N-(1-Pyrene)maleimide: A Fluorescent Cross-Linking Reagent†

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ABSTRACT: N-(1-Pyrene)maleimide is nonfluorescent in aqueous solution but forms strongly fluorescent adducts with sulfhydryl groups of organic compounds or proteins. The conjugation reactions of N-(1-pyrene)maleimide are relatively fast and can be monitored by the increase in fluorescence intensity of the pyrene chromophore. In cases where primary amino groups are also present in the system, we have observed a red shift of the emission spectra of the fluorescent adducts subsequent to the initial conjugation, as characterized by the disappearance of three emission peaks at 376, 396, and 416 nm, and the appearance of two new peaks at 386 and 405 nm. Model studies with N-(1-pyrene)maleimide adducts of L-cysteine and cysteamine indicate that the spectral shift is the result of an intramolecular aminolysis of the succinimido ring in the adducts. Evidence from both chemical analysis and nuclear magnetic resonance studies of the addition products supports this reaction scheme. N-(1-Pyrene)maleimide adducts of N-acetyl-L-cysteine and β-mercaptoethanol, which have no free amino group, do not exhibit a spectral shift. Among several protein conjugates only the N-(1-pyrene)maleimide adduct of bovine serum albumin (PM-BSA) shows the spectral shift resembling that of PM-cysteine. N-(1-Pyrene)maleimide reacts with the sulfhydryl group of the single cysteine residue at position 34 in BSA. The finding that the α-amino group of the N-terminus in PM-BSA is blocked after the spectral shift is completed strongly suggests that N-(1-pyrene)maleimide cross-links the N-terminus and the cysteine residue in BSA. The relative proximity of the sulfhydryl and amino groups is very critical in the cross-linking as demonstrated by the observation that the spectral shift observed with PM-BSA can be prevented by addition of denaturating reagents such as 1% sodium dodecyl sulfate immediately after labeling, and by the failure of PM-glutathione to undergo the intramolecular aminolysis. Since the intramolecular rearrangement of PM adducts is associated with characteristic fluorescence changes, N-(1-pyrene)maleimide can serve as a fluorescent cross-linking reagent which provides information about the spatial proximity of sulfhydryl and amino groups in proteins.

Fluorescent probes are very useful in studying structure and dynamics of macromolecules. A variety of information can be obtained using fluorescent probes: (a) site-directed fluorescent labeling of macromolecules with polarity-sensitive probes will reveal the microenvironment of active sites; (b) multiple excited-state lifetimes are indicative of the heterogeneity of binding sites; (c) energy transfer from one fluorescent probe to another can be employed to measure the distance between specific sites; (d) spectral shifts or alterations in fluorescence quantum yield upon ligand binding may be interpreted as the ligand-induced conformational change of macromolecules; (e) fluorescent depolarization measurements can yield information about the size, shape, and flexibility of macromolecules; and (f) any of these fluorescent changes can be used to monitor the kinetics and equilibrium of ligand macromolecule or macromolecule–macromolecule interactions. In this communication, we report a novel application of a fluorescent probe: a probe which can serve as a spectroscopic indicator for cross-linking between sulfhydryl and amino groups in macromolecules. A fluorescent probe of this kind is N-(1-pyrene)maleimide.

N-(1-Pyrene)maleimide was first synthesized by Haugland (1970) and subsequently by Weltman et al. (1973). It does not fluoresce in aqueous solution but forms fluorescent adducts with organic compounds or proteins which contain sulfhydryl groups. Because of the long lifetime of its adducts, N-(1-pyrene)maleimide is useful for fluorescent polarization studies of high molecular weight proteins and other macromolecules.

During our studies on DNA-dependent RNA polymerase of *Escherichia coli*, we observed time-dependent changes in the fluorescence properties of N-(1-pyrene)maleimide-labeled σ subunit (PM-σ)[1] in Tris buffer. Further investigations of the adducts of N-(1-pyrene)maleimide with both small molecules and other proteins indicate that these spectral changes are associated with molecular rearrangements in the adducts. The mechanism of these chemical reactions and the possible usage of N-(1-pyrene)maleimide as a fluorescent crosslinking reagent to study proximity relationship between specific groups in macromolecules are discussed.

