EFFECTS OF THE STATE OF THE SUCCINIMIDO-RING ON THE FLUORESCENCE AND STRUCTURAL PROPERTIES OF PYRENE MALEIMIDE-LABELED $\alpha\alpha$-TROPOMYOSIN

YOSHIHARU ISHII AND SHERWIN S. LEHRER
Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114 and The Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Rabbit skeletal $\alpha\alpha$-tropomyosin was labeled at Cys-190 with pyrene maleimide to form (S-[N-(1-pyrene)succinimido])$_2$-tropomyosin (pyrene$_I$-Tm). The product with cleaved succinimido-rings, pyrene$_{II}$-Tm was also prepared by incubation of pyrene$_I$-Tm at pH > 7.5. The pH dependence of the rate of cleavage indicated that hydrolysis rather than aminolysis was the more likely reaction. Pyrene$_I$-Tm exhibited a loss in helix content and end-to-end polymerization compared with unlabeled Tm, which increased upon formation of pyrene$_{II}$-Tm. The cleavage resulted in increased interchain excited state excimer fluorescence originating from pyrene-pyrene interaction between the chains. Thus, increased pyrene-pyrene interaction at Cys 190 leads to an increase in unfolding, the effects of which appear to be transmitted to the ends of tropomyosin. The fluorescence properties of the two types of pyrene-succinimide adducts of dithiothreitol were very similar to the corresponding adducts of pyrene-Tm indicating excimer formation through ground state pyrene-pyrene interaction.

INTRODUCTION

Tropomyosin (Tm) is an essential component of the Ca^{2+}-regulatory thin filament complex of skeletal and cardiac muscle (Ebashi and Endo, 1968; Smillie, 1979; Leavis and Gergely, 1984) whose end-to-end interactions are considered to be important in the cooperative binding of myosin subfragment 1 and in the regulation of muscle contraction (Greene and Eisenberg, 1980; Hill et al., 1980; Hill et al., 1981; Lehrer and Morris, 1982).

Pyrene maleimide labeled-Tm exhibits excimer (excited dimer) fluorescence (Betcher-Lange and Lehrer, 1978; Lin, 1982) due to the proximity of the Cys 190 residues of each chain. The excimer fluorescence of (S-[N-(1-pyrene)succinimido])$_2$-tropomyosin (pyrene$_I$-Tm), the product of reaction at low pH in which the Cys bound succinimido-ring is intact, is much less than the excimer fluorescence of pyrene$_{II}$-Tm produced by cleaving the succinimido-ring by incubation at pH $\geq$ 7.5 (Betcher-Lange and Lehrer, 1978). From the pH dependence of the cleavage rate determined in our study, it appears that hydrolysis rather than aminolysis is the more likely cleavage mechanism.

The low salt polymerizability of pyrene$_{II}$-Tm is markedly reduced (Graceffa and Lehrer, 1980), indicating that end-to-end interactions are inhibited (Ooi et al., 1976; Ueno et al., 1976; Johnson and Smillie, 1977). Here we show how a loss in polymerizability is correlated with a small loss in helical content produced by the primary labeling reaction. Subsequent ring cleavage increases this perturbation, which is probed by an increase in excimer fluorescence.

These results are consistent with the observation that some localized chain separation and unfolding is required to form the excimer state since the pyrenes cannot interact around the outside of the molecule (Lehrer et al., 1981). The loss of polymerizability thus appears to be due to a small unfolding perturbation associated with the pyrenes at Cys 190, the effects of which are increased on succinimido-ring cleavage and are transmitted to the ends of the molecule.

EXPERIMENTAL PROCEDURES

Tropomyosin was prepared from rabbit skeletal muscle as previously described (Lehrer and Morris, 1982). The α and β chains were separated by the method of Cummins and Perry (1973) and $\alpha\alpha$-Tm was renatured and reduced at 37°C as described previously (Betcher-Lange and Lehrer, 1978).

