Interaction of P–N–P and P–C–P Analogs of Adenosine Triphosphate with Heavy Meromyosin, Myosin, and Actomyosin*

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ABSTRACT: The interaction of a β–γ-imido analog of adenosine triphosphate, adenylyl imidodiphosphate (AMP-PNP), in which a NH replaces the terminal bridge oxygen, has been studied with myosin systems. AMP-PNP is not hydrolyzed (<2%) by myosin or heavy mermyosin in 16 hr under conditions (pH 7.4, 0.05 M Tris·Cl–10 mM $CaCl_2$) in which ATP is 90% cleaved in 10 min. AMP-PNP is, however, the most potent competitive inhibitor of Ca^{2+} and Mn^{2+} moderated heavy meromyosin ATPase known (K_i = 3.1 × 10^{-6} and 8.8 × 10^{-8}, respectively). These K_i values are near the K_m values for adenosine triphosphate under these conditions and reflect the close structural similarity of the two compounds.

Conversely, inhibition in the presence of Mn^{2+} by the analogous methylene analog, adenylyl methylenediphosphate (AMP-PCP), is mixed with a much larger K_i (4.4 × 10^{-5} M). AMP-PCP with Ca^{2+}, however, is a competitive inhibitor of heavy meromyosin ATPase (K_i = 2 × 10^{-4} M) indicating the importance of the metal ion on the nature of inhibition. AMP-PNP with Mg^{2+} or Mn^{2+} and AMP-PCP with Mg^{2+} protect myosin against heat inactivation at 40° with concentration dependencies comparable to that observed in heavy meromyosin ATPase inhibition studies. AMP-PNP but not AMP-PCP effectively replaces ATP in dissociating Mg^{2+}·actomyosin in high ionic strength solutions. All these experiments point to a strong interaction of AMP-PNP with heavy meromyosin, myosin, and actomyosin. Contraction experiments, however, with glycerinated muscle fiber bundles or actomyosin gels with AMP-PNP at various pH's and with different metal ions were all negative. These findings would appear to rule out any theory of contraction in which the motivating force is based simply on the binding of a negatively charged substrate to a positively charged actomyosin–metal ion complex.

The nature of the interaction of ATP with myosin and probably with actin represents the key to understanding contraction in skeletal muscle and related contractile systems. To study this interaction and to gain a better insight into contraction we have synthesized analogs of ATP modified in the triphosphate chain; *e.g.*, adenosine sulfatopyrophosphate (Yount *et al.*, 1966a,b) γ-fluoroadenosine triphosphate (B. Haley and R. Yount, in preparation), and as described in the preceding paper (Yount *et al.*, 1971), adenylyl imidodiphosphate (AMP-PNP).[1]

We synthesized AMP-PNP following the lead of Myers and coworkers who first made the analogous carbon compound, AMP-PCP (Myers *et al.*, 1963) and studied its interaction with myosin and actomyosin systems (Moos *et al.*, 1960). Surprisingly, AMP-PCP had little effect on these latter systems. It was not a substrate, would not dissociate actomyosin or support contraction, and in the presence of excess Mg^{2+} was not an inhibitor of ATP cleavage. It was our belief that a P–N–P analog would more closely mimic ATP and allow us to answer a number of pertinent questions about the nucleotide–protein interaction in contractile systems and to test certain theories of contraction (Morales *et al.*, 1955; Davies, 1963), as well. We were also interested in using analogs of this type to define the nucleotide dependence of [^{18}O]phosphate-exchange reactions characteristic of myosin (Swanson and Yount, 1966; Benson *et al.*, 1967; Boyer, 1967).

The previous paper (Yount *et al.*, 1971) has described the synthesis of AMP-PNP and compared its chemical and physical properties to AMP-PCP and ATP. The close structural similarity of AMP-PNP and ATP predicted by X-ray crystallographic studies (Larsen *et al.*, 1969) is borne out by the biochemical studies reported in this paper describing the interaction of AMP-PNP, AMP-PCP, and ATP with heavy meromyosin, myosin, and actomyosin systems. AMP-PNP is shown to be uniquely useful in defining those interactions which depend on the binding of ATP rather than on the cleavage of its terminal phosphate group.

