Locking Regulatory Myosin in the Off-state with Trifluoperazine*

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Scallop striated adductor muscle myosin is a regulatory myosin, its activity being controlled directly through calcium binding. Here, we show that millimolar concentrations of trifluoperazine were effective at removal of all regulatory light chains from scallop myosin or myofibrils. More important, 200 μM trifluoperazine, a concentration 10-fold less than that required for light-chain removal, resulted in the reversible elimination of actin-activated and intrinsic ATPase activities. Unlike desensitization induced by metal ion chelation, which leads to an elevation of activity in the absence of calcium concurrent with regulatory light-chain removal, trifluoperazine caused a decline in actin-activated MgATPase activity both in the presence and absence of calcium. Procedures were equally effective with respect to scallop myosin, myofibrils, subfragment-1, or desensitized myofibrils. Increased α-helicity could be induced in the isolated essential light chain through addition of 100–200 μM trifluoperazine. We propose that micromolar concentrations of trifluoperazine disrupt regulation by binding to a single high-affinity site located in the C-terminal domain of the essential light chain, which locks scallop myosin in a conformation resembling the off-state. At millimolar trifluoperazine concentrations, additional binding sites on both light chains would be filled, leading to regulatory light-chain displacement.

Trifluoperazine (TFP),¹ a member of the phenothiazine class of drugs, is one of the strongest antagonists of calmodulin action known, capable of binding to calmodulin in the presence of calcium and preventing its stimulatory effects (1–3). The structure of the TFP-calmodulin complex in the presence of calcium has been determined at 2.45-Å resolution (4, 5), where it was shown that TFP induces a profound configurational change in calmodulin, converting the elongated dumbbell to a compact globular structure, analogous to the form obtained through the binding of calmodulin to a target peptide (6, 7).

This effect was accomplished when a single TFP molecule bound to the predominant binding site, a hydrophobic pocket within the C-terminal domain (4). Additional TFP-binding sites are apparent when the TFP concentration is raised (5). Recently, TFP-binding sites on the related calcium-binding protein troponin C have also been identified (8).

Myosin light chains belong to the same large family of calcium-binding proteins as calmodulin, to which they display a similar overall structure (9, 10). There are two types of light chain, termed regulatory (R-LC) and essential (E-LC); one member of each type binds to each of the two heads of conventional myosin II. In those regulatory myosins that are activated directly by calcium binding (for review, see Ref. 11), the exact relationship of the heavy chain to the light chains, in the presence of calcium, is now known in detail, the structure of the regulatory domain of scallop myosin having first been established at 2.8-Å resolution (12) and then refined to 2.0-Å resolution (13). Solution studies (14) indicate that TFP can bind to the isolated light chains: measurements of the circular dichroism indicated half-maximal binding of TFP with a dissociation constant in the range of 14–50 μM, the binding of which resulted in a significant change in secondary structure consistent with an increase in α-helical content, whereas EPR spectroscopy detected binding at sites of lower affinity with half-maximal effects yielding dissociation constants in the range of 370–800 μM.

The removal of R-LCs from scallop myosin through chelation of metal ions is a well known phenomenon (15, 16), complete dissociation being achieved in a reversible manner at elevated temperatures (17, 18). However, the same treatment has proven ineffective when applied to smooth muscle and other myosins (11). Recently, TFP was shown to facilitate both R-LC exchange and R-LC dissociation from smooth muscle myosin (19–21). It was therefore of interest to see whether or not TFP would prove effective at R-LC removal from scallop myosin. Here, we demonstrate that TFP in the millimolar range can remove R-LCs from scallop myosin. However, unlike the ensuing loss of regulation that accompanies metal ion chelation, brought about through an elevation of actin-activated MgATPase in the absence of calcium (17), TFP binding results in a monotonous decline in actin-activated MgATPase both in the presence and absence of calcium. Furthermore, we demonstrate that the loss of regulation brought about by TFP occurs at concentrations an order of magnitude lower than those required to effect R-LC dissociation. These results are discussed in the light of current structural knowledge: we formulate a hypothesis suggesting that TFP may act by interfering with the conformational relay mechanism operating through the interface between the C-terminal lobe of E-LC and regions of the heavy chain, thereby fixing the “off-state” of scallop myosin.