$\alpha\alpha$-Tm was labeled with N-(1-pyrene)maleimide (PM) (Polysciences Inc., Warrington, PA) as described previously to obtain pyrene$_I$-Tm and pyrene$_{II}$-Tm was prepared by incubating pyrene$_I$-Tm in 5 M GdmCl, 5 mM bicine buffer, pH 8.4, overnight at room temperature (Graceffa and Lehrer, 1980). Unlabeled-$\alpha\alpha$-Tm treated by the same procedure as described above in the absence of PM was used as a control to insure that no differences in properties were the result of the labeling and conversion procedure. These stock solutions were kept in low salt at pH 6.0 (2 mM Mes) for pyrene$_I$-Tm or pH 7.5 (2 mM Hepes) for pyrene$_{II}$-Tm and unlabeled-$\alpha\alpha$-Tm in the presence of 1 mM EDTA at 0°C (Lehrer and Morris, 1982) and diluted into appropriate solutions before use.

The concentration of $\alpha\alpha$-Tm was determined with $A_{277\text{ nm}} - A_{320\text{ nm}}$ (1 mg/ml) = 0.24 (Lehrer, 1975), the concentration of pyrene-Tm was

Address correspondence to Boston Biomedical Research Institute.

determined by the Lowry method using unlabeled-$\alpha\alpha$-Tm as a standard and the concentration of pyrene bound to Tm was determined with $\epsilon_{343\,nm} = 2.3 \times 10^4\ M^{-1}$ in the native state (Graceffa and Lehrer, 1980). The degree of labeling was 1.6 pyrene/$\alpha\alpha$-Tm, i.e., the Cys residues were 80% labeled.

The PM adduct of dithiothreitol (DTT) or 2-mercaptoethanol was prepared by dissolving PM in N,N-dimethylformamide (DMF) to a final concentration of 1–2 mM. DTT or 2-mercaptoethanol was reacted with PM in DMF at a ratio of 1.2 PM/SH-groups. Conversion to type II was carried out by diluting the type I adduct into 1 mM NaOH. For spectroscopic measurements, these stock solutions were diluted to ~1 μM into the desired buffered solutions.

The kinetics of conversion of pyrene$_I$-Tm to pyrene$_{II}$-Tm was followed by monitoring the fluorescence change associated with the conversion, which was initiated by diluting pyrene$_I$-Tm at pH 6.0 into solutions of greater pH (see Fig. 1). Data points of monomer and excimer fluorescence, F_M and F_E, vs. time were obtained and computer fitted (PDP 11-44) by a nonlinear, least-square's routine to a one exponential kinetic scheme, programmed by Dr. T. Scott in our department utilizing the following equations:

$$F_M(t) = F^f_M + (F^o_M - F^f_M)\exp(-kt)$$

$$\Delta F_M(t) = F_M(t) - F^f_M$$

$$\sqrt{F_E(t) - F^o_E} = \sqrt{F^f_E - F^o_E} \times [1 - \exp(-k't)]$$

$$\Delta F_E(t) = F_E(t) - F^o_E$$

to yield first order rate constants k, k' as well as initial and final values F^o, F^f. k and k' were plotted vs. pH and compared with the following theoretical curves which assumed either hydrolysis or aminolysis:

$$\log(k_{hydrolysis}) = pH + C$$

$$\log(k_{aminolysis}) = -\log(1 + 10^{-pH+pK_a}) + C,$$

where pK_a is the dissociation constant of the amino group involved. The constants C were determined for the best fit.

