Excimer Fluorescence of Equine Platelet Tropomyosin Labeled with *N*-(1-Pyrenyl)iodoacetamide[†]

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ABSTRACT: Tropomyosin from equine platelets was reacted with *N*-(1-pyrenyl)iodoacetamide, a sulfhydryl-specific fluorescent reagent, to give an average extent of incorporation of 1.12 pyrene (Py) groups per platelet tropomyosin (P-TM) chain. The predominant site of reaction on P-TM was the penultimate COOH-terminal residue, Cys-246. The high proportion of the total emission that is due to pyrene excimers and the pretransition observed in thermal denaturation of Py-P-TM point to a rather loose structure for the COOH-terminal amino acid residues of P-TM. The label on Cys-246 also reports on end-to-end overlap interactions that occur between two different tropomyosin molecules. Additions to a Py-P-TM solution at low ionic strength of unlabeled P-TM, rabbit cardiac tropomyosin (C-TM), or a carboxypeptidase A treated, nonpolymerizable derivative of C-TM all reduce the extent of excimer fluorescence from the sample. Addition of salt greatly reduces the effects of the unlabeled TM species on the Py-P-TM emission spectrum. Circular dichroism measurements indicate Py-P-TM still to be >95% helical. However, analysis of excimer fluorescence levels in samples that contained a constant protein concentration but different mole ratios of labeled to unlabeled P-TM suggests that the bulky pyrene group may diminish the tendency of Py-P-TM to polymerize in an end-to-end manner.

Striated muscle tropomyosin (TM)[1] is a highly helical protein composed of two polypeptide chains, each of molecular mass 33 kDa, coiled around each other to form a rod some 41 nm long [reviewed by Smillie (1979)]. Recently, a number of TM-like molecules have been identified in nonmuscle cells [reviewed by Côté (1983)]. In general, the nonmuscle TMs closely resemble muscle TMs in composition, structure, and stability. However, they are distinguished by being about one-seventh less massive and by having a reduced tendency to interact with each other in a head-to-tail manner.

To date, the best characterized nonmuscle TM is that purified from horse blood platelets (Côté & Smillie, 1981a,b,c; Lewis et al., 1983). Two similar P-TM species have been identified in equine platelets, α and β. The β form dominates, being present in at least a two-fold excess over the α form (Côté & Smillie, 1981a). The amino acid sequence of the β form (Lewis et al., 1983) can be aligned with that of muscle TMs to reveal extensive homology. At the ends of the chains, however, the homology becomes less strong. Alterations in these regions are thought to account for the weakened end-to-end overlap interactions between P-TM molecules (Côté et al., 1978).

N-(1-Pyrenyl)iodoacetamide (PIA) and *N*-(1-pyrenyl)-maleimide (PM) have been used to label muscle TM sulfhydryl groups (Betcher-Lang & Lehrer, 1978; Graceffa & Lehrer, 1980; Lehrer et al., 1984; Ishii & Lehrer, 1985; Lin, 1982). In particular, labeled Cys-190 residues on adjacent chains of a single TM molecule can approach sufficiently close to each other to permit pyrene excimer formation. Therefore, emission spectra of pyrene-labeled striated muscle TMs display a broad band of excimer fluorescence in the vicinity of 480 nm in addition to the usual sharp pyrene emission bands in the 375–425-nm region. The excimer fluorescence is affected by salt (Graceffa & Lehrer, 1980), which enhances hydrophobic interactions between pyrene groups, and by denaturation of the TM coiled coil by chemical (Betcher-Lang & Lehrer, 1978; Lin, 1982) or thermal (Graceffa & Lehrer, 1980) means. The denaturation studies reveal a pretransition in the separation of the TM chains that has been interpreted in terms of a localized unfolding of the molecule in the vicinity of the Cys-190 residues (Graceffa & Lehrer, 1980).

In this study, we have labeled P-TM with PIA under mild conditions that would allow only a limited degree of reaction with Cys-190 on muscle TMs. The prime site of labeling on P-TM is not Cys-153, the homologue of Cys-190 of muscle TMs, but rather Cys-246, the second to last residue on each P-TM polypeptide chain. The emission spectrum of pyrene-labeled P-TM (Py-P-TM) is distinctly different from those of pyrene-labeled muscle TM species. Changes in the emission properties of the label near the COOH terminus of P-TM in response to ionic strength, denaturation, and the presence of unlabeled TM molecules allow us to speculate on the structure of the COOH-terminal region of the molecule.

