Alteration of Actin-Tropomyosin Interaction in 2,4-Pentanedione-treated Rabbit Skeletal Myofibrils*

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In previous work, we (El-Saleh, S., Theiret, R., Johnson, P., and Potter, J. D. (1984) J. Biol. Chem. 259, 11014–11021) presented evidence that Ca²⁺ activation of skeletal myofilaments depends on a specific actin domain. We showed that rabbit skeletal thin filaments reconstituted with actin modified at Lys-237 activate heavy meromyosin-Mg²⁺-ATPase activity independently of the Ca²⁺ ion concentration. The modification, which apparently blocks the inhibitory effects of troponin-tropomyosin (Tn-Tm), on acto-heavy meromyosin-Mg²⁺-ATPase activity, consisted of conversion of Lys-237 to an enamine by reaction of purified actin with 2,4-pentanedione (PD). In experiments reported here, we have treated myofibrils with PD with the idea of altering actin in its native state within the myofilament lattice. Preparations of native and PD-treated myofibrils were insensitive to Ca²⁺ ion concentration. Results of steady-state and time-resolved x-ray diffraction studies are consistent with the idea that at rest Tm resides in a “blocking” configuration close to the groove of the actin helix and, that associated with Ca²⁺ activation, Tm moves out of the groove into a “nonblocking” configuration. This model is attractive in its simplicity, yet the significance of Tm movements as causal in the activation process has been brought into question by evidence that Tm and myosin heads do not compete for the same binding site on actin. It has been shown that binding of S-1 (2-4) or HMM (5) to actin-Tn-Tm occurs with about the same affinity whether the Ca²⁺ concentration is maximally activating or below the threshold for activation of ATPase activity. Other recent data provide evidence for a relatively moderate effect of Ca²⁺ on the blocking of HMM binding to actin by Tm-Tn (6-8). This result indicates that in solution the mechanism by which Tm-Tn inhibits the reaction of cross-bridges with actin may involve not only a steric hindrance, but also inhibition of an elementary step in the kinetics of ATP hydrolysis by actin and myosin (8).

Control of the actin-myosin reaction in vertebrate striated muscle by Ca²⁺ is known to depend on the presence of Tn-Tm (for review, see Ref. 1), yet exactly how this thin filament complex regulates myofilament contraction is unresolved. One line of evidence indicates that Tm may sterically block the reaction of actin with myosin. Results of steady-state and time-resolved x-ray diffraction studies are consistent with the idea that at rest Tm resides in a “blocking” configuration close to the groove of the actin helix and, that associated with Ca²⁺ activation, Tm moves out of the groove into a “nonblocking” configuration. This model is attractive in its simplicity, yet the significance of Tm movements as causal in the activation process has been brought into question by evidence that Tm and myosin heads do not compete for the same binding site on actin. It has been shown that binding of S-1 (2-4) or HMM (5) to actin-Tn-Tm occurs with about the same affinity whether the Ca²⁺ concentration is maximally activating or below the threshold for activation of ATPase activity. Other recent data provide evidence for a relatively moderate effect of Ca²⁺ on the blocking of HMM binding to actin by Tm-Tn (6-8). This result indicates that in solution the mechanism by which Tm-Tn inhibits the reaction of cross-bridges with actin may involve not only a steric hindrance, but also inhibition of an elementary step in the kinetics of ATP hydrolysis by actin and myosin (8).

Whatever the precise mechanism of Ca²⁺ activation of myofilament activity, it is clear that interactions between actin and Tm are an important element. We (9) recently showed that these interactions are altered following chemical modification of actin with 2,4-pentanedione, which specifically modifies Lys-237. Thin filaments (actin-Tn-Tm) reconstituted with this modified actin were reacted with HMM and the normal “off” state of actin-Tn, characteristic of the activity of actin-Tn-Tm-HMM-Mg²⁺-ATPase at pCa > 8, was altered to an “on” actin-Tn state. This state was “potentiated” in that the ATP hydrolysis by HMM reacting with modified actin-Tn-Tm was higher in the presence and absence of Ca²⁺ than the activity of unmodified preparations at maximally activating Ca²⁺.

