PHOTOMODULATION OF THE NUCLEATING ACTIVITY OF A PHOTOCLEAVABLE CROSSLINKED ACTIN DIMER

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SUMMARY

The ability to generate substrate concentration jumps through photo-deprotection of amine, carboxyl and phosphate groups has been an important development for investigations of protein activity in complex systems. To broaden the versatility and applications of photo-deprotection techniques for the photomodulation of protein activity we describe the synthesis and characterisation of a reagent for generating free thiol from thioether groups and a related photocleavable, heterobifunctional crosslinking reagent. Chemical and spectroscopic studies of a model thiol protected derivative were used to show some features of thiol group photo-deprotection. To demonstrate how the photocleavable crosslinking reagent may be used to modulate the activity of proteins we investigated the effect of light on the nucleating activity of crosslinked actin dimer; thus following near-ultraviolet irradiation of the actin dimer the crosslink was cleaved, presumeably at the thioether bond, resulting in the concomitant dissociation of dimer, loss of nucleating activity and creation of a concentration jump of polymerisable G-actin monomer. On the basis of this initial study we discuss applications and limitations of these reagents for the photomodulation of protein activity *in vitro* and *in vivo*.

INTRODUCTION

Light-mediated chemical bond cleavage of certain nitrobenzyl derivatives of biomolecules ("caged compounds") provides a simple and effective method to generate concentration jumps of substrates and ligands in complex biological medium (for a review see McCray & Trentham, 1989). Here we present a new photocleavable, heterobifunctional crosslinking reagent that can be used to modify the activity of a protein through covalent complex formation with some unrelated macromolecule (Senter *et al*, 1985); upon irradiation of the complex with near ultraviolet light, the crosslink is cleaved resulting in dissociation of the macromolecular complex and restoration of protein activity. In a similar manner, if the activity of a protein is enhanced in

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an artificial crosslinked complex, then, following photocleavage of the crosslink, dissociation of the complex will lead to a normal level of protein activity. Photocleavable crosslinked protein complexes of this nature may be regarded, somewhat loosely, as *caged* proteins. Our interests in developing these reagents and the technique of *photomodulation of protein activity* center on their application for generating rapid jumps of protein activity *in vivo*. In particular, we plan to use this technique to create rapid, localized concentration jumps of functional actin and actin binding proteins from their inactive crosslinked complexes within living cells and then follow the dynamics of actin filament assembly or disassembly using fluorescence image microscopy.