Experimental Procedures

Materials. N-(1-Pyrene)maleimide was synthesized as described by Weltman et al. (1973). N-Acetyl-1-aminopyrene was synthesized by reacting 1-aminopyrene with acetic anhydride in dimethyl sulfoxide. L-Cysteine, N-acetyl-L-cysteine, cysteamine, glutathione (reduced form), and β-lactoglobulin were purchased from Sigma. Bovine serum albumin and ovalbumin were obtained from Miles and Schwarz/Mann, respectively. *Bacillus pumilus* flagellin was a gift from Drs. R. W. Smith and K. Schanky. The σ subunit of *Escherichia*

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[1] Abbreviations used are: PM, N-(1-pyrene)maleimide; PM-BSA, pyrenemaleimide adduct of bovine serum albumin; PM-σ, pyrenemaleimide adduct of the σ subunit of *Escherichia coli* RNA polymerase; Bicine, N,N-bis(2-hydroxyethyl)glycine; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Me_4Si, tetramethylsilane; DDPM, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DDPS, N-(4-dimethylamino-3,5-dinitrophenyl)succinimide; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

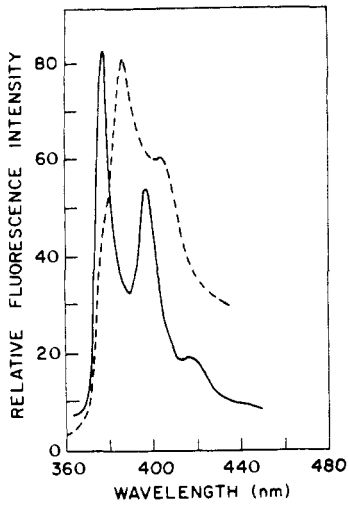

FIGURE 1: Corrected fluorescence emission spectra of PM-σ in Tris buffer. The wavelength of excitation was 342 nm. (—) Spectrum recorded 2 h after labeling; (- - -) spectrum recorded 2 days after labeling.

coli RNA polymerase was prepared by the method of Berg et al. (1971). Silica gel plates for thin-layer chromatography were obtained from Eastman Organic Chemical.

N-(1-Pyrene)maleimide Adducts of Proteins. Proteins (bovine serum albumin, β-lactoglobulin, ovalbumin, *Bacillus pumilus* flagellin, or the σ subunit of *Escherichia coli* RNA polymerase), 1–2 mg/ml in 0.05 M phosphate buffer at pH 6.0 or 7.5, were reacted with a twofold molar excess of *N*-(1-pyrene)maleimide at 23 °C for various time periods. Unreacted *N*-(1-pyrene)maleimide was removed by Sephadex G-25 gel filtration. The samples were then lyophilized and weighed. The stoichiometry of labeling was estimated from the absorption spectra of the solution of protein adducts assuming the molar absorptivity of 4×10^4 M^{-1} cm^{-1} at 345 nm for pyrene chromophore (Rawitch et al., 1969). The protein concentrations were determined by the method of Lowry et al. (1951).

Titrations of free sulfhydryl group in proteins or protein adducts were carried out spectrophotometrically with 1×10^{-3} M 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) in 0.05 M potassium phosphate buffer (pH 7.5), readings being taken at 412 nm against a blank lacking protein. A molar absorptivity of 1.36×10^4 M^{-1} cm^{-1} was used to calculate the concentration of reactive sulfhydryl groups (Ellman, 1959).

N-Terminal analysis of BSA and PM-BSA was performed using a dansylation method (Hartley and Massey, 1956; Gray, 1967) and the dansyl derivatives were identified on thin-layer polyacrylamide plates (Woods and Wang, 1967; Fragione and Milstein, 1968). BSA or PM-BSA (0.6 mg, 10 nmol) was first reacted with 0.05 ml of a 0.2 M solution of sodium bicarbonate and the reaction mixture was dried. Dansyl chloride (25 μl, 2.5 mg/ml) and water (25 μl) were added to the $NaHCO_3$-treated protein and the reaction mixture was incubated for 60 min at 37 °C. After the solution was taken to dryness, 50 μl of 6 N HCl was added, and the mixture was incubated for 18 h at 110 °C in a sealed tube. The dried sample was then dissolved in 20 μl of 50% pyridine–water, the solution was centrifuged, and the supernatant was applied to polyamide plates. Standard dansyl amino acids were used as references. The plates were subjected to two-dimensional chromatography in a three-solvent system. A solvent system (1.5% formic acid) was used in the first dimension for 50 min. A second solvent system [benzene–acetic acid (9:1, v/v)] was used in the second dimension for 60 min. The plates were then subjected to a third solvent system [ethyl acetate–methanol–acetic acid (20:1:1, v/v/v)] in the second dimension for another 50 min. The dansyl-labeled residues were visualized and identified under an ultraviolet light.

