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Individual States in the Cycle of Muscle Contraction

(psoas/rabbit/ATP analogs/myosin)

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ABSTRACT **By using appropriate analogs of ATP, isometrically-held glycerol-extracted psoas fibers from rabbits are forced successively into states corresponding to molecular species in the contractile cycle. In each state measurements are made of $P_\perp$, a fluorescence polarization parameter thought to relate to attitude of S-1 moieties of the myosin molecules. Also, the value of $P_\perp$ is measured during active tension development. It is suggested that this value is a time-average of the $P_\perp$ as S-1 moieties move through the various states of the cycle. Proposals are made concerning the sequence of states in the cycle.**

If actin filaments are propelled past myosin filaments (muscle contraction) by impulses that the myosin molecules deliver during a portion of their individual kinematic–chemical (ATPase) cycles, one must look for correlations between attitude in space of the moving parts [probably the S-1 moieties (1)] of crossbridges and the chemical state of these parts (i.e., complexed with ATP, with hydrolysis products, etc.). Such correlations are attempted in this work, using an as attitudinal parameter, $P_\perp$ [the polarization of the on-axis tryptophan fluorescence when a single fiber is excited by polarized light with plane perpendicular to the fiber axis (2, 3)], and studying successive chemical states in the cycle one at a time. All of the moving parts are forced into the same state and held there, either by well-known procedures (e.g., by imposing "rigor" at full overlap most S-1 moieties complex with actin), or by using two new classes af ATP analogs: disulfides that "affinity-label" the nucleotide binding site by forming a label-to-site disulfide bond (ref. 4; also Yount, R. G., Frye, J. S., and O'Keefe, K. R., unpublished), and nonhydrolyzable triphosphates in which a P-NH-P bridge replaces the usual β–γ oxygen bridge. Incidental studies on inorganic pyrophosphates, PP_i and PPP_i, and on ADP are also reported.

INSTRUMENTATION, MATERIALS, TECHNIQUES

Basically, the described (3) instrumentation for measuring $P_\perp$ was used, except that appropriate circuits now provided $P_\perp(t)$ as an analog signal. The force transducer signal was preamplified, then amplified separately at low and high gain to provide, respectively, the damped signal that is the tension, $T(t)$, and the undamped response to periodic (0.25–1.0%) length changes at 5 Hz, the amplitude [$\Delta T(t)$] of which is the dynamic stiffness. The functions of time, $I_{||}$, $I_\perp$, $P_\perp$, T, and ΔT, were displayed on separate channels of a polygraph. The circuit forming $P_\perp$ was calibrated and shown to be stable by passing through it known currents (ratios then calculable) similar to those generated by the photomultiplier tubes. This monitor insured the reproducibility and 3-figure accuracy of $P_\perp$.

The syntheses of the 6-SH analog ("HS-TP") (6), and of the unsplittable analog ("AMP-PNP") (5), were described, as have been some of their reactions with myosin (4, 7–9). Because the 6-SH analogs probably label through a disulfide intermediate (7), their disulfides such as PNP-P-S-S-P-PNP ("S_2-P-PNP") are able to label in a few minutes (Yount, R. G., Frye, J. S., and O'Keefe, K. R., unpublished). The ATPase of affinity-labeled myosin can be completely regenerated by thiol treatment (T. Tokiwa, unpublished), and according to J. A. Duke (personal communication), the nucleotide that is recovered after affinity labeling with either S_2-TP or S_2-DP is the diphosphate. These observations suggest that the labels fit "naturally" into the ATPase sites of myosin, as they cause no irreversible damage and may even be hydrolyzed while covalently attached. The analog, S_2-MP affinity labels heavy meromyosin (Yount, R. G., Frye, J. S., and O'Keefe, K. R., unpublished), but is ineffective on fibers in the time intervals used for S_2-TP or S_2-DP (see also ref. 4). Presumably because of its lesser acidity, AMP-PNP does not truly mimic ATP (in every respect except hydrolyzability) unless the pH is near 8.0; in this work, it was applied at pH 7.9, whereas with other substances the pH was 6.8–7.0.

Glycerinated *psoas* fibers were prepared and used as described (3). The fiber or fiber bundle, 8–10 mm long, was held isometrically, and the average sarcomere length was known. At the usual length (2.2–2.7 μm) nearly maximal overlap of thick and thin filaments obtained; in special instances fibers were first stretched (in relaxing solution) so as to minimize or eliminate overlap.

