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FLUORESCENCE SPECTROSCOPIC STUDIES OF PYRENE-ACTIN ADDUCTS

T.-I. LIN and R.M. DOWBEN

Department of Pathology, Baylor University Medical Center, Dallas, TX 75246, U.S.A.

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Reaction kinetic studies of the sulfhydryl-directed fluorescent probes *N*-(1-pyrene)maleimide (PM) and *N*-(1-pyrenyl)iodoacetamide with actin from rabbit skeletal muscle showed that there were three accessible sulfhydryl groups in actin. Fluorescence spectral studies showed energy transfer from aromatic amino acid residues to fluorophore reacted at Cys-373, as well as weak excimer fluorescence probably due to doubly labeled molecules at Cys-10 and Cys-373. These results provide further evidence that trytophan and tyrosine residues are located near the probe attached to Cys-373 or Cys-10 and the latter two thiols are in close proximity. In aged PM-labeled F-actin, the succinimido ring of PM underwent intramolecular aminolysis, resulting in large emission spectral changes and increased excimer fluorescence. Solvent perturbation studies indicate that the probes were located in a hydrophobic environment; their quantum yield and spectrum properties were very sensitive to changes in the microenvironment. Nanosecond-pulse fluorimetry studies revealed complex fluorescence emission decays with three intrinsic lifetimes in adducts with low molecular weight thiols as well as in labeled proteins. Fluorescence lifetimes were 17, 48 and 111 ns for the pyrenemaleimide adduct of actin, and 3, 14 and 60 ns for the pyrenyliodoacetamide adduct. Supporting evidence is given for the argument that multiple fluorescence lifetimes are an intrinsic property of the pyrene derivatives and are not due to the presence of impurity or heterogeneity in the protein reaction sites. Because of their high sensitivity and long lifetimes, pyrene derivatives are extremely useful.

1. Introduction

Pyrene derivatives are very useful for fluorescence studies of protein conformation. They also exhibit complex absorption and fluorescence emission spectra. The fluorescence lifetimes and quantum yield of pyrene adducts with proteins are very sensitive to their microenvironments. The fluorescence emission intensity of the several peaks depends upon the solvent environment, and the ratios may be used as indicators of its hydrophobicity or polarizability [1]. Changes in spectra, fluorescence intensity, lifetimes and quantum yields can be used to monitor conformational changes in pyrene-labeled proteins that accompany physiologically relevant process. The utilization of a long-lived probe such as pyrene offers many experimental advantages. Studies of the rotational motion of large macromolecules are best accomplished using a probe with a lifetime comparable to the rotational correlation times to be measured which are hundreds of nanoseconds for the contractile proteins. In energy-transfer experiments [2], it is also advantageous to use long-lived probes so that small changes in separation distance result in large changes in lifetime.

Actin is a major protein found in the thin filaments of muscle, and is a major constituent of nonmuscle cells exhibiting motility. Spherical monomeric G-actin molecules polymerize in the presence of KCl, divalent cations and nucleotide triphosphates to form a double-stranded helical F-actin filament. Each monomeric actin molecule contains five cysteine residues, some located in or

Abbreviations: GdnHCl, guanidine hydrochloride; DMAMS, 4-dimethylamino-4′-maleimidostilbene; MANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; PIA, *N*-(1-pyrenyl)iodacetamide; PM, *N*-(1-pyrene)maleimide; SDS, sodium dodecyl sulfate; TM, tropomyosin.

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near functionally important sites. For example, Cys-373 is located at the myosin-binding site, and Cys-10 and Cys-256 are near the sites involved in actin polymerization [3]. Two SH-reactive pyrene derivatives, *N*-(1-pyrene)maleimide (PM) and *N*-(1-pyrenyl)iodoacetamide (PIA), are frequently used to label proteins. Thus, the SH-directed pyrene probes can be attached near functionally important sites of actin as fluorescent indicators of changes in actin properties which accompany such physiologically important processes as G-F transformation, binding of nucleotide, binding of divalent cations, binding of thin filament regulatory proteins, and interaction with myosin.