MATERIALS AND METHODS

Proteins. Platelet tropomyosin was prepared from lyophilized horse blood platelets according to method 2 of Côté and Smillie (1981b). Rabbit cardiac tropomyosin (C-TM)

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[1] Abbreviations: PIA, *N*-(1-pyrenyl)iodoacetamide; PM, *N*-(1-pyrenyl)maleimide; Py, pyrenyl; TM, tropomyosin; P-TM, equine platelet tropomyosin; C-TM, rabbit cardiac tropomyosin; NPTM, nonpolymerizable rabbit cardiac tropomyosin; F_{485}/F_{385}, ratio of fluorescence intensity of a sample measured at 485 nm to that at 385 nm; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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and a carboxypeptidase A treated, nonpolymerizable derivative of C-TM (NPTM) were prepared as described previously (Mak & Smillie, 1981). Actin was purified from an acetone powder of rabbit back and leg muscles as described by Spudich and Watt (1971). All protein preparations were judged to be greater than 95% pure by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method of Laemmli (1970). Concentrations of proteins in solutions employed were determined by amino acid analysis of duplicate samples hydrolyzed for 20 h at 110 °C in tubes sealed under vacuum.

Optical Methods. Fluorescence spectra were recorded by using a Perkin-Elmer MPF-44B spectrofluorometer equipped with a thermostated cell. Excitation was at 344 nm. Some measurements also were made by using a custom-built fluorometer (Burtnick, 1982) in the laboratory of L.D.B. As this instrument uses a high-pressure 200-W Hg source, excitation was at either the 365- or 313-nm Hg spectral lines (indicated in the appropriate figure legends). The 313-nm line was more useful when it was necessary to shift the Raman scattering peak due to water away from the region of pyrene monomer fluorescence. The data reported here have not been corrected for instrumental response.

Absorption spectra were obtained with a Cary 115C recording spectrophotometer, and circular dichroism measurements were recorded with a Jasco J500C spectropolarimeter fitted with a DP500N data processing unit.

Prior to optical analysis, unpolymerized protein samples were clarified by centrifugation at 100000*g* for 20 min. Other samples were centrifuged in a microcentrifuge (Eppendorf) at 15000*g* for 10 min.

Labeling of Tropomyosin. To label tropomyosin, the protein was first dissolved at 2–4 mg/mL in 0.15 M KCl, 10 mM Tris-HCl, and 2.0 mM dithiothreitol, pH 8.0, and dialyzed overnight at 4 °C against the same buffer. Subsequently, dialysis was performed against 0.15 M KCl and 10 mM Tris-HCl, pH 8.0, without dithiothreitol, for 4–5 h. *N*-(1-pyrenyl)iodoacetamide (Molecular Probes) was dissolved in *N*,*N*-dimethylformamide and added slowly to the stirred tropomyosin sample until an approximate 10-fold molar excess of reagent to protein was achieved. The reaction was allowed to proceed overnight at room temperature in the dark on a mechanical rocker. The reaction mixture was centrifuged at 15000*g* for 10 min to remove the precipitated reagent and the supernatant put on to dialyze against 0.15 M KCl and 10 mM Tris-HCl, pH 8.0, to reequilibrate the protein and to remove the last traces of excess reagent.

The extent of labeling of tropomyosin with PIA was quantitated by independent determinations of the amounts of label and protein in a sample solution. Pyrene concentrations were determined spectrophotometrically by using a molar absorption coefficient of $2.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 344 nm (Kouyama & Mihashi, 1981). Protein concentrations were determined by amino acid analysis of aliquots of the stock sample solutions.

Tryptic Digestion of P-TM. Py-P-TM and P-TM at 2 mg/mL were dialyzed against 50 mM ammonium bicarbonate and then placed into test tubes in a 37 °C water bath. TPCK–trypsin (Worthington) was added to each to a weight ratio of TM/trypsin of about 10. Samples were incubated for 6 h and were then freeze-dried twice prior to subsequent analysis.

Carboxypeptidase Digestion of P-TM. P-TM was digested with diisopropyl fluorophosphate treated pancreatic carboxypeptidase A (Sigma) as described by Mak and Smillie (1981). The digestion proceeded for 6 h in 10 mM Tris-HCl, 0.1 M KCl, 1.0 mM dithiothreitol, and 0.01% sodium azide, pH 8.0, at 37 °C and was stopped by heating the solution to 85 °C for 3 min. During the incubation period, duplicate 100-μL samples were removed at 1.5 and 6 h. Each sample was put into 500 μL of 12% trichloroacetic acid. The protein precipitate was removed by centrifugation. Trichloroacetic acid was extracted from the supernatant with diethyl ether, and the remaining aqueous phase was freeze-dried and subjected to amino acid analysis.

