Suppression of Muscle Contraction by Vanadate

Mechanical and Ligand Binding Studies on Glycerol-extracted Rabbit Fibers

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ABSTRACT The suppression of tension development by orthovanadate (V\textsubscript{i}) was studied in mechanical experiments and by measuring the binding of radioactive V\textsubscript{i} and nucleotides to glycerol-extracted rabbit muscle fibers. During active contractions, V\textsubscript{i} bound to the cross-bridges and suppressed tension with an apparent second-order rate constant of $1.34 \times 10^3$ M\textsuperscript{-1}s\textsuperscript{-1}. The half-saturation concentration for tension suppression was 94 $\mu$M V\textsubscript{i}. The incubation of fibers in V\textsubscript{i} relaxing or rigor solutions prior to initiation of active contractions had little effect on the initial rise of active tension. The addition of adenosine diphosphate (ADP) and V\textsubscript{i} to fibers in rigor did not cause relaxation. Suppression of tension only developed during cross-bridge cycling. After slow relaxation from rigor in 1 mM V\textsubscript{i} and low (50 $\mu$M) MgATP concentration (0 Ca\textsuperscript{2+}), radioactive V\textsubscript{i} and ADP were trapped within the fiber. This finding indicated the formation of a stable myosin-ADP-V\textsubscript{i} complex, as has been reported in biochemical experiments with isolated myosin. V\textsubscript{i} and ADP trapped within the fibers were released only by subsequent cross-bridge attachment. V\textsubscript{i} and ADP were preferentially trapped under conditions of cross-bridge cycling in the presence of ATP rather than in relaxed fibers or in rigor with ADP. These results indicate that in the normal cross-bridge cycle, inorganic phosphate (P\textsubscript{i}) is released from actomyosin before ADP. The resulting actomyosin-ADP intermediate can bind V\textsubscript{i} and P\textsubscript{i}. This intermediate probably supports force. V\textsubscript{i} behaves as a close analogue of P\textsubscript{i} in muscle fibers, as it does with isolated actomyosin.

INTRODUCTION

A fundamental goal of muscle research has been to understand the relationship between the elementary biochemical reactions and the mechanical and structural states of the contractile machinery. Studies using isolated proteins have indicated that actomyosin hydrolyzes ATP via the following series of reactions (Lynn and...
Taylor, 1971; Trentham et al., 1976; Taylor, 1979; Eisenberg and Greene, 1980; Sleep and Hutton, 1980; Mornet et al., 1981):

\[
\begin{align*}
&\text{ATP} \\
&\text{AM} \rightleftharpoons \text{AM} \cdot \text{ATP} \rightleftharpoons \text{AM} \cdot \text{ADP} \cdot \text{P}_i \rightleftharpoons \text{AM} \cdot \text{ADP} \rightleftharpoons \text{AM} \\
&\text{P}_i \rightleftharpoons \text{AM} \cdot \text{ADP} \rightleftharpoons \text{AM} \cdot \text{ADP} \rightleftharpoons \text{AM} \\
&\text{M} \rightleftharpoons \text{M} \cdot \text{ATP} \rightleftharpoons \text{M} \cdot \text{ADP} \cdot \text{P}_i \rightleftharpoons \text{M} \cdot \text{ADP} \rightleftharpoons \text{M}
\end{align*}
\]

where A is actin, M is myosin, ATP is adenosine triphosphate, ADP is adenosine diphosphate, and P\textsubscript{i} is inorganic phosphate.

It is generally presumed that the cyclic interaction between actin and myosin (cross-bridge cycle) during energy transduction by a muscle fiber corresponds to this biochemical scheme. The M \cdot ADP \cdot P\textsubscript{i} intermediate is relatively stable, until it binds to actin (cross-bridge attachment), which promotes the release of P\textsubscript{i}. We have recently reported evidence that the P\textsubscript{i} release step in muscle fibers is closely coupled to force generation in the cross-bridge cycle (Hibberd et al., 1985a) and that P\textsubscript{i} release can be reversed by the binding of P\textsubscript{i} to an AM' \cdot ADP state (Webb et al., 1985).

One approach toward defining the relationship between muscle biochemistry and mechanics has been through mechanical and structural studies of well-defined but "frozen" biochemical states in muscle fibers (Reedy et al., 1965; Mannherz et al., 1973; Goody and Holmes, 1983). Several stable states of actomyosin that may correspond to intermediates of the cross-bridge cycle have been studied with the isolated proteins, but they have not yet been fully characterized in muscle fibers (Mornet et al., 1981; Wells and Yount, 1982). Among these is the stable complex formed by myosin, ADP, and orthovanadate (V\textsubscript{i}).

In studies with isolated myosin and actomyosin, V\textsubscript{i} acts as a tightly bound analogue of P\textsubscript{i}. The dynein ATPase, Na,K-ATPase of red cell membranes, and Ca-ATPase of sarcoplasmic reticulum, as well as several other enzymes, are inhibited by the formation of a stable enzyme-V\textsubscript{i} complex (Gibbons et al., 1978; Smith et al., 1980; Oriz et al., 1984, and references therein). The tight binding of V\textsubscript{i} to the phosphate site of the proteins is a widely applicable model.

V\textsubscript{i} blocks the myosin and actomyosin ATPase reactions by forming a stable M \cdot ADP \cdot V\textsubscript{i} complex (Goodno, 1979). This intermediate dissociates very slowly (rate constant \( \approx 2.5 \times 10^{-6} \text{ s}^{-1} \), Goodno, 1982), but binding to actin promotes V\textsubscript{i} and ADP release (Goodno and Taylor, 1982). V\textsubscript{i} also suppresses the contraction of skinned muscle fibers (Goody et al., 1980; Magid and Goodno, 1982). A more detailed mechanical and chemical characterization of muscle fibers suppressed by V\textsubscript{i} would provide a comparison with the states of the isolated proteins.

We have measured the binding of radioactive ADP, ATP, and V\textsubscript{i} to skinned muscle fibers to determine whether a stable M \cdot ADP \cdot V\textsubscript{i} complex forms as in solution. Cross-bridge attachment, force generation, and the kinetics of V\textsubscript{i} binding were studied in mechanical experiments. The results indicate that the interactions between V\textsubscript{i} and actomyosin in muscle fibers are closely analogous to
the corresponding behavior of the isolated proteins and that $V_i$ binding and dissociation affect cross-bridge force production. Thus, $V_i$ qualitatively behaves like $P_i$.