EXPERIMENTAL PROCEDURES

 Protein Preparation and EDTA Desensitization—Myofibrils and myosin from scallop striated adductor muscles were prepared as described earlier (22). Desensitization of scallop myofibrils and myosin through metal ion chelation was accomplished by standard procedures (17, 23). CaMg-S-1 was prepared by papain digestion (24), except that the reaction was terminated by the combined addition of N°-p-tosyl-l-lysine chloromethyl ketone (to 10 mM from a 0.5 mM stock in 50% ethanol) and leupeptin (to 10 mg/liter from a 0.5 mg/ml stock in buffer), so as to avoid covalent modification of S-1 as compared with the original procedure.

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The abbreviations used are: TFP, trifluoperazine; E-LC, essential light chain; R-LC, regulatory light chain; S-1, myosin subfragment-1; MOPS, 4-morpholinopropanesulfonic acid.

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Rabbit F-actin was prepared according to standard protocols (25) from actin acetone powder.

**TFP-induced Light-chain Dissociation**—Dissociation experiments involving TFP were performed as follows. Myosin (15 mg/ml in a total volume of 60 μl) in either high (0.5 mM KCl, 20 mM phosphate (pH 7.0), 2 mM MgCl₂, 3.0 mM NaN₃, and 10 μM CaCl₂) or low (0.1 mM KCl, 20 mM phosphate (pH 7.0), 2 mM MgCl₂, 3.0 mM NaN₃, and 10 μM CaCl₂) ionic strength buffer was stirred gently for 30 min on ice with TFP added to various final concentrations from a 20 mM stock solution. Upon completion of the reaction, 1.4 ml of water were added to each tube, and the material was allowed to stir on ice for 10 min, ensuring myosin filament formation, prior to centrifugation for 15 min at 13,000 rpm and 4 °C and lyophilization of the supernatant (∼10 μl) when scallop myosin was treated with increasing concentrations of TFP. Lanes 1–6, scallop myosin treated with 0, 667, 1333, 2000, 2667, and 3333 μM TFP, respectively. The initial reaction volume was 60 μl for each treatment and contained 0.9 mg of myosin in high ionic strength buffer. Approximately one-sixth (10 μl) of the resuspended pellet (a) or supernatant (b) was loaded onto each lane. Note that TFP selectively dissociated R-LC and did not bring about the release of E-LC into the supernatant. c, combined results from five sets of experiments, each representing a different myosin preparation. Because no E-LC was released during TFP treatment, results could be plotted graphically, with the R-LC/E-LC ratio being represented on the ordinate (equivalent to the ratio of R-LC to myosin heavy chain) and TFP concentration on the abscissa. •, ■, and △, experiments conducted at high ionic strength; × and ○, experiments conducted at low ionic strength. See “Experimental Procedures” for details.
concentration of 2.5 mg/ml and dialyzed against a buffer composed of 200 mM KCl, 30 mM MOPS, 15 mM $\beta$-mercaptoethanol, 2.0 mM MgCl$_2$, 0.6 mM EGTA, and 0.5 mM CaCl$_2$ (pH 7.0) at 4 °C. In these experiments, the free calcium concentration was calculated to be $2.1 \times 10^{-6}$ M.

Circular Dichroism—To determine if TFP directly elicited a change in the secondary structure of E-LC, far-UV CD spectra were obtained using a Jobin-Yvon JY-CD6 spectropolarimeter. E-LC (19 $\mu$M), in the presence or absence of 200 $\mu$M TFP, was incubated in 30 mM phosphate buffer (pH 7.0) at room temperature for 15–30 min. CD spectra were obtained at 20 °C using the following instrumental settings: range, 250–185 nm; scan rate, 5 nm/min; time constant, 2 s; and wavelength increment, 0.1 nm. Spectra of the buffer alone (without TFP), taken under identical conditions, were first subtracted from the corresponding protein spectra prior to obtaining a difference spectrum by subtracting the corrected E-LC spectrum (without TFP) from the corrected E-LC spectrum (with TFP). In addition, induction of the $\alpha$-helix was monitored as a function of increasing TFP concentrations (0, 40, 50, 80, 100, 120, 150, 200, 250, and 300 $\mu$M) at 208 and 222 nm using shorter scans (225–205 nm) run under identical conditions. Reproducible results were obtained from measurements taken using two separate preparations of E-LC purified by electroelution.

