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Inhibition of myosin ATPase by vanadate ion

(enzyme mechanisms/enzyme kinetics/active-site modification/metal toxicity/protein conformation)

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ABSTRACT **Inhibition of the myosin ATPase by vanadate ion (V_i) has been studied in 90 mM NaCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 8.5, at 25°C. Although the onset of inhibition during the assay is slow and dependent upon V_i concentration ($k_{app} \approx 0.3\ M^{-1}\ s^{-1}$), the final level of inhibition approaches 100%, provided the V_i concentration is in slight excess over the concentration of ATPase sites. Inhibition is not reversible by dialysis or the addition of reducing agents. The source of this irreversible inhibition consists of the formation of a stable, inactive complex with the composition M·ADP·V_i (where M represents a single myosin active site). The complex has been isolated, and its mechanism of formation from M, ADP, and V_i has been studied. Omission of ATP increases the rate of formation by about 35-fold ($k_{app} \approx 11\ M^{-1}\ s^{-1}$), yet this rate is still low in comparison with the rates of simple protein–ligand association reactions. This slowness is interpreted in terms of a rate-limiting isomerization step that follows the association of M, ADP, and V_i: M·ADP·V_i → $M^{\dagger}$·ADP·V_i († indicates the inactive product of the isomerization). The properties of $M^{\dagger}$·ADP·V_i are compared with those of the ATPase intermediate M**·ADP·P_i, and the possible role of V_i as an analog of P_i is discussed.**

Evidence is accumulating that suggests that enzymes involved in phosphotransferase or phosphohydrolase reactions are capable of accepting vanadate ion (V_i) as an analog of inorganic phosphate (P_i). To date, there is evidence that six enzymes exhibit this property: ribonuclease A (1), acid phosphatase (2), alkaline phosphatase (3), glyceraldehyde-3-phosphate dehydrogenase (4), Na^+,K^+-ATPase (5–7), and the dynein ATPase (8). Lindquist *et al.* (1) observed that V_i was a competitive inhibitor of ribonuclease and explained this finding in terms of the formation of a complex between V_i and uridine that resembled the intermediate uridine 2′,3′-phosphate. Van Etten *et al.* (2) found that acid phosphatase was competitively inhibited by V_i with a K_i value at least 100-fold lower than that for P_i. Evidence that V_i binds to the same site as P_i was provided by Lopez *et al.* (3), who found that V_i and P_i inhibit alkaline phosphatase in mutually exclusive fashion.

In studies on glyceraldehyde-3-phosphate dehydrogenase, DeMaster and Mitchell (4) made the remarkable observation that V_i appears to function as an alternative substrate for the enzyme in place of P_i. Recently, Josephson and Cantley (5) and Cantley *et al.* (6, 7) have shown that V_i strongly inhibits the Na^+,K^+-ATPase, and Gibbons *et al.* (8) have shown that it inhibits the dynein ATPase as well. A plausible explanation for all these results is provided by the suggestions of Lindquist *et al.* (1) and Van Etten *et al.* (2) that the tetrahedral vanadate ion is structurally analogous to P_i.

Although preliminary investigations by two groups concluded that V_i did not inhibit the myosin ATPase (5, 8), the mass of experimental results with other enzymes suggested that inhibition of myosin was likely to occur. In this investigation, a detailed examination was made of the effect of V_i on myosin. The results show that V_i is an effective inhibitor of the myosin ATPase, to the extent that stoichiometric concentrations of V_i produce almost total inhibition. The mechanism of inhibition by V_i was examined, and the results provide a simple explanation for the lack of inhibition observed in earlier studies.

MATERIALS AND METHODS

Materials. Na_2ATP and Na_2ADP were products of Sigma and [α-^{32}P]ATP and [^{3}H]ADP were products of New England Nuclear. Na_3VO_4 and V_2O_5 were supplied by Fisher. The indicator dye 4-(2-pyridylazo)-resorcinol (PAR), was a product of Aldrich. Other chemicals were of reagent grade.

