Vol. 201, No. 3, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 30, 1994 Pages 1213-1219

PHOTOCLEAVABLE NITROBENZYL-PROTEIN CONJUGATES

Stephen Thompson*, Julia A. Spoors, Marie-Claude Fawcett, and Colin H. Self*

Department of Clinical Biochemistry, The Medical School, Framlington Place, Newcastle Upon Tyne, NE2 4HH, United Kingdom

Received April 5, 1994

We have developed methods to allow the reversible binding of up to 15 nitrobenzyl residues per bovine serum albumin molecule and show 95% of these residues can be removed by exposure to UV light for 10 min. The general non-specific coating method is presented by means of a model system but is applicable to a wide range of proteins with important biological functions. Potentially, any protein could be coated with sufficient photo-removable groups to inhibit its biological function. Its activity may then be restored at will by exposure to UV light removing the coupled 2-nitrobenzyl groups.

Light-mediated chemical bond cleavage of certain nitrobenzyl derivatives provides a simple and efficient method for the remote activation of biomolecules in complex biological situations. Photocleavable 2-nitrobenzyl groups have been widely used as blocking groups in organic synthesis [1] and to 'cage' nucleotides in the study of intra-cellular dynamics [2,3,4]. Similar photocleavable protein crosslinking reagents have also been developed [5,6]. In all of these procedures a single 2-nitrobenzyl group is coupled per substrate molecule, so that the substrate is inactive when coupled, but fully active on decoupling after exposure to UV light.

When the substrate is a large molecule such as an enzyme, antibody or nucleic acid it is unlikely that one coupled nitrobenzyl group will hinder its function unless it is coupled in a highly specific site-directed manner. Mendel et al [7] have prepared a catalytically inactive lysozyme by specifically substituting an aspartyl β-nitrobenzyl ester into the active site of the enzyme. On irradiation with long wavelength UV light fully active lysozyme is obtained. However this procedure requires a detailed knowledge of the enzyme's active site and the site-specific incorporation of an unnatural amino-acid.

An alternative, simpler and more generally applicable procedure, may be to coat a protein with a sufficiently large number of photocleavable residues that it effectively becomes inert. Willner et al [8] have shown that papain activity may be photoregulated when photochromic azo groups (which reversibly flip between their cis

*To whom correspondence should be addressed (Fax: 091 222 6227).

0006-291X/94 $5.00

and trans forms) are anchored to the enzyme backbone. However, the enzyme activity is altered considerably in both the cis and trans forms and the photoregulateable azo groups remain on the protein. In contrast, we have investigated the synthesis and photo-degradation of simple 2-nitrobenzyl alcohol-protein conjugates in which bovine serum albumin (BSA) is non-specifically coated with 2-nitrobenzyl residues.

This paper compares the synthesis of 2-nitrobenzyl alcohol-protein conjugates by two different methods, using either the carbonylating reagent, 1,1' carbonyldiimidazole (CDI) or trichloromethylchloroformate (di-phosgene, DP) to couple the 2-nitrobenzyl alcohol hydroxyl groups to protein amine residues. The subsequent photolysis of the conjugates on exposure to long wavelength UV light is also examined.

METHODS

The coupling of 2-nitrobenzyl alcohol (NBA, Aldrich Chemical Co) and its α-methyl substituted derivative, 1-(2-nitrophenyl)ethanol(NPE) to BSA was studied.

Synthesis of 1-(2-nitrophenyl)ethanol. NPE was synthesised by the reduction of 2-nitroacetophenone with $NaBH_4$ as previously described (2).

Preparation of 2-nitrobenzyloxycarbonyl-protein conjugates

a) **With CDI**. 10mg (0.065mmol) NBA was reacted with 10mg (0.062mmol) of the carbonylating reagent, CDI (Sigma Chemical Co), in 1ml dry dioxan for 2h at 20°C [9]. This solution was then added to 5, 10 or 20mg BSA in 4 ml of 0.1M $NaHCO_3$ pH8.3 and allowed to react for 24h at 20°C. This mixture was dialysed for 16h at 4°C against 0.9% NaCl and then centrifuged at 600g for 10 min to remove insoluble complexes.

b) **With di-phosgene**. The procedure of Senter et al [5] was followed. 31.3μl (0.26mmol) of DP (Fluka Chemicals Ltd) was added to a solution of 40mg (0.26mmol) NBA and 20.6μl (0.26 mmol) pyridine in 1ml of dry dioxan. A white precipitate immediately formed and the reaction was shown to go to completion in 15 min by TLC. The reaction mixture was then evaporated in a stream of nitrogen for 45 min to remove unreacted material and the off-white nitrobenzyloxycarbonyl chloride was resuspended in 1ml of dioxan. Different aliquots of this solution (eg; 0.1, 0.2 and 0.3ml) were then added to varying concentrations of BSA in 4ml of 0.1M $NaHCO_3$ pH8.3 and allowed to react for 24h at 20°C. Each mixture was then dialysed for 16h at 4°C against 0.9% NaCl and white insoluble complexes were removed by centrifugation at 600g for 10 min.