RESULTS

Our principal measurements on fibers exposed to 5 mM ligands, or to reagents that attach irreversibly, are summarized in Table 1, but these data are best considered in groups:

Relaxation. When this physiological state is induced by Mg^{2+} + ATP, T is about 0, ΔT is small (about 2×10^7 dyne cm^{-2}), and $P_\perp$ about 0.127. Relaxation so defined is also achieved by, (*i*) substitution of AMP-PNP for ATP, and (*ii*) labeling with S_2TP, or S_2-P-PNP, or S_2-DP (Fig. 1*A*–*F*). Disulfide exchange generates SH-nucleotides absorbing at 300 nm; these must be washed away before $P_\perp(t)$ can be measured.

Pyrophosphate (Fig. 2*D*), PP_i, plus Mg^{2+}, reduces T (10), but, as White (11) found earlier, PP_i reduces ΔT by only 20–30%, while ATP reduces it by 70–85% (Table 1). Furthermore, PP_i produces $P_\perp$ values of only 0.103–0.109. Due to the limited

TABLE 1. *Characterization of steady states*

(a)	(b) $P_\perp$	(c) T	(d) ΔT	(e)
	"Standard" states:			
1. Nothing (rigor)	0.095 ± 0.002	0.94 ± 0.26	8.86 ± 2.37	− − +
2. ATP, Mg (relaxation)	0.127 ± 0.004	0.02 ± 0.11	2.18 ± 1.00	− + +
3. ATP, Mg, Ca (tension)	0.116 ± 0.003	1.90 ± 0.30	4.48 ± 0.91	+ − +
	States attained with ATP analogs:			
4. ADP, Mg	0.101 ± 0.004	1.25 ± 0.59	9.05 ± 3.31	− − +
5. AMP, Mg	0.099 ± 0.008	0.90 ± 0.01	8.30 ± 0.20	− − +
6. PP_i, Mg (post-rigor)	0.103 ± 0.003			
7. PP_i, Mg (post-ATP)	0.109 ± 0.004	0.14 ± 0.12	6.55 ± 1.68	− + +
8. PPP_i, Mg	0.101 ± 0.003	0.12 ± 0.13	6.25 ± 1.01	− + +
9. ITP, Mg	0.124 ± 0.001	0.04 ± 0.07	4.12 ± 0.46	− + +
10. ITP, Mg, Ca	0.109 ± 0.003	1.13 ± 0.58	4.68 ± 0.94	+ − +
11. AMP-PNP*	0.128	0.04	2.61	− + +
12. S_2P-PNP*	0.129	0.02	2.53	− + −
	0.127			
13. S_2TP*	0.125 ± 0.001	0	1.97 ± 0.70	+ + −
14. S_2DP*	0.130 ± 0.001	0 ± 0.05	2.13	− + −
15. S_2MP*	—	0.86	8.72	− − +

When a normal-length fiber or fiber bundle is exposed to a KCl–buffer solution made 5 mM with respect to the first eleven substances listed in column (*a*), then columns (*b*), (*c*), and (*d*) give the "steady value ± standard deviation" of the polarization, $P_\perp$, tension, T (dyne $cm^{-2} \times 10^{-6}$), and dynamic stiffness, ΔT (in dyne $cm^{-2} \times 10^{-7}$) respectively that characterize the resulting state. The three entries ("+" for "yes", and "−" for 'no") in column (*e*) answer respectively the questions, does isometric tension (unrelieved by slight shortening) develop? does the fiber relax? is the effect reversible? Similar information is given for the last four substances, but these are applied as potential affinity labels (see text).

* See *Methods* for structure.

solubility of the Mg^{2+}–PP_i mixtures exploration of a wider pH range is impossible.

Despite older reports (12), ADP does not relax a system in rigor, and probably has no *direct* effect on such a system (see below).

Rigor (Table 1 and Fig. 1*A*) develops when filament overlap is extensive and binding of actomyosin-dissociating ligands is avoided, either by not adding them or not adding their coligand (13), Mg^{2+}. However, this state can develop in the presence of Mg^{2+} and ADP or AMP, or after short exposure to S_2-MP. The state is characterized by a T that may be fairly high (typically, 0.9×10^6 dyne cm^{-2}), but is relieved by 1–3% shortening, by a high ΔT (e.g., 8.9×10^7 dyne cm^{-2}), and by P about 0.095. $P_\perp$ (rigor) is unaffected by ±1% changes in length.

