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EXCIMER FLUORESCENCE OF PYRENE-TROPOMYOSIN ADDUCTS

T.-I. LIN

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

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Studies of the fluorescence of *N*-(1-pyrene)maleimide and *N*-(1-pyrenyl)iodoacetamide adducts of rabbit skeletal muscle tropomyosin revealed the presence of excimer fluorescence characterized by a broad emission band at 480 nm with a shoulder at 505 nm. Monomer fluorescence decay exhibited different lifetimes, viz., about 3, 22 and 87 ns for the pyrenemaleimide adduct; about 2.5, 11 and 51 ns for the aminolyzed maleimide adduct; and about 2.5, 15 and 74 ns for the pyrenyliodoacetamide adduct. Almost identical excimer fluorescence lifetimes were found for all adducts; about 9, 35, and 65 ns. Excimer fluorescence was sensitive to changes in ionic strength and pH of the medium while monomer fluorescence did not change. The protein denaturants guanidine hydrochloride and urea caused dissociation of the two tropomyosin subunits and partial disappearance of excimer fluorescence, but not as effectively as the hydrophobic surfactant sodium dodecyl sulfate. The sensitivity of excimer fluorescence to changes in the microenvironment make these pyrene derivatives very useful probes for studying conformational changes and binding interaction of tropomyosin with other contractile proteins. The unique location of the excimer probe at tropomyosin Cys-190 and its characteristic long lifetimes could make it useful in time-resolved anisotropy studies and fluorescence energy-transfer experiments.

1. Introduction

One of the most interesting fluorescence properties of pyrene and its derivatives is excimer formation, first recognized to occur by Forster and Kasper [1] in solutions of concentration greater than 10^{-4} M. Excimers are homologous dimers that exist only within the lifetime of excited electronic states. They are detectable in the fluorescence emission spectra. Many aromatic chromophores are able to form excimers, e.g., naphthalene, perylene, anthracene, and many of their derivatives. The presence of excimer fluorescence sometimes can be demonstrated only under extreme conditions such as at very low temperatures or high pressures. The excimers of pyrene compounds appear to be those most easily demonstrable under ordinary conditions.

Intermolecular excimers are formed by translational diffusional collision of an excited fluorophore molecule with a molecule in the ground state. The process requires that the two fluorophore molecules are associated at least briefly, commonly in a stacking interaction. Thus, intermolecular excimer fluorescence requires a minimum concentration of fluorophore in the solution. Excimers exhibit a distinctive fluorescence emission maximum, and a fluorescence lifetime different from that of the monomer. In macromolecules, when two or more aromatic fluorophores can be attached to sites in proximity to each other, interaction between the fluorophores is more likely and intramolecular excimer formation is favored. In the latter case, excimer formation occurs even at low concentrations of fluorophore, and the intensity of excimer fluorescence is linearly proportional to the concentration of the intramolecularly

Abbreviations: DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; PIA, *N*-(1-pyrenyl)iodoacetamide; PM, *N*-(1-pyrene)maleimide; SDS, sodium dodecyl sulfate; TM, tropomyosin.

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associated fluorophores Specifically, excimer fluorescence can be observed at low protein concentrations when pyrene fluorophores are attached to two or more binding sites near one another. The equilibrium separation distance between the fluorophores in a pyrene excimer was estimated by Birks and Kazzaz [2] to be about 3.53 Å. The occurrence of intramolecular excimers in biological macromolecules is rare because of the stringent conditions required for their formation.

Intermolecular excimer fluorescence in a concentrated solution of the *N*-(1-pyrene)maleimide (PM) adduct of 1-butanethiol was first reported by Weltman et al. [3]. In two recent studies, Lovejoy et al. [4] reported excimer formation in human serum albumin labeled with pyrenebutyrylsulfuric anhydride at the free amino groups of lysine residues, and Betcher-Lange and Lehrer [5] found excimer fluorescence in rabbit skeletal muscle α,α-tropomyosin labeled at the cysteine residues with PM. In the previous studies, only the steady-state fluorescence properties of the pyrene excimer were investigated. Excimers of protein conjugates of pyrene derivatives typically exhibit a broad emission band at 480 nm, unlike the monomer fluorescence which is characterized by several sharp emission peaks in the 375–420 nm region.

Skeletal muscle tropomyosin (TM) is a long rod-shaped molecule consisting of two highly α-helical subunits (almost 100% helical content) of 284 amino acid residues oriented in the same direction and wound in a coiled-coil superhelix. There are two types of subunits, α-chains containing a single cysteine residue at 190, and β-chains containing two cysteines at 36 and 190. The α- and β-subunits are present in rabbit psoas muscle in a 3.53:1 ratio so that approximately half the TM molecules are $\alpha\alpha$ and half are $\alpha\beta$; in other types of muscle, the ratios of α- to β-subunits may be different. Because the two subunits are oriented in parallel and in register, the Cys-190 residues lie close to each other. Thus, when two pyrene molecules are linked to the two cysteine residues, they lie in such close proximity that excimer fluorescence is likely to occur.