In experiments described here, instead of preparing thin filaments with modified pure actin, we have treated myofibrillar preparations with 2,4-pentanedione with the idea of modifying actin in the myofilament complex. The results

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indicate that this treatment alters actin most likely at Lys-237 and induces the same potentiated "on" state of myofibrillar ATPase activity which is independent of Ca2+ ion concentration as found previously (9) with modified actin.

EXPERIMENTAL PROCEDURES

Materials—The reagent 2,4-pentanediene (Gold Label) was a product of Aldrich and was used without further purification. All other reagents were of analytical grade.

Preparation of Myofibrils—Skeletal muscle myofibrils were prepared essentially as described by Solaro et al. (10) except that the initial muscle homogenization step was extended to 2 min. "Desensitized" myofibrils (DMF), which are free of Tn-Tn, were prepared as described by Lehman (11). Tn-Tn complex was prepared as described by Murray (12). Before use, the myofibrils were washed by resuspension and centrifugation for 10 min at 8000 × g at 4 °C and washed with M-buffer (10 mM MOPS, pH 7.0, 2.0 mM EGTA, 0.2 mM DTT, 0.15 mM NaCl).

Modification of Myofibrils and DMF with PD—PD was added to modified myofibrils (5 mg/ml in M-buffer at 4 °C) at a 100-, 200-, and 400-fold molar excess to actin-lysine content which was computed based on a myofibrillar actin content of 27% by weight (13) and a lysine content of the total actin of 18.5% (18). During the addition of PD, the pH was maintained at 7.0 by the addition of 0.5 N KOH. The treatment with PD proceeded for 21 h with mixing of the myofibrillar suspension on a rotatory shaker at 4 °C. The reaction was stopped by diluting the myofibrillar suspension with 10 volumes of M-buffer. Excess reagent was removed by dilution at 9000 × g for 15 min, followed by three resuspensions and centrifugations in M-buffer (5 ml/mg myofibrils). The final pellet was homogenized in the ATPase buffer: 120 mM MOPS, pH 7.0, 5.0 mM MgCl2, 2.0 mM EGTA, 90 mM KCl, 0.2 mM DTT, 0.15 mM NaCl. As described under "Results and Discussion," only lysine residues were modified by this procedure. Desensitized myofibrils were modified as described above using a 100-fold molar excess of PD to actin-lysine, computed using an actin content of 33.8%.

Modification of Tm-Tn, Myosin, and Actin with PD—Preparations of Tm-Tn were modified with PD by reacting the proteins in M-buffer with the same volume: weight ratio (PD/Tm-Tn) as used in the modification of myofibrils. The volume:weight ratio was computed assuming Tn and Tn each constitute about 5% of the myofibrillar mass and exist in 1:1 stoichiometry (13). The Tm-Tn preparation was treated with PD for 21 h, as described above, and excess reagent was removed by exhaustive dialysis (4 liters of M-buffer with three changes). The absorbance of the modified complex was measured in the wavelength range 257-400 nm in order to determine the nature of the modified residues in the protein complex (9). Myosin was also treated with PD under the same conditions used for modification of Tm-Tn except that we used a myosin concentration equivalent to the myosin in myofibrils (43% by weight (13)). For removal of excess reagent, PD-treated myosin was centrifuged (8000 × g) and the pellet was washed further as described above for PD-treated myofibrils. Actin in M-buffer was modified as previously described (9) using PD at a 200-fold molar excess to actin-lysines. Under such conditions, Lys-237 of actin is the only residue that reacts with PD (9).