Experimental Section

Materials. The tetrasodium salts of AMP-PNP and AMP-PCP were prepared as described in the preceding paper (Yount *et al.*, 1971). Methyl triphosphate was a gift from Dr. H. Brintzinger. ATP, ADP, and Tris were from Sigma Chemical Co. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1963). Myosin was prepared by a modification (Yount *et al.*, 1966b) of the procedure of Kessler and Spicer (1952) and used within 7 days. Heavy meromyosin was prepared by modifications of the Szent-Györgyi (1953) procedure and stored at −20° after freeze-drying with an equal quantity of sucrose (Yount and Koshland, 1963). Glycerinated muscle fibers and natural actomyosin were prepared and stored at −20° according to Szent-Györgyi (1951). ATPase assays were run as previously described except no 2-butanol extraction was used (Yount and Koshland, 1963). Protein concentrations were measured by the $OD_{280\ m\mu}$ and calibrated by the trichloroacetic acid precipitation

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[1] Abbreviations used are: AMP-PNP, adenylyl imidodiphosphate; AMP-PCP, adenylyl methylenediphosphonate; HMM, heavy meromyosin.

and dry weight method of Hoch and Vallee (1953). Using this procedure 1 mg/ml of HMM or myosin gave $OD_{280\ m\mu}$ of 0.80 and 0.67, respectively.

[γ-^{32}P]ATPase Assays. Reaction tubes (6-ml final volume) were made up to the following concentrations: 2 mM divalent metal, 12.5 mM Tris·Cl (pH 7.4), 0.1 M KCl, and 0.1–1.0 μg/ml of HMM. HMM solutions were made up fresh daily in 0.6 M KCl with a 50- to 100-fold excess of bovine serum albumin added to prevent absorption of HMM on glass surfaces. The reactions were started by pipetting in the [γ-^{32}P]ATP solutions. At 0.5- to 1.0-min intervals five 1-ml aliquots were taken for each assay and were blown quickly into 2 ml of ice-cold 7.5% trichloroacetic acid containing 0.5 μmole of carrier P_i. Five milliliters of an acid molybdate solution (2.5 g of ammonium molybdate and 14 ml of concentrated H_2SO_4 per l. of solution) was added with mixing and the phosphomolybdate complex formed extracted with 10 ml of 2-butanol with exactly 15-sec shaking. The 2-butanol layer (6 ml) was pipeted into a counting vial containing 15 ml of scintillation fluid (700 ml of toluene, 300 ml of absolute ethanol, 4.0 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene). Each sample was counted in a Packard Tri-Carb spectrometer for a sufficient time to give 95% probability of less than 5% error. No more than 15% of the total ATP was hydrolyzed in any of the assays. The specific activity of the [γ-^{32}P]ATP solutions was determined as given above after acid hydrolysis of known amounts of ATP in 1 N HCl, 15 min at 100°. Knowing the rate of [^{32}P]P_i release, the specific activity of the [γ-^{32}P]ATP in the assay sample and the HMM concentration, the steady-state velocity was calculated for each sample. Quenching was corrected by adding known amounts of [^{32}P]P_i to representative sample tubes.

It was found important to filter dilute [γ-^{32}P]ATP solutions (10^{-6} M or lower) through 0.20-μ bacterial filters attached to disposable sterile plastic filter flasks (Nalgene) in order to prevent rapid microbial decomposition of the ATP. If this were not done, the background hydrolysis of ATP in "enzyme-free" controls was so high meaningful data were not obtainable. Dilute solutions of ATP kept frozen in the above containers were stable for at least 10 days.

Results

The ability of HMM or myosin to hydrolyze AMP-PNP was checked in the following manner. Reaction mixtures (0.5 ml) were made up containing 0.05 M Tris·Cl (pH 7.4), 0.05 M KCl, 5–10 mM divalent metal ion, 10 mM nucleotide, and 1 mg of HMM or myosin and incubated at 25°. Aliquots (0.05 ml) were taken at 0, 0.5, 1, 2, 4, and 16 hr and analyzed after either paper chromatography in 1-propanol–concentrated NH_3–H_2O (6:3:1, v/v) or paper electrophoresis at pH 6.8 in 50 mM sodium citrate buffer (Yount *et al.*, 1971). In no case in the presence or absence of Mg^{2+}, Mn^{2+}, or Ca^{2+} was any enzymic cleavage detected. As little as 1% cleavage representing 0.005 μmole of adenine nucleotide could have been detected with the ultraviolet quench system used (UV Products Chromatovue cabinet with transilluminator attachment). In all cases the enzyme-free controls showed no decomposition indicating the stability of AMP-PNP under these conditions. In control experiments with Ca^{2+}-HMM and ATP over 90% of the ATP was cleaved in less than 10 min under the above reaction conditions indicating the marked difference in susceptibility of AMP-PNP and ATP to enzymic cleavage.