Table I: Amino Acid Composition of the Major Pyrenyl Peptide of Py-P-TM

amino acid	peptide (mol/mol)	P-TM residues 228–247[a] (mol/mol)	amino acid	peptide (mol/mol)	P-TM residues 228–247[a] (mol/mol)
Lys	0.1		Ala		
His	1.0	1	½-Cys	nd[b]	1
Arg			Val	1.2	1
Asx	4.0	4	Met		
Thr	1.5	2	Ile	1.0	1
Ser	1.2		Leu	3.9	4
Glx	4.8	5	Tyr		
Pro			Phe		
Gly	1.5	1			

[a] From Lewis et al. (1983). [b] Not determined.

The cysteine contents of carboxypeptidase-digested and native P-TM were determined by performic acid oxidation of the proteins and determination of their cysteic acid contents (Moore, 1963).

Results and Discussion

Incorporation of Label. Our preparations of Py-P-TM contained 1.12 ± 0.37 (on the basis of nine labeling experiments) pyrene groups per protein chain. This degree of labeling was significantly higher than the maximum value of 0.1 mol of pyrene/mol of –SH we were able to attain when labeling C-TM under identical conditions.

Tryptic peptides of Py-P-TM in 0.1% trifluoroacetic acid (TFA) in water were subjected to high-performance liquid chromatography on Beckman C-3 or C-8 Ultrapore reverse-phase columns. Elution with gradients extending from 0.1% TFA in water to 40% acetonitrile and 0.05% TFA in water yielded about 25 partly to completely resolved peptide peaks (monitored by absorbance at 210 nm). Pyrene eluted in as many as five peaks (monitored at 344 nm). Only one of these fractions also corresponded to a peak in the 210-nm absorbance trace, had a high ratio of absorbance at 344 nm to that at 210 nm, and had an amino acid composition that could be correlated with that of a potential tryptic peptide of P-TM (at the COOH terminus, Table I). The peak eluted late in the gradient, at about 30% acetonitrile, consistent with the presence of the hydrophobic pyrene group attached to it. Compositions of the other, significantly less fluorescent peptide peaks could not allow confident differentiation between the two possible sulfhydryl sequences on each chain.

In order to establish more definitely the site of labeling of P-TM, we prepared carboxypeptidase-digested samples of P-TM and labeled the truncated protein with PIA. Carboxypeptidase A released three residues, Ile-247, Cys-246, and Asn-245 (determined as Ser by amino acid analysis), from the COOH terminus of each P-TM chain. Treatment of P-TM with a mixed set of carboxypeptidases A, B, and Y also produced a truncated P-TM devoid of Cys-246. The cysteic acid content of performic acid oxidized, intact P-TM was determined to be 1.86 per chain while that of carboxypeptidase-treated P-TM was 1.09 per chain, in agreement with the

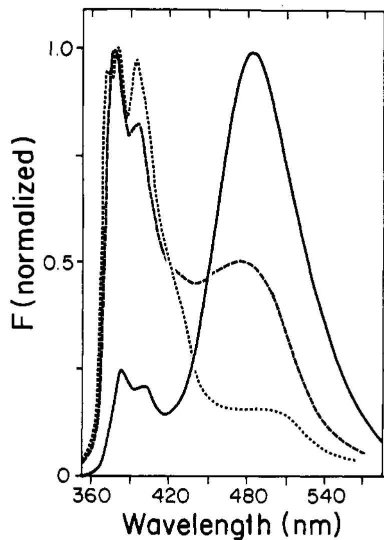

FIGURE 1: Emission spectra of Py-P-TM. Excitation was at 344 nm. The solid curve shows the emission of native Py-P-TM (1.13 mol of Py/mol of P-TM chains) at 4.2×10^{-5} M in 150 mM KCl and 10 mM Tris-HCl, pH 8.0. The dashed curve shows the emission of the same Py-P-TM after it had been subjected to tryptic digestion for 6 h at 37 °C. The dotted curve shows the emission of carboxypeptidase-treated P-TM, 4.3×10^{-5} M, that subsequently had been labeled with PIA, 0.31 Py per chain. Note that these spectra have been normalized so that their maxima reach 1.00. $T = 21$ °C.

proposed loss of Cys-246. Labeling experiments performed in parallel on intact and carboxypeptidase-digested P-TM samples revealed a much lower degree of labeling of the shortened P-TM, 0.31 Py per chain, than for the intact P-TM, 1.08 Py per chain. The low reactivity of digested P-TM with PIA indicates that the prime site of reaction under our conditions is Cys-246.