Proteins. Myosin and chymotryptic heavy meromyosin (HMM) were prepared according to the procedures of Perry (9) and Weeds and Taylor (10), respectively. The HMM fraction that precipitated between 45 and 60% saturated ammonium sulfate was dialyzed free of ammonium sulfate, centrifuged 30 min at 40,000 × *g*, and used within 10 days. The concentration of HMM was expressed in terms of the ATPase-site concentration, which was determined spectrophotometrically by using a value of $A_{280}^{1\%} = 6.47$ (11), assuming a molecular weight of 340,000 and two ATPase sites per molecule.

ATPase assays were carried out in buffer A (0.09 M NaCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 8.5) at 25°C by the addition of MgATP (final concentration, 1 mM) to HMM at 1–7 μM sites. Assay times ranged from 0.1 to 5 hr. The reaction was stopped in aliquots (1 ml) of the assay solution with 1 ml of 10% trichloroacetic acid. The aliquots were then clarified by centrifugation and half of each was analyzed for P_i by the procedure of Taussky and Shorr (12). V_i concentrations below 10 mM caused less than 1% interference.

Vanadium Analysis. Stock solutions of V_i were prepared from either Na_3VO_4 (adjusted to pH 10 with 6 M HCl) or V_2O_5 (adjusted to pH 10 with 10 M NaOH) and then boiled to destroy yellow polymeric species such as $V_{10}O_{28}^{6-}$ (13). Standard solutions were prepared by volumetric dilution. In order to minimize the pH-dependent polymerization of V_i, all studies were carried out under the alkaline conditions used for the ATPase assays (buffer A). UV-visible spectra of V_i standard solutions were obtained by using a Cary 14 spectrophotometer, and the extinction coefficient was determined: $\lambda_{max} = 265$ nm, $\epsilon_{265} = 2925\ M^{-1}\ cm^{-1}$. The V_i concentration was determined spectrophotometrically wherever possible.

Where this was unfeasible (e.g., in the presence of protein), vanadium was determined by a modification of the colorimetric procedure of Pribil (14), using the metallochromic dye PAR. To a 1-ml sample in buffer A was added 100 μl of 1 M imidazole (pH 6.0) and subsequently 100 μl of 2 mM PAR. After 30 min of color development, the absorbance was read at 550 nm. Vanadium determinations were sometimes made in the presence of 1% sodium dodecyl sulfate ($NaDodSO_4$), for which 63 μl of 20% $NaDodSO_4$ was added to the usual assay. Although

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Abbreviations: HMM, heavy meromyosin; M, single active site of the myosin ATPase; PAR, 4-(2-pyridylazo)-resorcinol; $NaDodSO_4$, sodium dodecyl sulfate; V_i, vanadate ion (unspecified degree of protonation)—i.e., VO_4^{3-}, HVO_4^{2-}, or $H_2VO_4^-$.

it was necessary to correct for small changes in absorbance produced by HMM and $NaDodSO_4$, the calibration curve was linear in the range of 1–20 μM V_i. Because of the sensitivity of the analysis, the vanadium content of glass posed an interference, which was circumvented by the use of plastic vessels.

M·ADP·V_i complexes (M denotes a single active site of myosin ATPase) were isolated at room temperature from incubation mixtures of HMM + ADP + V_i by two chromatographic procedures: Gel filtration (0.5–0.8 ml sample) was carried out by using a 1.5 × 22 cm column of Sephadex G-25, which was eluted at a flow rate of 0.5 ml/min. Ion exchange chromatography (1- to 5-ml sample) was carried out by using 0.5 × 2 cm columns of Dowex-1 × 8 (200–400 mesh), which were eluted at a flow rate of 0.2 ml/min. Both types of columns were equilibrated and eluted with buffer A. Controls with HMM + ADP and HMM + V_i confirmed the ability of these columns to remove at least 99% of unbound ADP or V_i.

The total vanadium content of the M·ADP·V_i complex was determined by the colorimetric procedure in the presence of 1% $NaDodSO_4$. The V_i sequestered by the complex was determined as the difference in apparent vanadium content of the complex before and after denaturation with 1% $NaDodSO_4$. Of the total vanadium in the isolated complex, 85–95% was found to be sequestered.