Extraction of Actin from Modified and Unmodified Myofilaments—Modified and unmodified myofilaments were first treated with a relatively high ionic strength phosphate buffer (0.3 M KCl, 0.15 M potassium phosphate, 20 mM EDTA, 5 mM MgCl2, 1 mM Na2-ATP, pH 7.0) to wash the bulk of myofilaments. The myofilaments were further treated with low ionic strength (20 mM KCl, 0.2 mM NaHCO3) to remove the Tm-Tn complex essentially as described by Murray (12), except that the final wash (12) was done with 2 mM MOPS, pH 7.0. This was necessary to minimize the effect of acidic pH on hydrolysis of any enamine products that may form in the proteins of the PD-treated myofilaments (16). The myofilament residue was then processed for preparation of an acetone powder as described by Pardee and Spudich (17) except that the high KCl extraction step was done at pH 7.0 and water extraction was replaced by a similar step using 2 mM MOPS, pH 7.0. These latter modifications are necessary to control the stability of enamine in the PD-treated samples (16). The extracted actin was analyzed spectrophotometrically as described above in order to determine the nature and stoichiometry of bound PD (9, 16).

A control experiment was done to determine whether actone treatment quantitatively affects the stability of the enamines in the PD-myofilaments. In this experiment, 0.5 ml of actin that had been modified with PD in the cold (for 18 h) and that had 0.5 mol of modified lysine residues/mol of actin was treated with 3 volumes of acetone. The samples were left overnight at room temperature and the dried actin was suspended in M-buffer. The soluble fraction of each actin sample was clarified by centrifugation at 16,000 × g for 15 min, and the supernatant fraction was assayed for its enamine content.

Reconstituted Preparations—Reconstituted "myofilbrils" were prepared by mixing modified or unmodified Tm-Tn complexes with 50 mg of modified or unmodified DMF at a weight ratio of 1:3 (Tm-Tm:DMF). KCl, MgCl2, and DTT were added to each sample to achieve final concentrations of 0.2, 5, and 1.0 mM, respectively. The mixture was then homogenized and incubated overnight at 0-4 °C with gentle rotary shaking. Following dilution with 5 volumes of water, the samples were pelleted by centrifugation (8000 × g for 30 min at 4 °C) to remove unbound Tm-Tn. The pellets were resuspended in 50 ml of the ATPase buffer, centrifuged, and finally homogenized in the ATPase buffer. The success of reconstitution was judged by the sensitivity of the ATPase activity to Ca2+ and by the relative binding of Tm-Tn (modified or unmodified) to DMF (using SDS-PAGE (18)).

Reversal of Modification by NH2OH Treatment of PD-Myofilbrils—PD-myofilbrils (at 1.0 mg/ml in ATPase buffer) were treated with 500-fold molar excess of NH2OH (using 0.1 M stock solution of NH2OH-HCl) to actin content in myofilbrils. The samples were mixed on a rotatory shaker for 1 h at room temperature, after which excess reagent was removed by centrifugation in a Beckman Model TJ-6 table centrifuge set at maximum speed and for 10 min. The pellets were homogenized in ATPase buffer and assayed for their ATPase activities as well as for their protein content.

Assays—ATP hydrolysis by various protein preparations was measured as previously described (19) from determination of P in reaction mixtures described in the figure legends. Protein concentrations were determined using the method of Lowry et al. (20) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