TABLE I: Effect of ATP Analogs on Turbidity of Actomyosin Gels.[a]

Nucleotide or Analog	Concn (mM)	Max. $\Delta A_{550\ m\mu}$, min	Temp (°C)	pH	System
ATP	0.1	0.156, 3	25	7.4	B
ATP	0.1	0.160, 4	25	8.0	B
ATP	0.12	0.360, 10	30	7.5	Z
AMP-PNP	0.1–1.0	0.002, 3	25	7.4	B
AMP-PNP	0.1–1.0	0.002, 4	25	8.0	B
AMP-PNP	0.1–1.0	0.003, 10	30	7.5	Z
AMP-PCP	0.1–0.5	0.002, 5	25	7.4	B
MeTPP[b]	0.5	0.033, 10	30	7.8	Z
MeTPP	0.1	0.026, 10	25	7.8	Z
None		0.002, 4	25	7.8	Z

[a] Conditions; system B, 0.06 M Tris·Cl–5 mM $MgCl_2$–0.1 mM $CaCl_2$. Actomyosin (0.25 mg/ml) in a 3.0-ml total volume, 1-cm light path using a Beckman-Gilford spectrophotometer with hand mixing. System Z, same as above except 0.11 mg/ml of actomyosin in a 10.0-ml total volume, 2-cm light path, Zeiss PMQ spectrometer equipped with a magnetic mixing device. [b] MeTPP is methyl triphosphate.

Contraction Experiments. The ability of AMP-PNP to support contraction was investigated with glycerol-extracted muscle fibers and actomyosin gels. Bundles of fibers 0.2–0.4 mm in diameter and about 2 cm long were bathed in a solution of 0.1 M KCl, 0.05 M Tris·Cl (pH 7.4), 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ at room temperature. Their rest length was determined and they were transferred to an identical solution containing 2 mM AMP-PNP. No shortening was observed in 20 min. Identical results were obtained at pH 8.0 where shortening of these model systems is optimal (Bowen, 1965) and AMP-PNP would be in principally the minus four net charge form. Varying the Mg^{2+} concentration from 1 to 10 mM or replacing Mg^{2+} with Mn^{2+} (2 mM) also did not induce contraction with AMP-PNP. Addition of ATP to 2 mM caused shortening at both pH's to less than 40% rest length indicating the fibers were functional. No attempt was made to see if AMP-PNP reduced the rate of shortening. AMP-PCP was likewise without effect on these systems as had been previously reported by Moos *et al.* (1960).

The effect of AMP-PNP on the turbidity of actomyosin gels at 550 mμ was also investigated since this is an especially sensitive model system for contraction studies (Yasui and Watanabe, 1965; Levy and Fleisher, 1965). Representative results are given in Table I. As can be readily seen neither AMP-PNP nor AMP-PCP had an effect on the gel particles. Varying the pH from 7.4 to 8.0 was also without effect. On the other hand addition of ATP gave an immediate increase in turbidity giving maximal absorbance changes in less than 10 sec. Small, slow but reproducible, increases in absorbance were seen with additions of methyl triphosphate, an analog known to be rapidly hydrolyzed by myosin (LeClerq, 1968; Imamura *et al.*, 1970). Whether these effects represent "true contraction" is a moot point since methyl triphosphate was without observable effects on glycerol-extracted muscle fibers.

Actomyosin Viscosity Studies. At high ionic strength ATP

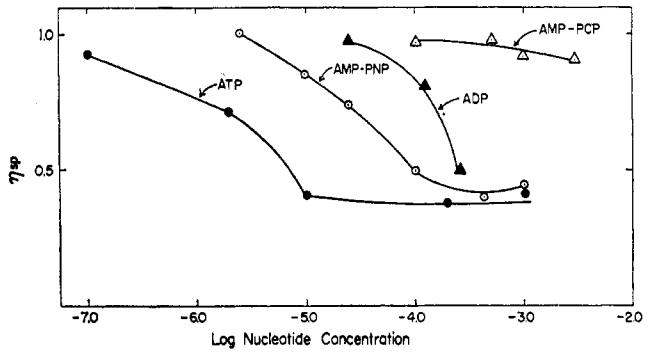

FIGURE 1: Effect of nucleotides on actomyosin viscosity at high ionic strength. Cannon-Manning semimicroviscometers (1 ml) with outflow times of 80–100 sec with buffer at 20° were used. Actomyosin solutions were clarified by centrifugation at 30,000 rpm for 45 min at 1–2° before using. Solutions contained 1.8 mg/ml of actomyosin, 0.06 M Tris·Cl (pH 7.4), 0.6 M KCl, 1.0 mM $MgCl_2$, and varying amounts of nucleotide. Specific viscosity values used were averages of three repeated measurements at each nucleotide concentration at 20°.