Incorporation of ADP into the complex was determined by using suitable dilutions of either [^{3}H]ADP or [α-^{32}P]ADP generated *in situ* from [α-^{32}P]ATP. [α-^{32}P]ADP was determined by Cerenkov counting in H_2O, and [^{3}H]ADP was determined by scintillation counting under standard conditions.

Fluorescence Studies. Fluorescence emission spectra of HMM and its complexes were obtained at an active site concentration of 3 μM in buffer A at 25°C on a Hitachi–Perkin Elmer MPF-44A fluorescence spectrophotometer. An excitation wavelength of 295 nm was used, and the emission maximum was at 335 nm. Although elevated concentrations of V_i interfered with fluorescence measurements, concentrations below 10 μM caused less than 1% interference.

RESULTS

ATPase inhibition studies were carried out at pH 8.5 to ensure that the predominant form of vanadate was the monomeric HVO_4^{2-}, rather than the various polymeric species that are favored at lower pH (13). Fig. 1 (curve A) shows that the ATPase assay was linear for at least 3 hr, indicating that the enzyme was stable under assay conditions. In the presence of 0.5 mM V_i (curve C), a slight initial inhibition was followed by a progressive inhibition of more than 80% over a period of 3 hr. The first-order plot (Fig. 1 *inset*) shows that the initial inhibition was about 16% and the observed rate constant (k_{obs}) for the progressive inhibition was 1.4×10^{-4} s^{-1}, corresponding to an apparent second-order rate constant (k_{app}) of 0.28 M^{-1} s^{-1}. The slow onset of inhibition was atypical of a simple reversible interaction between enzyme and inhibitor, suggesting that a reaction was occurring in addition to the hydrolysis of ATP. Three possible explanations for this type of behavior were considered: First, a slow reaction between HMM and V_i might be responsible for inactivation of the ATPase during the assay. Products of ATP hydrolysis might also play an obligatory role in inhibition by V_i, so that a requisite amount of ATP needed to be hydrolyzed before appreciable inhibition could occur. Finally, a slow reaction between ATP and V_i might lead to formation of a new chemical species (e.g. a vanadium–ATP complex) which was the true inhibitor, rather than V_i. These possibilities were evaluated by carrying out ATPase assays in various ways (Fig. 1).

The possibility of reaction between HMM and V_i was examined by preincubation of HMM with V_i prior to the assay. Fig. 1 (curve D) shows that this preincubation had no effect on the rate of inhibition (confirmed in later experiments in which a 2.5-hr preincubation was used), suggesting that a direct reaction between HMM and V_i did not occur. The possible role of ADP as an obligatory cofactor for inhibition was evaluated by the addition of excess ADP (equivalent to total hydrolysis of the ATP) to the assay. If this hypothesis were correct, the ADP should have produced immediate inhibition of about 80%. Curves F and G show that the excess ADP produced only a nominal acceleration of inhibition by V_i, whereas ADP alone (curve B) had no effect. Thus, the slow inhibition could not be explained by the accumulation of ADP. Neither could this inhibition be attributed to a slow reaction between ATP and V_i, because 1-hr preincubation of ATP and V_i produced no enhancement of the inhibition (curve E).