ATPase Activity—Treatment of myofilbrils with PD not only resulted in a loss of sensitivity of ATPase activity to Ca2+ but also was associated with a potentiation of the ATPase activity (Fig. 1). Maximal Ca2+-dependent Mg2+-ATPase activity of untreated control myofilbrils (PCa 4) was reduced by 80% when the free Ca2+ was reduced to less than 10-8 M by the addition of EGTA. However, in the case of the PD-treated myofilbrils, ATPase activity was not reduced at PCa > 8 and remained at 100-106% of the maximal Ca2+-stimulated Mg2+-ATPase of the control myofilbrils. At PCa 4, the ATPase activity of PD-myofilbrils was "potentiated" in that it was 110-120% of that of the control myofilbrils at PCa 4. Functionally, therefore, the PD treatment of skeletal myofilbrils produces ATPase states similar to those produced in our earlier studies using reconstituted thin filament work in which we measured ATPase activity of HMM reacting with reconstituted thin filaments containing actin that had been modified at Lys-237 by treatment with PD (9). Lysines were in fact the only residues modified in PD-myofilbrils. As shown in Fig. 2, the spectrophotometric scan of PD-myofilbrils exhibits a characteristic absorbance at 310 nm indicating the formation of enamine due to the interaction of lysine-amino groups with PD (9, 16). This was further confirmed following the hydrolysis of the products (and hence the disappearance of the 310 nm absorbance) after NH2OH treatment of PD-myofilbrils, a reaction that is specific for enamine hydrolysis (16). That the altered ATPase functions of PD-myofilbrils arise from modification of a single lysine in actin is discussed below.

The altered ATPase properties of PD-myofilbrils could be reversed following the hydrolysis of enamine by NH2OH. The results are shown in Fig. 3. The NH2OH-treated PD-myofilbrils were Ca2+-sensitive (78% relaxation compared to 75% in NH2OH-treated control myofilbrils). These results
were scanned at a concentration of 1 mg/ml. Myofibrils of their ATPase activities. Myofibrils of their ATPase activities. Myofibrills each at 5 mg/ml in M-buffer were treated with 100-fold molar excess of PD to actin-lysines in myofibrils. The reaction lasted for 21 h at 4 °C. Following removal of excess PD by centrifugations (8000 x g, for 15 min) and washing cycles with the M-buffer, the myofibrill pellets were homogenized and assayed for their ATPase activities at 30 °C in the ATPase buffer: 120 mM MOPS, pH 7.0, 5.0 mM MgCl₂, 2.0 mM EGTA, 90 mM KCl, 0.2 mM DTT, 0.15 mM NaN₃. Measurements were performed in the presence of Ca²⁺, pCa 4.0 (21). The ATPase reaction was initiated by the addition of ATP (using 0.1 M stock ATP, pH 7.0) to 2.0 mM and the reaction was terminated by the addition of 50% trichloroacetic acid to achieve a 10% final concentration. Released inorganic phosphate was measured according to the Fiske and SubbaRow (19) method. ATPase activities were normalized based on a 100% activity of control myofibrils at pCa 4.0. +Ca²⁺, pCa 4.0; -Ca²⁺, pCa 8.0.

indicate that the modification of lysine residues in myofibrils by PD is responsible for the alteration of the Ca dependence of their ATPase activities.

Site Responsible for Alteration of Ca²⁺ Sensitivity of Myofibrillar ATPase Activity—The possibility that PD modification of myofibrillar proteins, other than actin, might be responsible for the alteration of the ATPase activity was tested by reconstitution experiments involving Tm-Tn and myosin that were modified with PD under conditions similar to those used to produce the PD-myofibrils. In these proteins, only lysine residues were modified as determined from spectral analysis (scans not shown). Reconstitution of Ca²⁺-sensitive preparations from desensitized myofibrils was the same whether untreated or PD-treated Tm-Tn complexes were bound to actin in DMF to nearly the same extent as determined by densitometry (results not shown). Lane 1, DMF; lane 2, Tm-Tn; lane 3, PD-treated Tm-Tn; lane 4, DMF + Tm-Tn; DMF lanes PD-treated Tm-Tn.

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A Functionally Critical Site on Actin in Skeletal Myofibrils

Fig. 5. ATPase activities of myofibrils reconstituted with PD-treated and untreated Tm·Tn preparations. PD treatment of the Tm·Tn complex was performed under the same conditions used to produce PD-myofibrils. Desensitized myofibrils (DMF) were then mixed with PD-treated and untreated myofibrils as described under "Experimental Procedures." The resulting reconstituted myofibril preparations were then assayed at 30°C for their Ca2+-sensitive ATPase activities in the ATPase buffer and over the above physiological pCa range (21). ○, DMF + Tm·Tn; □, DMF + PD-treated Tm·Tn; ◦, DMF.