NPE was coupled to protein by the same procedures as NBA but 11 and 44mg of NPE were used instead of 10 and 40mg of NBA.

Photolysis of conjugates. Samples were irradiated in quartz cuvettes at a distance of 0.5cm with UV light for various times at room temperature. After irradiation the samples were dialysed for 16h against 0.9%NaCl to remove photocleaved products. A Spectroline EN-16/F UV lamp (Spectronics Corporation, Westbury, New York) with an emission peak of 365 nm was used as the source of the UV light.

Analysis of conjugates. The absorbance profiles of NBA and NPE protein conjugates between 230 and 350nm were measured on a Unicam 8700 scanning spectrophotometer. The average number of residues of NBA or NPE bound to protein, both before and after exposure to UV light, was estimated by taking the absorbance value of the NBA-BSA and NPE-BSA conjugates at 280nm. The absorbance due to the BSA was subtracted and the amount of NBA or NPE bound could then be calculated by comparison with the absorbance profiles of NBA and NPE standards. Absorbances >300nm are due to the amount of NBA (figure 1) or NPE (figures 2 & 3) coupled to the BSA. The small amount of absorbance >300nm remaining in irradiated NPE-BSA samples (figures 2 & 3) is probably due to contaminating NPE photolysis products. This absorbance can be removed by centrifugation at 110,000g for 30 min without significantly effecting the results. Protein concentrations were measured using bicinchoninic acid solution [10]. The coupling and removal by UV light of NBA or NPE to proteins was confirmed by isoelectric focussing and electrophoresis in non-denaturing conditions using the discontinuous buffer system of Laemmli [11].

RESULTS

NBA was reacted with CDI then coupled to 10mg BSA in 4ml of 0.1M $NaHCO_3$. A clear supernatant was obtained after dialysis and centrifugation and this contained 8mg of BSA as NBA-BSA conjugates. The absorbance profile of a diluted aliquot of these conjugates is shown in figure 1. There is an increase in the absorbance between 240-350nm due to an average of 12.5 NBA residues being bound per protein molecule. Around 7.5 of the residues cleave on exposure to UV light. On increasing the amount of albumin (to 20mg) or reducing the time given for the CDI to react with the alcohol to 1h instead of 2h, then the number of NBA residues bound fell to an average of 4 per protein. All of these were found to cleave from the protein on exposure to UV light.

NBA was also coupled to BSA using di-phosgene. 0.1ml of the nitrobenzyloxycarbonyl chloride in dioxan was added to 5, 10 and 20 mg BSA in 4ml of 0.1M $NaHCO_3$. A large white pellet was obtained after dialysis and centrifugation but the supernatant solution contained the majority of the protein (90%). Spectrophotometry showed that approximately 12/15/17 residues of NBA were bound per BSA molecule in each of the 20/10/5mg BSA samples respectively. On exposure to UV light only slightly more than 60% of the coupled nitrobenzyl residues could be removed.

The nitrosobenzaldehyde byproduct (I, scheme I) produced on the photolysis of NBA conjugates may react with and inactivate proteins [2,12]. It can also transform into azobenzene-2,2'-dicarboxylic acid (II) which can act as an internal light filter hindering effective photolytic cleavage [1]. This may explain why it was not possible to remove all the NBA groups when the BSA was highly substituted. The coupling of α-methyl substituted NBA (NPE) to protein was therefore studied. Its photolysis products should be less reactive and its rate of photolysis should be much faster [2,12].