Adducts of N-(1-Pyrene)maleimide with Small Molecules. Approximately 5 mg of the small molecule (L-cysteine, cysteamine, *N*-acetyl-L-cysteine, β-mercaptoethanol, or glutathione) was dissolved in 1 ml of H_2O and the pH of the solution was adjusted to the desired value (6.0 or 7.5). An equimolar amount of *N*-(1-pyrene)maleimide dissolved in 50% ethanol–acetone was added, and the mixture was allowed to stand at room temperature in the dark from 3–24 h. The resulting solution was evaporated to dryness (or near dryness) in vacuo. The precipitates were dissolved in acetic acid and recrystallized by addition of acetone or ether. The products were analyzed by ascending thin-layer chromatography on silica gel plates using the upper layer of 1-butanol–acetic acid–water (4:1:5, v/v/v) as the solvent. Chromatograms were dried in air, and the spots were visualized by the fluorescence emission under an ultraviolet lamp. Ninhydrin tests of the spots were performed by spraying the chromatogram with a 0.25% solution of ninhydrin in 1-butanol and examination of the color formation after dryness.

Spectroscopic Measurements. Absorption spectra were measured using a Cary 118-C recording spectrophotometer. Corrected fluorescence excitation and emission spectra were recorded on a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with a corrected spectra accessory. The solution used had an absorbance of less than 0.05 to obviate inner filter effects. All spectral measurements were carried out at 23 °C.

NMR spectra were observed at 100 MHz on a JEOL PF100 spectrometer. The reference was tetramethylsilane (Me_4Si). The solvent was dimethyl-d_6 sulfoxide. The samples were lyophilized before dissolving in the deuterated solvent.

Results

Time-Dependent Fluorescence Changes of N-(1-Pyrene)-maleimide-Labeled Proteins. *N*-(1-Pyrene)maleimide (PM) reacted with 1–2 sulfhydryl residues in the σ subunit of *Escherichia coli* RNA polymerase (Yarbrough and Wu, 1974). When the fluorescence emission spectrum of the labeled σ subunit (PM-σ) was recorded immediately after labeling, three well-defined peaks at 376, 396, and 416 nm were observed (Figure 1). After standing at 3 °C for 2–3 days in Tris buffer (0.05 M Tris-HCl, pH 8, 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol), a red shift of the emission spectrum appeared with the formation of two new peaks at 386 and 405 nm. No such spectral changes were found for PM-σ stored in phosphate or Bicine buffer at the same pH.

In order to characterize these spectral changes, several other protein conjugates with *N*-(1-pyrene)maleimide were prepared and their fluorescence properties examined. When an excess amount of *N*-(1-pyrene)maleimide was used to label bovine serum albumin (BSA), the stoichiometry of labeling was estimated to be about 0.42 mol of pyrene chromophore per mol of BSA. It has been known that the native BSA molecule contains 0.3–0.5 reactive sulfhydryl group as determined by Nbs_2 or *N*-ethylmaleimide (Means and Feeney, 1971). Using the procedure of Ellman (1959), we found that Nbs_2 reacted with 0.40 sulfhydryl group per mol of BSA in phosphate buffer (pH 7.5). Titration of PM-BSA with Nbs_2 gave evidence for the absence of a reactive sulfhydryl group (less than 0.05 mol per mol of protein). Thus *N*-(1-pyrene)maleimide reacted with a cysteine residue in BSA.

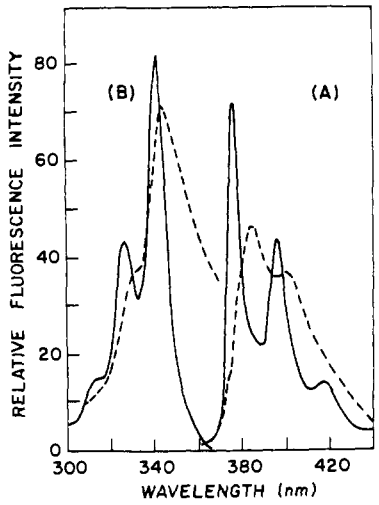

FIGURE 2: (A) Corrected fluorescence emission spectrum of PM-BSA in phosphate buffer (pH 7.5). The wavelength of excitation was 342 nm. (—) Spectrum recorded 5 min after labeling; (- - -) spectrum recorded 2 h after labeling. (B) Corrected fluorescence excitation spectrum of PM-BSA in phosphate buffer (pH 7.5). (—) Spectrum recorded 5 min after labeling; emission observed at 376 nm. (- - -) Spectrum recorded 2 h after labeling; emission observed at 386 nm.