Reports on the fluorescence lifetimes of pyrene and its derivatives are abundant in the literature (see the preceding paper [6]) but inconsistent. Also, there seems to be a growing concern as to whether multiple fluorescence decay behavior of pyrene derivatives is an intrinsic property of the pyrene probe itself. Problems of this kind can be best studied by nanosecond-pulse fluorimetry using single-photon-counting detection as employed in this report. The steady-state fluorescence properties and decay lifetimes of small thiol compound and actin labeled with PM or PIA are reported here. These studies were aimed primarily at exploring the usefulness of these fluorescent probes in the conformational studies of actin and its interaction with various muscle contractile proteins and in other types of more biophysically oriented experiments, viz., fluorescence energy transfer and time-resolved fluorescence depolarization studies. In the conformational studies, high sensitivity of the probe to the microenvironment is required. In the latter two types of experiments, a probe with long lifetimes is desirable. The present studies seem to indicate that pyrene derivative probes suit these requirements very well.

2. Materials and Methods

2.1. Reagents

Very high purity PM and PIA were purchased from Molecular Probes, Inc. (Plano, TX, U.S.A.). Ultrapure urea and GdnHCl were purchased from Schwarz-Mann (Orangeburg, NY, U.S.A.). dimethylformamide, methanol and dimethyl sulfoxide were spectro grade solvents obtained from J.B. Baker (Phillipsburg, NJ, U.S.A.). 2-Mercaptoethanol was purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). All other chemicals were reagent grade.

2.2. Proteins

Actin and tropomyosin were prepared from the back and leg muscles of rabbits. Actin was extracted from acetone powder stored at −20°C and purified as previously described [4]. Tropomyosin (TM) was extracted from an ethanol/ether powder using the method described by Briskey and Fukazawa [5] with modifications [6]. The purity of the proteins was checked by SDS gel electrophoresis. The protein stock solutions were contained in 1 mM NaN_3 for storage.

2.3. Preparation of PM and PIA adducts

To a solution of 40 μM actin in 0.15 M KCl, 1 mM $MgSO_4$ and 10 mM phosphate buffer, pH 8.4, were added aliquots of 20 mM PM or PIA dissolved in dimethyl sulfoxide or dimethylformamide to obtain a dye-to-protein ratio of 10:1. The final solvent concentration was kept below 2% to avoid denaturation of actin. The reaction with PIA was done in the dark overnight at 4°C, while the reaction with PM was monitored in a spectrofluorimeter at 25°C and completed in less than 2 h. The reaction was stopped by the addition of an excess of 2-mercaptoethanol. The labeled actin was depolymerized by dialysis against 0.2 mM ATP-$MgCl_2$, 2 mM phosphate buffer, pH 7.4, and the remaining dyes unreacted with the protein were then removed by chromatography on a Sephadex G-25 column. TM was lightly labeled with PM or PIA as decribed in the preceding paper [6].

PM and PIA adducts of 2-mercaptoethanol and *N*-acetylcysteine were prepared by allowing equimolar amounts of the pyrene reagent and SH compound to react at room temperature in 0.15 M KCl, 1 mM $MgSO_4$ and 10 mM phosphate buffer, pH 8.4. Precipitate after reaction, if any, was removed by filtration through a 0.45 μm Millipore

filter. PM adducts of cysteine and lysine were similarly prepared at two different pH values 7.0 and 11.6 for cysteine, and 7.2 and 10.4 for lysine.

2.4. Protein concentration

The concentration of unlabeled protein in solution was determined by measurement of its absorbance. The extinction coefficients used were 0.63 and 0.671 g^{-1} cm^{-1} for G-actin and F-actin at 290 nm, and 0.2341 g^{-1} cm^{-1} for TM at 280 nm. A scattered-light correction was always applied [4]. The protein concentration of probe-labeled actin was determined by the method of Lowry et al. [7] using unlabeled actin as standard. The molecular weight of actin was taken to be 42000 and that of TM to be 66000.

The concentrations of PM and PIA in the labeled proteins were determined as follows. Standard solutions of PM and PIA adducts of 2-mercaptoethanol were prepared at concentrations from 0.2 to 5 μM in 5% SDS. The fluorescence intensities of PM of PIA adducts measured at 377 and 386 nm, respectively, were found to be linearly proportional to the concentrations of labeled adducts in this range. Labeled proteins were dissolved in 5% SDS and the concentrations of PM and PIA were determined by comparing the fluorescence intensity with that of standard 2-mercaptoethanol adducts of PM or PIA. As PM-actin adducts aged, intramolecular aminolysis occurred (see section 3), accompanied by a shift of the fluorescence maximum from 377 to 386 nm. Therefore, the PM concentration in protein adducts was always determined on freshly prepared material. The molar labeling ratio of PM to actin was 0.83–1.45, and of PIA to actin 0.45–0.93.

