Photoactivated Heterobifunctional Cross-Linking Reagents Which Demonstrate the Aggregation State of Phospholipase A_2†

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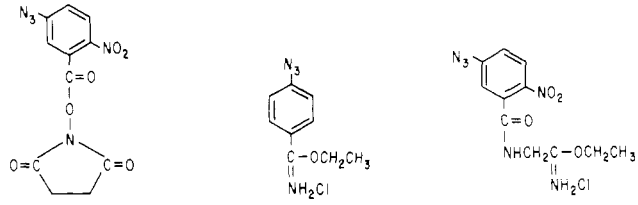
ABSTRACT: Two novel heterobifunctional cross-linking reagents, which can be used to attach photoactivatable nitroaryl azides to primary amino groups of proteins, have been synthesized. The two compounds, *N*-5-azido-2-nitrobenzoyloxysuccinimide and ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate·HCl, as well as ethyl 4-azidobenzimidate·HCl have been attached to lysine residues of cobra venom phospholipase A_2 without a loss in enzymatic activity. Subsequent illumination of the modified forms of the enzyme at appropriate wavelengths under conditions in which the native enzyme exists in an aggregated state led to the formation of covalently linked dimers and larger aggregates which could be separated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate.

Aryl azides have been used as substrate analogues to label the active sites of proteins covalently when mixtures of the two are illuminated (Knowles, 1972; Brunswick and Cooperman, 1971). They have also been incorporated into heterobifunctional reagents which have been used to cross-link proteins to membrane components (Erecinska et al., 1975; Ji, 1977). Erecinska et al. (1975) have shown that cytochrome *c* modified with 2,4-dinitro-5-fluorophenyl azide, becomes covalently attached to membrane preparations which contain cytochrome oxidase, when mixtures of the two preparations are irradiated with ultraviolet light. Moreover, the covalently linked cytochrome *c*–cytochrome oxidase complex was shown to carry out electron transport. Recently, Ji (1977) has shown that the illumination of concanavalin A, modified with methyl 4-azidobenzimidate·HCl, in the presence of its membrane receptor leads to the formation of cross-linked products.

Cross-linking of proteins modified with methyl 4-azidobenzimidate·HCl must be initiated by irradiation at 250–290 nm. This can lead to undesirable side reactions since it is known that tyrosine, tryptophan, and cystine residues in proteins are subject to photolysis at these wavelengths (Vladimirov et al., 1970). To circumvent the potential destruction of these residues during photoactivation, *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS)[1] and ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate·HCl (ANB-AI) were synthesized. The introduction of the nitro group onto the aromatic ring allows for initiation of photoactivation at 320–350 nm and increases the reactivity of the photogenerated arylnitrene. These two reagents, as well as ethyl 4-azidobenzimidate·HCl (AB-I), were used to demonstrate photoactivated cross-linking of monomers of phospholipase A_2 under conditions in which the enzyme exists in an aggregated state (Deems and Dennis, 1975).



ANB-NOS AB-I ANB-AI

Materials and Methods

Reagents. 5-Amino-2-nitrobenzoic acid, 4-aminobenzonitrile, and *N*-hydroxysuccinimide were obtained from Aldrich Chemical Co., aminoacetonitrile–H_2SO_4 was obtained from K&K Laboratories, and dicyclohexylcarbodiimide was obtained from Matheson Coleman and Bell. All other compounds were of reagent grade.

Synthesis Methods. The reactions described in detail below are summarized in Figure 1. The products were analyzed by thin-layer chromatography (TLC) on Eastman silica gel G plates with fluorescent indicator using a butanone–acetone–water (65:25:15) solvent system, except where noted. Aromatic compounds were detected three ways: by iodine vapor staining, observation of quenching of the fluorescent indicator, and in the case of nitroaryl azides, by the appearance of yellow spots following irradiation with long-wavelength UV light. ^1H NMR spectra of the products were obtained at 220 MHz with a Varian HR-220/Nicolet TT-100 pulse Fourier transform system. Samples were run as saturated solutions containing tetramethylsilane. Microanalyses of the products were performed by Galbraith Laboratories (Knoxville, Tenn.).