Attempts were made to couple NPE to BSA with CDI as had been successfully performed with NBA. All of the protein was recovered in the clear supernatant

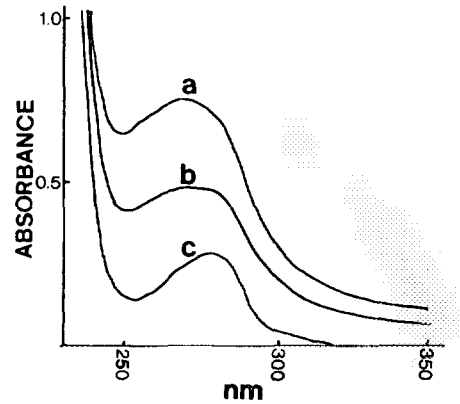
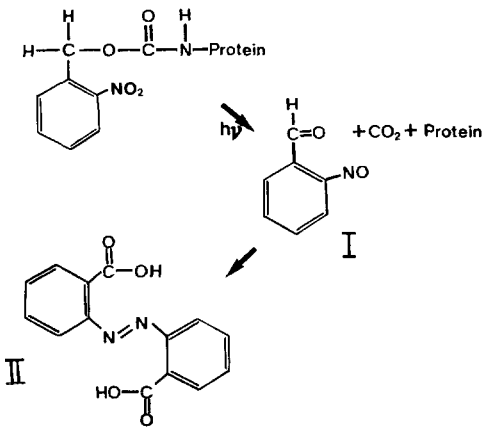


Figure 1. The absorbance profile of an NBA-BSA conjugate before (a), and after (b) irradiation with UV light for 30 min. A profile of a BSA standard with the same protein concentration (0.4 mg/ml) is also shown (c).



Scheme I. The products formed on the hydrolysis of NBA-protein conjugates.

solution, however, the absorbance profile of the supposed conjugates was identical to unconjugated BSA. The experiment was repeated twice but no NPE could be coupled showing, incidentally, that NPE did not non-specifically absorb to BSA. As the CDI coupling procedure was unsuccessful, the di-phosgene procedure was then attempted.

NPE reacted vigorously with di-phosgene to give a yellowish nitrobenzyloxycarbonyl chloride. This was resuspended in 1ml of dioxan and 0.25ml aliquots were added to 25 and 50mg BSA in 4ml 0.1M $NaHCO_3$. On centrifugation of the dialysed conjugates a large white pellet was obtained but all of the protein remained in solution. Spectrophotometry of diluted portions of the clear supernatants (figure 2) showed that there was an average of 14.8 NPE residues bound per protein molecule in the 25mg sample and 3 NPE residues per protein molecule in the 50mg sample. On

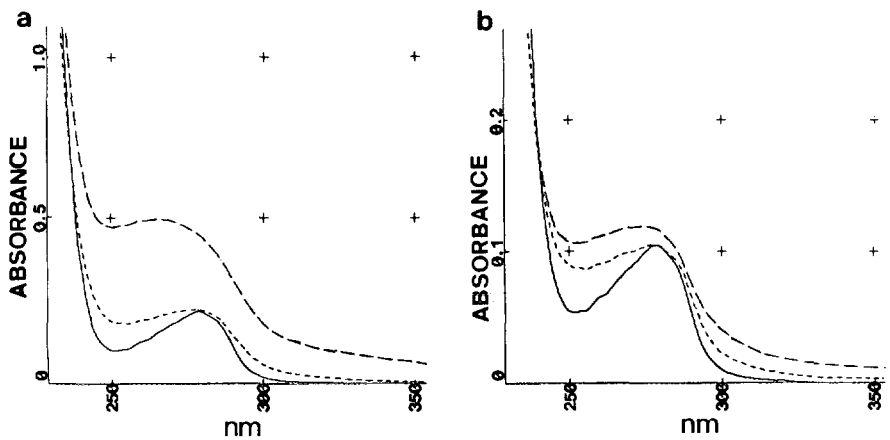


Figure 2. The absorbance profiles of diluted NPE conjugates to 25mg (a) and 50mg (b) BSA before (— — —) and after (- - - - -) irradiation with UV light. Unconjugated BSA standards are also shown (———).

exposure to UV light for 10 min 14/14.8 (fig 2a) and 3/3 (fig 2b) of the residues were cleaved from the protein. This proportional substitution of BSA with NPE residues was confirmed in other experiments by reacting increasing volumes of the nitrobenzyloxycarbonyl chloride solution with BSA. The absorbance profiles of the NPE-BSA conjugates (all diluted to 0.4mg/ml) correspond to an average 3.5, 6.9, 10.8 and 14.5 NPE residues per BSA molecule (figure 3). On treatment with UV light for 10 min, 2.5, 6.0, 10 and 14 of these residues were cleaved. If volumes of 0.4 ml (or greater), of the nitrobenzyloxycarbonyl chloride were reacted with BSA, most of the BSA was lost into the insoluble pellet. The BSA which remained in solution as NPE-BSA conjugates never exceeded an average of 15.3 residues of NPE per BSA molecule.