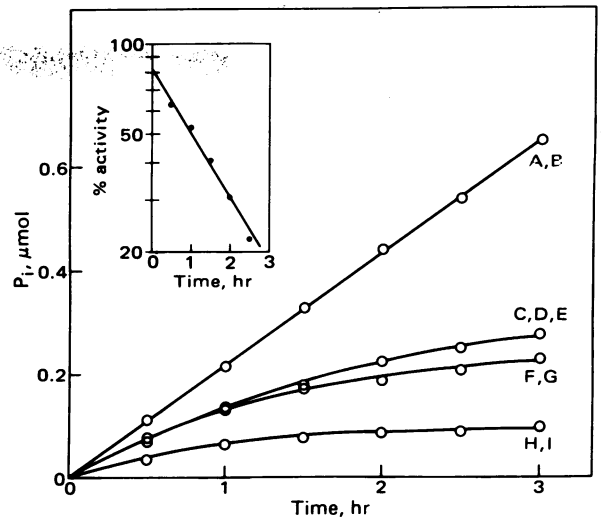


FIG. 1. Inhibition of HMM ATPase by V_i. ATPase assays were carried out with 2.5 μM HMM (sites) in 90 mM NaCl/5 mM $MgCl_2$/20 mM Tris·HCl, pH 8.5/1 mM MgATP (buffer A) at 25°C. When V_i and ADP were added, the final concentrations were 0.5 mM and 1.0 mM, respectively. After a 5-min preincubation of the HMM, the assay was begun by addition of ATP. Although more types of assays were carried out, the time courses were identical in several cases, leading to only four distinct classes of assay curves. Assays A and B contained no addition and added ADP, respectively. Assays C–E contained V_i alone: C, V_i added to assay; D, V_i added to preincubation mixture; E, V_i incubated with ATP for 1 hr before addition to assay. Assays F and G contained both ADP and V_i: F, V_i added to preincubation mixture and ADP added to assay; G, ADP added to preincubation mixture and V_i added to assay. Assay H contained ADP and V_i, both of which were added to the preincubation mixture. Assay I contained ADP and V_i which were incubated together for 1 hr prior to addition to the assay. (*Inset*) Plot of natural logarithm of the residual ATPase activity (%) versus time (data from assay C). The slope gives a value of $k_{obs} = 1.4 \times 10^{-4}$ s^{-1}, and the intercept corresponds to an initial inhibition of 16%.

The nature of the reaction was clarified by the finding that preincubation of HMM with both ADP + V_i resulted in an immediate inhibition of 50%, which approached 100% after 3 hr of assay (curve H). This suggested that ADP was, indeed, a cofactor for inhibition although its role was somewhat obscured by the ATP in the assay (see *Discussion*). Although the synergistic effect of ADP provided insight into the nature of the inhibitory complex, it left unexplained the fact that inhibition by V_i was still unexpectedly slow, even when excess ADP was present ($t_{1/2} \approx 5$ min). It was therefore necessary to consider the possibility that the true inhibitory species might be formed by a slow interaction between ADP and V_i. Preincubation of ADP and V_i was carried out for 1 hr prior to incuba-

tion with HMM. The assay (curve I) showed no acceleration in the rate of inhibition, indicating that the slowness of inhibition was not due to a slow reaction between ADP and V_i. The possibility that a new inhibitory species was formed by an enzyme-catalyzed side reaction was also evaluated. The accumulation of such an inhibitory species should have been reflected by the ability of previously inhibited HMM to accelerate the inhibition of fresh HMM. This was tested by incubation of HMM with ADP + V_i until the ATPase was almost completely inhibited. Fresh HMM was then assayed in the presence and absence of the inhibited HMM (Fig. 2). Although the inhibited HMM actually slowed the inhibition of fresh HMM (curves A and B), it was found that the inhibited HMM had a slight residual ATPase activity (curve C). When the appropriate correction was made, the rates of inhibition in both assays were almost indistinguishable, leading to the conclusion that either a new inhibitory species was formed but not released from the active site, or it was not formed at all.

The simplest explanation of the inhibition results, then, seemed to be that a ternary complex formed slowly between HMM, ADP, and V_i. Such a slowly formed complex would have to be relatively stable in order to be inhibitory at all. Thus, HMM was subjected to prolonged incubation with stoichiometric [α-^{32}P]ADP and excess V_i, followed (within 2 hr of the end of the reaction) by gel filtration of the products and analysis for bound ADP + V_i. Fig. 3*A* shows that ADP and V_i coeluted with the HMM in a mole ratio of 0.82 ADP and 0.92 V_i per mol of ATPase sites. When ADP and V_i were separately incubated with HMM (Fig. 3 *B* and *C*), only small amounts (about 0.15 mol per mol of sites) were associated with the HMM. Moreover, the HMM that was treated with ADP and V_i separately retained 100% of the untreated control ATPase activity, whereas the HMM treated with both retained only 11% activity, which was comparable with the fraction of unmodified ATPase sites. Thus, the inhibition of HMM by V_i was due to the formation of a stable complex with the composition M·ADP·V_i. Prolonged incubation of M, ADP, and V_i in the ratio of 1:1:1 produced similar results.