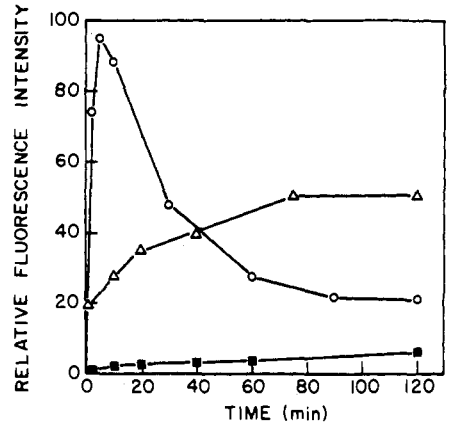

FIGURE 3: Kinetics of reactions of *N*-(1-pyrene)maleimide (10^{-5} M) with bovine serum albumin, β-lactoglobulin and *B. pumilus* flagellin in 0.05 M phosphate buffer (pH 7.5) at 23 °C. The concentration of the protein used was 2 mg/ml. Adducts were prepared as described in Experimental Procedures. Changes in fluorescence intensity were monitored at 376 nm. The wavelength of excitation was 342 nm. (○) Bovine serum albumin; (△) β-lactoglobulin; (■) *B. pumilus* flagellin.

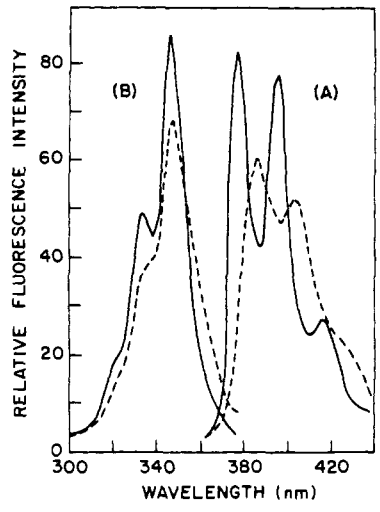

FIGURE 4: (A) Corrected fluorescence emission spectra of *N*-(1-pyrene)maleimide adduct of L-cysteine in phosphate buffer (pH 7.5) at 23 °C. The wavelength of excitation was 342 nm. (—) Spectrum recorded 2 min after reaction; (- - -) spectrum recorded 2 h after reaction. (B) Corrected fluorescence excitation spectra of *N*-(1-pyrene)maleimide adduct of L-cysteine. The wavelength of emission was 386 nm. (—) Spectrum recorded 2 min after reaction; (- - -) spectrum recorded 2 h after reaction.

The fluorescence emission spectra of PM-BSA recorded at 5 min and 2 h after the conjugation reaction are shown in Figure 2A. The emission spectrum of PM-BSA at 5 min resembles that of PM-σ recorded immediately after labeling. Similarly, a spectral shift was observed in the emission spectrum of PM-BSA taken after 2 h. These time-dependent fluorescence changes were also evident in the excitation spectrum when emission was observed at 386 nm (Figure 2B) as characterized by the disappearance of the peak at 328 nm and the red shift of the peak at 340 nm. In contrast to PM-σ, these spectral changes were observed in phosphate buffer and did not require the presence of Tris.

The fluorescence spectra of *N*-(1-pyrene)maleimide conjugated with other proteins such as β-lactoglobulin, ovalbumin, and *Bacillus pumilus* flagellin were essentially identical with that of PM-BSA recorded at 5 min (Figure 2), but no time-dependent spectral shifts were observed. Figure 3 shows the kinetics of reactions of *N-(1-pyrene)maleimide with BSA, β-lactoglobulin, and B. pumilus* flagellin in 0.05 M phosphate buffer (pH 7.5) at 23 °C. The reactions were monitored by changes in fluorescence intensity at 376 nm (excitation at 342 nm). As shown in the figure, *B. pumilus* flagellin, which has no cysteine residues (Smith and Koffler, 1971), reacted very slowly with *N*-(1-pyrene)maleimide. The reaction of β-lactoglobulin reached a maximum at about 75 min as demonstrated by the increase in fluorescence intensity. No further change was detected thereafter. For BSA, the fluorescence intensity increased rapidly to a maximum within 5 min, decreased subsequently, and leveled off at about 90 min. The biphasic kinetic curve clearly demonstrates the occurrence of at least two sequential steps in the spectral change.