In this communication, spectral and lifetime studies of monomer and excimer fluorescence are reported of TM adducts with *N*-(1-pyrenyl)iodoacetamide (PIA) and PM. In PIA-labeled TM, excimer fluorescence occurred immediately upon attaching two probe molecules to the Cys-190 SH groups. In the case of PM, two probes were also attached to the Cys-190 SH groups, but excimer fluorescence did not occur until subsequent reaction with the ϵ-amino group of Lys-189 which caused intramolecular aminolysis of the succinimido ring and attachment of the pyrene moiety to two residues on the protein. The commonly used protein denaturants, GdnHCl, urea and SDS, caused complete dissociation of PM-labeled TM subunits and loss of excimer fluorescence; the PIA adduct was more stable and resistant to dissociation by these agents. Excimer fluorescence also was sensitive to changes in ionic strength and pH. Nanosecond-pulse fluorimetric studies showed fluorescence decay curves with three lifetime components for both monomer and excimer. Lifetime measurements were also performed under a variety of conditions to study the fluorescence characteristics of the pyrene-TM adducts.

2. Materials and methods

2.1. Proteins

TM was extracted from an ethanol ether-dried powder of rabbit skeletal muscle using the method of Briskey and Fukazawa [6] somewhat modified. TM was extracted with a solution of 1 M KCl, 2 mM DTT, 0.1 mM $CaCl_2$, and 25 mM Tris-HCl, pH 8.0. TM in the crude extract was purified by ammonium sulfate fractionation between 40 and 60% saturated, and repeated isoelectric precipitation at pH 4.6 and redissolution at pH 7.5. The protein was then chromatographed on hydroxyapatite (Bio-Rad, Richmond, CA, U.S.A.) to remove traces of troponin [7]. The TM was used intact and not separated into subunits. SDS gel electrophoresis showed about a 3.3:1 ratio of α-chain to β-chain of TM without visible protein impurities. Concentrations of TM solutions was determined by absorbance measurements at 280 nm using an extinction coefficient of 0.234 l g^{-1} cm^{-1}. The molecular weight of TM was taken to be 66000.

2.2. Preparation of pyrene-TM adducts

Before labeling, TM was reduced in a solution of 20 mM DTT, 1 M KCl, 1 mM $MgSO_4$, 2 mM EDTA and 10 mM phosphate buffer, pH 7.4, under a nitrogen atmosphere for 1 h at 25°C and then overnight at 4°C. After reduction, the excess DTT was removed by several isoelectric precipitations of the TM folowed by gel filtration on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column previously equilibrated with 1 M KCl and 10 mM potassium phosphate, pH 7.0, using 1 M KCl as eluant. PIA and PM were purchased from Molecular Probes, Inc. (Plano, TX, U.S.A.) and used without further purification. Small aliquots of 20 mM probe solutions in dimethylformamide were added to 30–40 μM solutions of TM immediately after reduction so that the molar dye-to-protein ratio was approx. 10:1. The mixture was adjusted to pH 8.4, sealed under nitrogen, and allowed to react at room temperature. For kinetic studies, the reaction was monitored in a spectrofluorimeter. The reaction was allowed to proceed for various time intervals giving preparations with different probe-to-protein ratios and stopped by adding an excess of 2-mercaptoethanol. Probe not covalently linked to protein was removed first by exhaustive dialysis and then by chromatography on a Sephadex G-25 column previously equilibrated with 0.15 M KCl and 10 mM phosphate buffer, pH 7.0. After the addition of sodium azide, 1 mM final concentration, the proteins could be stored for 2 months without deterioration.

The concentration of protein in the adduct was determined by the method of Lowry et al. [8] using unlabeled TM as standard. The concentration of dye in the labeled protein was determined as follows. PM and PIA adducts of 2-mercaptoethanol were prepared. The PM-mercaptoethanol and -protein adducts were aminolyzed by adding equimolar alkaline ammonia, and then neutralized after the reaction was completed. Standard solutions of the aminolyzed PM- and PIA-mercaptoethanol adduct ranging from 0.2 to 5 μM in 5% SDS were prepared; the fluorescence intensity at 386 nm was linearly proportional in this range. Labeled proteins were denatured in 5% SDS and the concentration of PM or PIA was determined by comparison of the fluorescence intensity at 386 nm. In lightly labeled TM, the ratio was less than 0.4; in heavily labeled TM, the ratio of dye to protein was in the range 0.87–1.30. The highest ratio was about 2.5 as expected for TM which contained 2.5 mol SH/mol protein.

2.3. Spectral measurements

Absorbance spectra were obtained on a Cary (Varian, Palo Alto, CA, U.S.A.) Model 219 absorption spectrometer. Fluorescence spectra were obtained on a Farrand (Valhalla, NY, U.S.A.) Mark I spectrofluorimeter at 25°C. Routinely, four slits with 2.5 nm bandwidth resolution were used at the entrance and exit of both the excitation and emission monochromator. We found that the use of 0.5 or 1 nm bandwidth slits did not further refine spectral resolution. All spectra were corrected against a blank containing an identical concentration of unlabeled protein, but were not corrected for differences in the xenon lamp intensity or photomultiplier response at different wavelengths.