The properties of this complex were investigated by several methods: Additional procedures were evaluated for isolation of the complex, and chromatography on Dowex-1 was found to be an effective alternative to gel filtration. It was observed that the apparent V_i content of the isolated complex, as measured by the colorimetric procedure, was dependent upon the structural integrity of the enzyme. Measurements in the presence and absence of 1% $NaDodSO_4$ revealed that 0.85–0.97 mol of V_i was released per mol of sites when the complex was denatured. This indicated that the binding of V_i to native HMM was sufficiently strong to sequester it from reaction with the metal indicator dye. The specificity of this binding was suggested by the fact that HMM failed to sequester V_i when ADP was omitted from the reaction mixture. Additional evidence of this specificity was found in the steady-state fluorescence spectrum of the complex, which was found to be indistinguishable from that of HMM alone. When ATP was added to the complex, no fluorescence enhancement occurred, although controls of HMM + ATP, HMM + ATP + V_i, and HMM + ADP + ATP all showed 16–17% enhancement, comparable with that reported by Werber *et al.* (15). Thus, the reduction of fluorescence enhancement was additional evidence of the formation of the M·ADP·V_i complex. The composition of the

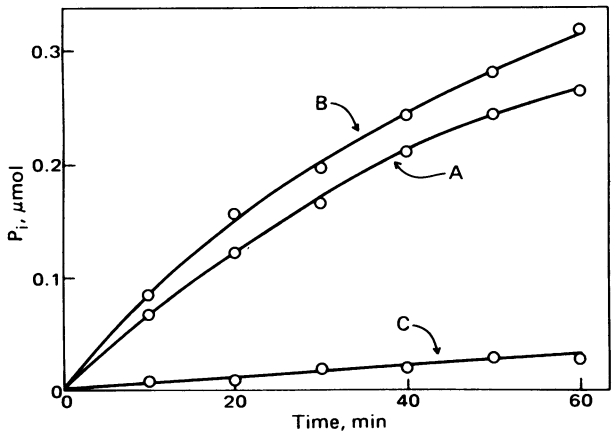


FIG. 2. Evidence against synthesis of a new inhibitor. The inhibition of HMM (6.9 μM sites) by 1 mM ADP and 1 mM V_i was studied in the absence of previously inhibited HMM (curve A) and in its presence (curve B). Assay conditions were otherwise identical to those described in the legend to Fig. 1. Inhibited HMM was prepared by preincubation of HMM (6.9 μM sites) with 1 mM ADP and 1 mM V_i for 10 min at 25°C. The residual ATPase activity of an aliquot of the inhibited HMM (curve C) was determined under the same conditions used for curve A. To another aliquot, fresh, concentrated HMM was added to 6.9 μM, and another assay was carried out (curve B). When the residual activity (curve C) was subtracted from curve B, progress curves A and B became practically identical.