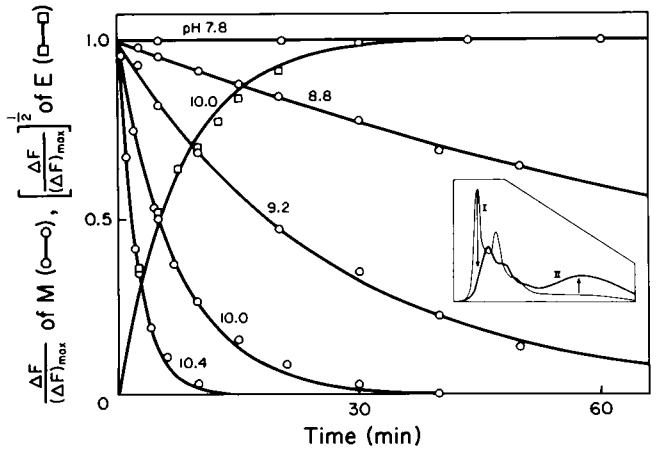

FIGURE 1 Kinetics of the conversion of pyrene$_I$-Tm to pyrene$_{II}$-Tm at various indicated pH values in the native state. (○), changes in the type I monomer fluorescence at 375 nm (↓ in *insert*); (□), changes in the square root of the excimer fluorescence at 480 nm (↑ in *insert*). (—), first order rate processes fitted to the data. Conditions: 0.5 M NaCl, 1 mM EDTA, and 5 mM bicine at 25°C. pH was adjusted by adding NaOH. *Insert* shows uncorrected fluorescence spectra of pyrene$_I$-Tm(—) and pyrene$_{II}$-Tm (—) at 0.031 mg/ml in 0.5 M NaCl, 1 mM EDTA and 20 mM Hepes buffer, pH 7.5, 25°C. Excitation was 340 nm and bandwidths were set at 5 nm for both excitation and emission.

The fluorescence measurements were performed in the thermostated cell holders of a Perkin-Elmer (Norwalk, CT) MPF-4a or a Spex (Edison, NJ) fluorolog 2,2,2 spectrofluorometer in the ratio mode. Circular dichroism measurements were performed on an updated Cary 60 instrument (Aviv Associates, Lakewood, NJ). For all thermal unfolding experiments the measurements were made 15 min after reaching desired temperature. The concentration of Na^+ for the low salt solutions was determined by conductivity. Viscosity measurements were performed in a viscometer (model 75; Cannon Instrument Co., State College, PA) with a water flow time of 125 s at 25°C.

RESULTS

Kinetics of the Conversion of Pyrene$_I$-Tm to Pyrene$_{II}$-Tm

The product of the reaction of pyrene maleimide with $\alpha\alpha$-Tm at low pH (6.0–7.0) is (S-[N-(1-pyrene)succinimido])$_2$-Tm, (pyrene$_I$-Tm or type I) i.e., the pyrene moiety is attached to Tm via an intact succinimido-ring. Incubation of pyrene$_I$-Tm at higher pH values results in an irreversible change in the spectrum associated with the production of pyrene$_{II}$-Tm (Betcher-Lange and Lehrer, 1978) (Fig. 1, *insert*). To determine if the conversion from type I to type II pyrene-Tm involves hydrolysis or aminolysis of the succinimido-ring the rates of fluorescence change at different pH values were studied. If hydrolysis is involved the rate should be proportional to [OH^-], if reaction with a neighboring amino-group is involved (aminolysis), the rate should be proportional to the degree of dissociation of the amino-group (Wu et al., 1976). In both cases, the resulting spectra are similar (Liburdy, 1978). The loss of monomer fluorescence showed first order rate processes at all pH values measured (Fig. 1). Pyrene$_I$-Tm denatured in 5 M GdmCl also showed a first order rate process for the conversion to pyrene$_{II}$-Tm with the rates about 5× greater than in the native case. In the case of the excimer increase at pH 10.0, a plot of the square root of the fluorescence increase gave a first order plot with $k' = 0.133\ min^{-1}$ similar to the rate constant for the monomer fluorescence decrease ($k = 0.135\ min^{-1}$). This indicates that the increase in excimer is proportional to the probability that both pyrenes of a molecule are randomly converted to type II, in agreement with the bimolecular mechanism of excimer fluorescence.

The rate constants were plotted as a function of pH in semilogarithmic plot in Fig. 2. The log of the rate constant is approximately proportional to the pH (with a slope of unity) in agreement with the hydrolysis mechanism. Curves that apply to the aminolysis mechanism were generated for different values of the pK_a of the hypothetical amino-group involved (dashed curves, Fig. 2). The data could only fit the aminolysis mechanism if the pK_a of the amino-group would be greater than ~10.5. Since the average pK_a of lysine groups in Tm have been reported to be 9.9 (Iida and Imai, 1969), it appears more likely that the succinimido-group is cleaved by hydrolysis than aminolysis.