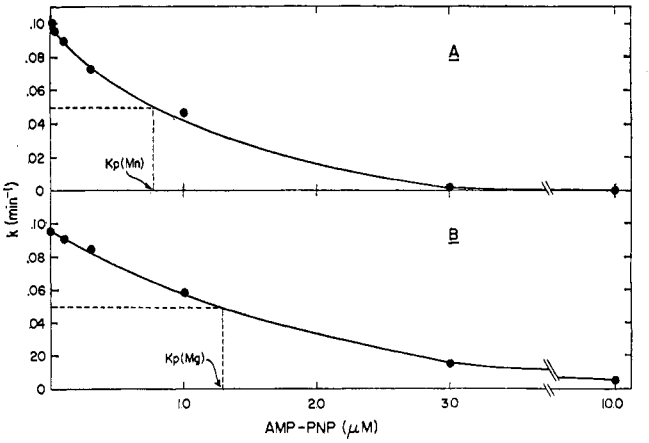

FIGURE 3: Effect of AMP-PNP on the rate of inactivation of myosin in presence of Mn^{2+} or Mg^{2+} at 40°. Conditions and treatments were as described in the text.

is known to dissociate actomyosin into actin and myosin. Figure 1 shows the effects of ATP in comparison with AMP-PNP, AMP-PCP, and ADP on the specific viscosity of actomyosin solutions in 0.6 M KCl. As can be seen, AMP-PNP is more effective than ADP but less effective than ATP in reducing the specific viscosity of actomyosin solutions. AMP-PCP is without significant effect. Despite the fact both ATP and possibly ADP (*via* myokinase) are subject to hydrolysis by this system, repeated viscosity measurements of the same solutions agreed in all cases within 1% of each other. It may also be important to note that Mg^{2+} must be present for both AMP-PNP and ADP to have any effect on actomyosin viscosity whereas ATP works in the presence or absence of Mg^{2+} (D. Ojala and R. Yount, unpublished observations).

Heat-Stability Studies. ATP is known (Ouellet *et al.*, 1952; Blum, 1960) to protect myosin against heat inactivation. To test the ability of AMP-PNP and AMP-PCP to act similarly the following experiments were performed. Reaction mixtures (2 ml) containing 10 mM $MgCl_2$ (or 2 mM $MnCl_2$), 0.1 M KCl, 50 mM Tris·Cl (pH 7.4), 3 mg/ml of myosin, and varying amounts of AMP-PCP or AMP-PNP were incubated at 40° for 15 min. Control solutions without nucleotide were kept at 0°. The heated solutions were then cooled in an ice bath and diluted with 8 ml of cold H_2O to precipitate the myosin. The myosin was collected by low-speed centrifugation and washed two times with 10 ml of ice-cold 0.03 M KCl before being redissolved in 3 ml of 0.6 M KCl. The optical densities of each myosin solution taken at 260 and 280 mμ indicated that all nucleotide had been removed and that over 95% of the enzyme was recovered. ATPase assays were performed at 25°, pH 7.4, in 0.05 M Tris·Cl, 0.1 M KCl, 10 mM $CaCl_2$, and 5 mM ATP taking five aliquots for each determination. The rate constant of inactivation, k (Burton, 1951), was then determined from

$$k = 1/t \ln (a_0/a') \quad (1)$$

where a_0 is the activity of the 0° control sample, a' is the activity remaining after incubating at 40°, and t is the time of incubation at 40° in minutes. A plot of these rate constants against AMP-PCP concentration is given in Figure 2. A protection constant, K_p can be determined from this plot at the point where $k = 1/2(k_0 + k_\infty)$, where k_0 is the rate constant of inactivation in the absence of nucleotide and k_∞ is the minimum rate of inactivation at the highest nucleotide concentration. Both rate constants are assumed to be first order. Under certain conditions this protection constant may equal the dissociation constant and/or the Michaelis constant (Burton, 1951). Here it indicates AMP-PCP binds

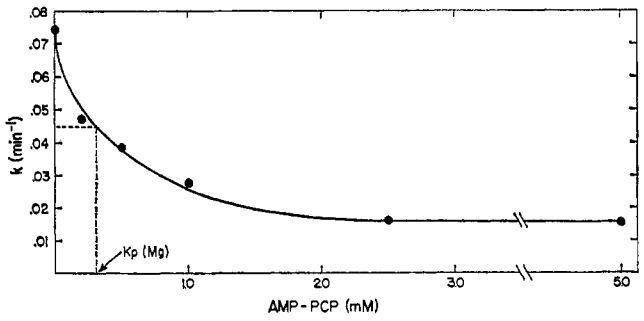

FIGURE 2: Effect of AMP-PCP on rate of inactivation of myosin in presence of Mg^{2+} at 40°. Conditions and treatment were as described in text.