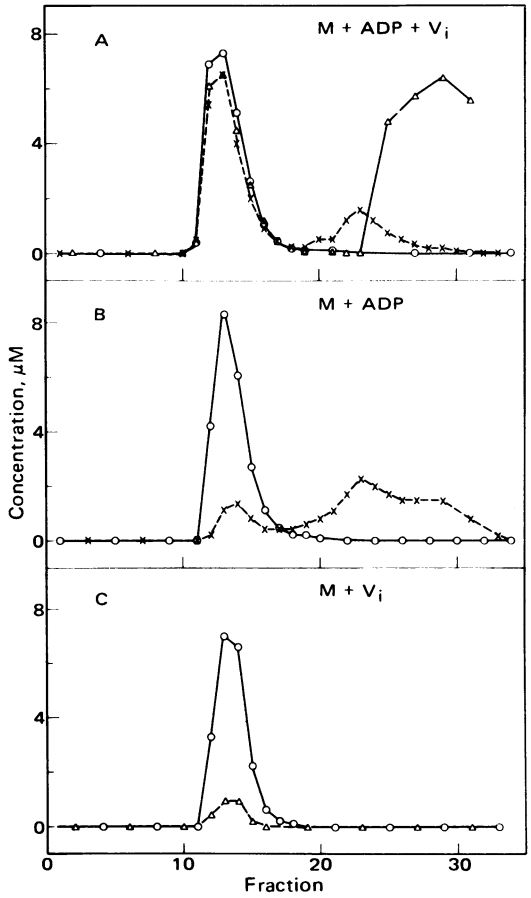


FIG. 3. Gel filtration of M·ADP·V_i complex. HMM (52 μM) in buffer A was preincubated 10 min at 25° with 52 μM [α-^{32}P]ATP to effect quantitative conversion to [α-^{32}P]ADP. V_i (200 μM) was then added, and the reaction was allowed to proceed for 2.5 hr at 25°C. An aliquot of the reaction mixture (0.75 ml) was applied to a 1.5 × 22 cm column of Sephadex G-25 and eluted at 0.5 ml/min with buffer A (1.5-ml fractions). Column fractions were analyzed for: O, protein (A_{280}); X, ADP (scintillation counting); and, Δ, V_i (colorimetric assay). (*A*) Elution profile of complete reaction mixture containing HMM, ADP, and V_i. (*B*) Elution profile of control containing only HMM and ADP. (*C*) Elution profile of control containing only HMM and V_i. V_i eluted in fractions 55–90. See *Discussion* for a possible explanation of early V_i elution in *A*.

Table 1. Composition of stable M·ADP·V_i complex

Method	ADP/M	V_i/M
Gel filtration	0.83	0.91
Dowex-1 chromatography	0.92	1.08
Sequestered V	—	0.95

The complex was prepared and isolated by gel filtration as described in the legend to Fig. 3 ([α-^{32}P]ADP was used). For chromatography on Dowex-1, the complex was prepared in the same manner, except that [^{3}H]ADP was used, and chromotography was carried out on a 0.5 × 2 cm column of Dowex-1 after the addition of a 30-fold excess of unlabeled ATP. Sequestered vanadium was determined from the difference in vanadium content of native and $NaDodSO_4$-denatured samples of the complex, as measured with the metallochromic dye PAR.

complex, as determined by several methods, is summarized in Table 1.

Preliminary studies of the rate of formation of the M·ADP·V_i complex were made by determination of the rate at which V_i was sequestered (data not shown). Under pseudo-first-order conditions for V_i (100 μM Vi_i, 20 μM ATPase sites) the rate constant was a hyperbolic function of ADP concentration, with a plateau attained near stoichiometric ADP. In the presence of excess ADP (500 μM ADP, 100 μM V_i, and 5 μM ATPase sites) the first-order plot was linear through about 70% modification, giving k_{obs} of 1.1×10^{-3} s^{-1} (k_{app} = 11 M^{-1} s^{-1}). Under these conditions inhibition of ATPase activity occurred with k_{obs} of about 1.6×10^{-3} s^{-1}, which was within the experimental error for the rate of modification. The rate constant for inhibition increased linearly with low concentrations of V_i but began to level off above 300 μM. At 400 μM, the highest V_i concentration examined, a k_{obs} of 5.7×10^{-3} s^{-1} was obtained.

Dissociation of M·ADP·V_i was examined by fluorescence, and no dissociation was found after 2.5 hr (no fluorescence enhancement with ATP). Dissociation was also examined by extensive dialysis of the complex (Fig. 4). Approximately 20% of the V_i was lost with a $t_{1/2}$ of about 1 day, and a plateau of about 80% incorporation was approached after 2 days, suggesting that about 80% was retained for a considerably longer period of time. The control retained only 10% stoichiometric V_i. Recovery of the ATPase activity was examined by incubation of the complex with the reducing agents 2-mercaptoethanol, dithiothreitol, and sodium ascorbate for periods ranging up to 25 hr. No reactivation was found, however.

