the fluorophore and then modified with NVOC-Cl. This may be a result of NVOC labeling of lysine 372 which may hinder modification of the thiol. The absorption spectrum of the TMR-labeled caged actin is shown in Figure 3B (solid line). The near-ultraviolet absorption band of the NVOC group is clearly separated from

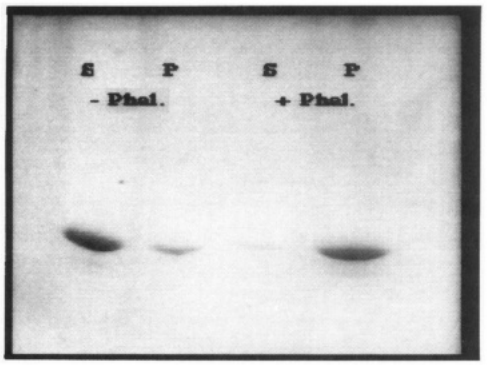

FIGURE 4: SDS–PAGE analysis of the 100000*g* supernatent and pellet fractions of 8 μM caged G-actin in PIPES-based F-buffer in the absence and presence of 12 μM phalloidin.

the S_0–S_1 transition of the TMR group. However, TMR does absorb some light in the near-ultraviolet region, since an orange fluorescence was observed from TMR-labeled caged G-actin during the photoactivation reaction (result not shown). The absorption spectrum of a lightly labeled caged fluorescein maleimide-labeled caged G-actin is shown in Figure 3C (solid line); the S_0–S_1 visible absorption band of fluorescein is lost in the lactone form and reappears after the photoisomerization reaction (Mitchison, 1989) Figure 3C, dotted and dash–dotted lines).

Photoactivation of G-Actin from Caged G-Actin. The extent of photoactivation of 500 μL of a 25 μM solution of caged G-actin in a PIPES-containing F-buffer, pH 6.9, with 5 mM DTT as a function of near-ultraviolet irradiation time is shown in Figure 5. In this reaction time course, 50 μL aliquots of the protein sample were removed at the defined exposure times and, after a 90 min period at 20 °C in the dark to allow for polymerization, the samples were centrifuged in an airfuge at 22 psi for 60 min at 4 °C to pellet F-actin. The supernatent was removed and the pellet dissolved in 50 μL of F-buffer. SDS–PAGE electrophoresis was then performed on the supernatent and pellet fractions. A visual inspection of the actin bands shows about 50% of polymerization competent G-actin is released after 2 min of irradiation and 95% after 12 min. Evidently caged G-actin, as I describe it, is polymerization incompetent not because the NVOC-Cl reaction produces denatured actin but because one or more amino acid residues essential for the polymerization reaction have been protected with the NVOC group, and these residues are removed following irradiation with near-ultraviolet light, liberating polymerization competent G-actin. From an analysis of four different caged G-actin preparations, the photoactivation yield of F-actin following a 12 min irradiation period was found to be between 60% and 95%. Evidence of cross-linked and degraded protein products was found in caged G-actin samples subjected to very long periods of ultraviolet irradiation, whereas photoactivation reactions conducted at pH 8.0 or higher gave a lower yield of polymerizable G-actin, as did caged G-actin stored for more than a week at 4 °C (data not shown).

As was discussed earlier, the absorption spectrum of caged G-actin shows that the low-energy S_0–S_1 spectrum of NVOC (Figure 3A, solid line) is centered at 350 nm where it is well separated from the protein absorption. Upon irradiation of caged G-actin with near-ultraviolet light, the intensity of the 350 nm band is significantly reduced, presumably because of a photoisomerization of the (2-nitrophenyl)ethyl group to the corresponding 2-nitrosoacetophenone as illustrated in Figure 1. A caged G-actin sample irradiated for 10 min was separated, after a 90 min polymerization period, into its polymerization competent and incompetent fractions by high-speed centrifugation. The absorption spectrum of the polymerized F-actin fraction (Figure 3A, dotted line) shows a marked attenuation in the absorption intensity beyond 300 nm; the residual near-ultraviolet-absorbing species may originate from NVOC groups that have not undergone the photoisomerization reaction, in which case over 90% of the NVOC groups are removed from caged G-actin. On the other hand, the absorption spectrum of irradiated caged G-actin which does not sediment following a high-speed centrifugation Figure 3A, dash–dotted line) is red-shifted with respect to that of NVOC and displays a new strong absorption band between 300 and 340 nm which is likely to originate from the water soluble nitrosoacetophenone photoproduct or its adduct with DTT.

As I showed earlier, caged G-actin in F-buffer in the presence of phalloidin is capable of polymerizing into filaments (Figure 4); the in vitro motility assay was used to show that these stabilized filaments move at a velocity of 3 μm/s on HMM, which is similar to that found for unlabeled F-actin stabilized with TMR-phalloidin (data not shown). Evidently NVOC labeling of G-actin has little or no effect on the ability of phalloidin-stabilized caged G-actin filaments to interact with HMM; nor does it affect the ATPase activity of HMM. Similar results have been found with other chemical modifications of G-actin (Prochniewicz & Yanagida, 1990). F-actin filaments prepared from photoactivated F-actin also form filaments that interact with HMM as shown by TMR-phalloidin staining. In this case, there was also no change in the sliding velocity of 3 μm/s (Figure 6), which suggests that for F-actin photogenerated from caged G-actin, chemomechanical transduction activity is unaffected by the photochemical reaction.