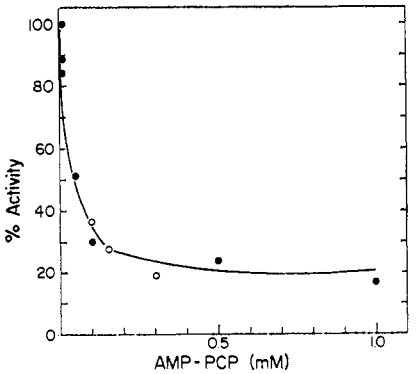

FIGURE 4: Effect of increasing AMP-PCP concentrations on HMM·Mn^{2+}ATPase. Assays were run with 5×10^{-8} M [γ-^{32}P]ATP, 0.2 μg of HMM/ml, 0.012 M Tris·Cl (pH 7.4), 2 mM $MnCl_2$, and 0.1 M KCl, as described in the text.

TABLE II: AMP-PNP Inhibition of HMM·ATPase Activity.[a]

Metal Ion	Inhibitor	[^{32}P]ATP Concn Used (μM)	K_m (μM)	V_m (μmole of P_i/min per mg)	K_i[b] (μM)	Type of Inhibn
Mn^{2+}	AMP-PNP	0.05–20	0.09	0.05	0.088 ± 0.010	Competitive
Ca^{2+}	AMP-PNP	1–10	1.2[c]	0.90	3.1 ± 0.42	Competitive
Ca^{2+}	ADP	1–10	3.5[c]	0.62	52 ± 6	Competitive

[a] Conditions: assays were run as described in the text: 2 mM divalent metal ion, 0.10 M KCl, 0.25–1.0 μg of HMM/ml, and 12.5 mM Tris·Cl (pH 7.4), 25°. [b] Averages plus and minus standard deviation of K_i values determined at three concentrations of inhibitor and at five concentrations of ATP. [c] K_m values for Ca^{2+}ATPase appear to vary somewhat with different HMM preparations. More recent determinations (B. Haley and R. G. Yount, unpublished results) give values closer to 3×10^{-5} M as found in the ADP experiment.

relatively tightly to Mg^{2+}-myosin ($K_i = 3.0 \times 10^{-4}$ M). Similar studies with AMP-PNP for Mn^{2+}-myosin and Mg^{2+}-myosin are given in Figure 3A,B. The protection constants ($K_i = 1.3 \times 10^{-6}$ and 8.0×10^{-7} M for Mg and Mn, respectively) indicate AMP-PNP binds to myosin some 200 times more strongly than AMP-PCP. They also show AMP-PNP binds somewhat more tightly to myosin in the presence of Mn^{2+} than with Mg^{2+}. Attempts to obtain similar protection constants for ATP were unsuccessful due to the rapid hydrolysis at the low concentration of ATP needed.

HMM Inhibition Studies. HMM was chosen over myosin to study the effects of AMP-PNP on ATP hydrolysis because (i) its active site appears to be essentially identical with that of myosin and (ii) it is possible to freeze-dry HMM and store it at −20° almost indefinitely. Myosin, on the other hand, is inactivated by freeze-drying.

AMP-PNP was shown to be a potent competitive inhibitor of both Mn^{2+}- and Ca^{2+}-ATPase activity. A summary of the kinetic constants is given in Table II. The K_i value for Mn^{2+}·AMP-PNP (8.8×10^{-5} M) is within experimental error identical with the K_m value for ATP (9.0×10^{-5} M) under these conditions. Similarly, AMP-PNP strongly inhibited HMM·Ca^{2+}ATPase activity with a K_i value of 3.1×10^{-6} M compared to a K_m value of 1.2×10^{-6} M. For comparison the inhibition by ADP was determined (Table II) and shown to be some ten times weaker than with AMP-PNP ($K_i = 5.2 \times 10^{-5}$ M). Thus AMP-PNP with either Mn^{2+} or Ca^{2+} as a cofactor effectively mimicked ATP in its interaction with the active site of HMM and represents the most potent competitive inhibitor of HMM yet found.

Prior studies (Moos *et al.*, 1960) had indicated that AMP-PCP was an inhibitor of myofibril ATPase activity only in the absence of divalent metal ions. This was somewhat surprising in view of the structural similarity of AMP-PCP to ATP and the potential of AMP-PCP to inhibit myosin systems was reinvestigated using HMM at the much lower ATP concentrations found to be necessary in studying AMP-PNP inhibition. Figure 4 shows that AMP-PCP does inhibit HMM·Mn^{2+}ATPase activity markedly but surprisingly the inhibition levels out above 0.2 mM inhibitor concentration. The same effect was also observed with HMM·Mg^{2+}ATPase but with an actual increase in activity occurring at AMP-PCP concentrations of 0.5 mM and higher. In addition, with concentrations of AMP-PCP above 0.5 and 2 mM Ca^{2+} a visible precipitate of AMP-PCP and metal ion occurs. Inhibition studies then were run at AMP-PCP concentrations below 0.2 mM. Figure 5 shows the results of varying AMP-PCP on HMM·Mn^{2+}ATPase activity at four ATP concentrations plotted according to Dixon (1953). The inhibition is of a mixed type; that is, it is neither strictly competitive nor noncompetitive. This can also be shown by replotting these data in double-reciprocal plots. The K_i value determined from the point of intersection is 4.4×10^{-5} M, indicating a relatively high affinity of AMP-PCP for HMM·Mn^{2+}.