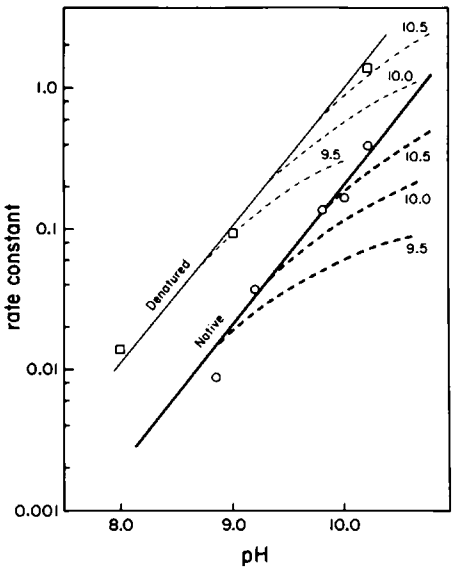

FIGURE 2 pH dependence of the pseudo-first order rate constants (min^{-1}) in the native (O) and denatured states (□). The experiment in the denatured state was made in the presence of 5 M GdmCl. Theoretical curves are plotted assuming hydrolysis (—, —), or aminolysis (---, —) with various indicated pK_a values.

Effects of the Label on Thermal Unfolding

Comparison of the effects of the type I and type II labels on the stability of Tm was made by studying the thermal unfolding profiles in both high and low salt concentrations by circular dichroism measurements at 222 nm. The studies were performed at pH 7.5, where the hydrolysis of type I is very slow. In 0.5 M NaCl, the unlabeled-$\alpha\alpha$-Tm showed a small pretransition near 35°, as well as a main transition near 50°C, as described earlier for rabbit skeletal Tm (Satoh and Mihashi, 1972; Woods, 1976; Lehrer et al., 1981) (Fig. 3). Two effects of the label at Cys 190 were observed; (*a*) the pretransition was shifted to lower temperature (to 30° for $pyrene_I$-Tm, and to 25°C for $pyrene_{II}$-Tm without much effect on the main transition, and (*b*) at low temperature below the pretransition there was a loss of helix (a few percent loss for $pyrene_I$-Tm, and ~10% for $pyrene_{II}$-Tm).

In low salt ($[Na^+]$ = 4 mM) the unlabeled- Tm showed only one steep main transition at 38°C (Fig. 4). Earlier studies also showed a single transition for rabbit cardiac $\alpha\alpha$-Tm (Betteridge and Lehrer, 1983). Two effects of the label were also seen at low salt; (*a*) the transition was shifted to lower temperature (33° for $pyrene_I$-Tm, and 30°C for $pyrene_{II}$-Tm), and (*b*) at low temperatures below the transition, similar losses of helix were observed for the labeled systems, with a greater effect for the type II label. The labels also caused a broadening of the transition. It is possible that an appreciable source of the broadening is the heterogeneity of the labeled Tm present (singly and doubly labeled) since the degree of labeling was not 100%.

Intramolecular Excimer Fluorescence in Pyrene-Tm

In high salt ([NaCl] = 0.5 M), the excimer fluorescence of $pyrene_{II}$-Tm increases from an initial value at low temperature to a maximum in a temperature range correlating with the pretransition described above and then decreases (Graceffa and Lehrer, 1980) due to chain dissociation at higher temperatures (Holtzer et al., 1983) (Fig. 5). The behavior of the fluorescence of $pyrene_I$-Tm differed from that of $pyrene_{II}$-Tm in several ways; (*a*) the initial excimer intensity was much less and the initial monomer intensity was greater, (*b*) there was only a small increase in intensity associated with the helix pretransition which was of similar magnitude for both types of pyrene-Tm, and (*c*) the

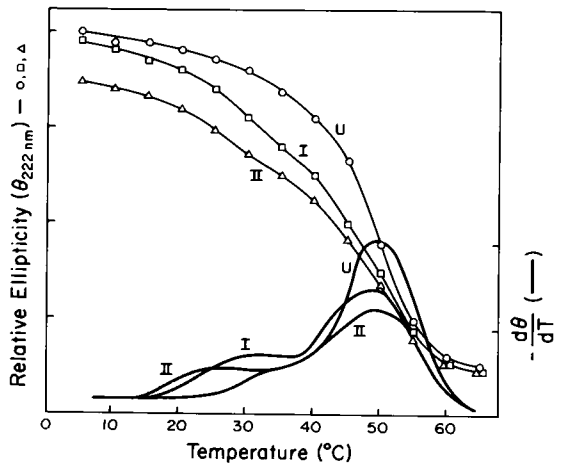

FIGURE 3 Comparison of the thermal unfolding of unlabeled-Tm (U) (O), $pyrene_I$-Tm (I) (□) and $pyrene_{II}$-Tm (II) (Δ) in high salt. Top curves show relative ellipticity at 222 nm (—) and bottom curves show their slopes (—). Conditions: 0.65 mg/ml in 0.5 M NaCl, 1 mM EDTA, 20 mM Hepes buffer, pH 7.5.