5-Azido-2-nitrobenzoic Acid. 5-Amino-2-nitrobenzoic acid, 1.4 g (wet, approximately 5.4 mmol), was suspended in 8 mL of 12 M HCl and cooled in a methanol–ice bath to −10 °C. To this solution 0.6 g of $NaNO_2$ (8.7 mmol) in 3 mL of water was added slowly with stirring and not allowing the temperature to rise above 0 °C. Then 8.0 mL of cold glacial acetic acid was added after which 0.6 g of NaN_3 (9.2 mmol) in 2.5 mL of water was added dropwise, with stirring, at a rate slow enough to keep the temperature below 10 °C. All reactions subsequent to azide formation were carried out in vessels shielded from light. After the NaN_3 was added, 15 mL of cold H_2O was added and the reaction mixture was stirred an additional 30

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[1] Abbreviations used are: ANB-NOS, *N*-5-azido-2-nitrobenzoyloxysuccinimide; ANB-AI, ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate·HCl; AB-I, ethyl-4-azidobenzimidate·HCl; DCCD, dicyclohexylcarbodiimide; NOS, *N*-hydroxysuccinimide; HOAc, acetic acid; TLC, thin-layer chromatography; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; Me_2SO, dimethyl sulfoxide; UV, ultraviolet; IR, infrared.

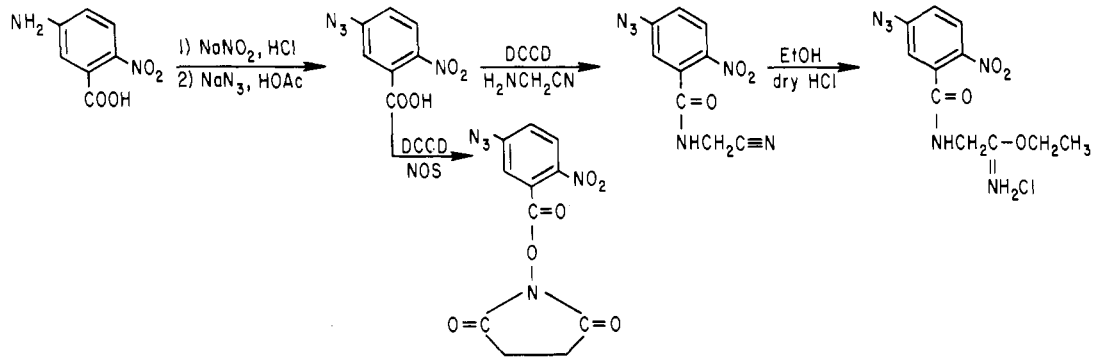

FIGURE 1: Pathway for the synthesis of ANB-NOS and ANB-AI.

min. The product forms a tan precipitate. The reaction mixture was then diluted to 100 mL with cold water and the product was removed by filtration. The product was washed with cold water and then air dried. The yield was 0.9 g (4.3 mmol dry). The product shows a single spot on TLC with an R_f of 0.31 while the starting material has a R_f of 0.12. The dried product melts at 163–164 °C dec. The IR spectrum ($CHCl_3$) shows a characteristic N_3 stretch at 2070 cm^{-1}.