Photoactivation of TMR-Labeled Caged G-Actin and Caged Fluorescein-Labeled Caged G-Actin. TMR-labeled caged G-actin is also capable of being photoactivated following

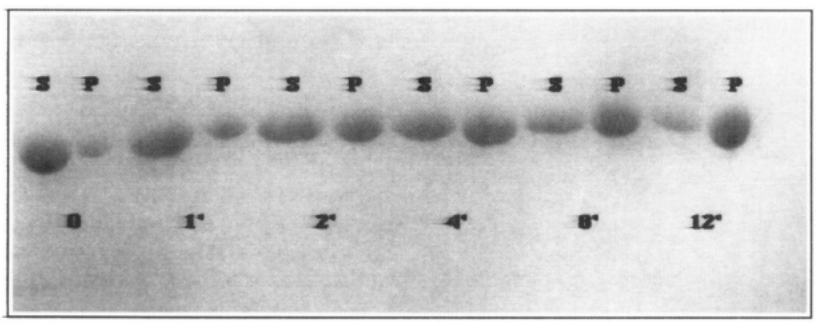

FIGURE 5: SDS–PAGE analysis of the 100000*g* supernatent and pellet fractions of 20 μM caged G-actin in PIPES-based F-buffer as a function of near-ultraviolet irradiation time.

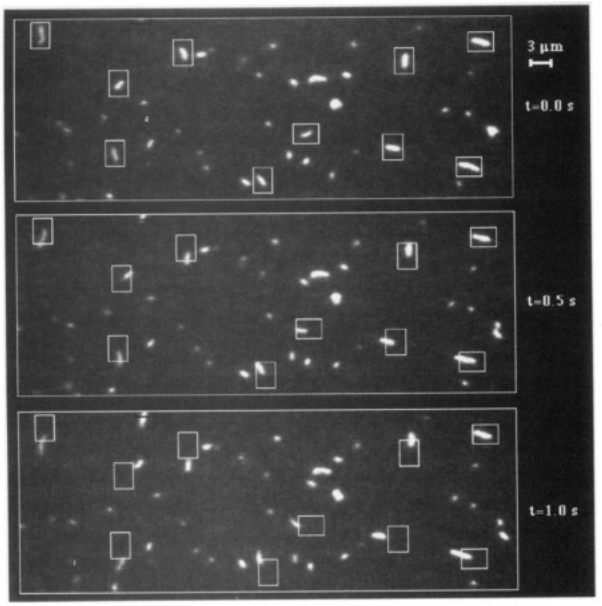

FIGURE 6: In vitro motility assay of TMR-phalloidin-labeled decaged F-actin filaments on a heavy meromysoin surface in the presence of 1 mM ATP. Sliding of filaments was monitored for 1 s. The relative positions of several filaments are shown at $t = 0$, 0.5, and 1.0 s. The average sliding velocity of the filament was calculated to be 3.0 μm/s.

irradiation with near-ultraviolet light. In the spin-down assay, SDS–PAGE analysis of Coomassie-stained protein in the supernatent and pellet centrifugation fractions of photoactivated TMR-labeled caged G-actin shows 60% of the TMR-labeled caged G-actin was liberated during the 10 min irradiation period (Figure 7), whereas analysis of the TMR maximum absorption intensity of the supernatent and pellet fractions suggests only 40% of the actin is polymerization competent (Figure 3B). This discrepancy can be explained if TMR-IA labels G-actin at more than one residue, which is expected to yield a G-actin conjugate with a higher critical concentration than that of singly labeled actin; the absorption spectrum of photoactivated TMR-labeled F-actin is similar to that of TMR-labeled G-actin (Tait & Frieden, 1984). The ratio of the absorption values at 556 and 350 nm increases following photoactivation, presumably because of removal of NVOC molecules from caged G-actin. Photoactivated TMR-labeled F-actin retains its fluorescence, a property that should be useful to image the relaxation kinetics of G-actin concentration jumps in living cells. Caged fluorescein maleimide-labeled caged G-actin can be photoactivated to fluorescein maleimide-labeled F-actin (Figure 7) with about a 60% efficiency following a 10 min irradiation period as judged by the spin-down assay. Interestingly, fluorescein fluorescence is generated within seconds of near-ultraviolet illumination of the caged G-actin conjugate, whereas complete activation of caged G-actin requires up to 10 min of illumination; evidently, although an identical photo-deprotection group is used in the protection of fluorescein and actin, photocleavage of the ether derivative is much more rapid than that of the carbamate derivative.