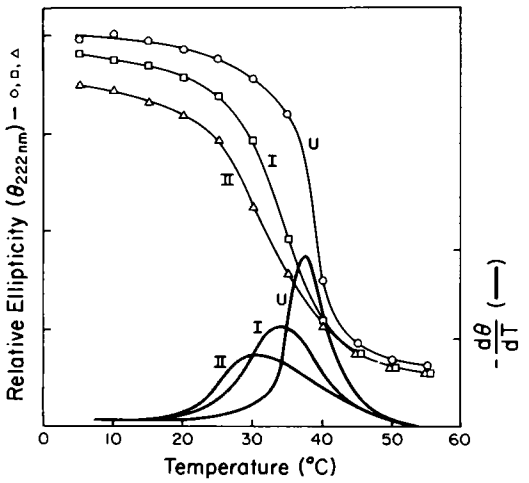

FIGURE 4 Comparison of the thermal unfolding of unlabeled-Tm (U) (O), $pyrene_I$-Tm (I) (□) and $pyrene_{II}$-Tm (II) (Δ) in low salt. Conditions: 0.65 mg/mL in 1 mM EDTA and 2 mM Hepes, pH 7.5. The measured $[Na^+]$ was 4.2 mM.

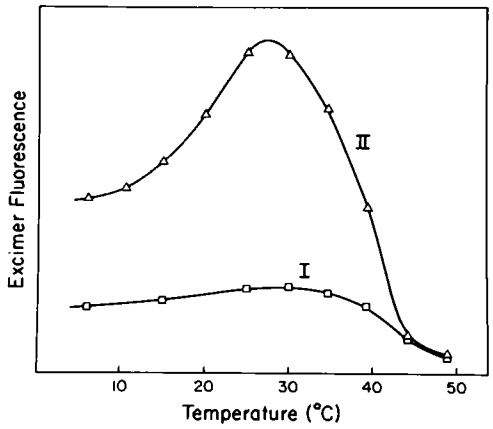

FIGURE 5 Temperature dependence of excimer fluorescence of pyrene$_I$-Tm (I) (□) and pyrene$_{II}$-Tm (II) (△) in high salt. Condition as for Fig. 3 except for [Tm] = 0.031 mg/ml. Excitation and emission were 340 and 480 nm, respectively.

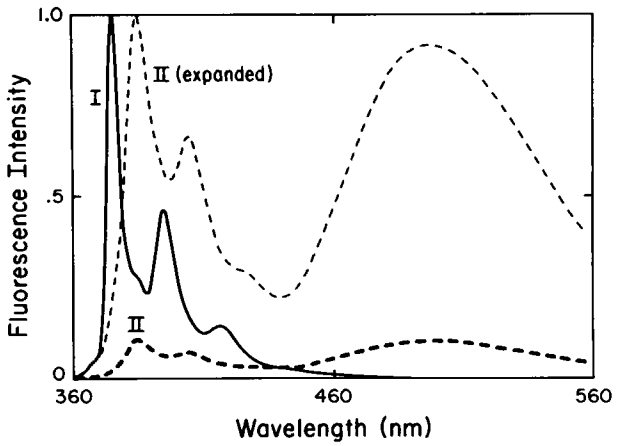

FIGURE 6 Fluorescence spectra of pyrene$_I$-DTT (—) and pyrene$_{II}$-DTT (---, —). [pyrene-DTT] = 1 μM in 20 mM Mes, pH 6.0 at 25°. The excitation wavelength was at 340 nm. Bandwidths = 1.25 nm for both emission and excitation.

temperature of maximum excimer fluorescence was somewhat greater. The excimer fluorescence from type I was not due to a contribution from some converted to type II, since the excitation spectrum remained characteristic of type I. Similar conclusions were obtained from the change in excimer fluorescence associated with GdmCl-induced unfolding profiles at low temperature (Graceffa, Ishii, and Lehrer, unpublished data).