EXPERIMENTAL PROCEDURES

All chemicals and solvents were obtained from Tokyo Kasei Company (Tokyo, Japan). Solvents were stored over molecular sieves. Mass spectra were recorded using a Hitachi M-80 instrument. 400 MHz NMR spectroscopy (JOEL, JNM-GX 400) was performed with highly purified samples dissolved in deuterated chloroform with tetramethylsilane as an internal reference. Infrared spectra of samples suspended in Nujol were recorded with a JASCO A202 instrument. Absorption spectra were recorded using a Shimadzu UV350A double-beam spectrophotometer and fluorescence measurements performed on an Hitachi F-4010 instrument.
Syntheses. 4-bromomethyl-3-nitrobenzoic acid (BNBA): 5 g of 4-bromomethylbenzoic acid was gradually added to 50 ml of fuming nitric acid at -11°C. After a further two hours the reaction mixture was poured onto crushed ice and the product recrystalised from methylene chloride-heptane. Mass spectrophotometric analysis of the product showed the expected molecular ions of 269 and 271 m/z and a debrominated fragment of 190 m/z.
4-bromomethyl-3-nitrobenzoic acid succinimide ester (BNBA-SE): 2.5 g of BNBA was dissolved in 20 ml of dry acetonitrile followed by 1.05 g N-hydroxysuccinimide and 2.15 g of dicyclohexylcarbodiimide and the reaction mixture stirred overnight at 20°C. After removal of the dicyclohexylurea and solvent, the product was recrystalised from methylene chloride-heptane yielding 2.02 g of white solid. Mass spectrophotometric analysis revealed molecular ions of 356 and 358 m/z debrominated fragment of 277 m/z. NMR: δ 2.94 (4H, s, succinimide methylene); δ 4.87 (2H, s, bromoalkane methylene); δ 7.778 (1H, d, aromatic); δ 8.339 (1H, dd, aromatic); δ 8.783 (1H, d, aromatic).
4-(2-hydroxyethylmercaptylmethyl)-3-nitrobenzoic acid: 100 mg of BNBA and a 5 fold molar excess of B-mercaptoethanol in 5 ml tetrahydrofuran was heated to reflux for 2 days. The product was purified by silica gel chromatography in chloroform-methanol (2:1 v/v).
Crosslinking of F-actin: Crosslinking was achieved by incubating a solution of F-actin at 25 μM in thiol free F-buffer (2 mM Tris/HCl, 0.1 M KCl, 0.2 mM ATP, 0.2 mM calcium chloride, 1 mM magnesium chloride, pH 8.0) with BNBA-SE at 12.5 μM for 1 hour at 20°C. The protein was centrifuged at 300,000 g, the pellet resuspended in g-buffer (2 mM Tris/HCl, 0.2 mM ATP, 0.2 mM calcium chloride, pH 8.0) containing 1 mM mercaptoethanol, dialysed against the same buffer for 24 hours at 4°C. Following a short sonication at 4°C the protein was re-centrifuged at 300,000 g, the supernatant set aside and the entire depolymerisation procedure repeated. Crosslinked actin dimer was purified from the monomer and higher order oligomers contained in a combined supernatent fraction by Sephadex G-150 size exclusion chromatography (1.6x 90 cm) in G-buffer.
Photocleavage of actin dimer: Photocleavage was performed on 400 μL samples of purified BNBA-actin dimer at a concentration of 0.85 μM in G-buffer containing 5 mM dithiothreitol at 20°C in (4 x 10 mm) quartz cuvettes using the direct and unmodified output of pulsed 355 nm laser light of a Nd-YAG laser (Spectra-Physics) operating at maximum power.

RESULTS AND DISCUSSION

The thiol photo-deprotection reagent, 4-bromomethyl-3-nitrobenzoic acid (BNBA), and the related crosslinking derivative, 4-bromomethyl-3-nitrobenzoic acid succinimide ester (BNBA-SE), are shown in Fig. 1a; the nitrobenzyl group introduces a photocleavable character into the molecule through excitation of its forbidden n-π* transition (McCray & Trentham, 1989; Walker *et al*, 1988). Photocleavage properties of thioether derivatives of BNBA were investigated using the model thiol protected compound 4-(2-hydroxyethylmercaptylmethyl)-3-nitrobenzoic acid. Three experimental observations are consistent with the conclusion that near ultraviolet irradiation of this compound results in the cleavage of the crosslink at the thioether bond: first, irradiation decreases the intensity of the 1530 cm⁻¹ infra-red band of the nitrobenzyl group (data not shown); second, irradiation results in the appearance of a new, red shifted absorption spectrum with the concomitant loss of starting material [Fig. 2; also compare the spectra of pre- and post-irradiated N-(α-carboxy-2-nitrobenzyl)carbamoyl choline (Milburn *et al*, 1989)]; third, irradiation of solid, odorless samples of the model compound resulted in the emanation of an unpleasant mercaptan odor, a sign that the free thiol was liberated. Certain similarities in the photocleavage properties of the nitrobenzylether group (Zehavi *et al*, 1971) with the nitrobenzylthioether compound described above points to a common reaction mechanism, which is known to occur via photoisomerization of the nitrobenzyl group for the former case (McCray & Trentham, 1989; Zehavi *et al*, 1971). We note that the application of BNBA as a thiol deprotection reagent may be limited by two possible secondary reactions of the photoproduct, 4-formyl-3-nitrosobenzoic acid; first, through nitroso- group coupling of the deprotected thiol (this reaction can be prevented in the presence of nitroso- group scavengers (Walker *et al*, 1988)), and second, through its transformation to azo-benzoic acid-2-2'-dicarboxylic acid, which may act as a light filter (Pillai, 1980). We have been unable to observe the long wavelength absorption band expected for the azo derivative (Ried & Wild, 1954) which suggests this reaction does not occur to the same extent as that found for unsubstituted 2-nitrobenzyl derivatives (Pilai, 1980), a possible reflection of the influence of the substituted carboxylic acid on the reactivity of the nitroso and aldehyde groups. Taken together these results suggest that BNBA may be used to inhibit protein activity through modification of essential cysteine residues although these conjugates may be reactivated following irradiation with near ultraviolet light.