Miscellaneous—Urea-acrylamide electrophoresis and SDS-acrylamide gel electrophoresis were performed as described previously (17). Protein concentrations were determined either spectrophotometrically (for myosin, $E_{1}^{1%} = 5.3$ (22); for S-1, $E_{1}^{1%} = 8.0$ (24)) or by the Bio-Rad protein assay (28). Free calcium concentrations were computed using an iterative procedure (17).

**RESULTS**

**Effect of Trifluoperazine on Regulatory Light-chain Dissociation from Scallop Myosin**—Incubation of scallop myosin (25 $\mu$M) for 30 min at 0 °C in high ionic strength buffer with increasing concentrations of TFP (up to 3.3 mM) resulted in the loss of R-LCs, as monitored by urea gel analysis of the resulting pellets and supernatants (Fig. 1, a and b), performed as described under "Experimental Procedures." Densitometry of lanes from urea gels on which pellets from TFP-treated myosin had been loaded indicated that R-LC dissociation was 60% complete following 2.0 mM TFP treatment (TFP/myosin molar ratio 5:80) and complete at 3.0 mM TFP (Fig. 1c). No E-LC dissociation was observed during this process (Fig. 1b). Similar results were obtained upon exposure of myosin to TFP at low ionic strength (Fig. 1c). EDTA-desensitized scallop myofibrils,

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**Fig. 2.** Trifluoperazine attenuates the rate of actin-activated MgATP hydrolysis by scallop myosin. The actin-activated MgATPase rate of scallop myosin was measured in the presence of increasing concentrations of TFP, both in the presence and absence of $2.1 \times 10^{-6}$ M free calcium. Each point shown represents an average of two individual determinations. Note that TFP inhibited the normal rate of hydrolysis irrespective of the presence or absence of calcium. Details are described under “Experimental Procedures.” The inset shows the same data replotted as the normalized actin-activated MgATPase ($100 \times (V - V_{\text{min}})/(V_{\text{max}} - V_{\text{min}})$), where $V$ is the measured actin-activated MgATPase rate, and $V_{\text{max}}$ and $V_{\text{min}}$ are the maximal and minimal rates for the series of measurements, respectively.

**Fig. 3.** Effect of trifluoperazine on the rate of ATP hydrolysis by intact and EDTA-desensitized scallop myofibrils. The actin-activated MgATPase rate of intact (■ and □) and EDTA-desensitized (▲ and △) scallop myofibrils was measured in the presence of increasing concentrations of TFP, both in the presence (□ and △) and absence (■ and ▲) of $2.1 \times 10^{-6}$ M free calcium. Each point shown represents an average of two determinations. Note that TFP inhibited ATP hydrolysis in both intact and EDTA-desensitized myofibrils, irrespective of the presence or absence of calcium. Details are described under “Experimental Procedures.” The inset shows the same data replotted as the normalized actin-activated MgATPase ($100 \times (V/V_{\text{max}})$), where $V$ is the measured actin-activated MgATPase rate, and $V_{\text{max}}$ is the maximal rate for the series of measurements).
from which all R-LCs had been removed, were also treated with increasing concentrations of TFP (data not shown), yet only traces of E-LC were displaced (<5%), the vast majority remaining tightly associated with the heavy chain. Results were invariant of the presence or absence of calcium. Taken together, these results indicate that millimolar concentrations of TFP are effective in the specific removal of R-LCs from scallop adductor muscle myosin, as has been found for smooth muscle myosin (19–21).

