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Articles

Comparison of the Fluorescence and Conformational Properties of Smooth and Striated Tropomyosin†

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ABSTRACT: In contrast to previous conformational studies with rabbit skeletal and cardiac tropomyosins, (i) when the cysteine side chains of chicken gizzard tropomyosin were reacted with 5,5′-dithiobis(2-nitrobenzoate), an interchain disulfide cross-link was *not* produced, (ii) when they were labeled with pyrenylmaleimide, excimer fluorescence was *not* observed, and (iii) when they were labeled with didansylcystine, a long-lived fluorescence component did *not* appreciably contribute to the fluorescence decay over a large temperature range including the major unfolding transition. In addition, the temperature dependence of the ellipticity at 222 nm did *not* reveal a pretransition prior to the main helix unfolding transition. This indicates that gizzard tropomyosin does not exhibit a localized chain-open state in the region of its cysteine residues, analogous to that seen with cardiac and skeletal tropomyosins, nor in any other region of the molecule. As a consequence, these observations suggest that gizzard tropomyosin is more rigid than striated tropomyosin.

Tropomyosin is a component of striated and smooth muscle thin filaments (Small & Sobieszek, 1983). In striated muscle systems, in conjunction with troponin, it is involved in the Ca^{2+}-dependent thin filament regulation of contraction (Ebashi & Endo, 1968), whereas its role in smooth muscle is less clear since regulation appears to be accomplished via a Ca^{2+}-dependent phosphorylation of myosin on the thick filament (Chacko et al., 1977; Gorecka et al., 1976; Sobieszek & Small, 1976).

Rabbit skeletal and cardiac tropomyosins consist of two parallel α-helical chains (subunits) that interact in register (Johnson & Smillie, 1975; Stewart, 1975; Lehrer, 1975) to form a rodlike molecule about 400 Å long (Cohen et al., 1973; Phillips et al., 1979). The skeletal molecule consists of two types of subunits, α and β, which differs slightly in amino acid sequence (Stone & Smillie, 1978; Sodek et al., 1978; Mak et al., 1979) combined into two principle molecular species, $\alpha\alpha$ and $\alpha\beta$ (Eisenberg & Kielley, 1974; Yamaguchi et al., 1974; Lehrer, 1975), while the cardiac molecule only contains α subunits (Cummins & Perry, 1973). Studies of smooth tropomyosin from fowl gizzard tissue have thus far indicated that it consists of two chains, α and β, which differ from each other and from striated tropomyosin chains in electrophoretic mobility on sodium dodecyl sulfate (SDS)[1] gels (Cummins & Perry, 1973) despite the same native molecular weight (Woods, 1969).

Smooth muscle tropomyosin shares many of the properties of skeletal tropomyosin; it self-polymerizes at low salt concentration, binds to F-actin under similar conditions, and can substitute for skeletal tropomyosin to confer Ca^{2+} sensitivity of actomyosin subfragment 1 ATPase in the presence of troponin (Dabrowska et al., 1980). Some differences in functional properties have been noted, however. In particular, smooth muscle tropomyosin activates skeletal actomyosin subfragment 1 ATPase more readily than skeletal tropomyosin and even activates under conditions that skeletal tropomyosin inhibits (Sobieszek & Small, 1981; Sobieszek, 1982; Yamaguchi et al., 1984).

In attempting to relate the conformation of tropomyosin to its function, we have been using fluorescence probes to study skeletal and cardiac tropomyosin conformations (Lehrer et al., 1981) and have been defining the way in which skeletal tropomyosin affects acto-S1 ATPase (Lehrer & Morris, 1982).