In order to confirm the spectrophotometric results the binding and removal of the NPE groups to BSA was also examined by electrophoresis in 8% polyacrylamide gels (figure 4). The BSA molecules migrated faster and as a more diffuse band as the number of NPE residues coupled to the BSA increased (lanes 2-4). This showed both that the NPE was coupling to the BSA amine residues, effectively leaving the BSA with a higher negative charge, and that the labelling was heterogeneous. On exposure to UV light for 10 min (lane 5) nearly all the NPE groups cleaved and the regenerated BSA migrated as control unlabelled BSA (lane 1). Two BSA bands are seen in each lane as some of the BSA migrates as a dimer. This effect is very common when proteins are electrophoresed under non-denaturing conditions.

Heavily coated NPE-BSA conjugates were also separated by isoelectric focussing in pH 4 to 8 gradients. The pI of the BSA altered from an initial value of pI 5.05 to pI

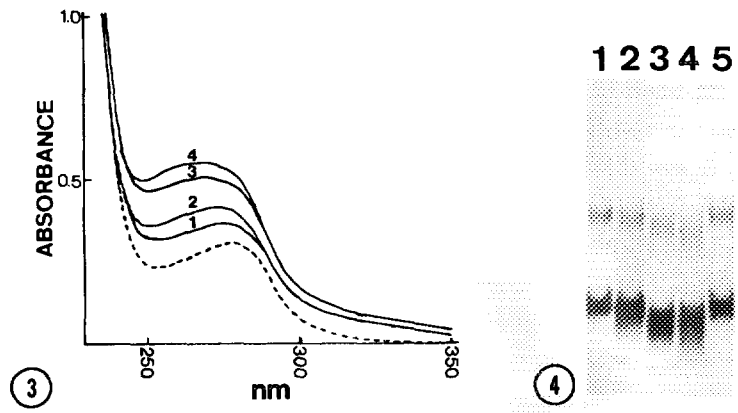


Figure 3. The absorbance profiles of the NPE-BSA conjugates (1, 2, 3 and 4) obtained when 0.1, 0.2, 0.3 and 0.2ml of NPE carbonylchloride solution were mixed with 40, 40, 40 and 20mg of BSA, respectively. All the samples gave virtually identical profiles after exposure to UV light and one is given for comparison (- - -).

Figure 4. A Coomassie blue stained polyacryamide gel of NPE-BSA conjugates. Lane 1 is control unlabelled BSA. Lanes 2-4 are NPE-BSA conjugates with 3.5 (lane 2), 10.8 (lane 3) and 14.5 (lane 4) NPE residues per BSA molecule. Lane 5 is the sample in lane 4 after 10 min exposure to UV light. All samples had approximately 6μg of protein loaded per lane.

4.87 when it was heavily coupled and returned to 5.05 when the NPE-BSA samples were irradiated with UV light. As a pI change of 0.01 is roughly equivalent to a change in charge of 1 unit for most proteins (13) this would approximate to 18 NPE substitutions. Given these assumptions and the very different methodology this value is in very good agreement with the results obtained by spectrophotometry.

Although wavelengths >340nm leave cell lines fully viable after 7 min exposure [6], irradiation with UV light may be damaging to some proteins. To investigate how quickly NPE could be removed from NPE-BSA conjugates a highly coupled NPE-BSA conjugate was exposed to UV light and aliquots were removed after 0,1,2,5 and 10 min. On spectrophotometry the NPE-BSA aliquots were found to have an average of 15.1, 10.8, 9.0, 5.0 and 0.8 residues of NPE per BSA molecule. Approximately, one-third of the coupled NPE residues were therefore cleaved from the BSA in 1-2 min and two-thirds after 5 minutes.

DISCUSSION

We show in this report that it is possible to reversibly bind considerable numbers of nitrobenzyl residues to BSA. The di-phosgene coupling procedure worked well with both alcohols, and is probably applicable for the coupling of all small molecules with hydroxyl groups to proteins. However, the carbonylating reagent, CDI, only coupled the primary alcohol NBA to protein. No coupling of the secondary alcohol, NPE, to protein could be detected. This was presumably due to steric hindrance preventing the CDI from reacting with the hydroxyl group of the NPE.