N-5-Azido-2-nitrobenzoyloxysuccinimide. 5-Azido-2-nitrobenzoic acid, 1.0 g (approximately 3.5 mmol), was dissolved in 20 mL of dioxane containing 5 g of anhydrous $MgSO_4$ which was removed by filtration after the starting material was dissolved. Then 0.4 g of *N*-hydroxysuccinimide (3.5 mmol) and 0.7 g of dicyclohexylcarbodiimide (3.6 mmol) were added with stirring. After 30 min a sample of the reaction mixture was examined by TLC. If starting material remained, small amounts of the other two reagents were added until starting material was no longer observed on TLC. After the reaction was complete the dicyclohexylurea was removed by filtration. The filtrate was concentrated under reduced pressure until a slight precipitate became apparent. Four volumes of ether was then added and the solution was cooled to −20 °C. The precipitated product was removed by filtration and washed with cold ether. The reaction yield was 1.0 g (3.2 mmol). The *N*-5-azido-2-nitrobenzoyloxysuccinimide has a R_f of 0.81 and a mp of 133–135 °C dec. The IR ($CHCl_3$) shows the N_3 band at 2100 cm^{-1} and the amide at 1745 cm^{-1}. The UV spectrum has maxima at 237 and 312 nm in $CHCl_3$. ^1H NMR spectra of ANB-NOS in CD_3OD showed the following peaks (reported as ppm downfield from internal tetramethylsilane): 8.25 (d, 1 H, benzene 3 H), 7.55 (m, 2 H, benzene 4 and 6 H), and 2.91 (s, 4 H, succinimide CH_2–). Microanalysis of the compound produced the following result. Anal. Calcd for $C_{11}H_7N_5O_5$ (305.23): C, 43.28; H, 2.32; N, 22.95. Found: C, 43.13; H, 2.40; N, 22.77.

N-5-Azido-2-nitrobenzoylaminoacetonitrile. 5-Azido-2-nitrobenzoic acid (0.8 g; approximately 2.6 mmol) was dissolved in 20 mL of dioxane and dried with 5 g of anhydrous $MgSO_4$ which was removed by filtration before the addition of the other reagents. Then 0.4 g of aminoacetonitrile–H_2SO_4 (2.6 mmol), 0.5 mL of 1 M KOH, and 0.5 g of dicyclohexylcarbodiimide (2.6 mmol) was added, and the reaction mixture was stirred for 30 min. Dicyclohexylurea was removed by filtration. The filtrate was evaporated to dryness under reduced pressure. This dried filtrate was extracted with 20 mL of acetone and filtered. This extract was evaporated to dryness under vacuum. The dried extract was washed with 50 mL of ether twice, redissolved in a minimal amount of acetone, and vacuum evaporated. *N*-5-Azido-2-nitrobenzoylaminoacetonitrile has a R_f of 0.78 on TLC. The yield was 0.6 g.

Ethyl N-5-Azido-2-nitrobenzoylaminoacetimidate·HCl. Vacuum-dried *N*-5-azido-2-nitrobenzoylaminoacetonitrile (0.5 g) was suspended in 5 mL of absolute ethanol in a Y-tube fitted with a drying tube and a gas inlet. The suspension was cooled to −10 °C in a methanol–ice bath. Then HCl, dried by bubbling through a concentrated H_2SO_4 trap, was bubbled into the ethanol suspension with stirring until the ethanol was saturated with HCl. This was detected by the escape of gas through the drying tube. At this point the nitrile was dissolved completely. The HCl inlet was then removed and the Y-tube stoppered. The reaction mixture was stirred at −10 °C for 4 h and was then placed at 4 °C overnight. Dry ether (30 mL) was added and the resulting suspension was cooled to −20 °C. The product was then filtered and washed with cold ether. The yield was 0.5 g (1.5 mmol). Since the product is hygroscopic it was stored under vacuum at 4 °C. Ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate·HCl has a R_f of 0.88 and a mp of 143–145 °C dec. The IR (KBr) shows N_3 at 2100 cm^{-1} and C=N at 1675 cm^{-1}. The UV spectrum shows maxima at 240 and 310 nm in ethanol. Since the product hydrolyzes rapidly in CD_3OD ^1H NMR spectra of ANB-AI had to be taken immediately after dissolving the solid in Me_2SO-d_6. Spectra in Me_2SO-d_6 showed these characteristics: 8.14 (d, 1 H, benzene 3 H), 7.45 (m, 2 H, benzene 4 and 6 H), 4.49 (q, 2 H, OCH_2CH_3), 4.33 (d, 2 H, $HNCH_2$), and 1.40 (t, 3 H, OCH_2CH_3). There were minor peaks which were shown to be hydrolysis products. When a small amount of D_2O was added to the sample the ethyl peaks of the reagent disappeared and the minor peaks increased concurrently. Analysis was consistent with $C_{11}N_6H_{13}O_4Cl$ containing a small percentage of H_2O.