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[1] Abbreviations: SDS, sodium dodecyl sulfate; $(Nbs)_2$, 5,5′-dithiobis(2-nitrobenzoate); PAGE, polyacrylamide gel electrophoresis; Mops, 4-morpholinepropanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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We have obtained evidence that skeletal tropomyosin exists in two states in solution under physiological conditions, a more stable high α-helical "chain-closed" state and a somewhat less stable "chain-open" state in which the α-helical chains are locally unfolded and separated near Cys-190. This evidence came from the observation of excimer fluorescence from skeletal tropomyosin specifically labeled with pyrenylmaleimide at the two adjacent Cys-190 residues (Betcher-Lange & Lehrer, 1978; Graceffa & Lehrer, 1980), and also with the study of a long-lived dansyl fluorescence component from dansylcystine-labeled cardiac tropomyosin at Cys-190 (Betteridge & Lehrer, 1983). It was also suggested that the ability of $(Nbs)_2$ to form a disulfide cross-link between subunits of both skeletal and cardiac tropomyosins is due to the presence of the chain-open state (Lehrer et al., 1981). This chain-open locally unfolded state would be expected to impart considerable flexibility to skeletal and cardiac tropomyosins (Lehrer, 1978; Betteridge & Lehrer, 1983). Evidence for selective instability in the carboxyl-terminal region containing Cys-190 has also been obtained by other techniques (Woods, 1977; Chao & Holtzer, 1975; Pato & Smillie, 1978; Krishnan et al., 1978; Wahl et al., 1978; Lehrer, 1978; Potekhin & Privalov, 1978, 1982; Phillips et al., 1979; Edwards & Sykes, 1980; Williams & Swenson, 1981; Graceffa & Lehrer, 1982).

In the work reported below, we performed similar studies on tropomyosin purified from chicken gizzard smooth muscle as was originally done on striated tropomyosin. We found that gizzard tropomyosin specifically labeled at Cys groups with pyrenylmaleimide and didansylcystine did not show pyrene excimer fluorescence and did not exhibit an appreciable long-lived dansyl fluorescence component, respectively. We also found that $(Nbs)_2$ reacted with the Cys of gizzard tropomyosin but did not produce a disulfide cross-link in contrast to cardiac and skeletal tropomyosins. These studies indicate that gizzard tropomyosin exists primarily in a chain-closed state and may therefore be expected to be more rigid than skeletal and cardiac tropomyosins. Recent comparative studies have suggested that this greater rigidity, in part, may explain the difference in the way that gizzard tropomyosin affects actomyosin subfragment 1 ATPase activity (Lehrer & Morris, 1984).

Experimental Procedures

Smooth muscle tropomyosin was extracted and purified from acetone powders of chicken gizzard tissue the day of sacrifice at a local poultry house or from frozen tissue (Pelfreeze) by using standard procedures (Cummins & Perry, 1973). Samples were reduced and kept in low salt solutions to retard oxidation of SH groups (Morris & Lehrer, 1982). The cysteine content was determined by the method of Ellman (1959). Determination of protein concentration by the Lowry technique using skeletal tropomyosin as a standard and analyses of near-UV absorption spectra yielded an extinction coefficient at 277 nm, $E(277\text{ nm})$, of 0.19 $(mg/mL)^{-1}$ cm^{-1}, where the absorption spectra were measured in solutions with at least 0.2 M salt to minimize aggregation and the absorption at 320 nm was subtracted from the absorption at 277 nm to approximate the small amount of light scattering ($\sim$1%). The lower extinction coefficient compared to striated tropomyosin where $E(277\text{ nm}) = 0.24$ $(mg/mL)^{-1}$ cm^{-1} (Lehrer, 1975, 1978) is in relative agreement with the lower tyrosine content (Cummins & Perry, 1974).

Gizzard tropomyosin was labeled with pyrenylmaleimide at pH 6, and the succinimido ring of the cysteine adduct was opened to form the type II product by incubation at pH 8.4 as described earlier for skeletal tropomyosin (Graceffa & Lehrer, 1980). The degree of labeling (Graceffa & Lehrer, 1980) was found to be 90%.