2.4. Lifetime measurements

Fluorescence lifetimes were determined using a modified Ortec (Oak Ridge, TN, U.S.A.) Model 9200 single-photon-counting system. The excitation source was high-voltage air-gap discharge lamp pulsed at about 16 kHz. Narrow band-pass filters were used to select the excitation wavelength, and to isolate the excitation light from the emission pathway. Excitation and emission decay profiles were collected in a multichannel analyzer with 512-channel full addresses. The total number of counts collected for an emission decay profile typically was about 2×10^7 with $4-10 \times 10^4$ counts in the peak channel. The time base in the multichannel analyzer was calibrated using an Ortec Model 425 NIM delay unit with a variable range from 1 to 31 ns and two Tektronix (Beaverton, OR, U.S.A.) Type 113 delay cables with 60 ns delay time for each cable.

2.5. Lifetime data analysis.

Deconvolution of the lamp flash impulse from the observed decay profile and analysis of the emission decay curve were performed using a modified method of moments program [9] that permitted determination of the number of components in the decay as well as their lifetimes and amplitudes. For decays with multiple components, the fluorescence emission function $F(t)$ was assumed to be a sum of exponentials,

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i)$$

where α_i is the amplitude and τ_i the lifetime of component i. The number of exponential components in the decay curve was determined by a matrix conditioning number as well as by the computation determinant. The relative intensity ratio of each component R_i was calculated by

$$R_i = \alpha_i \tau_i / \left(\sum_i \alpha_i \tau_i \right)$$

The calculations were carried out at various moment displacement (MD) indices (choice of 0, 1 or 2) and various depression parameters (DP) between 0 and 0.8. For each moment displacement tried, the best-fit depression parameter was determined which gave the smallest root-mean-square error of the moments (ten moments were calculated) for a reconvoluted decay as compared to the observed decay. When the calculated lifetimes were compared using different moment indices and the corresponding best-fit depression parameters, there was remarkably good agreement.

3. Results

3.1. Reaction kinetics of PM with tropomyosin

The reaction of PM with TM was monitored in a spectrofluorimeter as depicted in fig. 1. The kinetic curve was analyzed as described in the following paper [10]. The results suggest that there were probably three reactive SH groups.

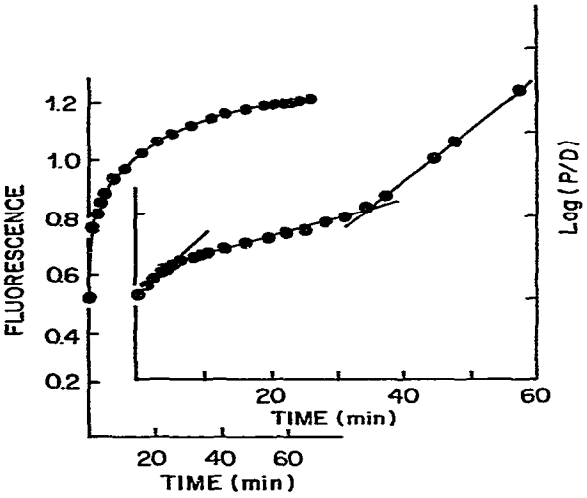
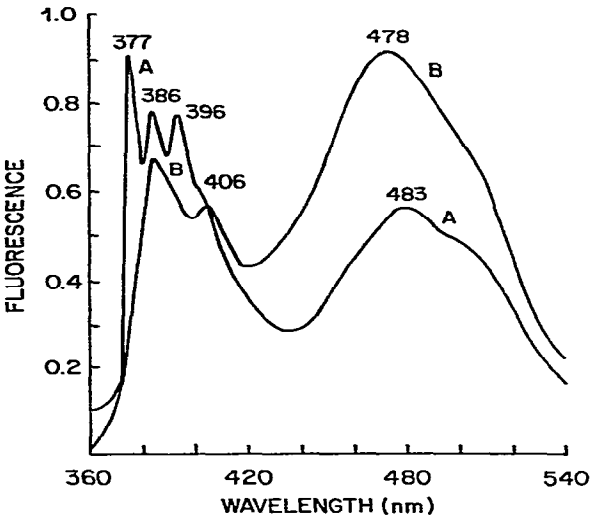


Fig. 1. Kinetics of reaction of PM with TM at 25°C. Initial concentrations: 3.5 μM TM, 10 μM PM, 1 M KCl, 1 mM $MgSO_4$, 2 mM EDTA and 10 mM phosphate, pH 7.4. Fluorescence was monitored by exciting at 340 nm and measuring emission at 377 nm.

3.2. Fluorescence emission spectra of pyrene-labeled TM

Fluorescence emission spectra of PM-labeled and PIA-labeled TM are shown in fig. 2, curves A and B, respectively. The complex spectral pattern in the region 360–440 nm is characteristic of all pyrene adducts; PM-TM and PIA-TM gave spectra that resembled adducts with cysteine and other low molecular weight SH compounds and with actin and other proteins (spectra not shown; see ref. 10). Typically, monomer fluorescence appeared in the 360–420 nm region of the spectrum, PM-TM adducts showing emission maxima at 376 386 and 396 nm and a shoulder at 405 nm, while PIA-TM adducts showed only two emission maxima at 386 and 406 nm. Excimer fluorescence appeared as a broad emission band in the region 435–560 nm for both PM- and PIA-protein adducts. Lightly labeled TM, i.e., a brief reaction time yielding an adduct with a molar dye-to-protein ratio of less than 0.4, did not exhibit emission



in the excimer region nor did PM- or PIA-cysteine adducts at the same dye concentrations.