2.5. Spectral measurements

Absorption spectra were recorded on a Cary (Varian, Palo Alto, CA, U.S.A.) Model 219 Spectrometer. Absorbance was measured in a Hitachi-Perkin-Elmer (Norwalk, CN, U.S.A.) Model 139 spectrophotometer. Fluorescence spectral studies were made in a 1 cm light path quartz cell using a Farrand (Valhalla, NY, U.S.A.) Mark I Spectrofluorimeter with constant-temperature water circulating through the cell compartment. Spectra were not corrected for wavelength-dependent differences in xenon lamp intensity nor photomultiplier tube response. However, fluorescence intensity measurements were partially corrected using a suitable blank. Protein and dye adduct concentrations were kept sufficiently low to minimize self-absorption effects.

2.6. Lifetime measurements

Fluorescence lifetimes were measured by single-photon-counting detection using a modified Ortex (Oak Ridge,TN, U.S.A.) Model 9200 system. A free-running 9.5 kV air discharge lamp pulsed at 16 kHz was used as the excitation source. Narrow band-pass filters were used to select the excitation and emission wavelengths and to isolate the emission path from the excitation path. The lamp flash was deconvoluted from the fluorescence decay which was then analyzed using the method of moments program [8–10] modified by Eisenfeld and Chang [11] which permits a determination of the number of components in the decay and the individual lifetimes and amplitudes of each component. Details of lifetime measurements and decay data analysis will be published elsewhere.

3. Results

3.1. Reaction kinetics of PM with F-actin

PM is virtually nonfluorescent but fluoresces intensely after the maleimide functional group has reacted to form a succinimido ring. Thus, the reaction of PM with F-actin was monitored conveniently in a spectrofluorimeter as depicted in fig. 1. The maleimide reacts specifically with free SH groups at neutral pH, with increased reactivity at pH 8.4 when the SH residues are ionized.

While rigorous quantification of the kinetics was not possible because it was not possible to determine differences in quantum yield of the probe bound to different sites, kinetic data did suggest that of the five SH groups in F-actin, only three reactive sites were accessible with different reactivities.

Table 1

Fluorescence emission spectral characteristics of pyrene conjugates

Solutions contained 2 μM PM-labeled protein or 4 μM PIA-actin, 0.5 M KCl, 1 mM $MgSO_4$, 10 mM phosphate, pH 7.4. Lysine and cysteine adducts were prepared at the pH indicated, and neutralized and filtered before measurement. Pyrene was dissolved in methanol. Excitation wavelength 345 nm, spectral resolution 2.5 nm. A spectral shoulder is denoted by s.

Compound	Emission peaks (nm) (intensity ratio)
(A) Small conjugates	
Pyrene	374 (1.00), 379 (0.54), 385 (0.86), 389 (0.704), 393 (0.778), 404 (0.440), 415 (s) (0.320)
PM-mercaptoethanol	374 (1.00), 382 (s) (0.242), 396 (0.437), 416 (s) (0.131)
PM-*N*-acetylcysteine	376 (1.00), 384 (0.797), 396 (0.772), 416 (s) (0.367)
PM-cysteine (pH 8.4)	376 (1.00), 385 (0.716), 396 (0.761), 416 (s) (0.403)
PM-cysteine (pH 11)	376 (1.00), 385 (1.08), 396 (1.307), 405 (s) (1.091), 416 (s) (0.886), 426 (s) (0.727)
PM-lysine (pH 10.4)	376 (1.00), 385 (0.988), 396 (0.911), 405 (s) (0.757), 416 (s) (0.485), 426 (s) (0.355)
PIA-mercaptoethanol	386 (1.00), 406 (0.625), 426 (s) (0.250)
(B) Protein adducts	
PM-actin (fresh)	376 (1.00), 382 (s) (0.321), 396 (0.510), 416 (0.163)
PM-actin (84 days)	376 (1.00), 386 (0.878), 396 (0.858), 405 (s) (0.675) 460 (s) (0.327)
PM-TM (monomer)	376 (1.00), 382 (s) (0.335), 396 (0.525), 415 (s) (0.178)
Aminolyzed PM-actin	386 (1.00), 406 (0.700), 426 (s) (0.317)
PIA-actin	386 (1.00), 406 (0.738), 425 (s) (0.422), 460 (s) (0.305)