Fluorescence Spectra. Typical emission spectra of Py-P-TM are shown in Figure 1. Excitation of Py-P-TM was at 344 nm, and emission was monitored over the 360–530-nm range. It is apparent immediately from the large, broad emission peak centered near 485 nm that a large portion of the total fluorescence arises from pyrene excimers. We find the relative heights of the 485- and 385-nm peaks (F_{485}/F_{385}) of our Py-P-TM preparations to approach 4 in 0.15 M KCl and 20 mM Tris-HCl, pH 8.0 at room temperature. In comparison, Lin (1982) reports a ratio of about 1.4 for muscle TM labeled with PIA, Betcher-Lang and Lehrer (1978) report a ratio of about 0.4 for muscle TM, and Lehrer et al. (1984) found a value near zero for gizzard TM labeled with PM. These data suggest the contribution of excimer fluorescence to total fluorescence in Py-P-TM to be much greater than for other types of TM. According to model-building studies by Graceffa and Lehrer (1980), Cys-190 of skeletal muscle TM is located in a portion of the TM molecule that, if it were in a coiled-coil conformation, would not permit the pyrene–pyrene interactions required to allow excimer formation. The observation of excimer emission contributes to the evidence these authors use to propose that Cys-190 lies in a part of TM that can undergo localized unfolding. Such unfolding would permit sufficiently close approach of the two pyrene-labeled –SH groups to allow excimers to form. We would extend this model of local unfolding of the TM coiled coil to the platelet molecule. Cys-246 residues, which occupy positions "c" in the repeating heptapeptide scheme for tropomyosin molecules [see Figure 4 of Côté (1983)], would be diametrically opposed in a P-TM cross section. In order for pyrene excimers to form, considerable flexibility of the COOH-terminal region of P-TM would be required. In support of this claim, nuclear magnetic resonance studies of skeletal muscle TM also suggest that various regions, the COOH terminus in particular, of the molecule may exist in states other than that dictated by the coiled-coil model (Edwards & Sykes, 1980; Stewart & Roberts, 1983) and may be rather flexible. The proposed flexibility of the COOH terminus of P-TM and the outward-facing positions of the Cys-246 residues would account for their relative ease of labeling.

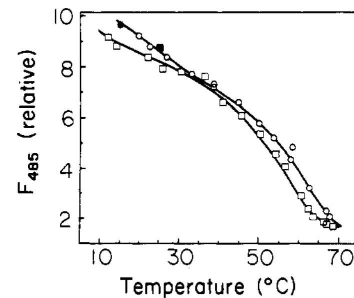

FIGURE 2: Thermal denaturation of Py-P-TM. Excitation was at 344 nm. Emission intensities at 485 nm, F_{485}, were recorded as a function of temperature for two samples of Py-P-TM at 1.5×10^{-6} M, one in 150 mM KCl and 10 mM Tris-HCl, pH 8.0 (○), and the other in 10 mM Tris-HCl, pH 8.0 (□). The solid symbols represent F_{485} values for samples recooled to the indicated temperatures at the end of the experiment.

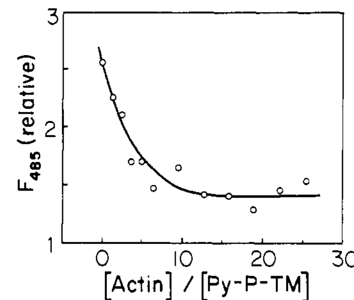

FIGURE 3: Addition of actin to Py-P-TM. To 12 identical 600-μL samples of Py-P-TM in 150 mM KCl and 10 mM Tris-HCl, pH 8.0, were added increasing amounts of G-actin in 2.0 mM Tris-HCl, 0.5 mM dithiothreitol, 0.2 mM ATP, and 0.2 mM $CaCl_2$, pH 8.0. All volumes were made up to 1.00 mL by addition of the buffer in which actin was dissolved. Final [Py-P-TM] = 1.8×10^{-6} M in each sample. One hour after the addition of 5 μL of 1.0 M $MgCl_2$ to each tube (final [Mg^{2+}] = 5 mM), the F_{485} values of the samples were read on excitation at 365 nm. $T = 21$ °C.