Binding of Nucleoside Diphosphates. In rigor at full overlap, when the S-1 moieties are largely bonded to actin, addition of ADP + Mg^{2+} causes small changes in T, ΔT, and $P_\perp$ resembling those caused by small additions of ATP. These changes, however, probably result from unavoidable myokinase contamination (14), for they are repressed if the ADP is accompanied by 10 mM AMP; in other words, the true effect of ADP on the bonded system appears to be nil. To investigate whether ADP has an effect on the S-1 moieties in the absence of actin, ADP–Mg^{2+} was added to fibers that had first been stretched beyond overlap, then equilibrated with rigor medium. A system so stretched has a $P_\perp$ (about 0.100) that approaches, but does not attain, that of relaxation, and when ADP + Mg^{2+} are added no change in $P_\perp$ is observed, even though the ADP is presumably binding to the S-1 moieties, without competition from actin§ (Fig. 3). Thus the attitude of S-1 moieties with a diphosphate noncovalently bound (Fig. 3) is very different from the attitude with a diphosphate covalently bound (Fig. 1*C*,*F*).

State of Active Tension Development. Fibers exposed to ATP-Mg^{2+}, or ITP + Mg^{2+}, plus Ca^{2+}, develop and maintain for minutes tension that is not abolished by small contractions. Usually, $T = 1.9 \times 10^6$ dyne cm^{-2}, $\Delta T = 4.5 \times 10^7$ dyne cm^{-2}, and $P_\perp$ about 0.116 (intermediate between $P_\perp$(rigor) and $P_\perp$(relaxation)) (Fig. 1*A* and Table 1).

DISCUSSION

The chemical–mechanical–attitudinal states identified above can now be redescribed, preparatory to assembling them in a cycle, e.g., 1,2, . . . etc. Of the following, the first three are defined with considerable assurance:

(*1*) *Myosin binding nucleoside triphosphate and having little or no affinity for actin; $P_\perp = 0.127$.* This state is achieved in conventional ATP relaxation, but we have shown that it can also be achieved by either a noncovalently- or a covalently-bound triphosphate (AMPPNP or $-S_2$PPNP).

§ From a few preliminary experiments, it appears that when such a stretched system is exposed to "contracting solution" there is likewise no change in $P_\perp$; on the other hand, when "relaxing solution" is added, $P_\perp$ moves toward the value usually assumed in relaxation at full overlap. It has been reported (15) that when live fibers are stretched, then stimulated (presumably like adding "contracting solution"), there are changes in x-ray diffraction qualitatively like those at full overlap.