tophan to pyrene, and the higher excitation peak at 278 nm may be attributed to possible energy transfer from tyrosine. TM lacks tryptophan completely which may account for the absence of an excitation peak at 291 nm in pyrene-labeled TM. Intrinsic protein fluorescence in the 340–350 nm region attributable to tyrosine and tryptophan residues was substantially less in the pyrene-labeled adducts than in unlabeled actin. These results suggest fluorescence energy transfer from tyrosine

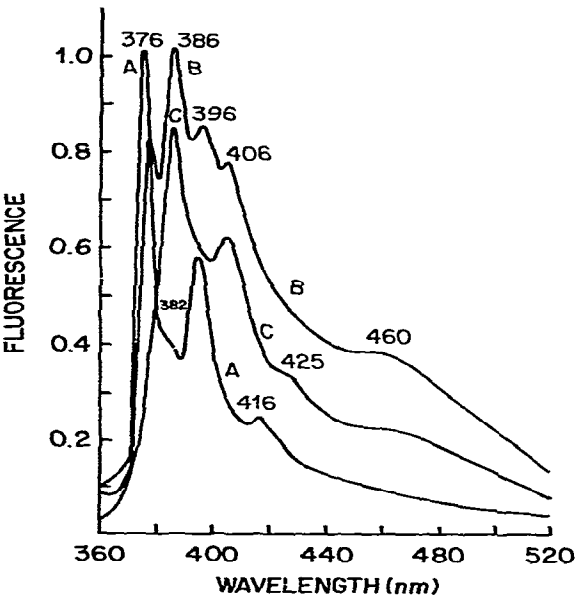

Fig. 2. Uncorrected fluorescence emission spectra. PM-actin (curve A), intramolecularly aminolyzed PM-actin (curve B) and PIA-actin (curve C). Conditions: 4 μM labeled actin in 0.15 M KCl, 1 mM $MgSO_4$ and 10 mM phosphate, pH 7.0, 25°C. Excitation at 345 nm; spectral resolution 2.5 nm. Dye-to-protein ratio: 1.45 in curves A and B, 0.93 in curve C.

and tryptophan residues in proximity to the pyrene via a mechanism similar to that proposed by Lin [3] in dansylaziridine-labeled actin, and also in MANS- and DMAMS-labeled actin (Lin and Dowben, in preparation). However, in the case of PIA-labeled actin (table 2), there was no indication of energy transfer from aromatic amino acid residues, suggesting a different spatial disposition of the pyrene moieties from that in the PM-labeled adducts.

3.3 Aminolysis of PM-labeled actin

Comparison of the emission spectra (table 1A) shows that the PM adduct of 2-mercaptoethanol displays a shoulder at 382 nm while the adduct of cysteine shows a peak at 385 nm. The peak at 385 nm increased with time and was greater at higher pH as a result of aminolysis of the succinimido group with the primary amino group. Reaction of

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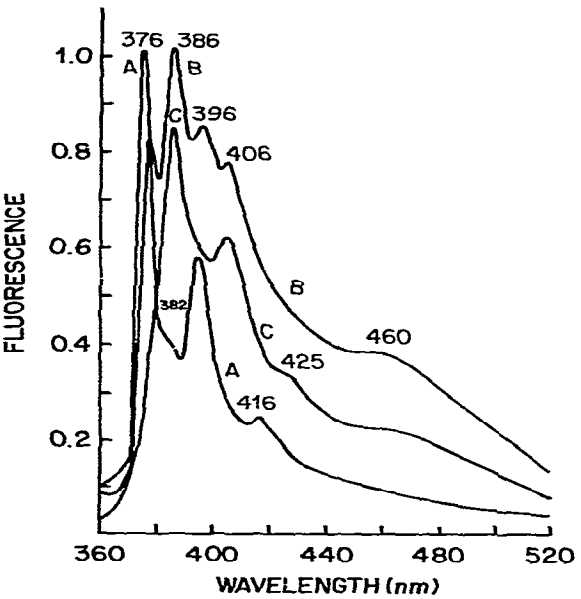