Having shown some evidence that mercaptans can be photo-deprotected from 4-bromomethyl-3-nitrobenzoic acid derivatives, we synthesized BNBA-SE as a photocleavable crosslinking reagent for the photomodulation of protein activity. Since our interests centre around the mechanisms of actin polymerisation we decided to use this reagent to modulate the nucleating activity of crosslinked actin dimer (Knight & Offer, 1980; Mockrin & Korn, 1982; Handel *et al*, 1990). Purified BNBA crosslinked actin dimer was prepared as described earlier.
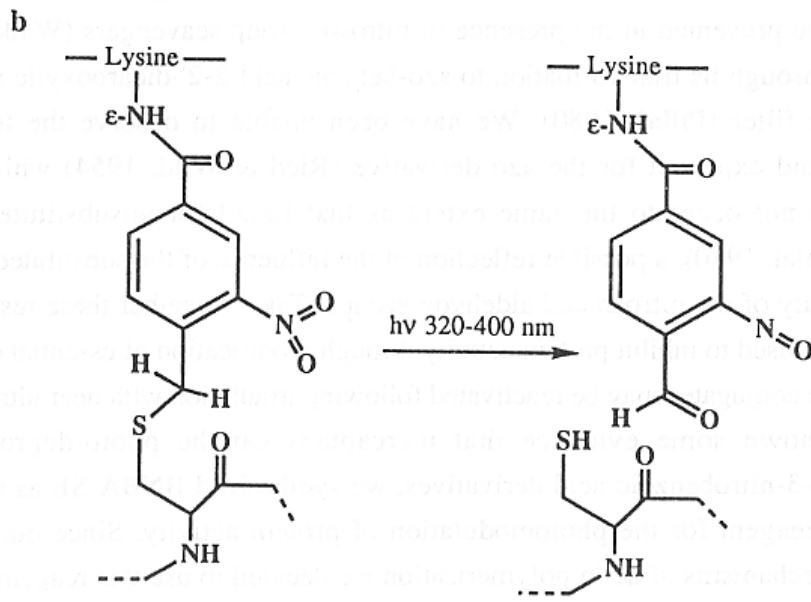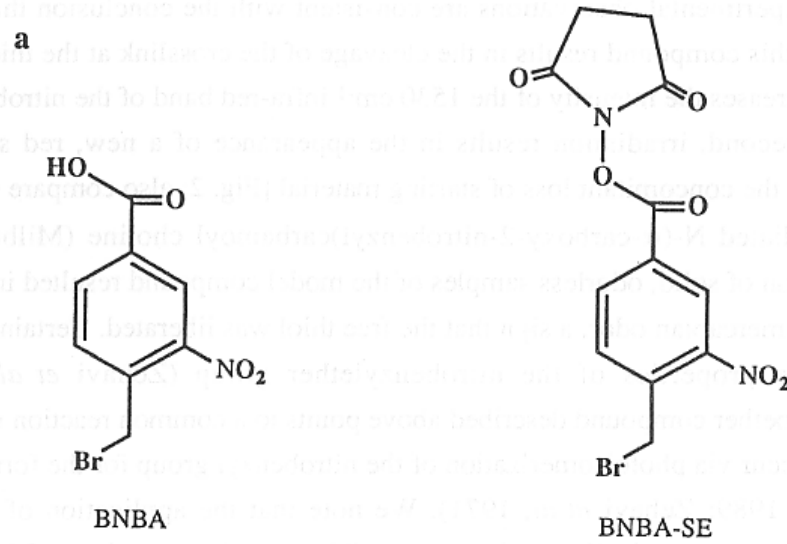
Fig. 1a: Molecular structures of BNBA and BNBA-SE.