Ethyl 4-Azidobenzimidate·HCl. 4-Azidobenzonitrile was synthesized from 4-aminobenzonitrile by the same procedure as that described above for the synthesis of 5-azido-2-nitrobenzoic acid from 5-amino-2-nitrobenzoic acid. Ethyl 4-azidobenzimidate·HCl was prepared by a procedure identical with that described above for the synthesis of ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate·HCl. The yield was 0.8 g (3.5 mmol). The R_f with ethyl acetate as running solvent was 0.65. The IR ($CHCl_3$) showed N_3 at 2100 cm^{-1}, and C=N at 1675 cm^{-1}. ^1H NMR spectra of AB-I in $CDCl_3$ showed the following characteristics: 8.07 (d, 2 H, benzene 2 and 6 H), 7.32 (d, 2 H, benzene 3 and 5 H), 4.62 (q, 2 H, OCH_2CH_3), and 1.60 (t, 3 H, OCH_2CH_3). Analysis calculated for $C_9H_{11}N_4OCl$ (226.69): C, 47.68; H, 4.90; N, 24.72; Cl, 15.63. Found: C, 47.63; H, 4.89; N, 24.66; Cl, 15.52.

Modification of Phospholipase A_2 with the Heterobifunctional Cross-Linking Reagents. Phospholipase A_2 from cobra venom was prepared by the procedure of Deems and Dennis (1975) as modified by Roberts et al. (1977a). The en-

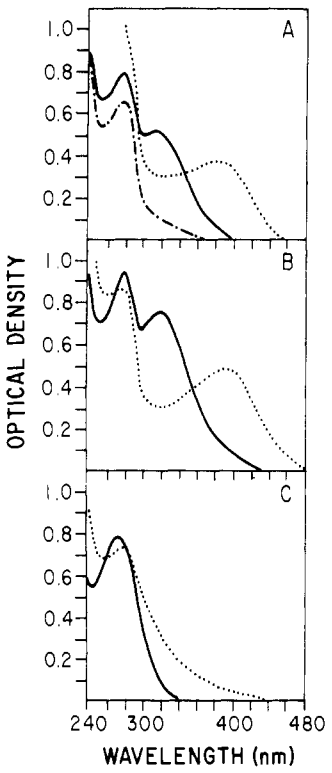

FIGURE 2: Spectra of the modified phospholipases before and after photoactivation: (A) phospholipase A_2 modified with ANB-NOS; (B) phospholipase A_2 modified with ANB-AI; and (C) phospholipase A_2 modified with AB-I. The spectra before photoactivation (—) and after photoactivation (···) are shown. In A is also shown the spectrum of unmodified phospholipase A_2 which was irradiated under the same conditions as the other samples (---). Photoactivation was carried out for 30 min at 300 nm at an enzyme concentration of 1.0 mg/mL in 0.05 M K_2HPO_4 (pH 8.0). To determine the spectra, samples were diluted 1:1 with buffer.

zyme was assayed with egg phosphatidylcholine as substrate in mixed micelles with Triton X-100 by the pH stat method (Dennis, 1973). Protein concentrations were determined by the method of Lowry et al. (1951). The enzyme was modified in 0.1 M Na_2CO_3 (pH 9.0) at a protein concentration of 1 mg/mL (0.09 mM) with a tenfold molar excess of each reagent (0.90 mM). *N*-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) was added in dioxane. The final dioxane concentration in the reaction mixture was 10%. This reaction mixture was incubated at room temperature for 4 h. Excess reagent was removed by gel filtration on a Sephadex G-25 column which was equilibrated with 50 mM sodium phosphate buffer (pH 8.0). The gel-filtered enzyme was lyophilized. The phospholipase was modified with ethyl 4-azidobenzimidate (AB-I) and ethyl *N*-5-azido-2-nitrobenzoylaminoacetimidate (ANB-AI) by the addition of the solid reagents to a stirred solution of the enzyme in order to minimize hydrolysis of these readily soluble compounds. These reaction mixtures were incubated overnight at room temperature. They were gel filtered, lyophilized, and stored at 4 °C as 1–2 mg/mL solutions.