Effect of Trifluoperazine on the Rate of ATP Hydrolysis by Scallop Myosin—Increasing concentrations of TFP gave rise to a decline in actin-activated MgATPase of scallop myosin both in the presence and absence of calcium (Fig. 2). Actin-activated MgATPase rates were halved by the addition of ~0.1 mM TFP (Fig. 2), whereas R-LC removal was half complete at ~1.5 mM TFP (Fig. 1). It may be noted that the relationship of TFP inactivation to R-LC removal is completely different from the situation resulting from EDTA treatment of scallop myosin. Whereas EDTA treatment leads to a decline in actin-activated MgATPase in the presence of calcium and a rise in actin-activated MgATPase in the absence of calcium (17), TFP treatment brings about a decline in actin-activated ATPase rates irrespective of calcium levels. Furthermore, EDTA-induced dissociation of R-LCs is causative with respect to desensitization (15, 17), whereas the effect of TFP on actin-activated MgATPase is independent of and precedes any effect on R-LC loss (compare results shown in Figs. 1–3).

A decline in the actin-activated MgATPase rate of scallop myofibrils in the presence or absence of calcium could also be demonstrated (Fig. 3). Significantly, increasing TFP concentrations also resulted in the attenuation of the actin-activated MgATPase activity of EDTA-desensitized scallop myofibrils (Fig. 3), demonstrating that the effect of TFP action on ATP hydrolysis does not require the presence of R-LC for its mediation.

Reversibility of the effect of TFP on ATPase activity was demonstrated through exhaustive dialysis of scallop myofibrils treated with 200 μM TFP (Table I), a concentration that leads to a significant reduction of actin-activated MgATPase, yet does not displace R-LC (Fig. 1). Measurement of the actin-activated MgATPase of these TFP-treated myofibrils, before and after dialysis, demonstrated reversibility; rates of activity were restored to intact levels both in the presence and absence of calcium (Table I). By contrast, exhaustive dialysis of myofibrils treated with either 2 or 4 mM TFP could not restore any activity even when incubated with a 12× molar ratio of R-LC to myosin throughout the dialysis period (data not shown).

Increasing concentrations of TFP gave rise to a gradual decline in the intrinsic rate of K⁺EDTA-ATP hydrolysis, an indicator of active-site functionality, as seen using preparations of myosin and intact or EDTA-desensitized myofibrils (Fig. 4). These rates were diminished by ~70% at 250 μM TFP, a concentration where inhibition of actin-activated MgATPase was complete (compare results shown in Figs. 2 and 4).

A limited number of determinations were made to ascertain the effect of increasing concentrations of TFP on the actin-activated MgATPase of scallop myofibrils. Increasing concentrations of TFP gave rise to a gradual decline in the intrinsic rate of K⁺EDTA-ATP hydrolysis, an indicator of active-site functionality, as seen using preparations of myosin and intact or EDTA-desensitized myofibrils (Fig. 4). These rates were diminished by ~70% at 250 μM TFP, a concentration where inhibition of actin-activated MgATPase was complete (compare results shown in Figs. 2 and 4).

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**Table I.**

Reversibility of the effect of 200 μM TFP on the actin-activated MgATPase of scallop striated adductor muscle myofibrils

| Condition* | Calcium* | Rate* µmol/min/mg% | Untreated value |
|------------|----------|---------------------|----------------|
| Intact myofibrils | − | 0.065 | 100 |
| | + | 0.595 | 100 |
| Intact myofibrils treated with 200 μM TFP | − | 0.035 | 54 |
| | + | 0.329 | 55 |
| Intact myofibrils treated with 200 μM TFP, then dialyzed exhaustively | − | 0.069 | 106 |
| | + | 0.562 | 94 |

*Conditions used are described under “Experimental Procedures.”