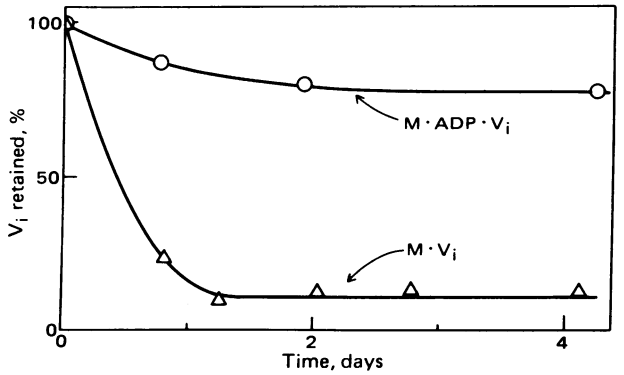


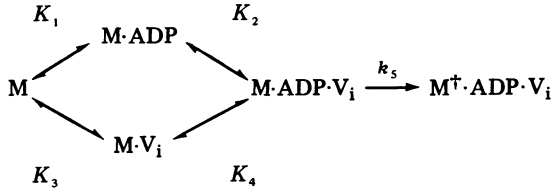
FIG. 4. Dialysis of M·ADP·V_i complex. HMM (69 μM) was inactivated by a 10-min incubation at 25°C with 0.5 mM ADP and 5 mM V_i in buffer A. The M·ADP·V_i complex was purified on a 0.5 × 5 cm column of Dowex-1 and found to have a V_i to site ratio of 0.9. Dialysis of 10 μM M·ADP·V_i was carried out at 4°C against 50 vol of buffer A (changed daily). Aliquots (1 ml) were taken for vanadium analysis. The control, which consisted of 10 μM HMM + 10 μM V_i, was dialyzed in the same way.

DISCUSSION

Vanadate ion is an effective inhibitor of the myosin ATPase. During the assay, when ATP is present, however, the onset of inhibition is slow ($t_{1/2} \approx$ 1.5 hr with 0.5 mM V_i). Although pretreatment of HMM with V_i has no effect, pretreatment with V_i + ADP causes much more rapid inhibition than that observed during the assay. These observations are explained by the fact that myosin forms a stable, inactive, complex with the composition M·ADP·V_i. In the presence of ATP, formation of M·ADP·V_i is slow, whereas in the presence of ADP formation is about 35-fold faster. The slowness of formation of the inhibitory complex under assay conditions is probably the reason that the preliminary studies of Josephson and Cantley (5) and Gibbons *et al.* (8) failed to detect inhibition of myosin by V_i.

One of the principal questions regarding this inhibition is whether V_i is the true inhibitor. There is evidence that V_i may complex with the ribose (1) and phosphate (16) moieties of nucleotides. Possible, though inconclusive support of this notion is found in the gel filtration studies (Fig. 3), which show that V_i (free of HMM) partially coelutes with ADP ahead of the usual V_i elution peak. If a complex of ADP and V_i were essential for inhibition, the concentrations of ADP and V_i would have parallel effects on the rate of formation of the enzyme–inhibitor complex. When the concentration dependence was examined, however, it was found that the rate approached a plateau at a stoichiometric ratio of ADP and myosin sites, whereas the variation with V_i did not plateau even at a 10-fold greater stoichiometric level. Moreover, preincubation of the V_i with ADP had no effect on the rate of ATPase inhibition. These results indicate that a complex of ADP and V_i is not the inhibitory species. It is also unlikely that a polymeric form of V_i is the inhibitor, because virtually complete inhibition of the ATPase is produced by incorporation of a single vanadium atom per active site. Therefore, free V_i is the most plausible inhibitor.

The simplest mechanism adequate to explain the incorporation of ADP and V_i into a stable ternary complex consists of equilibrium binding followed by a slow isomerization:



In this mechanism the formation of M·ADP·V_i is rapid and reversible, but the isomerization (k_5) is essentially irreversible, so that $M^\dagger$·ADP·V_i is the stable complex († indicates the inactive product of the isomerization). Preliminary evidence of a rapidly formed complex is found in the small, immediate inhibition that V_i produces in the ATPase assay (Fig. 1). Several types of evidence support the hypothesis that the stable complex is formed by an isomerization: The V_i in the complex is sequestered, and the fluorescence enhancement produced by ADP is quenched. Moreover, the rate of sequestration of V_i (k_{app} = 11 M^{-1} s^{-1}) is unusually low in comparison with the rates of simple protein–ligand association reactions, which have second-order rate constants in the vicinity of 10^7 M^{-1} s^{-1} (17, 18). Because the reaction approaches completion even at a stoichiometric ratio of M, ADP, and V_i, it is unlikely that this slowness is due to unfavorable equilibria in steps 1–4. It is more plausible that an intrinsically slow isomerization step occurs after the reversible formation of a M·ADP·V_i complex.