Similar studies with Ca^{2+}-activated HMM (Figure 6) indicates that AMP-PCP also inhibits in the presence of this metal ion. In this case, double-reciprocal plots of $1/v$ *vs.* 1/[S] at various AMP-PCP concentrations (not given here) shows AMP-PCP to inhibit competitively with a $1/V_m$ value of that shown in Figure 6. In a Dixon plot, the K_i of a competitive inhibitor can be obtained from the intersection of a line representing the dependence of $1/v$ on I at a given substrate concentration with a line drawn parallel to the abcissa through the $1/V_m$ value. In Figure 6, lines representing the dependence of $1/v$ on AMP-PCP at three ATP concentrations and a line drawn through the $1/V_m$ intersect to give a K_i of 2×10^{-4} M. Thus a change in the divalent metal ion cofactor markedly changed both the nature and extent of the inhibition with AMP-PCP.

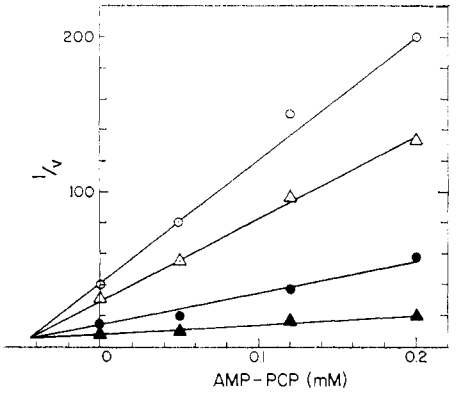

FIGURE 5: Inhibition of HMM·Mn^{2+}ATPase by AMP-PCP. Conditions were the same as in Figure 4. Assays were run with (▲) 5×10^{-6} M, (●) 5×10^{-7} M, (△) 1×10^{-7} M, and (○) 5×10^{-8} M [γ-^{32}P]ATP. $K_i = 4.4 \times 10^{-5}$ M. v is μmoles of P_i released per min per mg of HMM.

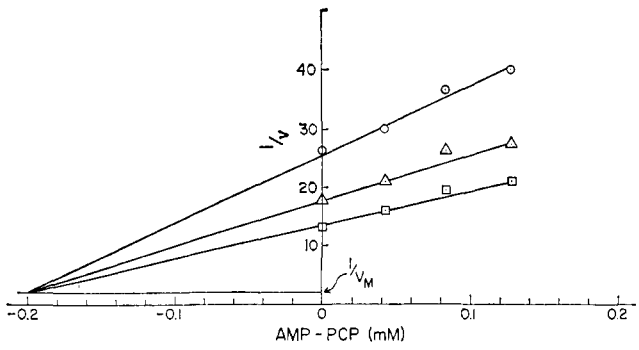

FIGURE 6: Inhibition of HMM·Ca^{2+}ATPase by AMP-PCP. Conditions were the same as Figure 4 except 2 mM $CaCl_2$ replaced $MnCl_2$. Assays were run with (□) 1×10^{-7} M, (△) 7.5×10^{-8} M, and (○) 5×10^{-8} M [^{32}P]ATP. $K_i = 2 \times 10^{-4}$ M. v is μmoles of P_i released per min per mg of HMM. The $1/V_m$ value came from the ordinate intercept of double-reciprocal plots of this same data.

Discussion

The stability of AMP-PNP to both enzymic and nonenzymic degradation has allowed the characterization of its interaction with muscle contractile proteins. All the enzyme studies reported here confirm the close similarity of AMP-PNP and ATP predicted by chemical and physical studies of the two compounds (Larsen *et al.*, 1969; Yount *et al.*, 1971). The analogous methylene analog of ATP, AMP-PCP, which is structurally less similar was shown to have much weaker interactions with all systems studied.

A comparison of the inhibitor and protection constants of AMP-PNP and AMP-PCP is given in Table III. Mn^{2+}, in particular, was used as a cofactor in these studies, because of its prominent role as a cofactor in myosin- and HMM-catalyzed $H_2O \rightleftharpoons [^{18}O]P_i$ exchange (Swanson and Yount, 1966; Herrman, 1970). It can be seen that the K_i values of AMP-PNP with either Ca^{2+} or Mn^{2+} are very similar if not identical with the K_m values for ATP under the same conditions. In addition, the low concentrations of AMP-PNP needed to protect myosin against heat inactivation at 40° with either Mn^{2+} or Mg^{2+} is indicated by the small K_p values of 0.8×10^{-6} and 1.3×10^{-6} M, respectively. While the exact significance of K_p values is unknown, they are useful in revealing binding differences of analogs to myosin and in judging the effects of variables such as divalent metal ion cofactors. Thus, AMP-PNP binds some 200 times more tightly to Mg^{2+}·myosin than does AMP-PCP and more tightly still in the presence of Mn^{2+} than with Mg^{2+}. The marked ability of AMP-PNP to stabilize myosin against heat inactivation at 40° may mean that ATP performs a similar function in the muscle cell at physiological temperatures.