**Fig. 4.** Effect of trifluoperazine on the intrinsic rate of ATP hydrolysis by scallop myosin. The K⁺EDTA-ATPase rates of scallop myosin (○), myofibrils (■), or EDTA-desensitized myofibrils (▲) in 2.0 mM EDTA, 0.1 mM KCl, and 1.0 mM ATP (pH 7.0) were monitored as a function of increasing TFP concentration to assess whether TFP had any effect on the intrinsic hydrolytic mechanism at the active site. Points shown are the average of duplicate ATPase determinations from two different preparations. Details are given under “Experimental Procedures.” The inset shows the same data replotted as the normalized K⁺EDTA-ATPase activity (100 × (V/Vmax)), where V is the measured rate, and Vmax is the maximal rate for the series of measurements.)
activated MgATPase activity of scallop CaMgS-1 (Table II). These data demonstrate that increasing TFP concentrations led to a decline in the actin-activated MgATPase activity of myosin S-1. However, the effect of TFP was not as pronounced as that observed with myosin; whereas a concentration of 250 μM TFP resulted in complete elimination of myosin actin-activated MgATPase (Fig. 2), at least 30% activity remained in the case of S-1 (Table II).

Effect of Trifluoperazine on Calcium-specific Binding to Scallop Myosin and CaMgS-1—Calcium-specific binding (2.1 μM free calcium in the presence of 1 mM MgCl2) (17) was performed on scallop myosin over a range of TFP concentrations (Fig. 5). At 200 μM TFP, a concentration where the elimination of actin-activated MgATPase was >80% complete (Fig. 2) yet R-LC losses remained minimal (Fig. 1), ~70% of the normal calcium-specific binding capacity was retained following TFP treatment (Fig. 5). Full restoration of normal calcium binding values was observed (data not shown) in sister aliquots treated with 200 μM TFP followed by overnight dialysis to remove TFP and to facilitate R-LC re-binding.

Treatment of scallop CaMgS-1 with TFP did not perturb calcium binding to the calcium-specific binding site (Fig. 5), which remains intact on this proteolytic fragment despite cleavage of 11 residues from the N terminus of R-LC (12, 24). Under conditions of 2.1 μM free calcium in the presence of 1 mM free magnesium, calcium bound to S-1 at a ratio of ~1 mol/mol, and this level of binding remained unchanged throughout TFP titration (Fig. 5), even when the range was extended to include 1.5 mM TFP (data not shown).

Effect of Trifluoperazine on the Circular Dichroic Spectra of the Scallop Essential Light Chain—Far-UV CD spectra were obtained to determine if TFP could elicit, directly, a change in the secondary structure of scallop E-LC. A difference spectrum (Fig. 6) obtained through subtraction of the corrected E-LC spectrum (−TFP) from the corrected E-LC spectrum (+TFP) indicates the induction of the α-helix by the addition of 200 μM TFP. This change was also monitored (data not shown) at the characteristic wavelengths associated with far-UV CD α-helical extrema, 208 and 222 nm, as a function of increasing TFP concentration and shown to be maximal following the addition of 100 μM TFP.

**DISCUSSION**

The selective and total dissociation of R-LC from scallop myosin by TFP treatment (Fig. 1) is reminiscent of R-LC removal from scallop myosin as a consequence of 10 mM EDTA treatment at elevated temperatures (17). Furthermore, increasing concentrations of TFP gave rise to a decline in actin-activated MgATPase in the presence of calcium (Figs. 2 and 3), superficially also similar to the effect of R-LC loss through metal ion chelation (17). However, the effects of TFP and EDTA on scallop myosin are not the same and differ from each other in several ways, as described below.