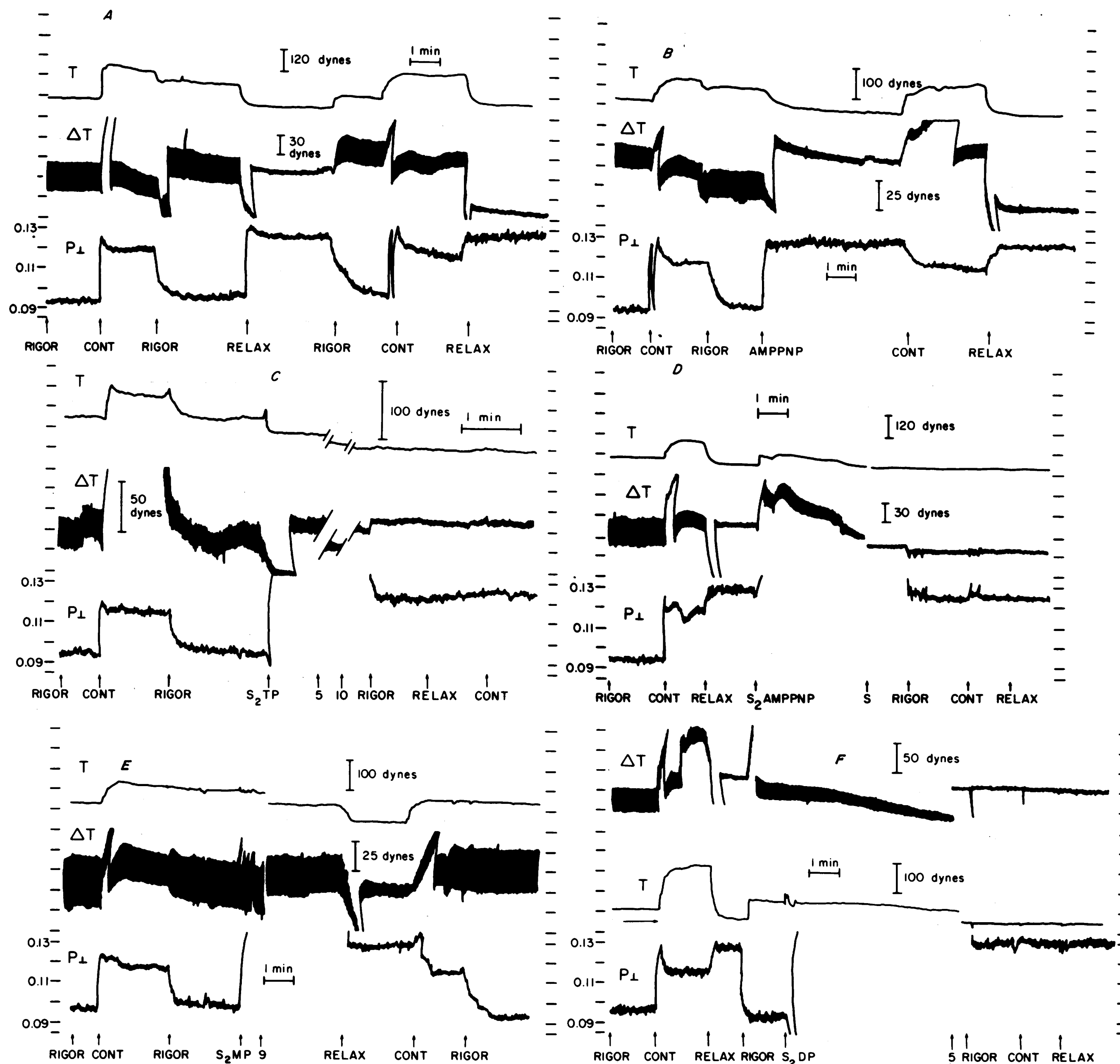

FIG. 1. Behavior of T, ΔT, and $P_{\perp}$ of fibers exposed to 40 mM KCl, 10 mM histidine, and various additions; pH 6.8–7.0 (unless otherwise stated) and room temperature. *Rigor* was produced without further addition, and was unchanged on adding 2 mM EGTA or 5 mM $MgCl_2$. *Relaxation* was produced by adding 5 mM (ATP + $MgCl_2$). *Contraction* (active tension) was produced by adding to the preceding solution enough $CaCl_2$ to fix pCa between 8 and 5. Analogs of ATP were added at 5 mM, with 5 mM $MgCl_2$. (*A*) Attainment and reversal of "standard" states. (*B*) Identity of conventional ATP relaxation with state produced by addition of 5 mM AMPPNP–10 mM $MgCl_2$ (pH 8.0). (*C*) Effect of affinity labeling by 11 min of exposure to S_2-TP. "5" and "10" indicate min of record removed. After exposure, the analog solution is removed; fibers are now unresponsive and in permanent "relaxation". (*D*) Effect of affinity labeling with S_2-PPNP. (*E*) Unsuccessful attempt to affinity label with S_2-MP; fibers still respond after analog solution is washed away. (*F*) Effect of affinity labeling with S_2-DP. Extent of "relaxation" is the same as with S_2-TP [see (*C*)], but is attained more slowly. *Horizontal arrow* indicates zero tension.

(*2*) *Myosin binding nucleoside diphosphate and having little or no affinity for actin; $P_{\perp}$ = 0.127.* "Relaxation" can also be achieved by a covalently bound diphosphate ($-S_2DP$), or by labeling with a triphosphate (S_2-TP) that may be hydrolyzed while attached.

(*4*) *Myosin binding actin; $P_{\perp}$ = 0.095.* This state is achieved in rigor at full overlap; it is unaffected by fiber length oscillations of small amplitude.

(*3*) *Myosin binding ADP; $P_{\perp}$ 0.10–0.11.* This state is achieved by first stretching the fiber overlap in "relaxing" solution (to remove actin), then equilibrating with ADP + Mg^{2+}.

FIG. 2. Dependence of T, ΔT, and $P_\perp$ on concentration of adenine nucleotides and PP_i. 40 mM KCl–10 mM Tris-maleate–2 mM EGTA (pH 7.0) are common; [$MgCl_2$] = [phosphate ligand]. (*A*) ATP, (*B*) ADP (text develops that these effects are indirect), (*C*) AMP, and (*D*) PP_i.

(*5*) *Myosin free of ligands; $P_\perp$ 0.10–0.11.* This state is achieved by first stretching the fiber beyond overlap in "relaxing solution" (to remove actin), then equilibrating with "rigor solution."