The ratios of fluorescence intensity at the emission peaks relative to the maximum at 376 or 386 nm are listed in table 1. When excited at 290 nm, monomer fluorescence was minimal but excimer fluorescence was prominent (see fig. 3); thus, when excited at 290 nm, emission in the 360–420 nm region contained contributions due to excimer fluorescence. When different excitation wavelengths were used, e.g., 345 nm as opposed to 290 nm

Fig. 2. Uncorrected fluorescence emission spectra of pyrene-labeled TM. PM (curve A) or PIA (curve B). Conditions: 2 μM PM-TM or 4 μM PIA-TM in 0.15 M KCl, 1 mM $MgSO_4$ and 10 mM phosphate, pH 7.0, at 25°C. Excitation at 345 nm, spectral resolution 2.5 nm.

Table 1

Fluorescence spectral characteristics of pyrene-labeled TM

Solutions contained 2 μM PM-TM or 4 μM PIA-TM, 0.15 M KCl, 1 mM $MgSO_4$ and 10 mM phosphate, pH 7.0. Aminolyzed PM-TM was prepared by adding Tris, 25 mM final concentration, adjusting the pH to 11.2, gently stirring for 1 h, and finally neutralizing the solution to pH 7.0. Monomer fluorescence from PM-TM was obtained using lightly labeled protein with a dye/protein ratio of 0.4. Spectral resolution 2.5 nm. A spectral shoulder is denoted by s.

Adduct	(A) Emission		(B) Excitation	
	Excitation (nm)	Emission peaks (nm) (intensity ratio)	Emission (nm)	Excitation peaks (nm) (intensity ratio)
PM-TM	345	376 (1.00), 386 (0.825), 396 (0.806), 405 (s) (0.609), 480 (0.469), 505 (s) (0.391)	376	343 (1.00), 328 (0.684), 315 (s) (0.301), 278 (0.410), 268 (s) (0.222), 245 (0.202)
	290	377 (1.00), 386 (1.145), 396 (1.031), 405 (s) (0.899), 480 (1.658), 505 (s) (1.425)	480	345 (1.00), 334 (s) (0.858), 282 (0.418), 249 (0.256)
PIA-TM	345	386 (1.00), 406 (0.846), 478 (1.370), 505 (s) (1.00)	386	342 (1.00), 328 (s) (0.579), 315 (s) (0.280), 278 (0.403), 268 (s) (0.233), 245 (0.181)
			480	346 (1.00), 334 (s) (0.768), 282 (0.363), 249 (0.193)
Aminolyzed PM-TM	345	386 (1.00), 406 (0.766), 478 (0.869), 505 (s) (0.722)	386	343 (1.00, 330 (s) (0.613), 316 (s) (0.274), 278 (0.359), 268 (s) (0.194), 248 (0.190)
			480	346 (1.00), 334 (0.879), 282 (0.383), 249 (0.227)
Monomer PM-PT	345	376 (1.00), 382 (s) (0.335), 396 (0.525), 415 (s) (0.178)	376	344 (1.00), 328 (0.650), 315 (s) (0.269), 278 (0.396), 268 (s) (0.206), 245 (0.191)

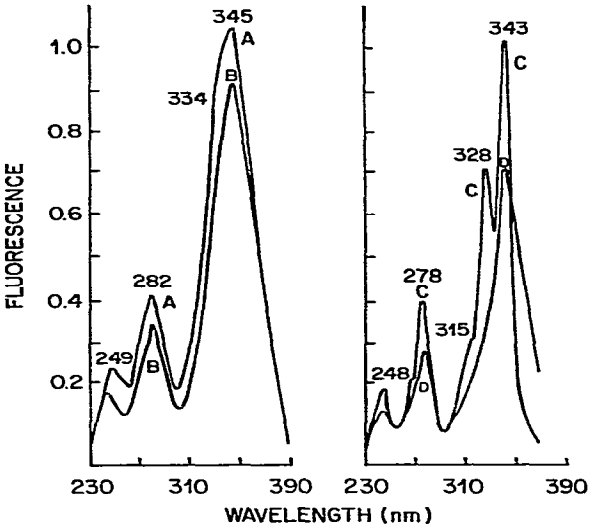


Fig. 3. Uncorrected excitation spectra of excimer fluorescence. PM-TM (curve A) and PIA-TM (curve B); monomer fluorescence of PM-TM (curve C) and PIA-TM (curve D). Emission measured at 480 nm for excimer, and at 377 nm for PM monomer and 386 nm for PIA monomer fluorescence. Same conditions as in fig. 2.

(table 1A), the intensity ratios of the peaks in the 360–420 nm emission region changed, as did the ratio of the 480 nm peak to that at 376 nm. These results suggest that the emission spectra in the 360–420 nm region were different for monomer and excimer. On the other hand, the intensity ratio of the peak at 480 nm to the shoulder at 505 nm did not depend upon the excitation wavelength, because the fluorescence in this spectral region could be attributed only to excimer.