DISCUSSION

NVOC-Cl, the photo-deprotection reagent used in this study, was originally developed by Patchornik's group for use in solid-phase peptide synthesis and carbohydrate chemistry (Amit et al., 1974). NVOC-Cl readily reacts with amino

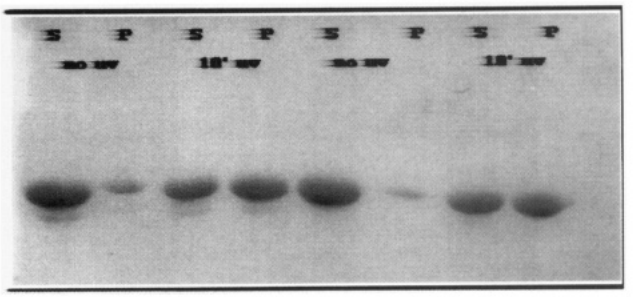

FIGURE 7: SDS–PAGE analysis of the 100000*g* supernatent and pellet fractions of photoactivated TMR-labeled caged G-actin (lanes 1–4) and caged fluorescein-labeled caged G-actin (lanes 5–8).

groups of lysine residues in amino acids, peptides (Fodor et al., 1991), and proteins (present work) in basic, aqueous solution to form the corresponding carbamate. This property suggested to me that NVOC-Cl could serve as a site-directed photo-deprotection reagent of essential lysyl residues of proteins. Lysine 61 of G-actin forms part of an actin–actin interface in F-actin, and its modification with fluorescein isothiocyanate prevents G-actin polymerization in physiological salt solutions [Burtnick, 1984; Miki, 1987; although see McKenna et al. (1985) for contradictory results]. On the basis of these earlier findings, reaction conditions were established to prepare a NVOC-labeled G-actin conjugate that is unable to undergo salt-induced polymerization, presumably by modification of the amino group of lysine 61. Deprotection of the NVOC group from caged G-actin was achieved using near-ultraviolet irradiation with subsequent recovery of polymerization activity. Photoactivation required the presence of 5 mM DTT to limit secondary reactions and photoproducts.

Caged protein conjugates such as caged G-actin are much more easily prepared using the NVOC-Cl conjugation approach compared to the use of the photocleavable cross-linking reagents introduced by Senter et al. (1985) and Marriott et al. (1992). In fact, the methodology described in this report can be applied to cage any protein whose activity is dependent on the presence of a lysine residue, although it is not known if other amino acid residues such as serine or threonine are also modified with NVOC-Cl. A number of factors must be considered in designing an optimum reaction condition for the preparation of caged G-actin or other caged proteins. First, the labeling should not be so high that the protein conjugate is denatured or becomes insoluble; second, if the labeling ratio is too low, the yield of caged G-actin will also be low since, in the case of G-actin, the essential lysine residue that needs to be modified may not be the most reactive in the protein; third, if the G-actin is overlabeled, then NVOC groups may still remain on essential lysine residues after irradiation, resulting in a poor photoactivation yield. Following the workup and photo-deprotection of caged G-actin as described in the methods section, an optimal labeling ratio of three to five NVOC groups/actin monomer was determined. This degree of labeling requires a 40-fold excess of NVOC-Cl over G-actin in a mildly basic buffer between pH 8.5 and 9.5. The photoactivation reaction, on the other hand, was found to increase in a neutral to slightly acidic pH buffer, which would be consistent with a photoisomerization mechanism that includes a protonation step (McCray & Trentham, 1989). Inclusion of DTT to 5 mM was necessary to decrease secondary reactions of the nitrosoacetophenone photoproduct which leads to inactivation of G-actin and/or chemical cross-linking. The

absorption spectrum of F-actin photoactivated from caged G-actin and SDS–PAGE analysis suggest that over 90% of the NVOC groups can be removed from G-actin following a 10 min period of near-ultraviolet irradiation, a yield that is comparable to the photo-deprotection efficiency of NVOC from amino groups in amino acids and peptides (Fodor et al., 1991). Taken together, these results lend support to the hypothesis that polymerization competent G-actin is derived from caged G-actin by an efficient photo-deprotection reaction that removes up to five NVOC groups. Characterization of photoactivated F-actin by the in vitro motility assay and electron microscopy (data not shown) showed that light-based photoactivation of caged G-actin is an effective method to trigger polymerization of G-actin in solutions that normally promote filament assembly and reduce the level of G-actin to its critical concentration.

Fluorescent labeling of G-actin at cysteine 374 provides a convenient way to follow the distribution and dynamics of F-actin filaments in cells (Wang & Taylor, 1979). The results presented herein suggest only 60% of TMR-labeled caged G-actin was photoactivated following a 10 min irradiation period; the unpolymerized G-actin showed evidence of having more than one TMR group/actin monomer. Although TMR-labeled G-actin was purified by a round of polymerization–depolymerization, this procedure used labeled G-actin at 50 times the critical concentration, a condition that may allow, what one might call, poor G-actin to polymerize. Better preparations of polymerization competent TMR-labeled actin may be obtained by reducing the fluorescent labeling ratio or through choice of a fluorescent dye known to have minimal effects on actin polymerization, such as Prodan (Marriott et al., 1988). Despite these reservations, TMR-labeled caged G-actin and caged fluorescein-labeled caged G-actin should be useful reagents to generate concentration jumps of G-actin in living cells and their fluorescence exploited in order to image the fate of the G-actin. Investigations along these lines are now underway.

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