Fig. 1b: Proposed photocleavage reaction of BNBA crosslinked protein complexes.
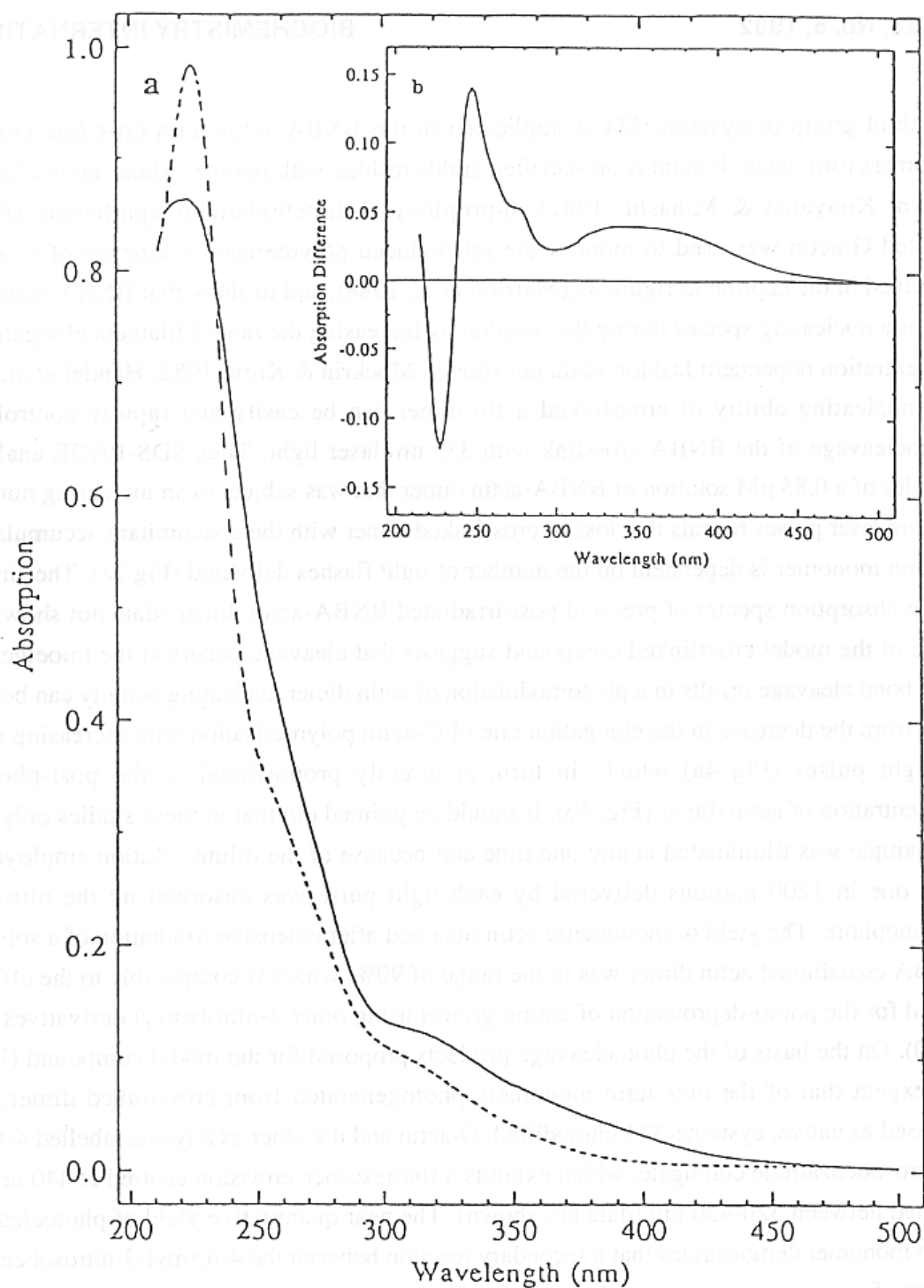
Fig. 2a: Absorption spectra of a 100 μM solution of 4-(2-hydroxyethylmercaptylmethyl)-3-nitrobenzoic acid dissolved in 0.1 M sodium phosphate buffer, pH 7.2 before (dotted line), and after (continuous line), irradiation for 100 minutes with 365 nm light as obtained from a hand held mercury lamp.