The fluorescence spectrum of carboxypeptidase-digested P-TM that subsequently was reacted with PIA shows a very small excimer contribution (Figure 1). This spectrum is similar to those of pyrene-labeled muscle TMs, suggesting that the signal comes from label at Cys-153. Labeling of incompletely removed Cys-246 also could contribute.

Excimer fluorescence from Py-P-TM can be eliminated either by tryptic digestion (Figure 1) or by thermal denaturation of the molecule (Figure 2). Both methods result in separation of the two P-TM chains. The thermal denaturation data also suggest that salt ions, to a limited extent, both enhance excimer formation and stabilize the structure of Py-P-TM. At both low and high ionic strengths, a pretransition can be observed in the melting profile for Py-P-TM. A similar pretransition has been observed in the melting of striated muscle TM species (Lehrer et al., 1984). The presence of the pretransition found in each curve indicates that localized weakening of the coiled-coil structure occurs prior to chain separation.

Interaction of Py-P-TM with Actin. Figure 3 shows the effects of adding muscle actin to Py-P-TM solutions on the excimer fluorescence intensity of the sample. The emission

intensity drops as the actin to Py-P-TM mole ratio increases and levels off in about the range of 6 or 7 actin molecules per P-TM molecule. This is consistent with results based upon cosedimentation studies reported earlier by Côté and Smillie (1981a), who found that P-TM could interact with muscle actin in the presence of Mg^{2+} ions.

As suggested earlier, interaction between pyrenes on two adjacent TM chains in P-TM requires that the chains have considerable freedom of movement. The binding of actin to Py-P-TM apparently places constraints on the flexibility of the chains and reduces the chances of excimer formation.

Interaction of Py-P-TM with Other TM Molecules. The tendency for muscle TM molecules to interact with each other in a head-to-tail manner is decreased as the ionic strength of the solution increases (Kay & Bailey, 1960; McCubbin & Kay, 1969). The somewhat shorter nonmuscle TM molecules, including P-TM, show a much reduced tendency to polymerize relative to muscle TM species, even at low ionic strengths (Côté et al., 1978). As PIA labels P-TM very near its COOH terminus, we attempted to see whether Py-P-TM could report in any way on the end-to-end interactions of TM molecules.

In these studies, samples of P-TM, Py-P-TM, C-TM, Py-C-TM, and NPTM were dialyzed extensively against 10 mM Tris-HCl, pH 8.0, prior to fluorescence analysis. Py-C-TM was prepared as described under Materials and Methods except that the initial dialysis was performed against a buffer that contained 10.0 mM dithiothreitol in order to reduce more extensively the Cys-190 residues. The second stage of dialysis, just prior to addition of PIA, was performed against the same solvent, but with 0.5 mM dithiothreitol. These modifications to the method resulted in a moderate improvement in the degree of labeling to 0.31 pyrenes per C-TM chain, a level sufficiently high to observe excimer fluorescence in emission spectra from such samples.

When KCl or NaCl was added to a Py-P-TM sample, the F_{485}/F_{385} ratio for the sample increased. The addition of KCl to 110 mM to a Py-P-TM sample having 1.13 pyrenes per protein chain caused an increase in the ratio from 3.47 to 3.60. Increasing the KCl concentration to 275 mM did not result in a further increase in the ratio. The increase in contribution of excimer fluorescence to the total emission is smaller than that observed by Graceffa and Lehrer (1980) for muscle TM. They suggest that the increase observed was due to an enhancement of the hydrophobic interactions of adjacent pyrenes. This effect could explain the small increase we observe as well. The magnitude of the increase we observe may not be as large simply because a large portion of the total emission that we observe is already due to excimers. Also, because the label on Py-P-TM is near the COOH terminus, we must consider the effects of salt on pyrene–pyrene interactions not only directly but also indirectly because salt affects the end-to-end overlap interactions of TM molecules.