Of fundamental importance are our observations of the effect of TFP on actin-activated MgATPase in the absence of calcium: the inhibited state of intact scallop myosin. In the case of R-LC removal through divalent cation chelation, a biphasic rise in this ATPase activity as a function of R-LC loss was observed (17, 18), providing insight into the cooperative nature of regulatory myosins. By contrast, TFP treatment of scallop myosin or myofibrils led to a monotonic decline in actin-activated MgATPase (−Ca2+) (Figs. 2 and 3). Furthermore, the sensitivity of actin-activated MgATPase activity to TFP took place over

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**TABLE II**

| [TFP]a | Calciuma | Rateb | Intact rate |
|-------|----------|------|-------------|
| μM    |          | μmol/min/mg | %          |
| 0     | −        | 0.435 | 100         |
| 0     | +        | 0.420 | 100         |
| 100   | −        | 0.198 | 46          |
| 100   | +        | 0.227 | 54          |
| 250   | −        | 0.159 | 37          |
| 250   | +        | 0.125 | 30          |

| Conditions used are described under “Experimental Procedures.” |
| Median values are from three separate determinations. |

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**FIG. 5.** Effect of trifluoperazine on calcium-specific binding to scallop myosin and CaMgS-1. Calcium-specific binding (determined in the presence of 2.1 × 10⁻⁶ M free calcium and 1.0 mM free magnesium) was examined for scallop myosin and scallop myosin CaMgS-1 as a function of increasing concentrations of TFP. Points shown are the median of three determinations from three separate myosin preparations (●) or the average of two determinations from two separate CaMgS-1 preparations (○). Bars are indicative of the spread of data points between experiments. Whereas calcium-specific binding to CaMgS-1 remained unaffected by TFP treatment over the concentration range examined, <60% calcium-specific binding remained at 500 μM TFP in the case of scallop myosin. Calcium-specific binding to scallop myosin was ~75% of intact values at 150 μM TFP, a concentration where actin-activated MgATPase was inactivated by >50% (Fig. 2). Details are given under “Experimental Procedures.”
Myosin MgATPase occurred at 100–150 μM, both in the presence and absence of calcium, 50% inactivation of myosin MgATPase was complete at 300 μM TFP (Table I). Only 40–50% calcium-specific binding remained in the case of scallop myosin, whereas calcium-specific binding to Ca-Mg-S-1 remained unimpaired (Fig. 5). This result emphasizes the functional dislocation of regulation from activity in scallop Ca-Mg-S-1, a molecule in which regulatory capability is completely impaired (15, 33). Scallop Ca-Mg-S-1 is also known to be more refractory to EDTA-induced dissociation of R-α-LC as compared with the two-headed myosin structure (24).

TFP binding is manifest over two distinct concentration ranges, indicative of at least two different sets of binding sites. Whereas 300 μM TFP is required to eliminate actin activation and regulation (50% inactivation at 100–150 μM TFP), 3000 μM TFP is required to remove R-LCs (50% inactivation at 1300–1600 μM TFP). Spectroscopic studies on TFP binding to skeletal muscle myosin light chains have demonstrated the presence of two types of TFP-binding site, differing in affinity by at least one order of magnitude. Measurements of the far-UV CD spectra indicated that TFP elicited increases in helical content with half-maximal effects at ∼20–50 μM TFP for LC1 and LC3 and ∼14 μM for LC2 (14). By contrast, paramagnetic spin probes attached to the same light chains exhibited a change in rotational correlation time upon TFP addition, the effect being half-maximal at ∼370–809 μM TFP, an order of magnitude higher than the CD results (14). Here, through far-UV CD analysis (Fig. 6), we have confirmed that micromolar concentrations of TFP have a direct effect in inducing an increase in α-helicity in isolated scallop E-α-LC; the effect is maximal by ∼100 μM. The higher affinities of the two sets of TFP-dependent transitions determined from spectroscopic data on isolated light chains as compared with the two ranges of TFP concentration required for the two different functional results obtained here on scallop myosin may reflect the fact that functional effects can be assessed on only the multichain structure.