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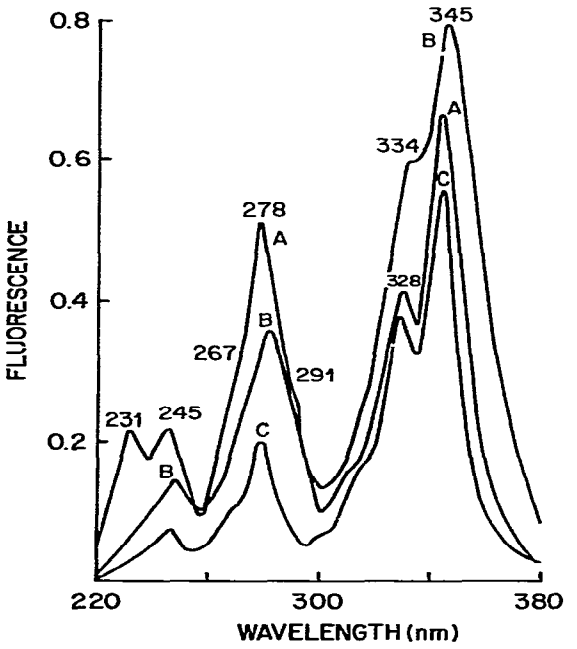

Fig. 3. Uncorrected excitation spectra of monomer fluorescence. PM-actin (curve A), aminolyzed PM-actin (curve B) and excimer fluorescence of PM-actin (curve C). Emission measured at 377 nm for monomer and 460 nm for excimer fluorescence. Same conditions as for fig. 2.

PM with the α-amino group of lysine yielded adduct with an emission maximum at 376 nm. The peak at 386 nm increased and became maximum as the pH was raised. Therefore, the emission spectral changes accompany the breaking of the succinimido ring and are not a result of reaction with the amino group per se. Aminolysis of the succinimido ring is characterized by the disappearance of three emission peaks at 376 (maximum), 396 and 416 nm and their replacement by new peaks at 386 (maximum) and 406 nm and a shoulder at 426 nm.

Freshly prepared PM-labeled actin exhibits a spectral shoulder at 382 nm (fig. 2, curve A). As the labeled protein aged, the fluorescence intensity of the 382 nm shoulder increased gradually while the intensity of the 376 nm maximum decreased pari passu. In 84-day old adduct (table 1b and fig. 2, curve B) there was a red shift of the 382 nm

Table 2

Fluorescence excitation spectral characteristics of pyrene adducts

Conditions same as for table 1. Emission wavelengths were 374 nm for PM-2-mercaptoethanol, 376 nm for PM-TM, 400 nm for PM-actin, and 386 nm for all other solutions.

Adducts	Excitation peaks (nm) (intensity ratio)
PM-mercaptoethanol	342 (1.00), 327 (0.612), 314 (s) (0.234), 278 (0.343), 267 (0.158), 245 (0.172)
PM-actin (fresh)	344 (1.00), 328 (0.642), 314 (s) (0.250), 291 (s) (0.340), 278 (0.773), 267 (s) (0.395), 245 (0.329), 231 (0.320)
PM-TM (monomer)	344 (1.00), 328 (0.650), 314 (s) (0.269), 278 (0.396), 268 (s) (0.206), 245 (0.191)
Aminolyzed PM-actin	345 (1.00), 330 (s) (0.575), 283 (0.730), 248 (0.183), 236 (0.133)
PIA-mercaptoethanol	348 (1.00), 332 (s) (0.578), 286 (0.492), 276 (0.236)
PIA-actin	346 (1.00), 332 (s) (0.625), 284 (0.680), 248 (0.319), 232 (0.263)
PIA-TM	342 (1.00), 328 (s) (0.858), 315 (s) (0.280), 278 (0.403), 268 (s) (0.233), 245 (0.181)