In one set of studies we attempted to limit variations in pyrene concentrations in the samples by adding aliquots of concentrated stock solutions of unlabeled TM species to dilute Py-TM solutions. The addition of unlabeled P-TM to a Py-P-TM sample in 10 mM Tris-HCl, pH 8.0 (Figure 4), results in a drop in the F_{485}/F_{385} ratio of the sample. At a P-TM/Py-P-TM mole ratio of 2.4, the drop is about 20%. This change is in the opposite direction to that induced by salt when added to Py-P-TM samples. The data suggest that some COOH- to NH_2-terminal interaction does occur between adjacent Py-P-TM and P-TM molecules at low ionic strength. Therefore, we suppose that end-to-end interactions also occur between adjacent Py-P-TM molecules. The addition of KCl

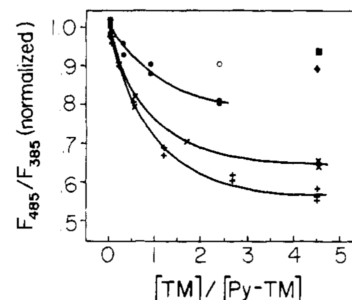

FIGURE 4: Addition of unlabeled TMs to Py-P-TM. All samples were dialyzed against 10 mM Tris-HCl, pH 8.0. For each experiment involving the addition of a different type of TM, two identical Py-P-TM samples were used. To one, the unlabeled TM was added while to the other, buffer alone was added. Spectra were recorded on excitation at 344 nm after the addition of each aliquot of unlabeled TM. The spectrum of the sample to which buffer alone was added was used to correct the spectrum of the sample to which TM was added for dilution effects. Because the initial concentrations of each solution varied somewhat, the data have been normalized such that the average value of two readings of a starting solution is 1.00. Symbols: (●) addition of stock P-TM (9.9×10^{-6} M) to Py-P-TM (3.5×10^{-7} M); (×) addition of stock C-TM (5.5×10^{-5} M) to Py-P-TM (4.8×10^{-7} M); (+) addition of stock NPTM (1.9×10^{-5} M) to Py-P-TM (6.3×10^{-7} M). The symbols ○, ■, and ♦ represent values measured after the addition of KCl to a final level of 275 mM to the P-TM-, C-TM-, and NPTM-containing samples, respectively. $T = 21$ °C.

to 110 mM restores the F_{485}/F_{385} ratio more than halfway back to its initial value. These effects suggest that end-to-end interactions between P-TM molecules can be disrupted by moderate salt concentrations. This is in agreement with earlier viscosity and sedimentation equilibrium results (Côté et al., 1978). The effects of addition of salt to Py-P-TM solutions reflect largely a disruption of end-to-end interactions between TM molecules, and the promotion of hydrophobic interactions is a less important effect.

The addition of C-TM to Py-P-TM also results in a reduction of the F_{485}/F_{385} ratio of the sample, but to a more significant extent than the addition of P-TM. Figure 4 shows a 35% reduction in F_{485}/F_{385} at a mole ratio of C-TM/Py-P-TM of 4.54. The reduction expected at a mole ratio of 2.4 would be about 32%. The larger drop than observed with the addition of P-TM suggests that the COOH terminus of P-TM can more readily interact with the NH_2 terminus of C-TM than with the NH_2 terminus of another P-TM. This may be the result of additional residues at the NH_2 terminus of P-TM that are not present in muscle TM molecules (Lewis et al., 1983). In addition, the COOH-terminal sequence of P-TM is strongly homologous with the COOH terminus of smooth muscle TM (Helfman et al., 1984; Sanders & Smillie, 1985), which readily undergoes end-to-end polymerization in low ionic strength solutions. Therefore, the COOH terminus of P-TM may interact better with the NH_2 terminus of a muscle TM than would the NH_2 terminus of P-TM with the COOH terminus of any other TM.

The interaction between the COOH termini of Py-P-TM and the NH_2 termini of C-TM molecules can be disrupted by salt. A KCl concentration of 100 mM restores about 75% of the drop in F_{485}/F_{385}, while higher concentrations of KCl (to 275 mM) cause no further dramatic alteration in the ratio.

The interaction between Py-P-TM and C-TM suggested to us that a complex should be able to exist that involves a Py-P-TM molecule whose COOH terminus overlaps the NH_2 terminus of a NPTM molecule. Indeed, addition of NPTM causes a fall in the F_{485}/F_{385} ratio of Py-P-TM samples in low ionic strength solutions. This drop was similar to that observed with the addition of C-TM (Figure 4). The somewhat stronger