TABLE III: Summary of Inhibitor and Protection Constants of AMP-PNP and AMP-PCP with HMM and Myosin.

Metal Ion	ATP	AMP-PNP		AMP-PCP	
	K_m[a] (μM)	K_p[b] (μM)	K_i[a] (μM)	K_p[b] (μM)	K_i[a] (μM)
Ca^{2+}	1.2		3.1		200
Mn^{2+}	0.090	0.80	0.088		44
Mg^{2+}	0.11	1.3		300	

[a] Conditions: 0.1 M KCl, 0.012 M Tris·Cl (pH 7.4), 2 mM divalent metal, 25°, with HMM. [b] Determined at 40° with myosin; details in text and Figures 2 and 3A,B.

The inhibition of HMM·ATPase by AMP-PCP deserves special comment. Both with 2 mM Mg^{2+} or Mn^{2+} the inhibition levels out or reverses above 0.5 mM AMP-PCP. With Mg^{2+} the maximum inhibition observed is about 60% and with Mn^{2+} about 80%. This leveling out is undoubtedly related to a similar observation in the heat protection studies (Figure 2). Here, even at saturating concentrations of AMP-PCP (5 mM), myosin is inactivated at 20% of the rate as when no nucleotide is present. This leveling out may represent the AMP-PCP interacting at a second weaker binding site which in turn modifies the active site in such a manner that AMP-PCP no longer binds as well. Alternately, AMP-PCP at high concentrations may form a two to one complex with Mg^{2+} or Mn^{2+} and as such no longer bind to the active site. It should be noted, however, that in all reactions tested Mn^{2+} and Mg^{2+} were always in excess of AMP-PCP and there is no evidence that a complex with two AMP-PCP and one Mg^{2+} or Mn^{2+} ever forms under such conditions. A satisfactory explanation awaits further experimentation but the data do point to possible complications of using high concentrations of AMP-PCP to do inhibition studies with myosin systems.

AMP-PCP, contrary to results with myofibrils (Moos *et al.*, 1960), does inhibit HMM·ATPase in the presence of excess divalent metal ions. The myofibril experiments, though, were run at much higher ATP and AMP-PCP concentrations than were used in this study and comparisons are difficult for the reasons given above. However, even with HMM and low concentrations of AMP-PCP the inhibition with Mn^{2+} was complex and indicated that AMP-PCP binds in a fashion other than as a simple competitive inhibitor. With Ca^{2+} as a cofactor, AMP-PCP is a competitive inhibitor, indicating the importance of the divalent ion in controlling the nature of the inhibition with this analog. With AMP-PNP only competitive inhibition was observed with either Ca^{2+} or Mn^{2+} as cofactors. In general, AMP-PNP was found to bind some 100–500 times more tightly than AMP-PCP.

AMP-PNP appears to be uniquely suited to test those theories of contraction in which the binding of ATP to myosin or actomyosin rather than its hydrolysis to ADP and P_i supplies the driving force of contraction. One such concept as developed by Morales and Botts (1952) (see also, Morales *et al.*, 1955) involved a "myosin" thread held in an elongated ("relaxed") state by the electrostatic repulsion of protein bound Mg^{2+} ions. ATP^{4-}, being highly negatively charged at pH 7.4, on binding to Mg^{2+} sites would neutralize the excess positive charge and contraction would occur by the "myosin" thread collapsing due to entropy forces. One of the original purposes of synthesizing AMP-PCP was, in fact, to test this hypothesis (Moos *et al.*, 1960). AMP-PCP, however, is not enough like ATP in its interaction with muscle fibers to give a valid answer. AMP-PNP, however, meets all the criteria a substrate should possess in order to test mechanisms of this type. It is (i) structurally very similar to ATP as judged by the crystal structures of imidodiphosphate and pyrophosphate, (ii) it is not a substrate, (iii) its net charge is very similar to ATP, (iv) it is a potent competitive

inhibitor of ATP hydrolysis by HMM,[2] and (v) it binds Mg^{2+} as tightly as ATP. While the electrostatic theory in its original formulation is no longer considered a viable explanation of muscle contraction, the experiments reported here are additional evidence that this and other theories of this type in which large structural changes are postulated to occur on binding ATP are unlikely. Our studies, however, do not rule out possible small cyclic changes produced by ATP binding as could occur within the framework of our current knowledge of muscle structure and function (see below and Morales, 1970).