Irradiations were carried out in a Rayonet Photochemical Reactor with the 300-nm lamps except where noted. The apparatus contains 16 fluorescent tubes arranged around a cylinder with an output of 200 mW per tube at 300 nm with approximate photon flux of 1.5×10^6 s^{-1} cm^3 at the center, a distance of 8 cm. The samples in glass or quartz tubes rotate on a carousel that places them 2 cm from the fluorescent tubes. The samples were illuminated at a phospholipase concentration of 1 mg/mL in 0.05 M sodium phosphate buffer (pH 8.0).

The aggregation state of the irradiated samples was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) with cytochrome *c*, chymotrypsinogen, egg albumin, and bovine serum albumin used as marker proteins. Relative protein concentrations on the gels stained with Coomassie blue were determined from planimeter tracings of scans taken with a Gilford 2400 spectrophotometer. Absorption spectra were carried out on a Cary 118 recording spectrophotometer.

TABLE I: Activity and Aggregation State of Phospholipase A_2 after Modification and before Irradiation.[a]

Reagent	Rel act.	% Monomers	% Dimers	% Trimers
None	1.00	97	3	0
ANB-NOS	1.00	94	5	1
ANB-AI	1.37	93	6	1
AB-I	1.07	97	3	0

[a] The percentage of monomers, dimers, etc. was estimated from densitometer scans of gels stained with Coomassie blue. Using protein standards of known molecular weight, the relative mobility of monomers corresponded to a mol wt of 11 500, dimers to a mol wt of 21 000, trimers to a mol wt of 32 000, and tetramers to a mol wt of 42 000.

Results

Properties of the Modified Phospholipase before Photoactivation. The absorption spectra of the phospholipase modified by each of the three reagents are shown in Figure 2. The new absorption maxima exhibited by the modified phospholipases are very similar to those of the reagents used in the modifications. Based on the reagent extinction coefficient of $\epsilon = 9 \times 10^3$ M^{-1} at 312 and 318 nm which was calculated from the absorption spectra of ANB-NOS and ANB-AI, respectively, it is estimated that not more than 2 of the 5 lysines in the phospholipase have been modified by these reagents. Because the maximum of AB-I lies in the same region as that of the protein, it is not possible in this way to estimate the number of groups incorporated into the enzyme when it reacts with AB-I. The number of lysines in phospholipase modified by ANB-AI and AB-I has also been estimated by amino acid analysis following hydrolysis by 6 N HCl for 24 h at 105°C. Comparison of the analysis of the modified enzyme with that of the native enzyme indicates that the phospholipase modified by ANB-AI had about 1.7 less lysine residues after correction for hydrolysis of the amidine derivative (Reynolds, 1968). The phospholipase modified by AB-I had about 1.8 less lysine residues, but this number was not corrected for possible losses since the arylamidine hydrolysis rate was not available. Since the modification of lysine residues with ANB-NOS leads to the formation of amides which are readily hydrolyzed by 6 N HCl, the number of modified lysines in this case could only be estimated by the increase in extinction of phospholipase A_2 at 312 nm. This analysis suggests that 1.5 lysine residues were also modified.

The modified phospholipases were subjected to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ prior to illumination with ultraviolet light. The enzymatic activity of the modified phospholipases was also determined before illumination. The results of these experiments are summarized in Table I. No loss in enzymatic activity accompanies the modification of the phospholipase A_2 with each of the reagents in the absence of irradiation.

Properties of the Modified Phospholipases after Photoactivation. The absorption spectra of the modified phospholipases following irradiation are also shown in Figure 2. The

TABLE II: Activity and Aggregation State of the Modified Phospholipases after Photoactivation at 300 nm for 30 Min.