peak of 386 nm. The intensities at 396 and 415 nm also increased and the peaks shifted to 406 and 426 nm. Wu et al. [12] reported that in PM-labeled bovine serum albumin, the succinimido group can undergo intramolecular aminolysis with a nearby primary amine group, probably an ϵ-amino group of lysine. Our own results indicate that a similar intramolecular aminolysis of PM-labeled actin occurs upon storage. Searching the amino acid sequence of actin [13] for possible amino groups involved in the reaction with the succinimido ring of PM derivatives, we found only Cys-373 has an adjacent lysine residue at 372. Aminolysis also occurred in actin briefly labeled with PM, in which pyrene probe was attached predominantly at the most reactive Cys-373. Therefore, it seems that aminolysis probably occurred at Cys-373 involving the ϵ-amino residue of Lys-372. Intermolecular aminolysis of PM-labeled actin can be performed quite rapidly by adding 8 M urea, 5.5 M GdnHCl, or adding Tris and raising the pH above 10.5 to

ionize the amino group on Tris. The latter procedure produced changes in the emission spectrum characteristic of aminolysis; upon subsequent lowering of the pH to neutral vlaues, the emission spectrum remained altered and did not revert to its original form.

The emission spectrum of PM-labeled actin that had undergone aminolysis with storage resembled the emission spectrum of PIA-labeled actin (table 1B). In both cases, a shoulder at 460 nm was present, indicating intramolecular excimer formation similar to that observed with PM- and PIA-labeled TM [6]. Previously, Lin [3] suggested that Cys-10 and Cys-373 of actin are located in proximity to one another in the native protein. Because excimer fluorescence occurs only when two pyrene moieties are in close proximity, this finding provides new evidence that Cys-10 and Cys-373 are near to each other. The excitation spectrum obtained using emission at 460 nm (fig. 3, curve B) was attributed to excimer fluorescence and was very similar for both PM- and PIA-labeled actin, and resembled the pyrene excimer of TM adducts [6]. The rate of intramolecular aminolysis and the intensity of excimer fluorescence were much greater in labeled TM. In contrast, PM-labeled actin that had undergone aminolysis after addition of an exogenous amine and raising of the pH did not show an increase in excimer fluorescence. Upon denaturation by urea or SDS, the excimer fluorescence disappeared.

The excitation spectrum of aminolyzed PM-labeled actin (fig. 3, curve C) differed from that of fresh PM-labeled actin but resembled that of PIA-labeled actin (table 2). The shoulder at 291 nm in fresh material was absent in the aminolyzed material suggesting that there was no energy transfer from aromatic amino acid residues after aminolysis. Possibly the molecular rearrangement of aminolysis is accompanied by movement of the pyrene moiety away from the tryptophan and tyrosine residues and toward a nearby moiety to enable excimer fluorescence.

Table 3

Fluorescence lifetimes of PM and PIA adducts

Solution conditions same as for table 1. Excitation at 340 nm, emission at 400 nm. R, relative intensity ratio; τ, fluorescence lifetime; MD, moment displacement index; DP, depression parameter (see text).

Compound	R_1^*	τ_1 (ns)	R_2	τ_2 (ns)	R_3	τ_3 (ns)	DP	MD
(A) Small conjugates								
PM in dimethylformamide	0.21	1.41	0.16	11.0	0.63	47.3	0.4	0
PM-mercaptoethanol	0.02	2.64	0.04	20.9	0.94	64.8	0.38	0
PM-cysteine (pH 8.4)	0.39	1.35	0.44	7.16	0.18	26.6	0.35	0
PM-cysteine (pH 11)	0.24	1.34	0.60	6.17	0.17	30.2	0.37	0
PM-lysine (pH 7.2)	0.35	1.64	0.53	6.27	0.12	37.7	0.3	0
PM-lysine (pH 10.4)	0.40	1.27	0.48	6.12	0.12	29.5	0.35	0
(B) Protein adducts								
PM-actin (fresh)	0.19	16.94	0.50	48.3	0.31	110.8	0.70	2
PM-TM (monomer)	0.44	2.98	0.34	22.1	0.22	86.7	0.23	1
PIA-actin	0.51	2.92	0.44	13.5	0.05	59.8	0.25	0
PIA TM	0.67	2.51	0.27	15.2	0.06	73.8	0.11	0
PM-actin (60 days)	0.07	6.34	0.41	32.0	0.52	89.8	0.21	0
Aminolyzed PM-actin	0.23	7.52	0.38	23.3	0.39	66.6	0.21	0
Aminolyzed PM-TM	0.36	2.41	0.52	11.1	0.12	50.9	0.15	0
PM-actin in GdnHCl	0.51	4.08	0.36	15.2	0.13	40.6	0.21	0
PM-TM in GdnHCl	0.38	3.14	0.37	12.2	0.25	37.7	0.46	1
Aminolyzed PM-actin in GdnHCl	0.34	4.20	0.39	9.89	0.27	53.5	0.18	0