Preliminary results were presented to the Biophysical Society (Goldman et al., 1983).

**METHODS**

Glycerol-extracted single fibers from rabbit psoas muscle were prepared as described by Goldman et al. (1948a). T-shaped aluminum clips folded around the ends of the fiber held it horizontally in the apparatus (Goldman and Simmons, 1984). The fiber dimensions were determined by microscopy as described in Goldman and Simmons (1984).

**Mechanical Apparatus**

The method used for monitoring and recording tension and stiffness and the trough system for Figs. 1–6 were described in Goldman et al. (1984a). Briefly, tension was measured at one end of the fiber while a piezoelectric device imposed a low-amplitude (1 μm), 500-Hz sinusoidal length change. The steady tension and sinusoidal components of tension in phase and 90° out of phase with the length change were separated by a lock-in amplifier. The in-phase component (stiffness) is qualitatively related to the total number of cross-bridge attachments and the out-of-phase component (quadrature stiffness) indicates the presence of cross-bridges with quick stress-relaxation such as actively force-generating cross-bridges (Goldman et al., 1984a).

Stiffness was also determined by small step length changes (Fig. 7). In order to eliminate contributions of end compliance in those experiments, the sarcomere length change in a central region of the fibers was measured at high time resolution by a white-light diffraction method (Goldman, 1983).

When long incubation periods were required (Fig. 8), water evaporation and the consequent variation of the bathing constituents were prevented by covering the solution troughs with a layer of silicon oil. The solution exchange apparatus in this case has been described by Hibberd et al. (1985b).

**Solutions**

The constituents of the bathing solutions are listed in Table I. Vanadium pentoxide was purchased from Fisher Scientific Co., Pittsburgh, PA. A 5- or 10-mM stock of $V_3$ was prepared and then heated to $\sim 100^\circ C$ for 1 h while maintaining the pH at 10 by the addition of KOH. The stock was cooled and then retitrated to pH 10 at room temperature. This procedure was used to minimize $V_3$ polymerization (Pope and Dale, 1968; Goodno, 1979). Experimental solutions were made from the $V_3$ stock a few hours before each experiment.

**Initiation of Contraction**

**Ca-Jump Method (Figs. 1–3)** The Ca$^{2+}$ buffer gradient method of Moisescu (1976) was used to switch fibers rapidly from the relaxed to the actively contracting state. A fiber was first incubated for two periods of 2 min each in low-EGTA pre-activating solution to reduce the internal Ca$^{2+}$ buffering capacity of the fiber. The fiber remained relaxed in the pre-activating solution. The fiber was then transferred into a high-CaEGTA activating solution, and tension rose quickly to a plateau value. The contraction was terminated after 5–60 s by transferring the fiber to a 0-Ca$^{2+}$, high-EGTA relaxing solution.

**ATP-Jump Method (Fig. 4)** Fibers were also rapidly switched from rigor to active contractions by laser-pulse photolysis of caged ATP (Goldman et al., 1982, 1984a, b).
Fibers were transferred through the following series of solutions: (a) low-ATP relaxing solution (0.1 mM MgATP [Table I, A]); (b) rigor solution (0 Ca" [Table I, A]); (c) rigor solution with Ca" (without V; [Table I, A] or with V; [Table I, B]); and (d) rigor solution

### TABLE I
Composition of Solutions

| Solution name | Total MgCl₂ | Final MgATP | Total Na₂ATP | Total MgADP | Total ADP | Total Ca | Total EGTA | Total HDTA | Total Na₂CP | Total V; | Total CK |
|---------------|-------------|-------------|--------------|-------------|-----------|----------|------------|------------|------------|----------|----------|
| **(A) No-V; solutions** | | | | | | | | | | | |
| Activating | 6.76 | 5.0 | 5.49 | — | — | 25.0 | 25.0 | — | 19.49 | — | 1.0 |
| Pre-activating | 6.95 | 5.0 | 5.45 | — | — | * | 0.10 | 24.9 | 19.49 | — | 1.0 |
| Relaxing | 7.70 | 5.0 | 5.44 | — | — | * | 25.0 | — | 19.11 | — | 1.0 |
| Relaxing (low ATP) | 2.66 | 0.1 | 0.12 | — | — | * | 30.0 | — | 21.56 | — | 1.0 |
| Rigor | 3.23 | — | — | — | — | * | 51.48 | — | — | — | — |
| Rigor with Ca" | 1.27 | — | — | — | 20.0 | 20.0 | 32.63 | — | — | — | — |
| Rigor with ADP | 4.07 | — | 2.0 | 4.80 | — | — | — | — | — | — | — |
| **(B) V; solutions** | | | | | | | | | | | |
| Activating | 6.55 | 5.0 | 5.48 | — | — | 30.0 | 30.0 | — | 13.96 | 1.0 | 1.0 |
| Pre-activating | 6.75 | 5.0 | 5.43 | — | — | * | 0.10 | 29.9 | 13.96 | 1.0 | 1.0 |
| Relaxing | 7.67 | 5.0 | 5.43 | — | — | * | 30.0 | — | 13.51 | 1.0 | 1.0 |
| Rigor | 2.50 | — | — | — | — | * | 30.8 | 21.50 | — | 1.0 | — |
| Rigor with Ca" | 1.17 | — | — | — | 30.0 | 23.66 | — | — | — | — | — |
| Slow relaxing | 2.5 | 0.05 | 0.05 | — | — | — | 30.8 | 21.30 | — | 1.0 | — |
| Rigor with ADP | 4.47 | — | 2.05 | 5.0 | * | 30.7 | 14.73 | — | 1.0 | — | — |
| **(C) No-V; photolysis solutions** | | | | | | | | | | | |
| Rigor with CP | 2.65 | — | — | — | 20.0 | 13.58 | 20.0 | — | 1.0 | — | — |
| Rigor with CP and Ca" | 1.34 | — | — | — | 20.0 | 12.49 | 20.0 | — | 1.0 | — | — |
| **(D) V; photolysis solutions** | | | | | | | | | | | |
| Rigor with CP | 2.65 | — | — | — | 20.0 | 12.93 | 20.0 | 1.0 | 1.0 | — | — |
| Rigor with CP and Ca" | 1.87 | — | — | — | 20.0 | 13.28 | 20.0 | 1.0 | 1.0 | — | — |
| **(E) Radioactive binding solutions** | | | | | | | | | | | |
| ³⁴V; incubating | 1.2 | 0.05 | 0.05 | — | — | * | 5.0 | — | 0.5–1.0 | — | — |

[¹⁴C]ATP and [¹⁴C]ADP incubating solutions were the same as V; rigor (B) with labeled and unlabeled nucleotide added to above solution. 150 mM KCl was added to the [³⁴V; incubating solution to maintain an ionic strength of 0.2 M.