Modified phospholipase[a]	Rel act.	% Monomers	Dimers	Trimers	Tetramers
Native-PLA_2	1.00	97	3	0	0
PLA_2-ANB-NOS	0.48	6	23	29	43
PLA_2-ANB-NOS[b]		11	15	29	45
PLA_2-ANB-AI	0.26	21	30	29	30
PLA_2-ANB-AI[b]		17	28	30	25
PLA_2-AB-I	0.80	48	31	14	7

[a] Phospholipase A_2 is abbreviated PLA_2. [b] Irradiated in the presence of 1 mM *p*-aminobenzoic acid which was added as a nitrene scavenger.

ANB-NOS and ANB-AI modified phospholipases show nearly total loss of the 312- and 318-nm absorption and the appearance of new absorptions at 380 and 390 nm, respectively. The activity of both these enzyme derivatives decreased following irradiation as shown in Table II. Also, a large increase in covalently linked multimers was observed on the polyacrylamide gels (Table II). These bands correspond to molecular weights of 11 500 (monomer), 21 000 (dimer), 32 000 (trimer), and 42 000 (tetramer) as calculated using standards of known molecular weight. When irradiation times were varied from 15 to 60 min there were no changes in the polyacrylamide gel patterns. When *p*-aminobenzoic acid was added as a nitrene scavenger (Roeder, 1975), to prevent random cross-linking, little change in the gel patterns was observed. This indicated that specific photoactivated cross-linking had occurred. Irradiation of phospholipase modified by AB-I was performed at 254 and 300 nm. When irradiated at both wavelengths, the modified phospholipase showed some loss of absorbance at 268 nm which was accompanied by the appearance of a broad absorption band above 300 nm. The enzyme photolyzed at 254 nm was totally inactive and did not penetrate the 10% polyacrylamide gel in the presence of $NaDodSO_4$. When it was irradiated at 300 nm, however, only a partial loss of activity was observed. Well-defined multimers were formed as shown in Table II, although in much lower percentages when compared to the two other cross-linking reagents.

Discussion

The results show that heterobifunctional reagents containing aryl azides can be used effectively to study protein–protein interactions. These new reagents offer certain advantages over homobifunctional reagents, such as dimethyl suberimidate, which have been used extensively to study protein–protein interactions (Bickle et al., 1972; Coggins et al., 1976; Davies and Stark, 1970; Wang and Richards, 1974). Since the aryl azido group in the heterobifunctional reagents is relatively inert prior to irradiation, the amount of cross-linking reagent attached to the protein under study can be controlled. With these heterobifunctional reagents, cross-linking is initiated by irradiation which generates nitrenes from the aryl azides on the surface of the modified protein. The properties of nitrenes are very well suited for this kind of study. They have a lifetime of less than 10^{-4} s (DeGraff et al., 1974). This minimizes the problem of nonspecific or random cross-linking, especially when nitrene scavengers are included in the photolysis mixtures. Nitrenes can react with a number of amino acid side chains (Knowles, 1972). This eliminates the need for a particular amino acid side chain of an acceptor protein molecule to be near the site of attachment of a modified donor protein molecule in an interacting system.

One end of the reagents used in this study is specific for primary amino groups of proteins. Furthermore, these reagents appear to be reasonably selective: when phospholipase A_2 is treated with a tenfold molar excess of any of the reagents, no more than two of the five lysines are modified. The modification of these lysines has no effect on the activity of this enzyme. The reagents offer two types of lysine modification. ANB-NOS reacts with ϵ-amino groups to form amides. This reduces the positive charge on the protein under physiological conditions. On the other hand, the imidates react with ϵ-amino groups to form amidines which are positively charged under physiological conditions. The use of both reagents might provide an estimate of the importance of electrostatic interactions in a given interacting system.