3.4. Fluorescence lifetime measurements

Three lifetime components were found in the fluorescence decay of PM adducts with small thiols (table 3A). For cysteine and lysine adducts, the lifetime components were about 1.3, 6 and 30 ns. In the 2-mercaptoethanol adduct, the two longer lifetimes were 21 and 65 ns. Reaction of PM with cysteine and lysine at alkaline pH yielded adducts with almost identical lifetimes of the two shorter components, but the lifetime of the longest component was different dependent upon whether conjugation occurred at neutral or alkaline pH. At alkaline pH, there is the possibility that PM reacted with amino as well as SH groups.

The lifetimes of actin and TM adducts are listed in table 3B. All three lifetime components of PM-actin were considerably longer, about 17, 48 and 111 ns, and the intensity ratios were different from the comparable values obtained for PM-TM. The lifetimes of the first two components of PIA-actin and PIA-TM were about the same, but the third lifetime was shorter in the actin adduct than in PIA-TM. PM-actin stored for 84 days that underwent intramolecular aminolysis showed much shorter lifetimes of 6.3, 32 and 90 ns. On the other hand, if aminolysis was brought about by the addition of an exogenous amine and alkalinization, the lifetimes were different, 7.5, 23 and 66.6 ns. After denaturation in 5 M GdnHCl, the lifetimes were about 3, 12 and 38 ns. The values of lifetimes and intensities ratios were different from comparable values for PM-TM adducts. These results are consistent with the explanation that the lifetimes and relative intensity ratios of the pyrene fluorescence components are sensitive to changes in the microenvironment.

The finding of three lifetimes in the decay curves of all PM and PIA adducts contrasts with an earlier study [14] which showed only a single lifetime for small thiol adducts and two lifetime components for several protein adducts including actin. The discrepancy is probably due to better instrumentation and better data resolution in the analysis employed in the present study. Kawasaki et al. [15] also found three lifetimes for PM-labeled actin, but two of the lifetimes were shorter, probably because these investigators studied actin adducts after intermolecular aminolysis by Tris buffer. On the other hand, Kouyama and Mihashi [16] recently reported three lifetimes of 170 and 80 ns, and a minor component of very short lifetime in PM-labeled actin. We thoroughly studied PM-labeled actin under many conditions but never found a lifetime as long as 170 ns. In measurements involving multiple lifetime components that differ by an order of magnitude in lifetime and amplitude ratio, it is essential to choose an appropriate time base for the experiment. In general, a time base which can accommodate five decays of the longest lifetime component should be used. A difficulty inherent in using longer time bases is the fact that the late channels accumulate very few counts as compared to the peak channels, a problem which is worsened if the longest lifetime component is of weak intensity. In our own experiments, we found that the use of too long a time based resulted in an abnormally large calculated longest lifetime component.

3.5. Perturbation studies

The effects of GdnHCl, urea and SDS on fluorescence properties of pyrene-actin adducts were investigated, particularly on PM adducts which had undergone extensive intramolecular aminolysis. To minimize the effects of intermolecular aminolysis with amino groups uncovered by GdnHCl or urea, the spectra were obtained immediately after adding these denaturants. The relative quantum yields of the pyrene-protein adducts in the denaturing media were calculated by comparing the integrated areas of the emission spectra between 360 and 560 nm. The emission spectral properties and relative quantum yields of PM- and PIA-labeled actin are given in table 4. SDS was a powerful denaturant, unfolding the polypeptide chain and abolishing excimer fluorescence; fluorescence at 460 nm completely disappeared in 5% SDS. The relative intensity of excimer to monomer fluorescence of PM-actin adducts diminished by 71% in GdnHCl and by 40% in urea; the comparable figures for PIA-actin were a 14% relative decrease in excimer fluorescence in GdnHCl and a 12% increase in urea. As in the case of labeled TM, urea and GdnHCl were more