1 [Ca⁺⁺ < 10⁻⁶].

2 All photolysis solutions contained 10 mM caged ATP.

All concentrations are in millimolar except creatine kinase (CK), which is given in milligrams per milliliter. All solutions contained 100 mM TES, 1 mM free Mg⁺⁺, and 10 mM reduced glutathione (except [³⁴V; incubating solution, which did not contain glutathione). pH was titrated to 7.1 with KOH at 20°C. The ionic strength was 0.2 M. HDTA: 1,6-diaminohexane-N,N,N',N'-tetraacetic acid; TES: N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CP: creatine phosphate.

with Ca⁺⁺, creatine phosphate, and 10 mM caged ATP (without V; [Table I, C] or with V; [Table I, D]). 2 min was allotted for the diffusion of caged ATP into the filament lattice. Then a 50-ns, 100–150-mJ, 347-nm pulse from a frequency-doubled ruby laser photolyzed 0.75–1.1 mM caged ATP to ATP and 2-nitrosoacetophenone. The ATP was liberated
expansively with a time constant of ~10 ms, which led to a rapid contraction in the presence of Ca²⁺.

**Ligand Binding Experiments**

The multi-trough system used in these experiments was similar to that described by Goldman and Simmons (1984). However, the troughs were smaller (volume ≈27 µl) and Teflon-coated to facilitate the collection of samples and washing of the troughs.

Vanadyl chloride (⁴⁸VCl₅) and the ammonium salts of [U-¹⁴C]adenosine 5'-diphosphate ([¹⁴C]ADP) and [U-¹⁴C]adenosine 5'-triphosphate ([¹⁴C]ATP) were purchased from Amer sham Corp., Arlington Heights, IL. ⁴⁸V was added to an experimental solution containing 0.5–1 mM unlabeled V; (Table I, E). The solution was then heated to ~100°C for 3 h at pH 7.1 to depolymerize the radioactive vanadate (Pope and Dale, 1968; Goodno, 1979). 50 µM ATP was added when the solution cooled to room temperature. Labeled ATP and ADP solutions were made by adding [¹⁴C]ATP or [¹⁴C]ADP to a solution with Vi and carrier nucleotides at the concentrations listed in the text. The final solutions were used within 1 wk.

A fiber was incubated in a solution with radioactive ⁴⁸V, [¹⁴C]ATP, or [¹⁴C]ADP for 25 min. The fiber was then transferred to an unlabeled relaxing solution for ~1 s and then put through a series of unlabeled washout solutions for 1- or 3-min periods. Each 27-µl bath was collected into a scintillation bottle by a vacuum trap, followed by the rinsing of 1 ml of water through the trough into the scintillation vial. This procedure transferred at least 99.7% of the counts in a trough to the collection vial. The radioactivity was counted on an LS 7000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) after mixing with 15 ml of scintillation fluid. At the end of each experiment, the fiber was dissolved in 1 ml of 1 N NaOH and then treated as above to determine the counts remaining. The radioactivity within the fiber at a particular time in the washout period was calculated by summing the counts collected in the bathing media after that time point plus the final fiber radioactivity.

**RESULTS**

**Mechanical Measurements**

**Ca-jump experiments** Fig. 1A shows the tension and stiffness of a single glycerol-extracted rabbit fiber during an isometric contraction and relaxation cycle. The Ca-jump technique described in the Methods was used to rapidly increase the internal Ca²⁺ concentration. When the fiber was put into the activating solution, tension rose to a steady value of 161 ± 12 kN/m² (mean ± SEM, n = 10) with a half-time of <250 ms.

The addition of 1 mM V₅ to the activation medium during the next contraction (Fig. 1B) caused the suppression of active force generation as previously reported (Goody et al., 1980; Magid and Goodno, 1982). Stiffness was also suppressed. Transferring the fiber to a trough containing activating solution without V; (Fig. 1C) caused slow recovery to the fully active tension level. To ensure a thorough removal of V; the trough was exchanged twice again for fresh V; free activating solution during the recovery. The fiber was relaxed and a subsequent contraction and relaxation (Fig. 1D) showed that the fiber recovered fully from the suppressed condition. Thus, V; binds and is released on the time scale of seconds during active contractions.
When V$_i$ was added to the 0-Ca$^{2+}$ relaxing and pre-activating solutions before a contraction, and the fiber was then activated in the same V$_i$ concentration (Fig. 1E), tension and stiffness initially developed at normal rates but then decayed.

![Graph showing suppression of active tension and stiffness by vanadate in a skinned muscle fiber. Continuous chart recording of tension (lower traces) and stiffness (500 Hz, in phase with a 1-μm sinusoidal length change) during a series of active contractions in the absence and presence of V$_i$. Before a contraction, the fiber was washed twice with low EGTA pre-activating solution (Pre). The fiber was then immersed in a high-Ca EGTA activating solution (Act) and relaxed in a high-EGTA relaxing solution (Rel). (A) Control contraction in the absence of V$_i$. (B) The contraction was initiated as in A, but after 17 s the fiber was transferred to an activating solution containing 1 mM V$_i$. (C) Tension and stiffness recovery in 0-V$_i$ Act. To ensure a thorough washout of V$_i$, the solution was exchanged for 0-V$_i$ Act two more times. Transient decreases of tension immediately preceding each 0-V$_i$ Act wash were due to cooling of the fiber in air during the solution exchanges. (D) Control contraction illustrating full recovery of tension and stiffness. After relaxation, the fiber was washed once in 1 mM V$_i$ Rel and twice in 1 mM V$_i$ Pre. (E) Activation in a steady 1 mM V$_i$ concentration. Even though the V$_i$ concentration was not changing, a transient contraction preceded suppression of tension. Sarcomere length, 2.12 μm; fiber dimensions, 2.29 mm × 7,133 μm$^2$; temperature, 21°C.]
This transient contraction suggested that $V_i$ does not bind until the fiber is activated. Since the $V_i$ concentration in the fiber was constant during the transient contraction of Fig. 1 E, the rate of suppression was not limited by $V_i$ diffusion into the fiber. We took advantage of this characteristic to investigate the kinetic properties of $V_i$ binding.