Fig. 2b: Absorption difference spectrum between the pre- and post irradiated samples described above.

The thiol group of cysteine-374 is implicated in the BNBA actin-actin crosslink since few oligomers form when F-actin is pre-labelled at this residue with pyrene iodoacetamide (data not shown; Kouyama & Mihashi, 1981). 6-propionyl-2-dimethylaminonaphthalene (Prodan) labelled G-actin was used to monitor the salt-induced polymerisation kinetics of G-actin as described in the caption to figure 4a (Marriott et al, 1988), and to show that BNBA-actin dimer acts as a nucleating species during the reaction by increasing the rate of filament elongation in a concentration dependent fashion (data not shown; Mockrin & Korn, 1982; Handel et al, 1990). The nucleating ability of crosslinked actin dimer can be easily and rapidly controlled by photocleavage of the BNBA crosslink with 355 nm laser light. Thus SDS-PAGE analysis of samples of a 0.85 μM solution of BNBA-actin dimer that was subject to an increasing number of 355 nm laser pulses reveals that loss of crosslinked dimer with the concomitant accumulation of G-actin monomer is dependent on the number of light flashes delivered (Fig 3a). The similarity in the absorption spectra of pre- and post-irradiated BNBA-actin dimer (data not shown) with those of the model crosslinked compound suggests that cleavage occurs at the thioether bond. That bond cleavage results in a photomodulation of actin dimer nucleating activity can be clearly seen from the decrease in the elongation rate of G-actin polymerisation with increasing number of light pulses (Fig 4a) which, in turn, is directly proportional to the post-photolysis concentration of actin dimer (Fig. 4b). It should be pointed out that in these studies only part of the sample was illuminated at any one time and because of the dilute solution employed, less than one in 1200 photons delivered by each light pulse was absorbed by the nitrobenzyl chromophore. The yield of monomeric actin obtained after extensive irradiation of a solution of BNBA crosslinked actin dimer was in the range of 90%, which is comparable to the efficiency found for the photo-deprotection of amino groups using other 2-nitrobenzyl derivatives (Pillai, 1980). On the basis of the photocleavage products proposed for the model compound (Fig. 1b) we expect that of the two actin monomers photogenerated from crosslinked dimer, one is released as native, cysteine-374 unmodified, G-actin and the other as a lysine labelled 4-formyl-3-nitrosobenzamide conjugate, which exhibits a fluorescence emission centred at 440 nm when excited between 320-430 nm (data not shown). The near quantitative yield of photocleaved G-actin monomer demonstrates that a secondary reaction between the 4-formyl-3-nitrosobenzamide group of one actin monomer with the photo-deprotected thiol on the other monomer does not occur. SDS-PAGE analysis was used to show that the photogenerated actin can polymerise as native actin under physiological conditions to form actin filaments (Fig 3b). This is an interesting result since it suggests that fully polymerisable actin can be photogenerated from BNBA-SE crosslinked actin complexes in which the polymerisation activity of actin is modified or blocked. We are currently engaged in preparing *caged* actin complexes of this nature as well as other inactive yet photomodulatable cytoskeletal protein complexes which we believe, following their photomodulation in living cells, may help us unravel some of the complex mechanisms that lay behind the regulation of actin polymerisation.
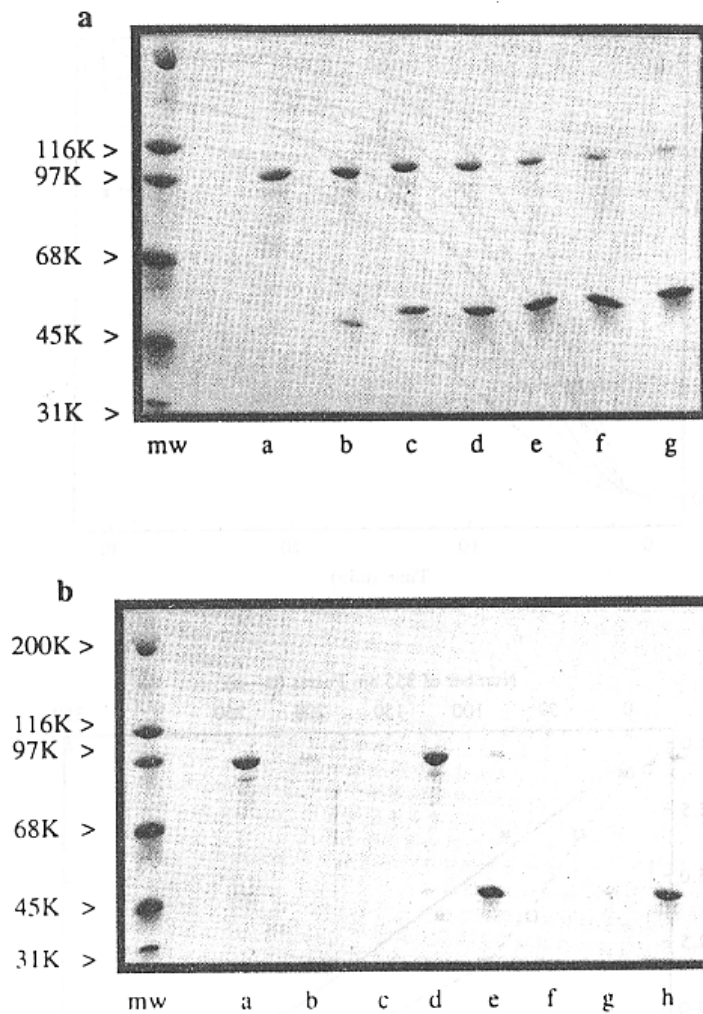
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116K >
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 31K >
        mw    a    b    c    d    e    f    g
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b