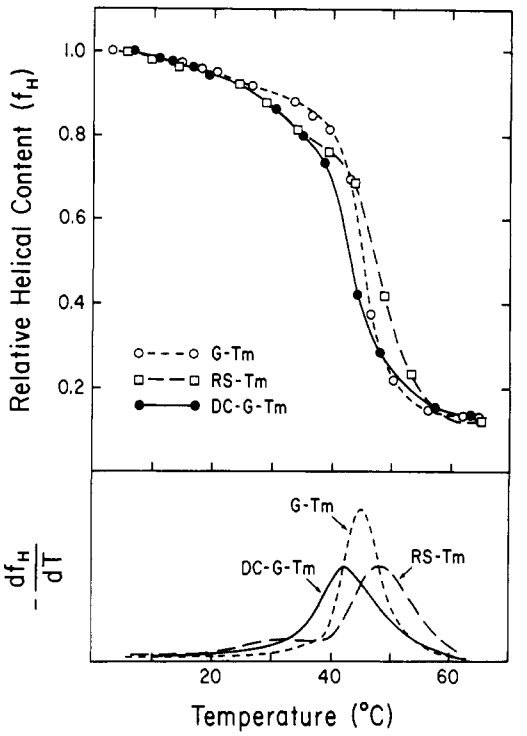

FIGURE 1: Comparison of the thermal unfolding of gizzard tropomyosin (G-Tm), dansylcystine-labeled gizzard tropomyosin (DC-G-Tm), and rabbit skeletal tropomyosin (RS-Tm). (Top) Normalized helical content obtained from the ellipticity at 222 nm; (bottom) slopes of upper curves. Conditions were 0.1 mg/mL in 20 mM Mops buffer, pH 7.5, 1 M NaCl, and 1 mM EDTA.

Gizzard tropomyosin was labeled with didansylcystine as described earlier for cardiac tropomyosin (Betteridge & Lehrer, 1983). The degree of labeling was found to be 80% by using absorption spectra as previously described (Betteridge & Lehrer, 1983).

Absorption spectra were obtained with a Beckman 25 double-beam or a Perkin-Elmer λ 3 spectrophotometer, fluorescence spectra with a Perkin-Elmer MPF-4a fluorometer, circular dichroism measurements with a Cary 60 spectrometer, and fluorescence lifetime measurements with a modified Ortec 9200 nanosecond fluorometer with excitation selected with a CS7-54 filter and emission with a CS3-72 filter. The excitation polarizer was set at 54.7° from vertical. The data were collected to a peak height of 20000 counts and analyzed by the method of moments procedure for a two-exponential decay (Isenberg & Dyson, 1969) as described previously (Betteridge & Lehrer, 1983). SDS–PAGE was performed on a Hoefer apparatus using 9% acrylamide (Laemmli, 1981).

Results

Thermal Unfolding Properties. There are two main differences in the thermal unfolding profiles of gizzard tropomyosin and rabbit cardiac tropomyosin in 1 M NaCl (Figure 1). Gizzard tropomyosin is somewhat less stable as judged by the midpoint of the main transition (approximated by the maximum slope, bottom panel of Figure 1), and gizzard tropomyosin does not exhibit a melting pretransition in the 20–40 °C range, as do both rabbit cardiac and skeletal tropomyosins (Woods, 1976; Betteridge & Lehrer, 1983). The dansylcystine probe on gizzard tropomyosin caused a small shift and a broadening of the transition but did not appear to cause any pretransition. Similar small effects of the label on the unfolding profile of cardiac tropomyosin were previously observed

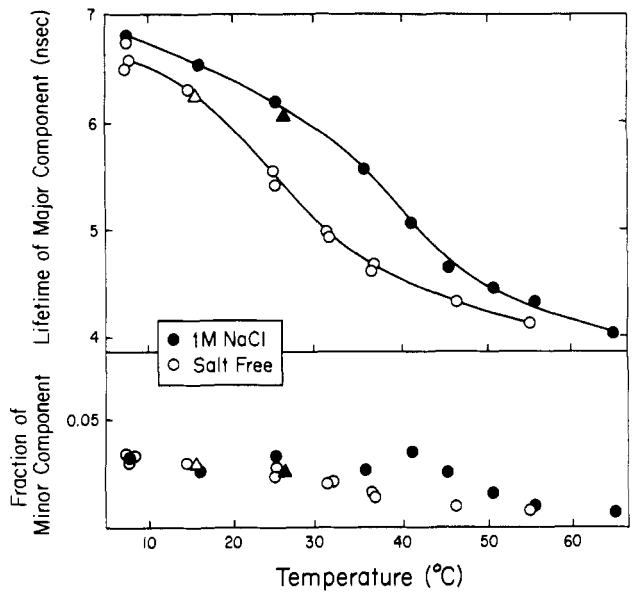

FIGURE 2: Temperature dependence of the fluorescence decay parameters of the two fluorescence components of dansylcystine-labeled gizzard tropomyosin. Conditions as for Figure 1. The lifetime of the minor component was approximately constant at 20 ns. (△, ▲) Reversed points.