Although both alcohols can be coupled to proteins, in practice only the α-methyl substituted alcohol, NPE, would be used because of its faster photolysis rates and because of the lower toxicity of its photolysis products. The 2-nitrosoacetophenone by-product of NPE-photolysis may still be damaging to some proteins but this effect can be eliminated by the addition of low levels of chemical compounds which contain thiol groups (2). If less than 8 NPE residues are bound, then all the residues cleave on exposure to UV light. If 8-15 residues are bound then about 95% of NPE residues are removed on irradiation by UV light for 10 min. This compares well with previous studies as >85% of photolabile mono-substituted antibody-toxin conjugate was cleaved by a 7 min exposure to UV light [5,6]. Shorter exposure times may well be possible as considerable amounts of NPE were removed from a highly coupled NPE-BSA conjugate on 1-5 min irradiation.

Photocleavable 2-nitrobenzyl residues have been used to protect amino acid amine and carboxyl groups in the synthesis of specific peptides [1,14,15] and similarly protected nucleotides have been used in the study of intracellular kinetics [2,3,4,12]. More recently novel photocleavable conjugates have been synthesized [5,6,16]. Antibody-toxin conjugates in which toxins are specifically linked via one of their amine groups through 2-nitrobenzyloxycarbonyl bridges to antibodies have been made [5]. On

exposure to UV light active toxin is released to kill the targetted cells [6]. A similar heterobifunctional molecule has been used to study the nucleating activity of actin dimers [16]. Actin is simultaneously bound, either through a lysine residue to an N-hydroxysuccinimide ester, or through a photocleavable thiol linkage to a 2-nitrobenzyl compound. On UV irradiation active actin monomers are released.

Our results lead us to suggest that such complicated conjugates are not necessarily needed for the study of cellular and protein-protein interactions. Any protein that controls cellular growth (eg hormones, growth factors) could be potentially hidden from cells by simply non-specifically coating it with several 2-nitrobenzyl residues as is reported here for BSA. On exposure to UV light it would be rapidly released and available for utilisation by the cell. Enzymic and antibody reactions could be controlled in a similar fashion. We are currently studying the formation and photocleavage of NPE-antibody conjugates. This further work has already provided data demonstrating inhibition followed by restoration of antibody-antigen binding corresponding to coupling then removal of NPE groups from the antibody (Thompson & Self, unpublished). This particular application of the general method described in this paper will be the subject of future publications in this area.

REFERENCES

1) Pillai, V.N. (1980) Synthesis. 1-26.
2) Kaplan, J.H., Forbush III, B. and Hoffman, J.F. (1978) Biochemistry 17, 1929-1935.
3) Walker, J.W., Reid, G.P. and Trentham, D.R. (1989) Meth. Enzymol. 172, 288-301.
4) Dantzig, J.A., Hibberd, M.G., Trentham, D.R. and Goldman, Y.E. (1991) J. Physiol. 432, 639-680.
5) Senter, P.D., Tansey, M.J., Lambert, J.M. and Blattler, W.A. (1985) Photochem. Photobiol. 42, 231-237.
6) Goldmacher, V.S., Senter, P.D., Lambert, J.M. and Blattler, W.A. (1992) Bioconjugate Chem. 3, 104-107.
7) Mendel, D., Ellman, J.A. and Schultz, P.G. (1991) J. Am. Chem. Soc. 113, 2758-2760.
8) Willner, I., Rubin, S. and Riklin, A. (1991) J. Am. Chem. Soc. 113, 3321-3325.
9) Bethell, G.S., Ayers, J.S., Hancock, W.S. and Hearn, M.T.W. (1979) J. Biol. Chem. 254, 2572-2574.
10) Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olsen, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76-85.
11) Laemmli, U.K. (1970) Nature 227, 680-685.
12) Wootton, J.F. and Trentham, D.R. (1989) In photochemical probes in biochemistry (P.E. Nielsen, Ed.) NATO ASI Series C 272, 277-296, Kluwer, Dordrecht.
13) Anderson, N.L. and Hickman, B.J. (1979) Anal. Biochem. 93, 312-320.
14) Tjoeng, F-S., Staines, W., St-Pierre, S. and Hodges, R.S. (1977) Biochim. Biophys. Acta. 490, 489-496.
15) Henriksen, D.B., Rolland, M., Jakobsen, M.H., Buchardt, O. and Breddam, K. (1992) Peptide Res. 5, 321-324.
16) Marriot, G., Miyata, H. and Kinosita, K. (1992) Biochem. Int. 26, 943-951.