Table 4

Effects of denaturants on fluorescence emission of actin adducts

4 μM labeled protein in 0.15 M KCl, 10 mM phosphate (pH 7.4), 1 mM $MgSO_4$; PM-actin was stored in 1 mM NaN_3 and aged 84 days; 7 μM labeled proteins in 5% SDS or 5 M GdnHCl or 8 M urea. Spectral parameters same as in table 1.

Medium	Emission peaks (nm) (intensity ratio)	Relative quantum yield (%)
(A) PM-actin adducts		
KCl	377 (1.00), 386 (1.209), 396 (1.016), 406 (s) (0.905), 426 (s) (0.569), 460 (s) (0.455)	100
SDS	377 (1.00), 386 (0.797), 396 (0.722), 405 (s) (0.525)	76
GdnHCl	377 (1.00), 386 (0.938), 396 (0.837), 405 (s) (0.499), 426 (s) (0.298), 460 (s) (0.132)	18
Urea	377 (1.00), 386 (1.155), 396 (0.964), 406 (s) (0.836), 460 (0.274)	31
(B) PIA-actin adducts		
KCl	386 (1.00), 406 (0.733), 426 (s) (0.411), 460 (s) (0.264)	100
SDS	386 (1.00), 406 (0.626), 426 (s) (0.229)	45
GdnHCl	386 (1.00), 405 (0.804), 430 (s) (0.390), 480 (s) (0.231)	14
Urea	386 (1.00), 405 (0.731), 426 (s) (0.438), 480 (s) (0.305)	28

effective in dissociating the stacked pyrene rings in the PM adducts than in the PIA adducts.

The relative quantum yield decreased about 24% for PM-actin and 53% for PIA-actin in 5% SDS. The quantum yield decreases were about 82–86% in GdnHCl and 69–72% in urea. These results suggest that the fluorophores in both types of adducts were located in a hydrophobic environment, and upon denaturation became exposed to solvent which caused a marked decrease in quantum yield.

4. Discussion

Large fluorescence enhancement (about 3–10-fold) resulting from the reaction of PM with proteins provides a convenient way for studying the reactivities and determining of the number of accessible groups in proteins. In actin, there are maximally three thiols (out of a total of five) capable of reacting with PM.

The pyrene fluorophores attached to either Cys-373 or Cys-10 receiving fluorescence excitation energy transferred from nearby tryptophan and tyrosine residues via a nonradiative process (Förster energy transfer). The presence of weak excimer fluorescence in both PIA-actin and intramolecularly aminolyzed PM-actin provides further evidence that the two cysteine residues, when the pyrene derivatives are attached, are also near each other. The drastic changes in the emission spectrum, in addition to excimer fluorescence, accompanying the intramolecular aminolysis of PM-labeled adducts provide a useful means to probe the proximity of lysine to cysteine residue as well as the proximity of two cysteine residues in the protein. Excimer fluorescence in pyrene-labeled TM [6], is strong because the Cys-190 residues are very close owing to the parallel arrangement in register of the two TM subunits favoring stacking interaction of two pyrene moieties. Unfolding of protein by denaturants, particularly SDS, resulting in loss of excimer fluorescence.

Lifetime measurements of small thiol compounds and proteins conjugated with PIA or PM under various conditions all showed three lifetimes, a short lifetime ranging from 1.3 to 17 ns, a medium lifetime ranging from 6 to 48 ns, and a long lifetime ranging from 26 to 111 ns. Three lifetimes are always present whether the protein adduct is native or unfolded by denaturants. These data together with the observation of also three lifetimes in the excimer of PM- and PIA-labeled TM [6], which can only be formed under very unique and special conditions, strongly suggest

that multiple components in the decay curve can only be an intrinsic property of pyrene derivatives, and are not due to impurity or heterogeneity of the pyrene-protein adduct. The high sensitivity of fluorescence intensity and lifetimes of the pyrene probes to the microenvironment in the protein adducts and the presence of long lifetimes of the probe make the pyrene derivatives very useful.

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