The rate of reaction by this mechanism is given by k_5[M·ADP·V_i], in which [M·ADP·V_i] is determined by the various

equilibria. Because this is a first-order reaction, the k_{app}, which is an extrapolated quantity, loses its meaning; and only the k_{obs} is significant. Under conditions that appear to be near half-saturation for M·ADP·V_i (excess ADP and 0.4 mM V_i), k_{obs} is about 6×10^{-3} s^{-1}. Thus, k_5 may be as low as 0.01 s^{-1}, which is reasonable for a protein conformational change (19). Because it is experimentally difficult to monitor the rate of reaction above 0.3 mM V_i under the conditions used in these studies, this value of k_5 is only an order-of-magnitude estimate.

It is currently uncertain whether the association of ADP and V_i with M is truly random. Assuming so, it is possible to draw certain conclusions about the relative contributions of the M·ADP and M·V_i complexes. The dissociation constant for M·ADP is known to be in the neighborhood of 1 μM (20, 21) and the plateau in the reaction rate with stoichiometric ADP is consistent with such tight binding. The dependence of the rate on V_i concentration, however, begins to diverge from linearity only above 0.3 mM, indicating that the dissociation constant for V_i is probably in the neighborhood of 0.5 mM. Because ADP and V_i compete for free myosin sites, it is apparent that M·ADP will be the dominant binary complex so long as ADP is present above a stoichiometric level and V_i is below about 0.5 mM. The rate of reaction, then, is given by:

$$v = k_5[\text{M·ADP·V}_i] = k_5 \frac{[\text{M·ADP}][\text{V}_i]}{K_2 + [\text{V}_i]},$$

where K_2 is the dissociation constant for M·ADP·V_i. This predicts that the rate will be proportional to the concentration of M·ADP, which is in turn equal to the total active-site concentration when stoichiometric ADP is present. During the steady-state of ATP hydrolysis, however, M·ADP drops to about 4% of the total site concentration, because k_{cat} is 25-fold greater than the rate of ADP release (22). Under these conditions, the rate of irreversible inhibition by V_i drops to about 3%, suggesting that M·ADP is, in fact, the main binary intermediate. Because the experimental conditions of these studies ($V_i \leq 0.5$ mM) select for the M·ADP pathway, additional studies are necessary to determine whether the binding of ADP and V_i is truly random.

It is interesting to compare the properties of M†·ADP·V_i with those of ATPase intermediate M**·ADP·P_i (23). Direct evidence has been presented here for a slow isomerization step in the formation of M†·ADP·V_i from M, ADP, and V_i. A similar mechanism has been inferred by Trentham *et al.* (22) on the basis of an unusually low calculated rate constant for the formation of M**·ADP·P_i from M, ADP, and P_i. A notable difference between M†·ADP·V_i and M**·ADP·P_i is that the latter dissociates with a $t_{1/2}$ of about 12 sec, whereas the former has a $t_{1/2}$ of a day or more. Because the rate of dissociation of M**·ADP·P_i is controlled by the isomerization step M**·ADP·P_i → M*·ADP·P_i (22), it is plausible that the dissociation of M†·ADP·V_i is controlled by the step M†·ADP·V_i → M·ADP·V_i. Thus, the difference in rates probably derives from the ability of V_i to lock the myosin into the M†·ADP·V_i conformation. This locking might be rationalized in terms of the ability of V_i to form a coordination complex with nucleophilic residues at the active site. The tetrahedral vanadate ion has the capacity either to exchange ligands or to accept a fifth ligand (as in crystalline metavanadates) to form a trigonal bipyramidal complex (13), which resembles the transition state for phosphoryl transfer (2, 24, 25). Although there is no evidence of a five-coordinate V_i species in free solution (13, 26–28), such a complex might be stabilized at the myosin active site. Because ADP is required for V_i incorporation, it is further possible that V_i incorporation involves the formation of a binary transition-state analog at the active site (24, 29). This type of mechanism has been suggested by Milner-White and Watts (30) to explain the anomalous inhibition of creatine kinase by certain pairs of inhibitors.

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