Fig. 2 shows tension recordings from control contractions in the absence of $V_i$ (A and C) and Ca-jump activations in the presence of 125 $\mu$M (B) and 1 mM (D) steady $V_i$ concentrations. In the presence of $V_i$, tension rose at a rate similar to that in the absence of $V_i$ but then decayed to a lower steady level. As the $V_i$ concentration was raised from 50 $\mu$M to 1 mM, the final tension level decreased (Fig. 3A) and the observed rate of tension decay (obtained from semilogarithmic plots) increased (Fig. 3B). The $V_i$ concentration leading to half-saturation of active tension suppression was $94 \pm 23$ $\mu$M (mean ± SD, $n = 3$). This is close to the values obtained by Solaro et al. (1980) and Herzig et al. (1981) in skinned cardiac fibers and by Magid and Goodno (1982) in skinned skeletal muscle fibers. The slope of the observed rate of suppression extrapolated to zero $V_i$ concentration was $1.34 \pm 0.30 \times 10^3$ M$^{-1}$s$^{-1}$ (mean ± SD, $n = 3$).

**ATP-Jump Experiments** The fibers could also be activated rapidly from rigor by the ATP-jump technique using laser photolysis of caged ATP (see Methods). Fig. 4 shows tension (bottom traces), in-phase stiffness (middle traces), and out-of-phase (quadrature) stiffness (upper traces) during activation of a fiber by caged ATP photolysis. The timing of the laser pulse is shown by the arrowheads. After liberation of ATP, tension decreased briefly and then increased to a relatively stable high level. These transients suggest that cross-bridges initially
FIGURE 3. Dependence on V<sub>i</sub> concentration of tension suppression (A) and the rate of tension decay (B). Data are from the experiment illustrated in Fig. 2. In A, the steady value of suppressed tension is plotted as a percentage of maximal active tension. The ordinate in graph B is the reciprocal of time constants obtained from semilogarithmic plots of tension recordings as in Fig. 2, B and D. Hyperbolic curves were fitted to the data in A and B by least-squares regression.
detach and then reattach to produce active force (Goldman et al., 1984b). When a steady 1-mM concentration of $V_i$ was included in the medium, the initial tension dip and the first 20 ms of the tension rise were unaffected, but thereafter tension rose more slowly and then decayed. Panel B shows the same events on a slower time base. The exponential decay of tension in the presence of $V_i$ was comparable to the tension suppression in the Ca-jump experiments (Figs. 1-3). These results suggest that $V_i$ does not bind to rigor cross-bridges. In the ATP-jump experiment, as in the Ca-jump experiment, $V_i$ did not bind effectively until cross-bridge cycling was initiated.

In the presence of $V_i$, the in-phase stiffness decayed at approximately the same rate and to approximately the same extent as the tension, which qualitatively indicates that in the suppressed contractions, fewer cross-bridges were attached. The quadrature stiffness indicates the visco-elastic nature of the attached cross-bridges.

**Figure 4.** Stiffness and tension transients induced by photolysis of caged ATP within a muscle fiber. In each panel, the lower traces are tension, the middle traces are in-phase stiffness, and the upper traces are out-of-phase (quadrature) stiffness recordings in the absence (-) and presence (+) of $V_i$. Calibration bars for both stiffness signals are relative to in-phase rigor stiffness. (A and B) Fast and slow time base recordings of the same events. The arrowheads mark the time of the laser pulse that liberated 1.1 mM ATP in the presence of ~32 μM Ca$^{2+}$. Baseline tension and stiffness recordings were made after subsequent relaxation of the fiber in 5 mM ATP relaxing solution. Sarcomere length, 2.51 μm; fiber dimensions, 2.46 mm × 5.087 μm²; temperature 21°C. (C) Similar recordings from another fiber in the absence of Ca$^{2+}$ when 0.8 mM ATP was liberated. In this case, the baselines were recorded in the photolyzed solution after full relaxation. Sarcomere length, 2.19 μm; fiber dimensions, 1.78 mm × 7,965 μm²; temperature, 20–22°C.
bridges (Goldman et al., 1984a, b). Quadrature stiffness was relatively well maintained after activation by caged ATP photolysis in the presence of Vi. This observation suggests that the remaining cross-bridges retain the quick stress-relaxation characteristic of normal, actively force-generating cross-bridges.

In the absence of Ca$^{2+}$, photolysis of caged ATP within a rigor fiber leads to relaxation with complex kinetics (Goldman et al., 1982, 1984a). Vi caused only a minor slowing of this relaxation (Fig. 4C).

**Figure 5. Suppression of tension and stiffness from rigor.** Lower traces are tension; upper traces are (in-phase) stiffness. Each panel shows the following series of events: (1) a control rigor contraction and relaxation, (2) slow relaxation from rigor in the presence of 1 mM Vi and 50 μM MgATP, (3) a suppressed test rigor contraction, and (4, 5) two recovered contractions. In B, after the slow relaxation, the fiber was washed five times in Vi-free 0.1 mM MgATP relaxing solution before it was transferred it to the rigor solution. Note that the time base of the recording was slowed during these washes. All of panel A was recorded at the faster chart speed. In the experiment, panel B was recorded first. The equivalent time point is marked with a dingbat on both tension traces. Sarcomere length, 2.65 μm; fiber dimensions, 2.67 mm x 5,766 μm²; temperature, 21°C.