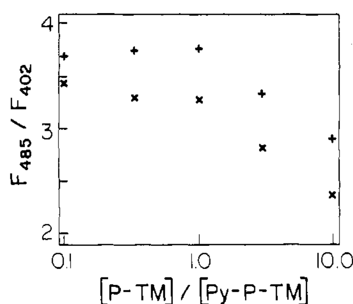

FIGURE 5: Interaction of unlabeled P-TM with Py-P-TM. Stock solutions of P-TM and Py-P-TM were dialyzed against 10 mM Tris-HCl, pH 8.0. Appropriate volumes of stock solutions and dialysate were mixed such that the final total concentration of protein present was 2.0×10^{-6} M. Spectra were recorded for each sample before (×) and after (+) the addition of KCl to 390 mM, and the ratio of fluorescence intensities at 485 and 402 nm, F_{485}/F_{402}, was calculated. Excitation was at 313 nm. $T = 22$ °C.

effects of NPTM over C-TM at the same mole ratios to Py-P-TM reflect the fact that NPTM molecules do not interact with each other in an end-to-end manner. Therefore, more NH_2 termini of NPTM than of C-TM would be available at a given TM concentration to complex with Py-P-TM. Again, salt disrupts the polymer as in the cases already discussed.

The addition of C-TM or of NPTM to Py-P-TM produces a decrease in F_{485} and an increase in F_{385}. It is important to note that in both cases of addition of unlabeled TM an isoemissive point near 437 nm becomes evident. The presence of an isoemissive point suggests that the data can be explained in terms of a two-state model in which pyrenes on Py-P-TM exist either as excimers or as noninteracting monomers. It is not necessary to invoke environmental perturbations of the probes due to alterations in the conformation of P-TM in order to explain the data.

To test whether some of the observations we made could be the result of the reporting of a small amount of pyrene label attached to Cys-153 on Py-P-TM, we labeled C-TM with PIA and monitored the fluorescence of this species as a function of added unlabeled P-TM. As mentioned, the level of excimer fluorescence in our Py-C-TM samples is low: $F_{485}/F_{385} = 0.20$ for this case. This addition of P-TM to a Py-C-TM sample in 10 mM Tris-HCl, pH 8.0, at mole ratios of P-TM/Py-C-TM up to 0.64 did not produce any change in the Py-C-TM emission. As Cys-153 of P-TM is homologous with Cys-190 of C-TM, we assume that a small amount of label at that site would not be able to report on interactions at the termini of the P-TM molecule and, therefore, that the observations made are the result of the labeling of the penultimate cysteine on P-TM. An increase in the ionic strength of Py-C-TM solutions causes a small rise in the F_{485}/F_{385} ratio from 0.20 to 0.24, consistent with the enhancement of hydrophobic interactions between pyrene moieties at higher ionic strengths.

In the above experiments, the pyrene concentrations in the samples in a given experiment varied by 8% at most. However, the total protein concentration in a given experiment varied by as much as 10-fold. To ensure that this large change in protein concentration was not the cause of the observed effects, we performed experiments in which P-TM/Py-P-TM and C-TM/Py-P-TM ratios were varied from 0 to 10 while the total tropomyosin concentration of the samples was kept constant. To measure the extent of excimer formation in samples with very low pyrene concentrations, we changed the excitation wavelength to 313 nm in order to eliminate artifacts due to a large Raman scattering peak due to water from the vicinity of pyrene monomer emission. This change in excitation wavelength did not affect the shapes of emission spectra of samples of Py-P-TM. In addition, due to the response properties of the custom-built fluorometer used in these particular studies, the monomer emission was followed at 402 nm instead of 385 nm. Therefore, the data were recorded as the change in F_{485}/F_{402} with change in ratio of unlabeled TM to Py-P-TM.

Samples were prepared to contain different mole ratios of unlabeled C-TM to Py-P-TM in 10 mM Tris-HCl, pH 8.0, at a total tropomyosin concentration of 2.0×10^{-6} M. As the mole ratio of C-TM to Py-P-TM increased from 0 to 10, the extent of excimer emission dropped off while that of monomer emission increased. Furthermore, when KCl was added to each sample to a final concentration of 390 mM, the ratio of excimer to monomer contributions increased. As in the previously reported experiments (Figure 4), the ratio was not restored completely to the low ionic strength values.

A similar experiment was performed by using mixtures of labeled and unlabeled P-TM at a total tropomyosin concentration of 2.0×10^{-6} M. Because the total concentration of labeled plus unlabeled P-TM was held constant, there should have been no change in the ratio of excimer to monomer fluorescence. However, the extent of excimer fluorescence was found to be least in solutions that contained the highest mole ratios of unlabeled to labeled P-TM (Figure 5). This suggests that the two forms of P-TM are not identical in their tendencies to polymerize. The presence of the pyrene groups diminishes the tendency for P-TM to interact in an end-to-end manner. Similar disruptive effects of pyrene groups have been reported (Ishii & Lehrer, 1985) for muscle TM labeled at Cys-190. The overall structural effects of pyrene on P-TM may be less than on muscle TM. Circular dichroism measurements reveal that P-TM remains >95% helical after labeling. Pyrene labeling results in a 10% drop in the helical content of muscle TM (Ishii & Lehrer, 1985).