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116K >
 97K >

 68K >

 45K >

 31K >
        mw    a    b    c    d    e    f    g    h
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Fig. 3a: Extent of photocleavage of a 0.85 μM solution of BNBA crosslinked actin dimer after irradiation with pulsed 355 nm light (a) 0 pulse; (b) 30 pulses; (c) 60 pulses; (d) 90 pulses; (e) 150 pulses; (f) 250 pulses; (g) 400 pulses.

Fig. 3b: Polymerisability of pre- and post irradiated BNBA crosslinked actin dimer. 95 μL samples of a 0.85 μM solution of BNBA crosslinked actin dimer in G-buffer containing 5 mM dithiothreitol was mixed with either 5 μL of g-buffer or 20x F-buffer. After 2 hours at 20°C the samples were centrifuged at 300,000 g. Supernatent fractions containing G-buffer (a), and F-buffer (c), were decanted and their corresponding pellets (b), and (c) were suspended in 100 μL of G-buffer and F-buffer, respectively. Similarly, 95 μL samples of an 0.85 μM solution of BNBA crosslinked actin dimer in G-buffer containing 5 mM dithiothreitol that had been subjected to 400 laser pulses at 355 nm was mixed with either 5 μL of G-buffer or 20x F-buffer. After 2 hours at 20°C the samples were centrifuged at 300,000 g. The supernatant containing G-buffer (e), and F-buffer (g), were decanted and their corresponding pellets (f), and (h) were suspended in 100 μL of G-buffer and F-buffer, respectively.
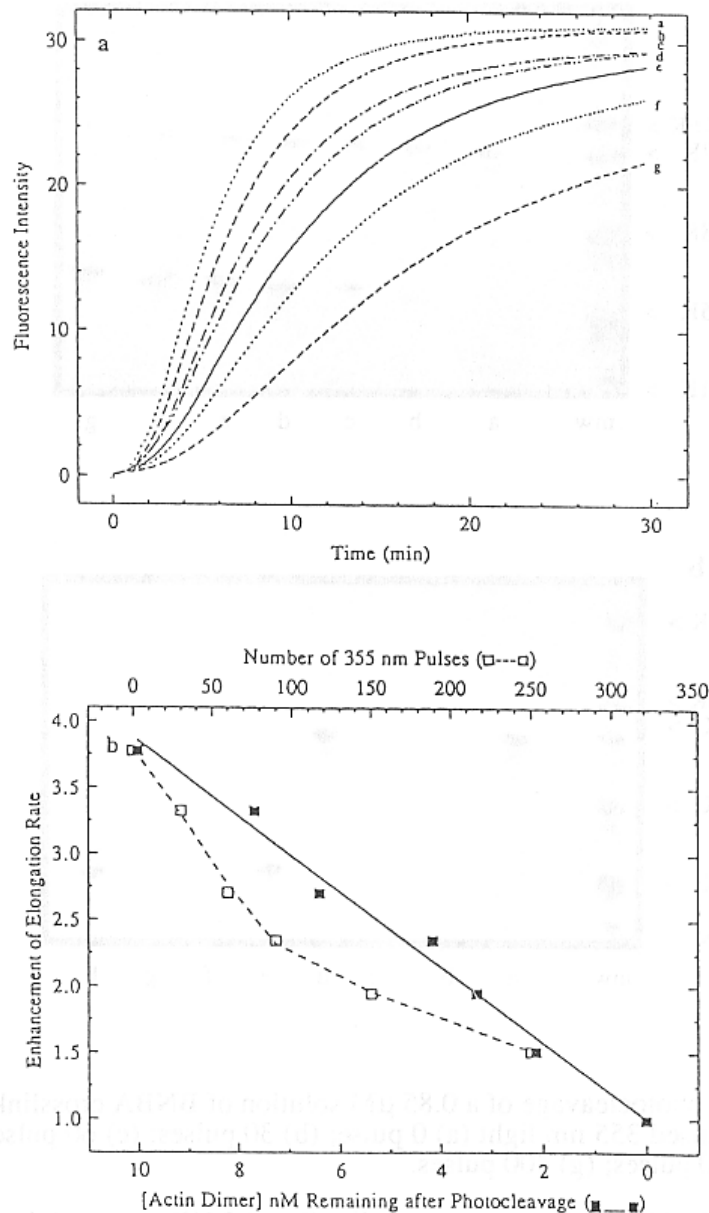
Fig. 4a: Effect of BNBA crosslinked actin dimer and its photocleavage products on the rate of G-actin polymerisation. 195 µL of a 3.4 µM solution of Prodan labelled G-actin in G-buffer at 20°C was mixed with 5 µL of a 0.85 µM BNBA crosslinked actin dimer solution that had been subjected to irradiation with (a) 0 pulse; (b) 30 pulses; (c) 60 pulses; (d) 90 pulses; (e) 150 pulses; (f) 250 pulses with 355 nm light as described in fig 3b, and in sample (g), dimer was replaced with 5 µL of 0.85 µM G-actin. Polymerisation of actin was initiated by the addition of 200 µL of two strength F-buffer and fluorescence emission intensity measured as a function of time with excitation at 400 nm (1.5 nm bandpass) and emission at 450 nm (5 nm bandpass).

Fig. 4b: Dependence of the elongation rate of Prodan labelled G-actin polymerisation (calculated from the data of fig 4a) on (i), the number of light flashes delivered (broken line, open squares) and (ii), the concentration of BNBA crosslinked actin dimer remaining in solution after delivery of a defined number of light pulses (solid line, filled squares). BNBA actin dimer concentration was determined by scanning the polyacrylamide gel described in fig. 3a. The enhancement in elongation rate is relative to that in the absence of actin dimer, the data of which was calculated from curve g in fig. 4a.

Direct photomodulation of biomolecular activity is expected to become an increasingly important technique for investigations of the intracellular function and regulation of proteins in complex systems. Since we have shown that protein activity can be triggered with one thousandth-fold less photocleavage than that required using *caged* substrates (McCray & Trentham, 1989) considerable attenuation in laser excitation power is possible thus limiting irradiation damage to living cells. On a related topic, in microscope based studies employing *caged* proteins the optically thin samples encountered will require photocleavable crosslinking reagents exhibiting higher extinction coefficients than BNBA-SE and also a means to visualize the distribution of the crosslinked protein within the cell. Our approach to this goal is centered around the synthesis of crosslinkers incorporating one or two methoxy groups in the nitrobenzyl ring and a fluorescent tag.

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