Fig. 6. ATPase activities of myofibrils reconstituted from PD-treated DMF and PD-treated and untreated Tm·Tn. DMF and Tm·Tn each was treated with PD and reconstituted into myofibrils as detailed under "Experimental Procedures." ATPase measurements were performed as described in Fig. 1. J, PD-DMF; 2, DMF; 3a, PD-DMF + Tm·Tn (−Ca2+); 3b, PD-DMF + Tm·Tn (+Ca2+); 4a, PD-DMF + PD-Tm·Tn (−Ca2+); 4b, PD-DMF + PD-Tm·Tn (+Ca2+); 2a, 2b, 2c and 2d are, respectively, the same as 1a, 1b, 1c, and 1d except that DMF were not treated with PD.

alteration in Ca2+ sensitivity of the Mg-ATPase activity.

On the other hand, as shown in Fig. 7, modification by PD strongly inhibited the specific Mg2+-ATPase activity of myosin. Moreover, modified and unmodified actin was able to produce only a low level of activation of the Mg2+-ATPase of PD-myosin compared to actin activation of unmodified myosin (Fig. 7). These results indicate that functional changes in myosin ATPase activity are not due to the modification of myosin in these myofibrils. It is apparent that treatment of isolated myosin with PD may directly affect the catalytic site by reacting with lysine residues that are protected under treatment conditions, when myosin is present in native or desensitized myofibrillar preparations. Results of spectrophotometric analysis (not shown) of PD-treated myosin indicated that only lysine residues reacted with the reagent. It is known that myosin contains a reactive lysyl localized to the catalytic domain, and that modification of this group with 2,4,6-trinitrobenzene sulfonate impairs myosin ATPase activity (22).

It is likely that treatment with PD modifies only Lys-237 of actin in the myofibrils. One line of evidence for this comes from studies in which we extracted actin from PD-myofibrils, as described under "Experimental Procedures." Spectrophotometric analysis showed that this extracted actin contained 0.5 mol of enamine residues/mol of actin. Since the isolation of actin was done by extraction of the myofibrils with acetone, we tested whether preparation of the acetone powder was associated with loss of enamine label from actin. This was done by treating pure actin containing 0.5 mol of enamine/mol with acetone. There was no loss of label, and we interpret this result as evidence in support of our conclusion that actin in PD-treated myofibrils is modified only at Lys-237. Additional evidence for this conclusion comes from: (a) our previous studies showing that direct treatment of pure actin with PD results in modification at a single lysine (Lys-237) and (b) results (Fig. 1) showing that PD treatment of myofibrils produces functional changes in ATPase activity and its regulation by Ca2+ similar to those produced in preparations reconstituted with Tn·Tm, PD-actin, and HMM (9). It is apparent that these changes in Ca2+ regulation of the actomyosin interaction induced by PD modification of actin at Lys-237 are a result of transformation of the actin·Tm interaction from one regulated between an "on" and "off" state by Ca2+ to one in which the actin·Tm state remains on and potentiated with respect to activation of the Mg2+-ATPase activity of myosin.

The significance of data presented here is that they show that this altered state of actin·Tm may be produced not only in systems reconstituted with PD-actin, but also in native myofilament preparations. PD should therefore be a useful reagent for "freezing" thin filament functional states and in studies aimed at understanding the nature of thin filament protein-protein interactions producing on, off, and potentiated states and their relation to triggering of these reactions by Ca2+ binding to troponin-C. Presumably, these studies can also be extended to skinned fiber preparations, where the mechanical effects of these modifications may also be studied.

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