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Dihydrolipoamide Dehydrogenase from Halophilic Archaebacteria: Purification and Properties of the Enzyme from *Halobacterium halobium*[†]

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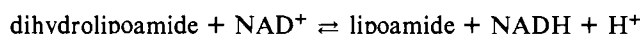
ABSTRACT: Halophilic archaebacteria possess dihydrolipoamide dehydrogenase activity but apparently lack the 2-oxoacid dehydrogenase multienzyme complexes of which it is usually an integral component [Danson, M. J., Eisenthal, R., Hall, S., Kessell, S. R., & Williams, D. L. (1984) *Biochem. J. 218*, 811–818]. In this paper, the purification of dihydrolipoamide dehydrogenase from *Halobacterium halobium* is reported. The enzyme is a dimer with a polypeptide chain M_r of 58 000 (±3000). The amino acid composition of the enzyme is compared with those of the eubacterial and eukaryotic dihydrolipoamide dehydrogenases, and evidence is presented to suggest that the N-terminal amino acid of the *H. halobium* enzyme is blocked. Chemical modification with the trivalent arsenical reagent (*p*-aminophenyl)dichloroarsine indicates the involvement of a reversibly reducible disulfide bond in the enzyme's catalytic mechanism. The possible metabolic role of this dihydrolipoamide dehydrogenase in the absence of 2-oxoacid dehydrogenase complexes is discussed.

Dihydrolipoamide dehydrogenase (EC 1.6.4.3) catalyzes the NAD-dependent oxidation of dihydrolipoamide [reviewed by Williams (1976)]:



$$\text{dihydrolipoamide} + NAD^+ \rightleftharpoons \text{lipoamide} + NADH + H^+$$

The enzyme fulfills this function in the pyruvate, 2-oxoglutarate, and branched-chain 2-oxoacid dehydrogenase multienzyme complexes. It is an integral component of each of these multienzyme structures, which are found in most eubacteria and all eukaryotes (Reed, 1974; Perham, 1975; Pettit et al., 1978). However, archaebacteria have been found to convert pyruvate and 2-oxoglutarate to their corresponding acyl-CoA thio esters via less complex oxidoreductases (Kerscher & Oesterhelt, 1982). In the halophiles and thermoacidophiles, ferredoxin, and not NAD$^+$, serves as the electron acceptor in these enzymes (Kerscher & Oesterhelt, 1981a,b; Kerscher et al., 1982), and methanogens use the deazaflavin derivative F_{420} (Zeikus et al., 1977). Lipoic acid is absent from the oxidoreductases, emphasizing a fundamental difference between these enzymes and the 2-oxoacid dehydrogenase multienzyme complexes.

Considering these data, it is unexpected that the halophilic archaebacteria possess a dihydrolipoamide dehydrogenase that, as is the case for the eubacterial and eukaryotic enzyme, is specific for NAD$^+$ and dihydrolipoamide (Danson et al., 1984). The basic enzymological and kinetic properties of this enzyme have been reported (Danson et al., 1984) although in the absence of the 2-oxoacid dehydrogenase complexes its function remained unclear. A more detailed molecular characterization of the archaebacterial dihydrolipoamide dehydrogenase was warranted, and in this paper we report the purification and properties of the enzyme from *Halobacterium halobium*.

Experimental Procedures

Materials

All chemicals used were of analytical grade. NAD$^+$, NADH, and aminopeptidase M were from C. F. Boehringer und Soehne, Mannheim, West Germany; DL-lipoamide was from British Drug Houses; DEAE-Sepharose CL-6B was from Pharmacia; hydroxylapatite–Bio-Gel HT was from Bio-Rad Laboratories, Richmond, CA; protamine sulfate (salmine) was purchased from Schwarz/Mann.

Methods

Bacterial Strain and Growth. *Halobacterium halobium* (C.C.M. 2090) was kindly provided by Dr. W. D. Grant (Department of Microbiology, University of Leicester, Leicester, U.K.). The organism was grown aerobically in liquid shake culture in the medium described by Payne et al. (1960).

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