In low salt, both types of pyrene-Tm showed similar values of initial excimer intensity as seen in high salt (data not shown). However, the initial excimer intensity decreased toward zero over a temperature range (20°–30°C) without any increase, corresponding to a highly cooperative helix-coil unfolding profile which does not exhibit a pretransition (Betteridge and Lehrer, 1983).

Intramolecular Excimer Fluorescence of Pyrene-Dithiothreitol

The fluorescence properties of pyrene-labeled dithiothreitol (pyrene-DTT) were studied as a model compound forming an intramolecular excimer for pyrene-Tm since it exhibited excimer fluorescence while 2-mercaptoethanol did not and the excimer to monomer fluorescence ratio did not depend on the pyrene-DTT concentration. The fluorescence spectra of both pyrene$_I$-DTT and pyrene$_{II}$-DTT (Fig. 6) were very similar to the previously reported corresponding pyrene-Tm types (Fig. 1 *insert*) (Betcher-Lange and Lehrer, 1978) with very little excimer present for type I. Conversion to type II pyrene-DTT by hydrolysis results in a red shift of the monomer peaks, broadening of the spectrum, and a large increase in excimer fluorescence.

The absorption spectra of both types of pyrene-DTT and pyrene-Tm in aqueous solution were broadened as compared to the corresponding types of pyrene-mercaptoethanol (data not shown), indicating ground-state pyrene-pyrene interaction. It appears, therefore, that despite the proximity of the pyrenes, the proper excimer configuration can not be attained in aqueous solution for pyrene$_I$-DTT and only partially attained for pyrene$_I$-Tm.

The excimer fluorescence of pyrene$_{II}$-DTT decreased monotonically with temperature. Between 5° and 70°C the decrease was approximately linear with a 23% drop over the complete temperature range. This provides further evidence that the changes seen for pyrene-Tm with temperature are due to Tm conformational changes rather than an intrinsic property of intramolecular pyrene excimer formation.

Effects of the Label on the Polymerizability of Tm

A comparison of the effects of the type I and type II pyrene-Tm on the salt dependence of viscosity was made in view of the previous observation that the end-to-end polymerizability of Tm is drastically reduced by the type II pyrene label (Graceffa and Lehrer, 1980). The viscosity of pyrene$_{II}$-Tm was only ~30% of the viscosity of unlabeled-Tm and decreased in the same manner as the salt concentration was increased at 25°C (Fig. 7). The viscosity of pyrene$_I$-Tm took intermediate values over the same salt concentrations. A part of the loss of viscosity of pyrene$_{II}$-Tm at 25° could be due to partial unfolding at low salt which results in a viscosity decrease above 20°C (Fig. 7 *insert*). However, the major part of the loss in viscosity appears to be related to the initial perturbation associated with the 10% loss in helix at temperatures below the pre- and main transitions, since a similar loss in viscosity was observed at low temperatures (Fig. 7, *insert*).

DISCUSSION

Earlier studies have shown that modification of Cys in rabbit skeletal Tm by pyrene maleimide under conditions where the covalently linked succinimido-ring is cleaved (Wu et al., 1976; Betcher-Lange and Lehrer, 1978) results

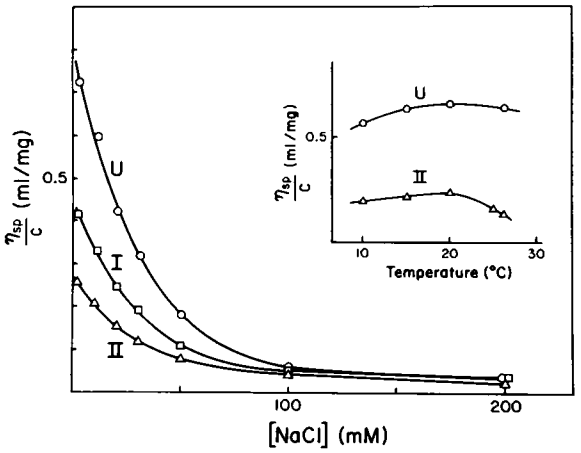

FIGURE 7 Comparison of the salt dependence of viscosity of unlabeled-Tm (U) (○), pyrene$_{I}$-Tm (I) (□) and pyrene$_{II}$-Tm (II) (△) at 25°C and temperature dependence of the viscosity in the absence of added salt (*Insert*). Conditions as for Fig. 4.