TFP binding to the hydrophobic pocket within the C-terminal lobe of E-α-LC would appear to be the most likely cause of ATPase inactivation. There is a monotonic decline in actin-activated MgATPase (±Ca2+) that is complete at 300 μM TFP, and prior removal of R-α-LC by EDTA still permits TFP inactivation of the desensitized myosin and exhibits the same concentration dependence (Fig. 3), suggesting that the heavy chain-E-α-LC complex can be induced to attain this extreme state.
FIG. 7. Location of the TFP-binding site within the crystal structures of TFP-calmodulin \((a)\), scallop myosin E-LC \((b)\), and the chicken gizzard motor domain-E-LC complex \((c)\) or within scallop myosin S-1 \((d)\). \(a\), location of the TFP-binding site within the C-terminal half of bovine brain calmodulin \((\text{Ser}^{81-\text{Ala}^{147}})\) \((4)\); \(b\), location of the putative TFP-binding site within the C-terminal half of scallop E-LC \((\text{Phe}^{84-\text{Gly}^{152}})\) as found within the regulatory domain structure \((12)\), as seen from the same perspective as for calmodulin \((a)\); \(c\), relationship of the putative TFP-binding site within E-LC as found within the smooth muscle myosin motor domain-E-LC ADP-AlF_4 complex \((35)\), illustrating its proximity to heavy-chain amino acid residues at the entrance to the nucleotide-binding pocket; \(d\), relationship of the putative TFP-binding site within E-LC as found within scallop adductor myosin MgADP-S-1 \((36)\), illustrating its proximity to heavy-chain amino acid residues within the SH3 domain. In \(c\) and \(d\), perspectives were chosen to illustrate the proximity of the E-LC C-terminal TFP-binding lobe to key heavy-chain surface features; the view of the converter region, which maintains its relationship to E-LC in both structures \((35)\), is approximately the same in both cases. Diagrams were drawn using RasMol \((\text{Version 2.4})\); coordinates were downloaded from the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University. Codes used were as follows: 1ctr \((\text{TFP-calmodulin complex})\) \((4)\), 1scm \((\text{scallop E-LC from the regulatory domain complex})\) \((12)\), 1br1 \((\text{chicken smooth muscle myosin motor domain-E-LC complex in the presence of AlF_4})\) \((35)\), and 1b7t \((\text{scallop adductor myosin S-1 in the presence of MgADP})\) \((36)\). Illustrations are rendered as wire-frame structures on which the \(\alpha\)-carbon backbone has been highlighted. Residues shaded green indicate amino acids known \((a)\) or inferred \((b–d)\) to be involved in TFP binding. Equivalent residues were determined through CLUSTAL analysis of the amino acid sequences of calmodulin \((50)\), scallop E-LC \((27)\), and chicken gizzard E-LC.
inhibited state. Also, TFP can induce, directly, a conformational change in isolated E-LC (Fig. 6). In the stoichiometric TFP-calmodulin complex, the hydrophobic tricyclic ring of TFP localizes to a hydrophobic pocket within the C-terminal lobe of calmodulin, entrapping TFP-calmodulin in the same conformation as that seen when calmodulin binds to a target peptide substrate (4, 6, 7). Although the C-terminal lobes of scallop R-LC and E-LC exhibit semi-open conformations, in contrast to the C-terminal lobe of calmodulin, which displays a fully open conformation (10), the required hydrophobic pocket remains available, albeit less deeply placed (Fig. 7, a and b). Of the 12 residues directly involved in TFP binding within the C-terminal lobe of calmodulin (4), 7 are identical to sequences found within either Argospecten irradians (27) or Pecten maximus (34). E-LC, and another two represent conservative replacements; furthermore, the spatial configuration of these residues appears to be conserved (Fig. 7, a and b). This places TFP within the hydrophobic pocket located between the two domains of the C-terminal lobe of E-LC (Fig. 7b).