**Rigor Experiments** Slow cross-bridge cycling can also be initiated in the absence of Ca$^{2+}$ at low ATP concentrations because remaining rigor bonds may activate the thin filaments (Bremel and Weber, 1972; Moss and Haworth, 1984). The suppression of tension with Vi was also observed during the cross-bridge cycling induced by low ATP concentrations. In the experiment illustrated in Fig. 5A, the fiber was put into rigor and then relaxed fully in 0.1 mM MgATP with creatine phosphate and creatine kinase (low-ATP relaxing [Table 1, A]). During
the subsequent rigor contraction, the fiber was transferred into a solution with 1 mM V; and 50 μM ATP (slow relaxing [Table I, B]). In the absence of creatine phosphate and creatine kinase, this ATP concentration is not sufficient to relax the fiber but results in cross-bridge cycling. However, when V; was included, tension and stiffness both decayed slowly to the relaxed level. The fiber remained in this solution for 9–10 min and was then transferred into the 0.1 mM MgATP relaxing solution without V; In the following rigor contraction, tension was markedly suppressed. Stiffness was suppressed, but less so than tension.

**FIGURE 6.** Time course of rigor tension recovery. Fibers were relaxed from rigor with 1 mM V; and 50 μM ATP (inset). The fiber was washed in V; free 0.1 mM MgATP relaxing solution for 1 min and was then transferred to rigor solution for various durations. The extent of recovery was determined by the amplitude of a subsequent rigor contraction. The data plotted are means ± SEM determined from nine fibers. Inset: sarcomere length, 2.35 μm; fiber dimensions, 2.45 mm × 6,491 μm²; temperature, 20°C.

The suppression of tension and stiffness was consistently observed in the rigor contraction after the incubation of the fiber in the V; containing, slow relaxing solution. Washing the fiber five times in V; free relaxing solution (0.1 mM MgATP; Fig. 5B) or allowing it to remain for up to 2 h in V; free relaxing solution (not shown) had no significant effect on this suppression. These findings suggest that after relaxing the fiber in the V; containing, slow relaxing solution, a stable intermediate was formed that produced less force in the subsequent rigor contraction. Recovery of tension and stiffness was observed in further active or rigor contractions (Fig. 5, A and B).

The extent of recovery from tension suppression increased with the duration of the rigor contraction. Fig. 6 (inset) shows the tension recording from an experiment in which the recovery of force development was tested after rigor
contractions of various durations. Full recovery was apparent after 10–20 min of rigor contraction (Figs. 5 and 6). However, a test rigor contraction was still 50% suppressed after a 2-min recovery (Fig. 6, graph). The recovery of rigor force seemed to depend on the total time in rigor rather than on the number of rigor contractions.

In the experiments of Figs. 1–5, the stiffness signals were reduced by mechanical compliance at the attachments between the fiber and the apparatus. To minimize the effects of end compliance, stiffness measurements were made under similar conditions, except that length changes were monitored in a central region of the fiber by a white-light diffraction method (Goldman, 1983). Step length changes were applied to a fiber at various stages of a V_i suppression and recovery protocol (Fig. 7): (a) in a normal rigor contraction, (b) in the low-tension (suppressed) contraction (Fig. 7A) after relaxation in 1 mM V_i, 50 μM ATP slow relaxing solution, and finally (c) in a recovered rigor contraction (Fig. 7B). The peak tension responses to the length steps are plotted against the observed sarcomere length changes in Fig. 7C. Stiffness, indicated by the slope of these force-extension curves, is decreased in the suppressed rigor contraction, but the tension indicated by the intercepts on the ordinate is decreased even more than stiffness. After a 10-min rigor contraction and relaxation, the subsequent rigor contraction had fully recovered tension and stiffness. These results are independent of compliances at the fiber attachments, since the length change was measured by light diffraction from a central region of the fiber. The experiment supports the data of Figs. 1–6, which indicate that tension and stiffness are suppressed by V_i. In rigor contractions, tension is suppressed more than stiffness.

Since our hypothesis derived from biochemical studies is that a stable M·ADP·V_i complex forms in the muscle fibers, we tested for tension suppression by adding V_i to rigor fibers pre-equilibrated in ADP (cross-bridge state AM·ADP). Fig. 8 shows a tension recording from this type of experiment. A control rigor contraction and relaxation were elicited first. The fiber was then transferred through the following series of bathing media: (a) rigor solution, (b) an extra rinse of rigor solution to remove traces of ATP, creatine phosphate, and creatine kinase, (c) rigor solution with 2 mM MgADP, a concentration sufficient to saturate the actomyosin binding sites with ADP (Marston, 1973; Dantzig et al., 1984), and (d) 2 mM MgADP, 1 mM V_i, rigor solution. After 2 h of incubation in ADP and V_i, no significant relaxation of tension occurred. A small length step (∆L) resulted in little tension recovery, which verified that the fiber was still in rigor. Relaxation in 5 mM ATP and then another rigor contraction showed that no appreciable tension suppression resulted from the procedure. This result required careful removal of ATP from the fiber and ADP stocks. Whenever cross-bridge cycling and active tension generation occurred, either from low levels of contaminant ATP or from intentionally added ATP (as in Figs. 1–7), V_i caused suppression of force.