Myosin Cycling among the States (system generating active tension) should exhibit $P_\perp = 0.116$, a time-average equal to,

$$\sum_j P_\perp(j)\Delta t(j)/\sum_j \Delta t(j), \qquad [1]$$

where $P_\perp(j)$ is the polarization statically measured in the j-th state, and $\Delta t(j)$ the time there spent by any individual S-1 moiety. But probably (17) expression [1] is replaceable by

$$\sum_j P_\perp(j)\bar{C}_j/\sum_j \bar{C}_j, \qquad [2]$$

where $\bar{C}_j$ is the steady-state concentration of the j-th species and—unlike $\Delta t(j)$—is operationally definable (being a function of measureable velocity constants). At present the $\bar{C}_j$ are unknown, so we cannot substitute our measurements of the $P_\perp(j)$ into [2] and equate to 0.116 in a quantitative test of our theory; we can only note that 0.116 lies between the extrema,

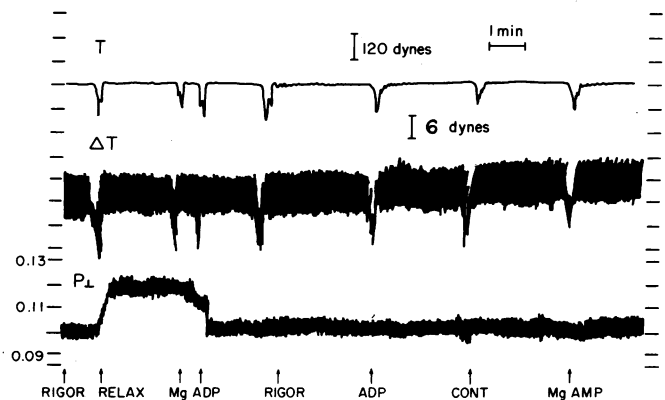

FIG. 3. Experiments on a fiber stretched beyond filament overlap. Note that in this condition "relaxation solution" alters $P_\perp$ without changing T. *Double arrows* indicate that fresh $MgCl_2$–ADP solution was introduced twice.

$P_{\perp}(1)$ and $P_{\perp}(4)$. We can, however, attempt to sequence the states by appealing to other information.

The Sequence of States. A first preliminary is to introduce, s, a space coordinate (linear or angular) for S-1 whose value at relaxation is s_0, and which cycles: $s_0 \rightarrow s_0 + \Delta s \rightarrow s_0 \rightarrow \ldots$ etc. A second preliminary is to consider an intermediate, M*ADP (with or without P_i), anterior to and distinct from M·ADP, the intermediate obtained by equilibrating M and ADP (18); M*ADP may account for certain ^{18}O (22, 23), and ^{32}P (20, 24), exchanges, and for certain spin-label (19) and fluorescence (21) experiments; possibly M-S-S-DP is an analog of M*ADP [in that case its interaction with actin would have to be weak (8)]. Letting M* denote deformed or displaced M, we suggest two sequences, depending on whether the "power stroke" (actin bonded to myosin) occurs during (*A*) $s_0 + \Delta s \rightarrow s_0$, or (*B*) $S_0 \rightarrow s_0 + \Delta s$:

State	$P_{\perp}$	Sequence *A*	Sequence *B*
(*1*)	0.127	M(*s*)·ATP	M(*s*)·ATP
(*2*)	0.127	M(*s*)*ADP	M(*s*)*ADP
(*3*)	0.110	M*($s + \Delta s$)·ADP	M(*s*)*ADP·Actin
(*4*)	0.095	M*($s + \Delta s$)·Actin	M*($s + \Delta s$)·ADP·Actin
(*5*)	0.095	M(*s*)·Actin	M*($s + \Delta s$)·Actin

Actin dissociates M·ADP (16), so states *A*(*3*) and *B*(*4*) are very short-lived. In both sequences the larger changes in $P_{\perp}$ are subsequent to hydrolysis. States (*4*) and (*5*) have the same $P_{\perp}$-value; in *A* because length oscillation in rigor does not change $P_{\perp}$ [flexibility in the myosin molecule (1)], and in *B* because addition of ADP in rigor does not change $P_{\perp}$. If, as widely reported, ADP desorption rate limits myosin ATPase, then *A* is clearly the better scheme, but if rate limitation resides in M*ADP → M*·ADP (25), then *B* is the better scheme. Moreover, *B* better explains why stretch [*B*(5) → *B*(4) → *B*(3)] increases the concentration of the ATP-^{32}P exchanging species, and more plausibly assigns the largest $P_{\perp}$ change [*B*(5) → *B*(1)] to the resetting of the system by ATP.

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