3.3. Fluorescence excitation spectra of pyrene-labeled TM

Fluorescence excitation spectra are depicted in Fig. 3 for PM-labeled TM (curves A and C) and for PIA-labeled TM (curves B and D). The excitation spectra obtained using emission at 480 nm (curves A and B) were attributed to excimer fluorescence and were very similar for both PM and PIA adducts. The excitation curves obtained using emission at 375 nm (curves C and D) were attributed to monomer fluorescence of the labeled TM. The fluorescence excitation spectra of PIA-labeled and PM-labeled TM were very similar and the peaks differed only slightly in their relative intensity ratios. The most notable difference is that PIA-TM had only a shoulder at 328 nm while PM-TM had a distinct peak in the monomer fluorescence spectra. Compared to monomer fluorescence, excitation peaks of the excimer were broader and shifted a few nanometers toward longer wavelengths (red shift) and the two shoulders at 268 and 315 nm were not distinct. These results agree with the previous report by Betcher-Lange and Leher [5] on PM-labeled $\alpha\alpha$-tropomyosin. tropomyosin.

3.4. Effects of aminolysis on PM-labeled TM

In studies of PM-labeled actin [10], we observed intramolecular aminolysis of the succinimido ring with amino groups of lysine residues with aging of solutions. The aminolysis was accompanied by disappearance of fluorescence emission peaks at 376, 396 and 416 nm (shoulder), and the appearance of two new peaks at 386 and 406 nm as well as a shoulder at 426 nm. Intermolecular aminolysis could also occur quite rapidly by adding an exogeneous primary amine to the protein adduct solution and raising the pH above 11. In contrast to actin, lightly labeled PM-TM (molar dye-to-protein ratio of less than 0.4) did not show much aminolysis and no fluorescence emission at 480 nm. Shortly after preparation, heavily labeled TM did not show aminolysis and had low fluorescence emission at 480 nm. However, as the labeled protein adducts aged, the fluorescence intensities at 386 and 405 increased, together with increased excimer fluorescence at 480 nm. Furthermore, the excimer band at 480 nm was also prominent after aminolysis was brought about by the addition of an exogenous amine such as Tris and subsequent alkalinization of the solution. These results suggest that excimer fluorescence occurred after intramolecular aminolysis with opening of the succinimido ring and attachment of PM to two points on the protein, as Betcher-Lange and Lehrer [5] concluded.

3.5. Fluorescence lifetime measurements

For adducts kept under nitrogen, the excimer fluorescence of the TM adducts could be observed uniquely in the 480 nm region, while monomer fluorescence was observed at 400 nm. Monomer fluorescence at 480 nm was negligible and did not affect the lifetime measurements. Table 2 sets out fluorescence lifetimes of PM-TM and PIA-TM observed at these two wavelengths. There are multiple components in the decay curves of both types of fluorescence. In PM-TM, the excimer decay has prominent components with lifetimes of about 66 and 35 ns. A third component of the decay curve with a lifetime of 8.9 ns has also been observed, but its intensity was low. The decay of monomer fluorescence was found to contain three components with lifetimes of about 87 ns, 22 ns and a highly intense short-lifetime component of about 3 ns. Aminolysis of PM-TM following addition of an exogenous primary amine and alkalinization did not result in a change in the lifetimes of excimer fluorescence, but those of the monomer fluorescence decreased to 51, 11 and 2.4 ns.

In PIA-TM, excimer fluorescence decay also had three components with the same lifetimes as in PM-TM with slightly different relative intensity ratios. However, the monomer fluorescence decay had shorter lifetimes for all three components. Also, the 74 ns component had a very low intensity, about 6% of the total.

It is well known that oxygen quenches pyrene fluorescence (see ref. 2). Oxygen did not seem to affect the excimer lifetimes of the pyrene-TM adducts greatly. Upon exposing PM-TM to air, a decrease in the long-lifetime component of monomer fluorescence was observed from 86 to about 75 ns and in the shorter-lifetime component from 22 to about 18 ns. The excimer fluorescence lifetimes were not affected at all.

3.6. Perturbation studies

Previous studies showed that both urea and GdnHCl at concentrations of greater than 4 M effectively denaturated TM, reducing the α-helical content by more than 90% [11,12], as did SDS [13]. The relative quantum yields of labeled adducts in various denaturing media were calculated by comparing the integrated areas of the emission spectra between 360 and 560 nm. The results are summarized in table 3.

In the case of PM-labeled TM, we observed the excimer fluorescence at 480 nm to disappear completely in the presence of 5 M GdnHCl or 7.3 M urea. SDS at concentrations as low as 0.03% caused excimer fluorescence to disappear. The relative quantum yield in solutions denatured by GdnHCl or urea was about 33 and 49%, respectively, compared to the undenatured adduct. However, in the presence of SDS, the quantum yield was 27% greater.

Table 2

Fluorescence lifetimes of monomer and excimer of labeled TM

Solution conditions same as for table 1. MD, moment displacement index; DP, depression parameter (see text).