The two new heterobifunctional reagents described here offer significant advantages over those described by Erecinska et al. (1975) and by Ji (1977). It has been shown that nitro groups ortho to aryl azides, as in 2,4-dinitro-5-fluorophenyl azide, react intramolecularly with the generated nitrenes to form benzofurazan *N*-oxides (Smith and Brown, 1951). ANB-NOS and ANB-AI were purposely synthesized with the nitro group para to the azide to avoid this problem. Because cross-linking of proteins modified with ANB-NOS and ANB-AI can be initiated at wavelengths from 300 to 350 nm, a region in which aromatic residues in proteins show negligible absorption, these reagents are more suitable for photoactivation studies than AB-I, which needs to be irradiated near 260 nm.

When unmodified phospholipase A_2 was illuminated for 30 min at 300 or 254 nm, it lost no activity. Even the modified phospholipases retained at least 25% of their activity when illuminated at 300 nm for 30 min. However, when the phospholipase was modified with AB-I and then illuminated at 254 nm, activity was totally abolished and large aggregates were formed which would not penetrate the 10% cross-linked polyacrylamide gels in the presence of sodium dodecyl sulfate.

The distance between the ϵ-N and the azido group when proteins are modified with ANB-NOS is approximately 6.5 Å, and that distance is approximately 10 Å when proteins are modified by ANB-AI. With the assumption that the Coomassie blue binds equally to all the modified proteins, the data suggest that the shorter range reagent, ANB-NOS, is more effective in cross-linking phospholipase subunits. This effect may be specific for phospholipase A_2 or it may reflect a general phenomenon. Because of the nonspecific reactions of nitrenes, an interpretation of chain length and optimal enzyme cross-linking (as has been made for homobifunctional reagents) is not straightforward at this time.

Phospholipase A_2 undergoes a concentration-dependent dimerization ($K_D \simeq 0.05$ mg mL^{-1}, ~5 μM) as detected by gel filtration and ultracentrifugation (Deems and Dennis, 1975). These techniques only determine an average molecular weight for the enzyme; they do not show the distribution or

range of the individual multimers (monomer, dimer, trimer, tetramer, etc.). Recently, it was postulated that phospholipase A_2 is active as a dimer or a higher order aggregate, and that this aggregation was induced by phospholipid and metal ions (Roberts et al., 1977b). Experiments in support of this used dimethyl suberimidate as a cross-linking reagent. This homobifunctional reagent will convalently cross-link enzyme subunits through lysine residues. The distance between the two reactive ends of the suberimidate is about 11 Å. Lysine residues on adjacent protomers in an aggregate must be within this distance for cross-linking to occur. Because of this requirement, cross-linking with dimethyl suberimidate may not reflect the true aggregation state of a protein in solution. When phospholipase A_2 at a concentration of 0.6 mg/mL is treated with dimethyl suberimidate, subsequent polyacrylamide gel electrophoresis shows the presence of monomers (37%), dimers (48%), and trimers (15%). The average molecular weight is 19 100. With the photosensitive heterobifunctional reagents, much more cross-linking is observed. The degree of aggregation shows some dependence on which cross-linking reagent is used. Phospholipase modified with ANB-NOS forms slightly larger aggregates (average mol wt of 33 000) than the enzyme modified with ANB-AI (average mol wt of 28 000). Both of these derivatives are much more aggregated than the phospholipase modified by AB-I (average mol wt of 19 500). Further experiments are in progress to determine if the attachment of the aryl azide moieties alters the aggregation equilibrium of phospholipase A_2, and if the cross-linked species produced by dimethyl suberimidate reflect the true aggregation state of phospholipase A_2.

This report appears to be the first demonstrating efficient photoactivated cross-linking of soluble proteins. Of the phospholipase modified with ANB-NOS or ANB-AI 80–90% was cross-linked and was shown to exist in discrete species varying from dimer to tetramer. The activity remaining after photoactivation does not correlate with any of the multimeric species. For ANB-NOS and AB-I the activity is significantly higher than the amount of monomer, however, indicating that a portion of the cross-linked proteins retains phospholipase activity. These features indicate the general usefulness of these new compounds for the investigation of protein–protein interactions.

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