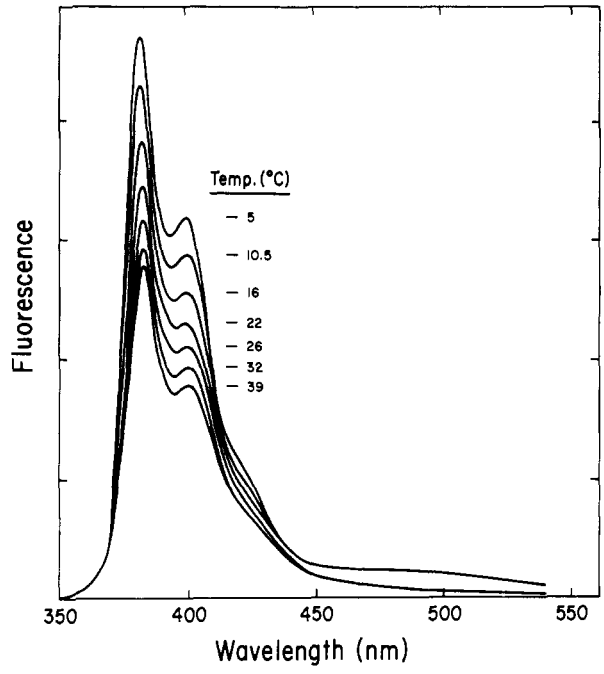

FIGURE 3: Temperature dependence of the fluorescence of doubly labeled pyrene-labeled gizzard tropomyosin. Conditions were 0.04 mg/mL in 5 mM Mops buffer, pH 7.5, 1 M NaCl, and 1 mM EDTA.

(Betteridge & Lehrer, 1983). In the absence of added salt, no pretransition was observed for either cardiac (Betteridge & Lehrer, 1983) or gizzard tropomyosin (data not shown); the transition midpoints for both were near 30 °C, and hardly any perturbation by the probe was noted.

Fluorescence Properties of Dansylcystine-Labeled Gizzard Tropomyosin. The fluorescence spectrum of dansylcystine-labeled gizard tropomyosin consisted of a broad band centered at 540 nm, very similar to the spectrum of dansylcystine-labeled cardiac tropomyosin, indicating that the average dansyl environment was very similar in the two tropomyosins. Fluorescence lifetime studies of dansylcystine-labeled gizzard tropomyosin showed that a minor contribution of a long-lived component (20 ns) was present in addition to a short-lived major component (7 ns). At low temperatures, the long-lived component only contributed about 3% to the fluorescence decay (Figure 2, bottom panel). No increase in the contribution of the long-lived component was observed over the complete temperature range of the unfolding, in contrast to the case of dansylcystine-labeled cardiac tropomyosin, where the contribution of the long-lived component reached 15% in the temperature range of the pretransition (Betteridge & Lehrer, 1983). Instead, only a monotonic decrease in the contribution of the long-lived component was observed which reached very low values at high temperature both in the presence of 1 M NaCl and in the absence of salt (Figure 2, bottom panel). The lifetime of the minor component did not significantly change over the complete temperature range. Changes in the lifetime of the major component, however, correlated with the unfolding transitions (see above) under both salt conditions (Figure 2, top panel), indicating that there were changes in the environment of the dansyl probe associated with the unfolding.

Fluorescence Properties of Pyrene-Labeled Gizzard Tropomyosin. The fluorescence spectrum of pyrenylmaleimide-labeled skeletal tropomyosin whose resulting succinimido ring was cleaved by alkaline incubation has been shown to consist of a broad excimer band at 480 nm as well as a structured monomer band with a major peak at 383 nm (Betcher-Lange & Lehrer, 1978). The contribution of the excimer of pyrene-labeled skeletal tropomyosin was shown to first increase and then decrease as the temperature was increased (Graceffa & Lehrer, 1980) in a manner qualitatively the same as the dependence of the long-lived fluorescence component of dansylcystine-labeled cardiac tropomyosin. In contrast to the fluorescence behavior of the skeletal system, pyrene-labeled gizzard tropomyosin essentially only showed monomer fluorescence over a large temperature range (Figure 3). The lack of excimer fluorescence could not be due to the lack of doubly labeled molecules since the degree of labeling was high (90%)). Despite the lack of excimer fluorescence, the monomer spectrum was very similar in both cases, indicating the similarity of the pyrene environment.