Currently, crystal structures of myosin heads from two regulatory myosins are available: the chicken smooth muscle myosin motor domain-E-LC complex, crystallized in the presence of MgADP-AlF$_4^-$ or MgADP-BeF$_4^-$ to 3.5- and 3.6-A resolution, respectively (35); and scallop (A. irradians) S-1, crystallized in the presence of MgADP to 2.5-A resolution (36). These are very different structures (Fig. 7, c and d). With respect to the chicken smooth muscle myosin structure (35), the G-helix within the TFP-binding lobe of E-LC abuts the motor domain close to the 25/50-kDa loop (Ala$_{198}$–Lys$_{204}$) (Fig. 7c), a region of the heavy chain implicated as a major determinant in the rate of ADP release (37–41). Additionally, other surface loop structures (Met$_{140}$–His$_{147}$, Ser$_{163}$–Asp$_{170}$, and Phe$_{256}$–Tyr$_{261}$ in the chicken smooth muscle myosin structure (35)) are capable of interaction with this TFP-binding lobe of E-LC. By contrast, within the scallop MgADPS-1 structure (36), the TFP-binding lobe of E-LC abuts the motor domain close to the N terminus (especially Ala$_{45}$–Lys$_{60}$) (Fig. 7d) within the so-called SH3 domain, which has been suggested to limit the potential swing of the lever arm (35). This region of contact is close to the heavy-chain region (Glu$_{21}$–Asn$_{37}$) found in the nucleotide-free skeletal myosin structure (42), where it abuts the F-helix within the C-terminal domain of E-LC (as described (35)). Although crystallized in the presence of MgADP, the point is well made (36) that scallop MgADPS-1 may be considered to be in an "ATP-like" or pre-power stroke state; consequently, this structure is classified as being in the weak binding state. Here, too, the F-helix of E-LC makes contact with heavy-chain residues, located within an α-helix close to the N terminus (Fig. 7d) (36). If the conformational off-stage locked by TFP corresponds to either of the above structures (Fig. 7, c and d), it can be seen that TFP will bind to a key location along alternative sequential conformational relays that carry information from the triggering site within the N-terminal domain of E-LC, via the C-terminal lobe of E-LC, to the ATP-binding pocket. We speculate that the interface between the 25/50-kDa loop and E-LC is most likely to participate in the off-state (as seen in Fig. 7c) because the 25/50-kDa loop is known to be a major determinant of the rate of ADP release (37–41), and the rate-limiting state in the absence of calcium is likely to be a weak binding ADP-P, intermediate (45, 46), not an ATP-like structure. Further work is needed to test this hypothesis through both structural and functional means. However, it is unlikely that either structure, alone, can account for all features of regulation, two heads being required (26, 43, 44).

Our results may also suggest that different mechanisms for the control of the off-state exist in regulatory myosins controlled either by direct calcium binding or through phosphorylation. Although the triggers are very different (calcium binds to scallop myosin E-LC (12, 47) whereas phosphorylation occurs on smooth muscle myosin R-LC (48)), conventional wisdom has been that the conformational sequelae of the two triggering mechanisms rapidly converge toward a common pathway in these conserved structures to facilitate full activation at the active site. However, smooth muscle heavy meromyosin lacking E-LC (LC17), produced through overexpression of recombinant baculo virus, exhibited a 75% reduction in the rate of displacement of actin filaments, yet this movement remained phosphorylation-dependent (49). Furthermore, smooth muscle myosin lacking LC17, produced through R-LC (LC20) readdition to light chain-depleted myosin, had reduced rates of superprecipitation and actin-activated MgATPase, yet these activities remained phosphorylation-dependent (21). If, as our results suggest, E-LC is vital for maintenance of the off-state, it is unlikely that regulated activity could remain in the absence of E-LC. Consequently, in addition to distinct triggering mechanisms, these two forms of myosin-linked regulation may also exhibit different conformational relays between their respective triggering sites and the active site.

In summary, we have shown that TFP has a dual effect on scallop myosin. Application of millimolar concentrations of TFP leads to dissociation of R-LC from scallop myosin. However, concentrations of TFP an order of magnitude lower than those required to displace R-LCs can eliminate the actin-activated MgATPase activity of scallop myosin, irrespective of the presence and absence of calcium. This occurs in a manner that is independent of the presence or absence of R-LC. Furthermore, TFP can directly affect the conformation of E-LC; comparison with calmodulin indicates that the putative binding site is conserved. The exact manner by which this inhibited state is maintained remains to be determined, but we hypothesize that this structure closely mimics the off-state of a regulatory myosin, which has, so far, eluded structural analysis.

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Locking Regulatory Myosin in the Off-state with Trifluoperazine
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