Fluorescence Properties of N-(1-Pyrene)maleimide Adducts with Small Molecules. As a model study for the spectral shifts observed with PM-labeled proteins, we have examined the fluorescence changes associated with the reactions of *N*-(1-pyrene)maleimide with L-cysteine, cysteamine, *N*-acetyl-L-cysteine, and β-mercaptoethanol. The fluorescence emission and excitation spectra of PM-cysteine recorded 2 min and 2 h after the reactions are shown in Figure 4A and 4B, respectively. PM-cysteamine showed similar changes in both emission and excitation spectra but the changes were faster and completed within 20 min. On the other hand, *N*-(1-pyrene)-maleimide adducts of β-mercaptoethanol and *N*-acetyl-L-cysteine, whose fluorescence spectra were almost the same as that of PM-cysteine recorded at 2 min, did not show the time-dependent spectral shift.

Mechanism of Spectral Changes Observed with PM-Cysteine and PM-Cysteamine. The model studies described above indicate that the amino group of L-cysteine, but not the carboxyl group, is essential for the spectral shift observed with PM-cysteine. Thus the mechanism for the reaction of *N*-(1-pyrene)maleimide with cysteine is proposed as shown in Figure 5. The first step is the rapid addition of the cysteine sulfhydryl group to the olefinic double bond in the maleimide moiety of *N*-(1-pyrene)maleimide to form *S*-[*N*-(1-pyrene)succinimido]cysteine (I). As the result of subsequent nucleophilic attack by the amino group on a carbonyl carbon, compound I undergoes a slower cleavage of the succinimido ring with a

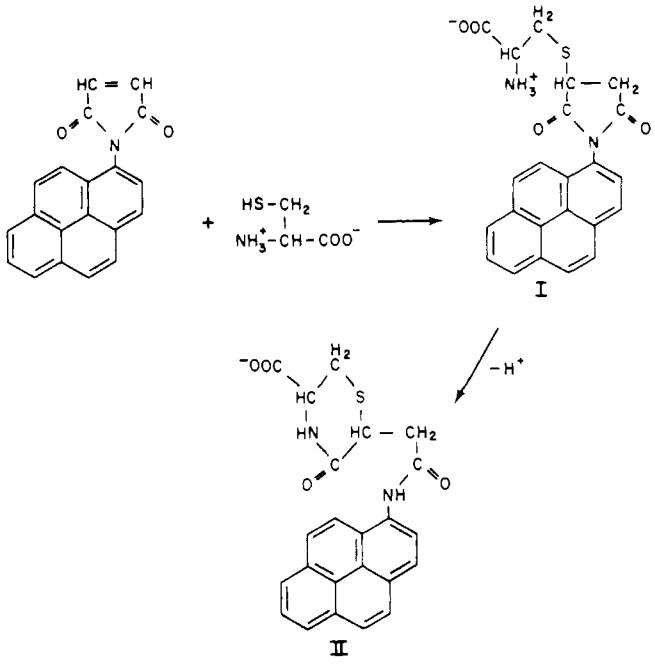

FIGURE 5: Mechanism of reaction of *N*-(1-pyrene)maleimide with L-cysteine.

concomitant cyclization to form a thiazane derivative (II). The validity of this mechanism was tested as follows. Since the intramolecular aminolysis is presumed to be initiated by the removal of a proton from the NH_3^+ group of compound I, no compound II should be formed at lower pH. In fact, at pH 6 or lower we detected no time-dependent spectral shift for *N*-(1-pyrene)maleimide adducts of L-cysteine and cysteamine. Furthermore, PM-cysteine, PM-cysteamine, and PM-glutathione were synthesized and purified at pH 6 and all these adducts gave a purple-colored reaction with ninhydrin as would be expected for compound I or other compounds containing a free amino group. When these addition products were allowed to stand at pH 7.5 and 23 °C for 2 h, PM-cysteine and PM-cysteamine readily rearranged into a thiazane derivative II, which showed the presence of a single, ninhydrin-negative spot on thin-layer chromatography indicating that the amino group had disappeared. However, we have found that PM-glutathione, which also possesses an amino group, failed to produce a ninhydrin-negative compound under the same conditions.

Other supporting evidence for the intramolecular aminolysis comes from NMR studies of PM-cysteamine synthesized at pH 7.5. The proton nuclear magnetic resonance spectrum of this compound in deuterated dimethyl sulfoxide solvent showed a peak at 10.4 ppm downfield from the tetramethylsilane (Me_4Si) standard. This peak was abolished by the addition of H_2O to the sample, suggesting that the resonance is due to an exchangeable proton. A similar peak at 10.3 ppm from Me_4Si was observed for *N*-acetyl-1-aminopyrene, while no such peak was seen for *N*-(1-pyrene)maleimide. Thus the 10.4-ppm resonance was assigned to the imido proton at the 1 position of the pyrene ring. The ratio of the observed resonance amplitude of the aromatic protons (~8.3 ppm downfield from Me_4Si) to the imido proton was about 9 to 1 for both *N*-acetyl-1-aminopyrene and PM-cysteamine. This is in agreement with structure II, which indicates that the intramolecular aminolysis has taken place in PM-cysteamine.