Reaction of Gizzard Tropomyosin with (Nbs)$_2$. (Nbs)$_2$ has been shown to react with skeletal and cardiac tropomyosins to produce a disulfide cross-link between adjacent Cys-190 residues (Lehrer, 1975). Treatment of gizzard tropomyosin with (Nbs)$_2$ under similar conditions and monitoring the change in absorption at 412 nm indicated that 1.8 SH groups per tropomyosin reacted, showing that there is approximately 1 SH group per chain in agreement with the amino acid composition (Cummins & Perry, 1973). Observation of the products of this reaction with SDS–PAGE indicated that no cross-links were produced (Figure 4) in contrast to the striated tropomyosin cases. Reaction of SH groups with (Nbs)$_2$ was also indicated by the slower migration of the (Nbs)$_2$-blocked monomer chains in the gel as compared to iodoacetamide-blocked monomer chains (Figure 4). Skeletal tropomyosin and cardiac tropomyosin whose SH groups were blocked with (Nbs)$_2$ in the denatured state where disulfide cross-links cannot be produced were also observed to have lower mobility on SDS gels.

Discussion

Our previous studies explored the conformational properties of rabbit skeletal and rabbit cardiac tropomyosins in the region of the Cys group at position 190 in the sequence (Lehrer et al., 1981). We have shown that the fluorescence decay of dansylcystine-labeled cardiac tropomyosin consists of two components: a short-lived (6-ns) component and a long-lived

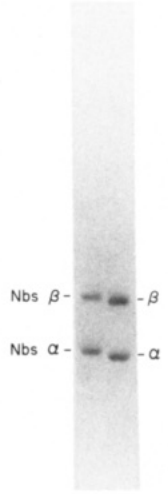

FIGURE 4: $(Nbs)_2$ treatment of gizzard tropomyosin blocks SH groups without producing S–S cross-links (9% SDS–polyacrylamide gels). Right slot, untreated; left slot, treated with $(Nbs)_2$. Reduced gizzard tropomyosin (0.56 mg/mL in 2 mM Hepes buffer, pH 7.5, 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM EDTA) was either reacted with 4 mM iodoacetamide to prevent oxidation of SH groups (untreated) or reacted with 2 mM $(Nbs)_2$ for 3 h at 25 °C and then with 4 mM iodoacetamide (treated) before denaturation in 0.5% SDS.

(17-ns) blue-shifted component. The contribution of the long-lived fluorescence component increased over the temperature range corresponding to a pretransition in the thermal unfolding profile before decreasing in the temperature range corresponding to the main unfolding transition (Betteridge & Lehrer, 1983). This was attributed to increased interaction between the probe and the hydrophobic ridge between the coiled coils as they locally separate and unfold. We have also shown that the excimer fluorescence of pyrene-labeled rabbit skeletal tropomyosin increased over the temperature range of the pretransition before decreasing in the main transition (Graceffa & Lehrer, 1980). Since space-filling molecular models showed that it was impossible for the adjacent pyrenes to interact to form an excimer unless the chains separate, the increase in excimer fluorescence was also interpreted as being due to increased localized chain separation which allowed the pyrene moieties to interact more favorably. We also suggested that the ability of these tropomyosins to form a disulfide cross-link between the chains via $(Nbs)_2$ reaction (Lehrer, 1975) or air oxidation (Johnson & Smillie, 1975; Stewart, 1975) is possible only if the chains are not staggered and there is localized chain separation (Lehrer et al., 1981). A model was proposed (Graceffa & Lehrer, 1980; Lehrer et al., 1981) which placed Cys-190 in the unfolded region of Woods' partially unfolded intermediate (Woods, 1976) and included chain separation. Thus, it appears that rabbit skeletal and cardiac tropomyosins equilibrate between a chain-closed state which predominates at low temperatures and a localized chain-open state which predominates at physiological temperatures.