Ligand Binding Studies

If V_i is a tightly bound analogue of P_i, a stable M·ADP·V_i complex would be formed in the fibers and the attachment of this complex to the thin filament
Figure 7. Tension and stiffness measurements using white-light diffraction to monitor the length change in a central region of the fiber. (A and B) Oscilloscope recordings of tension (lower traces), striation spacing (middle traces), and overall length (upper traces) during a series of step length changes. In A, rigor tension was suppressed after relaxation from rigor in 1 mM V_i, 50 μM ATP as shown in Figs. 5 and 6. The recordings of panel B were made in a recovered rigor contraction. Force-extension curves are plotted in C from an initial rigor contraction (sarcomere length, 2.68 μm [■]), the V_i-suppressed contraction corresponding to A (sarcomere length, 2.68 μm [▲]), and the recovered rigor contraction corresponding to B (sarcomere length, 2.59 μm [▲]). The ordinate indicates the peak tension deflection after each length change. The abscissa indicates the change in striation spacing. Fiber dimensions, 2.24 mm × 5,222 μm²; temperature, 19°C.
would promote \( V_i \) and ADP release. We tested this hypothesis by studying the binding of radioactively labeled \( V_i \) and nucleotides to fibers. Fig. 9A shows the time course of \(^{48}V_i\) washout from a single fiber, which was first loaded with \( V_i \) by slowly relaxing it from rigor, in a solution containing 50 \( \mu M \) ATP, 1 mM \( V_i \), and \(^{48}V_i\) (Table I, E). After 25 min, the fiber was transferred to a new trough containing \( V_i \)-free, low-ATP relaxing solution for 1 s, and then to troughs containing fresh relaxing solution every few minutes for 1 h. The fiber was then put into a rigor solution (tension was suppressed) and the trough exchanges were continued every few minutes. The contents of these troughs were collected and the radioactivity that had diffused out of the fiber was determined by liquid scintillation counting as described in the Methods. In relaxing solution, free and/or loosely bound \( V_i \) washed out of the fiber (Fig. 9A, open symbols). However, a component of \( V_i \) was trapped within the relaxed fiber and was released only when the fiber went into rigor (closed symbols). The burst of \( V_i \) released during the rigor contraction was equivalent to 164 ± 23 \( \mu \)mol \( V_i \) per liter fiber volume (mean ± SEM, \( n = 7 \)). The fiber was relaxed again for 12 min and a subsequent rigor contraction indicated that tension was fully recovered. There was no significant further release of \(^{48}V_i\).

After repeated washing for 1 h in \( V_i \)-free (low-ATP) relaxing solution, the \( V_i \) remaining in the fiber during the experiment of Fig. 9A could be trapped on a binding site with an extremely low desorption rate. Alternatively, it could be exchanging with free \( V_i \), but the tight binding could restrict its diffusion out of the fiber. In order to distinguish between these possibilities, we performed the "cold chase" experiment illustrated in Fig. 9B. The fiber was loaded with \( V_i \) as in the experiment of Fig. 9A, by relaxing it from rigor in 50 \( \mu M \) ATP and 0.5 mM \( V_i \), labeled with \(^{48}V_i\). After 25 min in the loading solution, the washout was determined by collecting 3-min effluent samples for 1 h in successive 0.1 mM

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**Figure 8.** Lack of tension suppression by MgADP and \( V_i \). Tension was recorded during a control rigor contraction and relaxation. The fiber was then transferred through the series of solutions labeled by arrows. After 2 h in 1 mM \( V_i \), 2 mM MgADP solution, a 0.1% length release was imposed (\( \Delta L \)) to test the mechanical condition of the fiber. The fiber was then relaxed in 5 mM MgATP relaxing solution. A subsequent rigor contraction showed no significant suppression of tension. Sarcomere length, 2.42 \( \mu \)m; fiber dimensions, 2.92 mm × 4,665 \( \mu \)m²; temperature, 19.5°C.

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Figure 9. Washout of $^{48}\text{V}_i$ from a skinned muscle fiber. The fiber was loaded with $^{48}\text{V}_i$ by the slow relaxation protocol shown in Figs. 5 and 6. The data points represent $^{48}\text{V}_i$ counts remaining in the fiber at the times plotted. A data point is plotted for each solution exchange. Open symbols indicate 0.1 mM MgATP relaxing solution washes; closed symbols indicate rigor solutions. The solutions were $V_i$-free during the washout runs, except that there was 1 mM $V_i$ present during the interval between the arrows marked “cold chase” and “0 $V_i$” on the upper curve in B. The final data point represents the number of counts that remained in the fiber. For A, the $^{48}\text{V}_i$ loading solution contained 1.0 mM carrier $V_i$, 150 μmol $V_i$ per liter fiber volume was released in the rigor contraction. Sarcomere length, 2.18 μm; fiber volume, 15.7 nl; temperature, 20°C. For B, the $^{48}\text{V}_i$ loading solution contained 0.5 mM carrier $V_i$, 110 and 120 μmol $V_i$ per liter fiber volume were released in the rigor contractions during the cold chase and control runs, respectively. Sarcomere length, 2.17 μm; fiber volume, 25.6 nl; temperature, 19°C.
MgATP, 0-Vi troughs. The fiber was then transferred into 1 mM Vi, 0.1 mM MgATP unlabeled solution (cold chase, Fig. 9B) for six 3-min washes. Little additional Vi washout occurred in the cold chase solution. The fiber was then transferred through several additional 0-Vi troughs and put into rigor (closed symbols). Tension was suppressed in this rigor contraction and 48Vi was released. Another rigor-relaxation cycle did not release further 48Vi. In a control run on the same fiber without the cold chase (lower curve), an equivalent amount of Vi was trapped within the fiber again and released by another rigor contraction.

In the cold chase experiment, if the remaining radioactivity within the fiber could exchange with free Vi, the radioactivity would have been released as unlabeled Vi occupied the binding sites. However, little radioactivity was released until the fiber was contracted in a rigor solution (Fig. 9B, closed symbols). Thus, Vi appears to be trapped within the fiber because its dissociation rate from myosin is slow on the time scale of minutes to hours. The results indicate that a stable complex including Vi is formed in the fiber and this complex is dissociated by the formation of rigor cross-bridges.

Similar experiments with radioactively labeled ATP and ADP were conducted to test whether nucleotide is trapped as well as Vi. In the experiment illustrated in Fig. 10, a fiber was relaxed in 1 mM Vi, 200 μM ADP, and 75 μM ATP to

**FIGURE 10.** Trapping of nucleotide in a fiber. The fiber was loaded with [14C]-ATP or [14C]ADP by relaxing the fiber from rigor with 1 mM Vi, 200 μM ADP, and 75 μM ATP. Nucleotide was trapped (60 μmol per liter fiber volume) when the ATP was labeled (○), but no significant amount of nucleotide was trapped when the ADP was labeled (□). Open symbols correspond to 0.1 mM MgATP relaxing solution baths and closed symbols correspond to rigor washes. The two sets of data were recorded from the same fiber. Sarcomere length, 2.33 μm; fiber volume, 20.3 nl; temperature, 20°C.
Two separate loading and washout runs were conducted on the same fiber and with equivalent solutions, except that the radioactive label was in the ADP during the first run and in the ATP during the second run. When the fiber was relaxed in the solution containing labeled ATP, a component of the label was trapped in the fiber, amounting to \(125 \pm 25 \mu\text{mol nucleotide per liter fiber volume}\) (mean \(\pm\) SEM, \(n = 5\)). This trapped nucleotide was released when a (suppressed) rigor contraction was elicited (Fig. 10, closed symbols). When the label was located in ADP, no significant amount of label was trapped within the fiber or washed out in rigor, even though the mechanical record was similar. These results suggest that the stable complex leading to tension suppression is \(M \cdot \text{ADP} \cdot V_i\), and that the trapped ADP is preferentially formed from ATP rather than from medium ADP. The experiment of Fig. 10 also corresponds to a cold chase situation in that unlabeled ATP was present during the first 45 min.