The time-dependent spectral shifts of PM-cysteine and PM-cysteamine may be explained based on the proposed reaction mechanism in Figure 5. The fluorescence spectra recorded immediately after reaction (Figure 4) are due to structure I while those observed 2 h later may be attributed to structure II. Compounds such as PM adducts of β-mercaptoethanol or *N*-acetyl-L-cysteine do not undergo the transformation from I to II and will, therefore, exhibit no spectral shift.

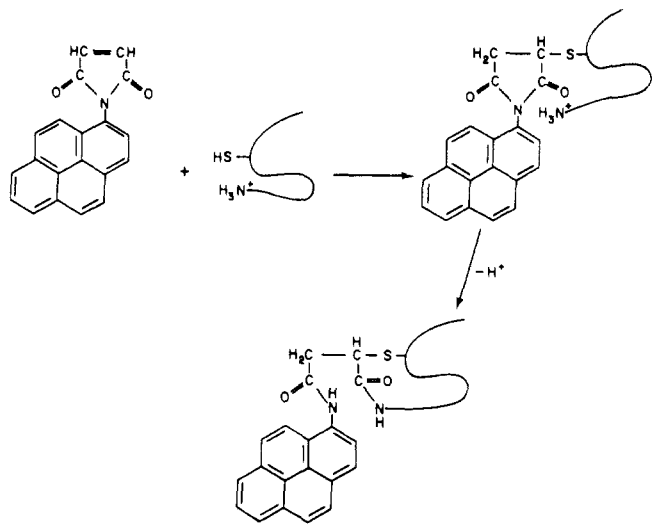

FIGURE 6: Intramolecular aminolysis of PM-BSA.

Evidence for Intramolecular Cross-Linking in PM-BSA. The similarity between the spectral shift observed with PM-BSA (Figure 2) and that of PM-cysteine (Figure 4) suggests an intramolecular aminolysis of PM-BSA, such as that illustrated in Figure 6, may have occurred. We have shown that *N*-(1-pyrene)maleimide reacted with a cysteine residue in BSA. Since the amino group of the cysteine residue in BSA is part of the peptide backbone and is not freely available for reaction, the amino group which attacks the succinimido ring must belong to an amino acid adjacent to the cysteine residue. If such intramolecular aminolysis does occur in PM-BSA, we would predict that (a) the native structure of BSA is important in maintaining the relative proximity of the cysteine and the residue which contains a free amino group and (b) the amino group must be in a deprotonated form and thereby the rate of intramolecular aminolysis should be dependent on the pH of the solution.

In accordance with our predictions, we found that PM-BSA, which was prepared at pH 6 and incubated with 1% sodium dodecyl sulfate for 1 h at the same pH, did not show a spectral shift at pH 7.5. To the contrary, no such effect was observed with sodium dodecyl sulfate on the spectral shift of PM-cysteine. Moreover, the rate of decrease in fluorescence intensity at 376 nm for PM-BSA is pH dependent. Figure 7A shows a semilogarithmic plot of the fractional fluorescence of PM-BSA vs. time at various pH. The linearity of the plot indicates that the first-order kinetics were obeyed in all cases. From the slopes of the lines, the values of first-order rate constant, k, were estimated and their dependence of pH is shown in Figure 7B. It can be seen that the plot is sigmoidal and has an inflection point at approximately pH 8.

In principle, the inflection point as determined above should give information about the pK value of the amino group involved. A pK of 9 was obtained for PM-cysteine by a similar analysis of the pH dependence of the rate of fluorescence change (data not shown). For PM-BSA, a pK of 8 suggests that the amino group may either be a high pK α-NH_2 group of the N-terminus, or a low pK ε-NH_2 group of a lysine residue

(Means and Feeney, 1971). To distinguish between these two possibilities, we have carried out N-terminal analysis of BSA and PM-BSA by a dansylation method (Hartley and Massey, 1956; Gray, 1967). In accord with a previous report (Thompson, 1954), the N-terminal amino acid residue of BSA was identified to be aspartic acid. After acid hydrolysis, the dansylated aspartic acid of BSA appeared as a single, bright fluorescent spot on a two-dimensional polyamide thin-layer chromatogram. In contrast, the corresponding spot produced by a comparable amount of PM-BSA, which had been prepared at pH 6 and allowed to complete the spectral shift (2 h at pH 7.5 and 23 °C), was much weaker in fluorescence. Thus, the N-terminal NH_2 group was at least partially blocked in PM-BSA.