Adduct	Emission (nm)	R_1	τ_1 (ns)	R_2	τ_2 (ns)	R_3	τ_3 (ns)	DP	MD
PM-TM	480	0.14	8.85	0.54	35.13	0.32	65.85	0.23	1
	400	0.44	2.98	0.34	22.14	0.22	86.71	0.17	0
Aminolyzed PM-TM	480	0.12	8.80	0.53	35.57	0.36	65.97	0.32	1
	400	0.36	2.41	0.52	11.10	0.12	50.91	0.15	0
PIA-TM	480	0.14	7.11	0.43	33.87	0.43	63.57	0.26	1
	400	0.67	2.51	0.27	15.24	0.06	73.83	0.11	0

Table 3

Effects of perturbants on fluorescence emission of labeled TM

PM-labeled protein was stored in 1 mM NaN_3 and aged 60 days. PIA-labeled adducts were denatured in GdnHCl or urea and allowed to stand for 12 h before measurement. Aggregated adducts were prepared by dialysis of 40 μM labeled proteins in 1 mM phosphate (pH 7.0) and diluted to 4 μM. Solution conditions: 4 μM labeled proteins in 0.15 M KCl, 1 mM $MgSO_4$, 10 mM phosphate (pH 7.0); 7 μM proteins in 5.5 M GdnHCl or 7.3 M urea; or 2 μM proteins in 0.03 and 5% SDS, respectively, for PM and PIA adducts. Spectral resolution 2.5 nm.

Medium	Emission peaks (nm) (intensity ratio)	Relative quantum yields (%)
(A) PM-TM adducts		
KCl	377 (1.00), 386 (1.158), 396 (0.979), 406 (s) (0.824), 482 (0.944)	100
SDS	378 (1.00), 386 (1.282), 396 (0.932), 406 (s) (0.816)	127
Urea	378 (1.00), 386 (1.430), 396 (1.100), 406 (s) (1.042)	49
GdnHCl	378 (1.00), 386 (1.190), 396 (1.110), 406 (s) (0.891)	33
1 mM P_i	377 (1.00), 386 (1.304), 396 (1.016), 406 (s) (0.957), 480 (0.516)	–
(B) PIA-TM adducts		
KCl	386 (1.00), 406 (0.846), 478 (1.370)	100
SDS	378 (s), (0.269), 386 (1.00), 406 (0.682), 430 (s) (0.332), 480 (0.265)	97
Urea	378 (s) (0.515), 387 (1.00), 406 (0.830), 430 (s) (0.553), 482 (0.557)	43
GdnHCl	378 (s) (0.681), 386 (1.00), 406 (0.698), 483 (0.872)	40
1 mM P_i	386 (1.00), 406 (0.725), 478 (0.738)	–

In the case of PIA-labeled TM, the fluorescence intensity ratio of excimer to monomer decreased from 1.37 to 0.872 in 5 M GdnHCl, and to 0.557 in 7.3 M urea, an approx. 40 and 60% decrease, respectively. The decrease in excimer fluorescence intensity caused by GdnHCl or urea was a slow process, taking several hours at room temperature to level off at its final value. On the other hand, SDS caused a rapid 80% reduction of excimer fluorescence. These results suggest that although GdnHCl and urea effectively unfold the subunit polypeptide chains of TM, they are less effective in separating the stacked pyrene moieties than is SDS. Comparison of the relative quantum yield of the PM- and PIA-TM adducts in the native and denatured states showed a relatively greater decrease for the PIA adducts and led to the conclusion that the PIA moiety is in a more slightly hydrophobic environment than the PM moiety on TM.

3.7. Effect of aggregation, ionic strength and pH

It is well-known that TM aggregates when the salt concentration of the solution is reduced. The rod-shaped TM molecules undergo both end-to-end and side-to-side association [14]. Aggregates were prepared by dialyzing labeled TM adducts against large volumes of water changed several times over 24 h. The viscous solutions of aggregated TM were diluted in 1 mM potassium phosphate buffer, pH 7.0, and fluorescence spectra were obtained immediately. Excimer fluorescence was about 45% lower for both aggregated PM- and PIA-labeled TM (Table 3). Subsequent addition of 50 mM KCl to PM-TM resulted in an increase in the fluorescence intensity ratio F_{480}/F_{377} from 0.516 to 0.588; further additions of salt did not cause any significant changes in either excimer or monomer fluorescence. For PIA-labeled TM, readdition of salt did not cause any appreciable changes

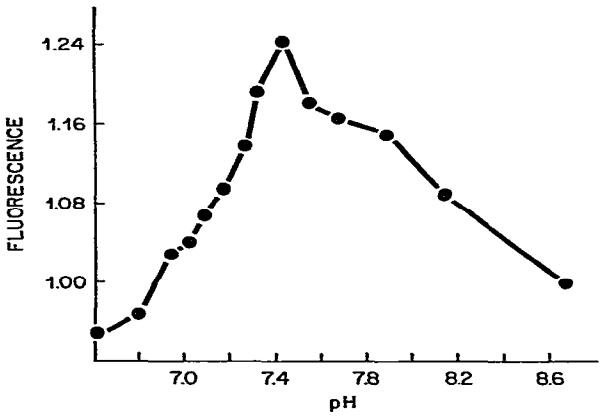


Fig. 4. pH dependence of excimer fluorescence of PIA-TM. Conditions: 4 μM PIA-TM in 0.15 M KCl, 1 mM $MgSO_4$ and 50 mM phosphate buffer. The pH was adjusted with HCl or KOH. Fluorescence measured at 480 nm.

in either excimer or monomer fluorescence. These results indicate that (1) the labeled protein adduct is more compact in solutions of high ionic strength than in water resulting in greater excimer fluorescence, and (2) side-to-side aggregation of TM did not increase excimer fluorescence presumably because the distance between two different TM molecules is too great to allow planar stacking interactions.