The release of trapped \(V_i\) and ADP during a rigor or active contraction is probably due to cross-bridge attachment. However, another possibility is that removal of ATP might lead directly to dissociation of the trapped ligands. To check this possibility, the test rigor contraction was performed with reduced...
overlap between the thick and thin filaments. A fiber was loaded with \(^{14}\text{C}\)-labeled nucleotide by relaxation from rigor in 1 mM \(V_i\), 50 \(\mu\)M ATP, and \([^{14}\text{C}]\text{ATP}\). After a few washes, it was stretched to a sarcomere length of 3.39 \(\mu\)m in (low-ATP) relaxing solution. A rigor contraction then released a smaller quantity of trapped ADP than it did at full overlap (Fig. 11, closed symbols). The fiber was relaxed and a second rigor contraction at the long sarcomere length released little further radioactivity. The fiber was returned to the initial length and a rigor contraction at full overlap released a further quantity of labeled ADP. In a repeat run with the same fiber maintained isometric at the short sarcomere length, a rigor contraction released trapped nucleotide in an amount equivalent to the total released in the trials with the altered sarcomere length. This experiment indicates that myosin heads in the overlap zone of the sarcomere release the trapped nucleotide when they attach to actin, but outside the overlap zone they retain the tightly bound nucleotide even when ATP is removed. Similar results were obtained for the release of trapped \(V_i\) at a reduced filament overlap.

**DISCUSSION**

Our ligand binding experiments provide strong evidence that suppression of tension by \(V_i\) in skinned muscle fibers is associated with the trapping of \(V_i\) and nucleotide on myosin. When myosin heads with tightly bound \(V_i\) and nucleotide attach to actin, the trapped ligands are released. These aspects of tension suppression by \(V_i\) are entirely analogous to the formation of a stable \(M\cdot\text{ADP}\cdot V_i\) complex (Goodno, 1979) that dissociates upon binding to actin in solution (Goodno and Taylor, 1982). In muscle fibers, the stable complex is also probably \(M\cdot\text{ADP}\cdot V_i\), since ATP is hydrolyzed more rapidly (Ferenczi et al., 1984) than tension is suppressed by \(V_i\) (Figs. 1–4). However, the ADP trapped in the fibers with \(V_i\) was preferentially derived from ATP (Figs. 8 and 10). The formation of a stable \(M\cdot\text{ADP}\cdot V_i\) complex in muscle fibers was postulated on the basis of previous mechanical and X-ray diffraction experiments (Goody et al., 1980; Magid and Goodno, 1982). The present experiments are the first direct demonstration of the stable complex in fibers.

In order to evaluate the reaction pathway leading to the formation of stable \(M\cdot\text{ADP}\cdot V_i\), it is useful to consider the normal ATPase reactions in the filament lattice. Myosin is thought to hydrolyze ATP slowly in a relaxed fiber via the bottom row of reactions of Scheme 1 (Marston, 1973). During active contraction, the major reaction flux for myosin is via the following series of steps:

(a) attachment of myosin to actin:

\[
\text{M}\cdot\text{ADP}\cdot\text{P}_i \rightarrow \text{AM}\cdot\text{ADP}\cdot\text{P}_i
\]

\(^1\) Note added in proof: Further ligand binding experiments using \([\gamma^{32}\text{P}]\text{ATP}\) and \([8-^3\text{H}]\text{ATP}\) have shown that trapped nucleotide is ADP (Bamrungphol, Dantzig, and Goldman, unpublished observation).
(b) product release and force generation:

\[
P_i \xrightarrow{\text{ADP}} \text{AM}\cdot\text{ADP}\cdot P_i \xrightarrow{\text{AM}'} \text{AM}\cdot\text{ADP} \rightarrow \text{AM}\cdot\text{ADP} \rightarrow \text{AM}.
\]

(c) detachment:

\[
\text{ATP} \xrightarrow{\text{A}} \text{AM} \xrightarrow{\text{AM}'} \text{AM}\cdot\text{ATP} \xrightarrow{\text{M}\cdot\text{ATP}} \text{M}\cdot\text{ADP}\cdot P_i.
\]

Many of these steps are probably reversible. In solution, the elementary ATP cleavage step occurs with dissociated myosin, \(\text{M}\cdot\text{ATP} \rightarrow \text{M}\cdot\text{ADP}\cdot P_i\), and also directly with actomyosin, \(\text{AM}\cdot\text{ATP} \rightarrow \text{AM}\cdot\text{ADP}\cdot P_i\). The contribution from the latter direct hydrolysis pathway in muscle fibers is not known. \(\text{AM}\cdot\text{ADP}\) can be formed by binding ADP to the rigor complex (AM). During ATPase activity, the population of a different state, \(\text{AM}'\cdot\text{ADP}\), has been inferred from studies of \(P_i\) binding to isolated actomyosin (Sleep and Hutton, 1980) and to muscle fibers (Webb et al., 1985). \(P_i\) binds readily to \(\text{AM}'\cdot\text{ADP}\) but not to \(\text{AM}\cdot\text{ADP}\).