The same approaches used in this study of the conformational properties of chicken gizzard tropomyosin indicated that localized chain separation does not occur in the region of the Cys groups. Dansylcystine-labeled gizzard tropomyosin did not have an appreciable long-lived fluorescent component, and pyrene-labeled gizzard tropomyosin showed little or nor excimer fluorescence over the entire thermal unfolding profile. Treatment of gizzard tropomyosin with $(Nbs)_2$ showed that one Cys per chain reacted without producing a disulfide cross-link under conditions that produced an interchain cross-link for the skeletal and cardiac tropomyosins. Thus, the rate of interchain sulfhydryl–disulfide formation of gizzard tropomyosin is much too slow to compete with external exchange with $(Nbs)_2$, in contrast to the striated systems (Lehrer, 1975). This can be considered evidence of the lack of localized chain separation for the gizzard system if the Cys groups are in equivalent positions on each chain. Evidence for the proximity of the Cys groups was obtained by Cu^{2+}-catalyzed air oxidation. We have found that it is possible to form disulfide cross-links with gizzard tropomyosin that approached 50% efficiency by incubation for 24–36 h at 35 °C, in agreement with the long reaction times used to cross-link gizzard tropomyosin with similar efficiencies in the earlier study (Strasburg & Greaser, 1976). Under the same conditions, skeletal tropomyosin is quantitatively cross-linked in 1 h. It is possible that the long times required for the gizzard system may increase the probability of cross-linking as a consequence of low-amplitude chain fluctuation which may bring about transient proximity of Cys residues.

It should be pointed out that the presence of a long-lived fluorescence component for dansylcystine-labeled cardiac tropomyosin, which correlates with localized unfolding, does not require Cys–Cys proximity (Betteridge & Lehrer, 1983). The absence of this component in the gizzard system therefore provides evidence for the lack of localized unfolding regardless of the disposition of the Cys residues.

The possibility that localized chain separation occurs in a region of the gizzard tropomyosin molecule that does not contain the SH groups must be considered. Although the complete amino acid sequence of the α or β chains has not yet been reported, some information regarding the location of the Cys residues is available. From analyses of polyacrylamide gels of peptides cleaved at Cys, it appears that the single SH group of each chain is not at position 190 but is located closer to the ends for both α and β gizzard (Betteridge et al., 1983). Thus, the Cys residues in gizzard tropomyosin do not appear to be located in the region of the molecule in which localized melting occurs for striated tropomyosin. No evidence of a thermal pretransition was obtained with circular dichroism studies, however, for both unlabeled and dansylcystine-labeled gizzard tropomyosins, in contrast to the cases of skeletal and cardiac tropomyosins, in agreement with earlier studies of Woods (1976). It appears, therefore, within experimental error, there is no region in gizzard tropomyosin that is locally melted prior to its major helix–coil transition. Since its helical structure is therefore largely maintained at 35–40 °C, gizzard tropomyosin may be expected to be more rigid than striated tropomyosin. It is interesting to note that these results indicate that Cys groups are not inherently found in regions of decreased stability in coiled-coil structures.

Most of the reported molecular properties of gizzard tropomyosin are very similar to those of skeletal and cardiac tropomyosins. The molecular weights are very similar (Woods, 1969), the amino acid composition shows a high proportion of hydropobic residues consistent with hydrophobic interactions that are involved in the stabilization between the coiled coils (Cummins & Perry, 1974), and paracrystals with similar stained banding patterns are obtained, indicating similar end to end and side to side interactions and similar molecular lengths (Cohen & Longley, 1966; Dabrowska et al., 1980; Yamaguchi et al., 1982). Gizzard tropomyosin can bind to skeletal F-actin under similar salt conditions as skeletal tropomyosin and can substitute for skeletal tropomyosin in a Ca^{2+}-regulated actomyosin (Dabrowska et al., 1980; Sobieszek & Small, 1981) ATPase system in conjunction with skeletal troponin. Some differences in the effect of smooth vs. skeletal tropomyosin on actomyosin ATPase in the absence of troponin

have been reported. In particular, gizzard has been shown to activate actomyosin subfragment 1 ATPase under conditions where skeletal tropomyosin inhibits (Sobieszek & Small 1981; Sobieszek, 1982). In separate comparative ATPase studies, we have proposed that the ability of gizzard tropomyosin to more readily activate actomyosin subfragment 1 ATPase is in part due to its greater rigidity (Lehrer & Morris, 1984).

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