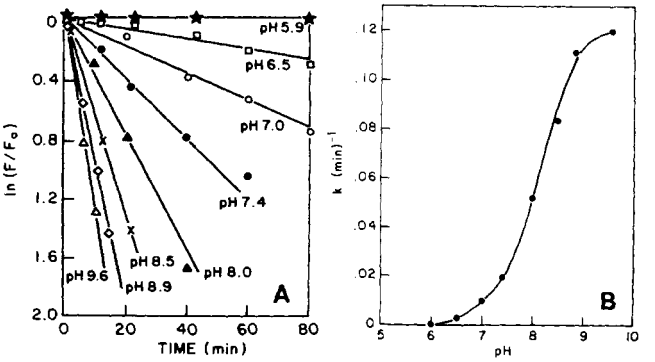

FIGURE 7: (A) Effect of pH on the rate of spectral shift observed with PM-BSA. Bovine serum albumin was labeled with *N*-(1-pyrene)maleimide at pH 6 as described in Experimental Procedures. After removal of free reagent, PM-BSA was added to phosphate or Bicine buffers, pH as indicated. Changes in the fluorescence intensity at 376 nm were monitored at 23 °C. The excitation wavelength was 342 nm. (B) The pH dependence of the first-order rate constant for PM-BSA. The rate constants were calculated from data shown in A.

Discussion

The conjugation reactions of *N*-(1-pyrene)maleimide with organic compounds or proteins which contain sulfhydryl groups are relatively fast and can be monitored by the increase in fluorescence intensity of pyrene chromophore (Figure 3). In some cases where primary amino groups were also present in the system, we have found that spectral shifts of the fluorescent adducts occurred following the rapid conjugation (Figures 2 and 4). In our model studies with PM-cysteine and PM-cysteamine, these spectral changes are interpreted as an intramolecular nucleophilic attack on the succinimido ring of the adducts by the primary amino group (Figure 5). We have presented both chemical (product analysis by ninhydrin test) and NMR evidence to support this reaction mechanism. Similar reaction mechanisms have also been reported for the cysteine adducts of *N*-ethylmaleimide (Smyth et al., 1960), *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (Witter and Tuppy, 1960), and *N*-[(*p*-(2-benzoxazolyl)phenyl)]-maleimide (Kanaoka et al., 1967). It should be noted that maleimides may also undergo a general base catalyzed hydrolysis to yield maleamic acids (Heitz et al., 1968; Smyth and Tuppy, 1968). However, the rates of such hydrolysis are too low to account for the observed spectral changes for PM-cysteine or PM-cysteamine at neutral pH. Thin-layer chromatography of these two adducts at pH 7.5 showed the presence of a single, ninhydrin-negative compound; the ninhydrin-positive hydrolytic products would appear only at alkaline pH (>9.5). This also agrees with our observation that PM adducts of *N*-acetyl-L-cysteine and β-mercaptoethanol, which have no primary amino groups, do not exhibit time-dependent spectral shifts.

Both PM-σ and PM-BSA showed time-dependent spectral shifts (Figures 1 and 2) similar to that observed for PM-cysteine. The fact that the fluorescence changes of PM-σ were observed only in Tris buffer, but not in Bicine or phosphate buffer, implies a possible intermolecular aminolysis of the succinimido ring by the amino group of Tris. For PM-BSA, the situation was quite different. Tris was not required for its spectral shift. In addition, the changes in spectral properties of PM-BSA probably did not reflect gross structural alteration in BSA molecules. Nanosecond fluorescence depolarization measurements performed with PM-BSA prepared at pH 7.5 yielded a rotational correlation time of 48 ns (Yarbrough and Wu, unpublished results). Similar values were reported for BSA labeled with various dyes (Weber, 1952; Harrington et al., 1956; Squire et al., 1968). Thus it appears that the spectral changes of PM-BSA are not due to intermolecular interactions between BSA molecules such as the formation of dimers or polymers.