In our muscle fiber experiments, \(V_i\) bound to cross-bridges during active cycling much more effectively than in relaxed or rigor conditions. Contractions were initiated by either a rapid increase of \(\text{Ca}^{2+}\) in a relaxed fiber (Figs. 1-3) or by a rapid increase of ATP concentration from rigor (with \(\text{Ca}^{2+}\), Fig. 4). In either case, when \(V_i\) was present at a steady 1-mM concentration, the initial development of tension was similar to the contraction without \(V_i\) but then tension decreased at a relatively slow rate. Thus, \(V_i\) does not seem to bind tightly to AM or to the myosin states in a relaxed muscle fiber. \(V_i\) also does not bind tightly to \(\text{AM}\cdot\text{ADP}\) since incubation of rigor fibers in ADP and \(V_i\) did not lead to relaxation or tension suppression of a subsequent contraction (Fig. 8) or to trapping of nucleotide in the fiber (Fig. 10, lower curve). \(\text{AM}\cdot\text{ATP}\) is present only briefly during active cross-bridge cycling (Goldman et al., 1984b) and the active site of myosin is unlikely to accommodate ATP and \(V_i\) simultaneously. These arguments leave \(\text{AM}'\cdot\text{ADP}\) as the sole intermediate of Scheme 1 to which \(V_i\) can readily bind to suppress tension. Therefore, our experiments suggest that stable \(\text{M}\cdot\text{ADP}\cdot V_i\) is formed by the following reaction:

\[
\text{AM}'\cdot\text{ADP} \rightarrow \text{AM}\cdot\text{ADP}\cdot V_i \rightarrow \text{M}\cdot\text{ADP}\cdot V_i,
\]

as suggested by Goody et al. (1980). Since the cross-bridge–nucleotide state capable of binding \(V_i\) (\(\text{AM}'\cdot\text{ADP}\)) is present during normal cross-bridge cycling, our experiments provide evidence that \(P_i\) dissociated before ADP. There is evidence for this order of product dissociation from experiments on isolated heavy meromyosin (Trentham et al., 1972; Sleep and Hutton, 1980), but the present experiments are the first strong indication in muscle fibers for release of \(P_i\) before ADP.

The present results and previous studies agree that \(V_i\) binds readily to the \(\text{AM}'\cdot\text{ADP}\) state populated in the actomyosin ATPase cycle. However, Goody et
al. (1980) and Goodno and Taylor (1982) observed slow binding of $V_i$ to AM-ADP formed by adding ADP to AM, which we did not observe (Fig. 8). Possible explanations for this difference include the fiber types, the duration of the incubations (Goody et al., 1980: insect fibers, 12-24-h incubation; present experiment: rabbit fibers, 2-h incubations), the ionic conditions, or low-level ATP contamination. With regard to the biochemical study with actomyosin subfragment-1 (Goodno and Taylor, 1982), the ionic conditions were different (much lower ionic strength) and their AM-ADP intermediate was presumably in equilibrium with M-ADP, to which $V_i$ could bind. However, this argument does not rule out the binding of $V_i$ to actomyosin subfragment-1-ADP.

Since the M-ADP-$V_i$ state is stable, $V_i$ might be expected to bind to M-ADP in the relaxed muscle fiber. This did not occur (Figs. 1, 2, and 9). The M-ADP state may be present only briefly in the relaxed ATPase cycle if ADP dissociates rapidly and ATP binds immediately to myosin (Goodno, 1982). Alternatively, if the M-ADP state is significantly populated in relaxed fibers, it may be in a conformation that does not bind $V_i$ tightly.

The kinetics of $V_i$ binding to AM'-ADP from the present results can be compared to $P_i$ binding to the same state. At a low $V_i$ concentration, the apparent second-order rate constant for $V_i$ binding and suppression of tension was $\sim 10^3$ M$^{-1}$s$^{-1}$. This value is close to the second-order rate constant, $5 \times 10^3$ M$^{-1}$s$^{-1}$, previously observed for tension reduction by $P_i$ binding to AM'-ADP (Hibberd et al., 1984, 1985a). At higher $V_i$ concentrations, the rate of suppression plateaued at rates in the range of 0.4–1 s$^{-1}$. This may indicate that M-ADP-$V_i$ detachment from actin is slow, which would be consistent with the slower relaxation in 0-Ca$^{2+}$, caged ATP experiments (Fig. 4C). Alternatively, the plateau rate of $V_i$ suppression could be limited by the overall cross-bridge cycling rate. Another possibility is that the free $V_i$ concentration may not increase concomitantly with the total added $V_i$ since it polymerizes (Pope and Dale, 1968) and is reduced to VO$^{2+}$ by glutathione (Macara et al., 1980).

The radioactive binding experiments showed that the amount of $V_i$ and nucleotide trapped was consistently lower than the expected myosin head concentration (≈200 μM). If the experimental conditions were not optimal, the concentrations of trapped ligands would be diminished. The above-mentioned uncertainty of the free $V_i$ concentration may have caused an underestimation of the trapped ligand concentration. However, if all the myosin heads were arrested as M-ADP-$V_i$, it would be difficult to explain the development of rigor tension and the release of trapped ligands upon removal of ATP. Another possibility is that cooperativity between the two heads of a myosin molecule might prevent the trapping of $V_i$ on one head if the other head has tightly bound ligands.

Can M-ADP-$V_i$ attach to actin? The recovery of tension and the release of trapped ligands by active or rigor contractions suggests strongly that M-ADP-$V_i$ can attach to actin. This cross-bridge attachment promotes $V_i$ and ADP release, behavior similar to that postulated in the normal cross-bridge cycle for the M-ADP-$P_i$ state. $P_i$ release from M-ADP-$P_i$ is relatively slow, but attachment to actin promotes $P_i$ release (Lynn and Taylor, 1971; Hibberd et al., 1985b).

The force generation by cross-bridges formed from M-ADP-$V_i$ is low (Figs. 1–7). If $V_i$ release from AM-ADP-$V_i$ is slower than $P_i$ release from AM-ADP-$
Pi, the equilibrium of the Vi dissociation step would be shifted toward AM·ADP·V.
This hypothesis would be consistent with the observation that tension is suppressed more than stiffness in rigor contractions (Figs. 5 and 7). Alternatively, M·ADP·V attachment to actin may be slower than M·ADP·Pi attachment. Either of these kinetic differences would explain the reduced force of contractions in the presence of Vi if Vi and Pi release are closely linked to the power stroke of the cross-bridge cycle.

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