Excimer fluorescence is sensitive to the pH of the solution, as depicted in fig. 4. Excimer fluorescence was greatest at pH 7.4. Lowering the pH to 6.6 caused at 24% fall in excimer fluorescence and increasing the pH to 8.7 caused a 20% decrease, while the intensity of monomer fluorescence remained essentially unchanged. These results suggest that the two pyrene moieties are stacked most compactly at physiological pH; slight changes in pH causing confomational changes of the protein that tend to separate the two pyrene rings.

4. Discussion

Excimers occur as an excited molecule interacts with a nearby molecule in its ground state to form a stable excited dimer. The excitation energy of the excimer, and consequently the extent to which the excimer fluoresces, depends upon the geometric arrangement of the paired monomers as in other types of electronic dipole coupling interactions. The quantum mechanical calculation by Murrell and Tanaka [15] for pyrene excimers shows that two pyrene molecules must be oriented parallel to each other with an interplanar distance of 3.6 Å.

The two highly α-helical polypeptide chains of TM are intertwined with an angle of 20° between them to form a coiled-coil superhelix. The center-to-center separation distance of the two chains is about 10 Å. The two chains are aligned in parallel with two Cys-190 residues in register. Therefore, the two Cys-190 residues are in close proximity, within 10 Å of each other. Lehrer [16] showed that the two cysteine residues could form an interchain S-S bond by oxidation; thus, the distance between the two cysteines could be as little as 1.9 Å, the S-S bond distance. A model building study based on the amino acid sequence of TM by Hodge et al. [17] showed that the hydrophobic residues in the two chains sequenced in a regular pattern with a seven-residue repeat. Hydrophobic interactions occur between these aligned residues in the two chains forming a continuous crest on the surface of the α-helix.

Since TM briefly labeled PM-TM showed only monomer fluorescence, PM presumably reacts first with the SH group of Cys-36 in the β-chain. Reaction with one Cys-190 occurs afterward. The succinimido ring of PM attached to Cys-190 then undergoes aminolysis with the adjacent ϵ-amino group of Lys-189 by the mechanism described by Wu et al. [18]. The latter reaction probably makes the bulky pyrene ring project outward from the already crowded SH-reactive site, thereby making room for a second PM molecule to react with the Cys-190 in the other chain. This mechanism is supported by the observation that excimer fluorescence increased as the protein aged and aminolyzed, or if the pH was raised. This was accompanied by a concomitant increase in the fluorescence at 386 nm that is characteristic of aminolyzed pyrenesuccinimide adducts. The recent study of Betcher-Lange and Lehrer [5] further showed that

intramolecular aminolysis involved only the same subunit because no evidence of interchain cross-linking was obtained when the aminolyzed PM-labeled TM was run on SDS gel electrophoresis.

One question which remains to be answered is whether excimer formation occurs after both pyrenesuccinimido rings at Cys-190 undergo intramolecular aminolysis, or after only one pyrenesuccinimido ring undergoes aminolysis. In the former mechanism, the two pyrene rings would be stacked in parallel allowing maximal interaction between the π-electrons of the two rings. In the latter mechanism, the two pyrene rings would be staggered and only partially overlapping. Intermolecular aminolysis upon addition of exogenous primary amine could result in the formation of additional excimer fluorescence and in changed lifetimes owing to the existence of two populations of excimers, one from intramolecular aminolysis with two pyrene rings stacked in parallel, and the other from intermolecular aminolysis with two pyrene rings staggered and only partially overlapping. The first alternative is supported by the following observations: (1) intermolecular aminolysis following the addition of exogenous amine and alkalinization did not affect excimer fluorescence nor lifetimes. (2) excimer fluorescence continued to rise as labeled protein aged (cf. Tables 1A and 3A), and (3) excimer fluorescence lifetimes were the same for both PIA- and PM-labeled TM.

The pyrene moiety in the PIA-labeled adduct differs from that in the PM-labeled adduct in several respects: (1) the distance between the pyrene moiety and the SH-attachment site is shorter in the PIA-labeled adduct by a C-C bond; (2) the rotation of pyrene in the PIA-labeled adduct around the C-S bond at the point of attachment is less hindered sterically than pyrene in the PM-labeled adduct which rotates around the C-C bond (compare the hydrogen atoms associated with the second carbon atom in the C-C bond case with the π-electrons associated with the sulfur atoms in the C-S bond case); and (3) pyrene in the PIA-labeled adduct has more freedom of translational motion in the adduct because it is attached at only one point, while PM after aminolysis has two points of attachment. The extra freedom of motion of the pyrene moiety in the PIA adduct may allow it to fit more easily into the hydrophobic crest on the surface of the helices or in the groove of the superhelix. The excimer formed is presumably stabilized by hydrophobic interactions between the stacked pyrene rings and their hydrophobic environment.