in a large degree of inhibition of the low salt polymerizability (Graceffa and Lehrer, 1980). Those results were confirmed and extended in this work, by studying changes of the properties of Tm resulting from: (*a*) the primary modification which links pyrene to Cys via an uncleaved succinimido-ring to form type I pyrene-Tm (pyrene maleimide reaction at pH 6), and (*b*) subsequent cleavage of the Cys-bound succinimido-ring at high pH to form type II pyrene-Tm. While the primary reaction resulted in some loss of polymerizability and helix content, further losses were observed after cleavage of the ring. These results show that the structural perturbation is not simply due to the blocking of SH-groups and suggest some correlation between helix loss initiated at Cys 190 and the interactions involved in polymerization. The helix pretransition was also somewhat affected with other modifications at Cys 190 (Betteridge and Lehrer, 1983; Graceffa and Lehrer, 1984), indicating that this region is particularly sensitive to perturbation.

In the original study we suggested that the type II product was the result of an aminolysis reaction with Lys 189 (Betcher-Lange and Lehrer, 1978). The pH dependence of the rate obtained in this study, however, indicates that the hydrolysis reaction is more likely. In any case, either type II product would result in more degrees of freedom of rotation of the pyrene moiety as compared to type I (around two additional single bonds between the pyrene and the S-atom) (Lehrer et al., 1981). This can explain the greater degree of excimer formation observed for the type II product for both pyrene-Tm and the model compound pyrene-DTT.

The presence of excimer fluorescence in type I and type II pyrene-Tm at low temperature, which originates from ground state pyrene-pyrene interaction (Betcher-Lange and Lehrer, 1978), indicates localized chain separation since the pyrenes can not stack around the outside of the molecule (Lehrer et al., 1981). Pyrene-pyrene hydrophobic interaction thus appears to be the cause of the perturbation at low temperature. At higher temperatures, in high salt, the unstable Cys 190 region was further destabilized by the presence of the probe. Further evidence for these effects being caused by the pyrene portion of the label was obtained by noting that type I and type II maleimide spin-labeled tropomyosin, which contained a nonhydrophobic nitroxide spin probe, only slightly perturbed the helix unfolding profile (Graceffa and Lehrer, 1980). Changes in initial excimer fluorescence have been used to study effects of actin and myosin on tropomyosin in the region of Cys 190. The initial excimer fluorescence of pyrene$_{II}$-Tm is only slightly reduced on binding to F-actin, but it is greatly reduced by the subsequent binding of low ratios of myosin subfragment 1 (S1) (Ishii and Lehrer, 1985). This indicates that S1 strengthens the chain-chain interaction, overcoming the probe perturbation. Thus, despite the perturbation that the introduction of the pyrene probe produced, useful information was obtained.

It is interesting to note that the excimer fluorescence of pyrene$_{I}$-Tm hardly increases in the pretransition temperature in contrast to the case of pyrene$_{II}$-Tm. Thus, although the absorption spectra indicate that the pyrenes interact on the ground-state the rotational constraints of the pyrenes in the type I linkage do not allow the pyrenes to optimally stack in the excimeric configuration even with the unfolding that occurs in this region of the molecule. A related observation was made regarding the relative mobility properties of type I and type II maleimide spin labels in a recent study (Graceffa and Lehrer, 1984).

The low salt polymerizability of Tm appears to primarily involve end-to-end interactions (Ooi et al., 1962; Johnson and Smillie, 1977; Ueno et al., 1976). The observation that the pyrene probes at Cys 190, located 2/3 from the amino terminal end, can inhibit polymerizability reinforces the previous result that the binding of pyrene$_{II}$-Tm to F-actin is reduced primarily through the weakening of end-to-end interactions (Ishii and Lehrer, 1985). Thus, perturbations can be transmitted over large distances in coiled-coil, rod-like molecules (Edwards and Sykes, 1980; Ueno, 1984; Lu and Lehrer, 1984). It has been postulated that Ca^{2+}-regulation involves troponin perturbation of Tm near Cys 190 (Lehrer et al., 1981), although a model involving direct interaction of troponin at the Tm ends has also been suggested (Pearlstone and Smillie, 1982, 1983). Further work will be necessary to clarify which mechanism is relevant.

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