BSA contains a single reactive cysteine residue located (position 34) relatively close to the N-terminus of the peptide chain (King and Spencer, 1972). The disappearance of the reactive sulfhydryl group in PM-BSA indicates the addition of *N*-(1-pyrene)maleimide to the sulfhydryl group of this cysteine residue to form a succinimide derivative. Since the spectral changes observed for PM-BSA (Figure 2) resemble those of PM-cysteine (Figure 4), they may be attributed to a reaction mechanism similar to that shown in Figure 5, in which the opening of the succinimido ring is caused by an adjacent amino group of one of the amino acid residues in BSA (Figure 6). By a dansylation method we have presented evidence for the blockage of the α-amino group of the N-terminal residue in PM-BSA after the spectral shift is completed. This strongly suggests that the terminal amino group is the one which participates in the intramolecular aminolysis. A similar study was carried out by Witter and Tuppy (1960) for the reaction of BSA with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM). Digestion of the reaction product with pepsin yielded two major yellow-colored *S*-DDPS-cysteine peptides. These two peptides had the same amino acid composition except that one lacked a free α-amino group. Interestingly, the blocked amino terminus could be cleaved by subtilisin suggesting that the blocking group probably involved an amide bond. These results were explained by an intramolecular acylation of the free terminal amino group due to the succinimido ring in one of the *S*-DDPS-cysteine peptides. Another similar intramolecular acylation has been postulated in experiments on the reaction of *N*-ethylmaleimide with hemoglobin (Benesch and Benesch, 1960).

The spectral shifts observed with PM-BSA could be prevented by addition of denaturating reagents such as sodium dodecyl sulfate immediately after labeling, indicating that the relative positions of the sulfhydryl and amino groups are very critical. This is also demonstrated by our finding that a 6-membered ring thiazane derivative was readily formed in PM-cysteine but an 11-membered ring derivative could not form in PM-glutathione. For PM-BSA, such intramolecular cyclization would involve the formation of a large size ring (between the N-terminus and the cysteine residue at position 34). Thus two residues far apart in the linear sequence of the protein yet closely positioned in space could be cross-linked by *N*-(1-pyrene)maleimide.

Some experimental conditions are crucial in performing the intramolecular cross-linking. For specific initial conjugation with sulfhydryl groups, the pH should be held neutral (~pH 7). At alkaline pH values, selectivity for sulfhydryl groups decreases and amino groups begin to react with maleimide (Brewer and Riehm, 1967). On the other hand, a pH value around 8 favors the subsequent intramolecular aminolysis (Figure 7). If the pH is too high (>9.5), the base-catalyzed hydrolysis of succinimide will occur. It is also apparent from the example of PM-σ given earlier that a primary amine, such as Tris, should not be used as buffer to avoid intermolecular aminolysis.

Although the pyrene chromophore is strongly fluorescent, *N*-(1-pyrene)maleimide shows no appreciable fluorescence in aqueous solution. In most aromatic compounds, the lowest energy electronic transition is of π,π^* nature. The nonfluorescent character of an aromatic compound containing a maleimide is presumably due to a low-lying n,π^* singlet state which involves the maleimide carbonyl groups (Kanaoka et al., 1970). Having longer lifetime, the n,π^* states are more susceptible to intersystem crossing at the expense of fluorescence (Hercules, 1966). Certain structural changes which affect the electronic state of the maleimide ring, e.g., saturation of the double bond to form succinimide derivatives, will restore the original strong fluorescence. Maleimide reagents of this type fluoresce only when covalently attached to sulfhydryl groups of proteins or organic compounds. We have found that certain adducts of one such reagent, *N*-(1-pyrene)maleimide, exhibit a red shift of emission maximum and a decrease in fluorescence intensity due to aminolytic reactions shown as in Figures 5 and 6. Comparison of structures I and II suggests that the possible factors which might contribute to these fluorescence changes are: (a) the rigidity of the molecular structure is decreased in the transition from I to II by opening the succinimide ring and, hence, the efficiency of luminescence may be decreased by increasing vibrational dissipation of electronic energy (Wehry and Rogers, 1966); and (b) the presence of an imido proton at the 1 position of the pyrene chromophore may facilitate the interaction of the chromophore with the solvent through hydrogen bonding or polarization effects. In aqueous solvent, these effects can lead to the red fluorescence shift of aromatic amines (Wehry and Rogers, 1966). For protein conjugates with *N*-(1-pyrene)maleimide, such characteristic fluorescence changes are indicative of cross-linking between a cysteine sulfhydryl group and an amino group. This amino group could either be the α-NH_2 group of the N-terminus or the ϵ-NH_2 group of a lysine residue. To distinguish between these two possibilities, one may measure the p*K* value of the amino group involved by carefully studying the pH dependence of the rate of the fluorescence changes. Thus *N*-(1-pyrene)maleimide can serve as a spectroscopic indicator for the spatial proximity of sulfhydryl and amino groups in proteins.

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