Perturbation studies confirmed that the excimer in the PIA-labeled adduct is more stable than that in the PM-labeled adduct. The two subunits of TM readily unfold and separate in 5 M GdnHCl or 7.3 M urea. In the case of PM-labeled TM these agents also cause the pyrene moieties to separate as indicated by the complete loss of excimer fluorescence. In the case of PIA-labeled TM, however, 40–60% of the excimer fluorescence remains over a period of 12 h, indicating a lack of complete separation of the pyrene rings. The surfactant SDS dissociates the pyrene rings of PM-labeled TM at concentrations as low as 0.03%: however, some residual excimer fluorescence can still be observed in PIA adducts even in 5% SDS. The measurements of relative quantum yield can also be interpreted to indicate that the microenvironment around the pyrene moieties of the PIA adduct is more hydrophobic than that of the PM adduct.

During the past two decades, extensive studies have been conducted on the fluorescence lifetimes of pyrene and its derivatives. The earlier studies suffered from poor instrumentation and inadequate methods for analyzing decay data that resulted in inaccurate evaluation of lifetimes and number of components in the decay. Recent advances in instrumentation have considerably shortened the data acquisition times and improved the accuracy and precision of the measurements. Improvements in data analysis, particularly extensions of the method of moments [9], have significantly reduced computation time and improved the precision, and allowed the analysis of decays. The earlier lifetime data for pyrene and its derivatives have been collected by Birks [19]. In organic solvents, pyrene exhibits a single, very long lifetime, ranging from 300 ns in benzene to 530 ns in absolute ethanol. The fluorescence of pyrene and its derivatives is extremely susceptible to quenching by oxygen with dramatic decreases in lifetimes. For example, Hautala et al. [20] found a lifetime

of 370 ns for pyrene in degassed cyclohexane, 202 ns in nitrogen-saturated solvent and 20 ns in oxygen-saturated solvent. The inconsistency of pyrene lifetimes reported in the early literature may well be due in part to variable quenching by oxygen. The oxygen-quenching effect has been exploited to obtained information about the permeability of micelles or membrane-like systems [21] and the accessibility of probe-binding sites in globular proteins [22–24]. Simple chemical modification of pyrene also decreases the lifetime, e.g., the lifetime of 4-methylpyrene in benzene is 90 ns. Incorporation of an electron-rich halogen also descreases the lifetime, e.g., 30 and 3 ns for 3-chloropyrene and 3-bromopyrene, respectively.

In the studies reported here, both PM- and PIA-labeled adducts of TM exchibited three lifetimes of approximately the same values as obtained by Kawasaki et al. [25]. The possibility that each lifetime represents PM bound to a different site seems unlikely for the following reasons. Lightly labeled TM in which PM is attached predominantly to Cys-36 of β-subunits shows three lifetime components in its decay. Similarly, PM- and PIA-actin adducts labeled predominantly at Cys-373, one of three reactive SH groups in actin, also show three lifetimes [10]. Weltman et al. [3] reported two lifetimes for PM-labeled bovine serum albumin which contains only a single reactive SH, Cys-34. It is likely that the very short lifetime component remained unresolved in the latter study, because the actin adduct also showed only two lifetime components in contrast to the three found in later studies.

Scouten et al. [26] and Hudson and Weber [27] pointed out that derivatives of polynuclear aromatic amines may possess more than one intrinsic lifetime. Pyrene-protein adducts all seem to exhibit three lifetime components, a very sort-lived component of about 2–17 ns, a medium component of 10–50 ns, and a long-lived component of 35 to over 100 ns. The fraction each component contributes to the total fluorescence varies considerably in different proteins, as does the total fluorescence which is a measure of the quantum efficiency. The fractional contribution of each lifetime component and the total fluorescence reflect microenvironmental effects on the fluorophore which can vary from protein to protein and even among different binding sites on a single protein. The general observation of three lifetime components in the decay of PM- and PIA-labeled protein suggests that multiple decay components of pyrene-protein adducts are an intrinsic property of the probe. In protein adducts having more than one binding site for pyrene derivatives, the three measured lifetimes presumably are the weighted averages of the different lifetimes within each class, i.e., short, medium and long, at each reactive site.

The strongest evidence for the existence of three decay components as intrinsic, nontrivial properties of pyrene-protein adducts comes from life-time measurements of excimer fluorescence. In all three adducts, PM-TM, aminolyzed PM-TM, and PIA-TM, the excimer lifetimes are almost identical and fall into the same ranges as the three components of monomer fluorescence. Because only one kind of excimer can exist in each of these three adducts, it is difficult to explain more than one lifetime component unless the three decay components are derived from the monomer. The excimer of pure pyrene exhibits only one lifetime (45–65 ns) as does the monomer. These observations of excimer fluorescence lifetimes also mitigate against heterogeneity in the pyrene probes as a cause for multiple lifetimes, because an impurity would be unlikely to form an excimer with three identical lifetimes.

Scouten et al. [26] recently investigated the problem of multiple lifetimes in several amine derivatives of polyaromatic ring compounds including PM. Despite their inability to resolve decay data with more than two lifetime components, in all the compounds they studied, more than one lifetime was found. Although they were not able to demonstrate unequivocally that the decay of PM adducts contained three components, their study did rule out the possibility that multiple lifetimes were the result of impurity or heterogeneity of the PM probe, or heterogeneity of the microenvironment of multiple binding sites.

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