Davies (1963) has proposed a detailed mechanism of muscle contraction in which cyclic changes between random coil and α-helical structures take place in the active-site region of myosin. These changes are moderated by ATP binding and hydrolysis and as such are reminiscent of aspects of the Morales–Botts theory. Hence, AMP-PNP should provide a valid test of this theory also. However, in this case the shortening per cycle is estimated to be very small, 100 Å/half-sarcomere length (see Davies (1967) for a discussion of these and related points). This is 1% or less of the total length of the sarcomere. It seems unlikely that actomyosin turbidity measurements or glycerol-extracted muscle fibers would reliably reveal changes of 1% or less in absorbency or length. If a test system could be devised to measure accurately 1% changes of tension or length, it should be possible to test this hypothesis directly with AMP-PNP. In fact, if such test systems cannot be found it seems unlikely this theory can ever be proven or disproven.

AMP-PNP has also proven useful in studies of relaxation. Chaplain and Frommelt (1968) have shown that AMP-PNP is almost as effective as ATP in relaxing muscle fibers. AMP-PCP was without effect, revealing the specificity of this interaction (R. Chaplain, personal communication, 1968). The studies reported here show that AMP-PNP is close to ATP in its effectiveness in dissociating actomyosin at high ionic strength. AMP-PCP is ineffective as Moos *et al.* (1960) have previously reported. Hamoir and coworkers (G. Hamoir, 1969, personal communication) have found AMP-PNP but not AMP-PCP will dissociate arterial actomyosin into actin and myosin at high ionic strength. Since these studies parallel to a large extent the studies on relaxation of intact fiber systems mentioned above, it seems likely that at least in this limited case, the dissociation of actomyosin is a good model for relaxation.

It is now clear that only the binding of ATP (or AMP-PNP) is required for relaxation (Chaplain and Frommelt, 1968) and to the extent that no ATP need be hydrolyzed, relaxation is a passive process. Other studies have also pointed to the passive nature of relaxation and to some of the specificity requirements for the purine base in the nucleotide triphosphate used to effect relaxation (Bendel, 1969). For example, while ITP will support contraction it will not cause glycerol-extracted muscle fibers to relax. Bendel (1969) explains the inability of ITP to relax fibers to the rapid hydrolysis of ITP by these systems. It is possible to test this hypothesis by using a β-γ-imido analog of ITP where the correct binding should occur but cleavage could not. If fibers fail to relax with this analog it seems clear a binding site more specific than the active site must be occupied for relaxation to occur. This would be of considerable interest in that kinetic experiments with HMM and actin (Eisenberg and Moos, 1970) would seem to rule out a second dissociating site for ATP. Many other problems concerning the nature of nucleotide-moderated actin-myosin interactions remain (Szent-Györgyi, 1968) and it would appear the use of the appropriate P-N-P analog is capable of resolving many of these.

[2] Repeated attempts were made to show AMP-PNP inhibited actomyosin ATP cleavage competitively under conditions required for contraction (see Table I). However, at the ATP concentrations near the K_m (assumed to be 10^{-5}–10^{-6} M, see Levy and Fleisher, 1965) the actomyosin concentrations must be 3–5 μg ml in order to measure the initial rate of [γ-^{32}P]ATP cleavage. At this protein concentration, actomyosin partially dissociates to actin and myosin and the activity observed is a mixture of myosin and actomoysin activities. Similar observations of the behavior of actomyosin systems at low concentrations have been made by Barany *et al.* (1967). Nonetheless it seems unlikely the active site of actomyosin is so different from HMM that AMP-PNP does not bind competitively and with an affinity comparable to ATP.

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pH-Dependent Inactivation of Nicotinamide–Adenine Dinucleotide Glycohydrolase by Its Substrate, Oxidized Nicotinamide–Adenine Dinucleotide*

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ABSTRACT: Nuclear NAD glycohydrolase activity decreases rapidly during incubation with its substrate NAD^+ at pH 7.4 and 37°. The possibility that this enzyme, as well as its microsomal counterpart, might be inactivated during the incubation has been investigated. The enzymatic hydrolysis of NAD^+ at pH 6.0 by nuclear or microsomal NAD glycohydrolase preparations proceeded at a nearly linear rate for 1 hr with a 50% utilization of the substrate. At pH 8.0, the rate of NAD^+ hydrolysis rapidly declined to zero after 10% of the substrate was used up. The inactivation of NAD glycohydrolase by NAD^+ in 25 min at 4° was pH dependent, and was 0% at pH 7.0, 30% at pH 7.5, and 60% at pH 8.0. At pH 8.0, the inactivation was related to the NAD^+ concentration and was 0% at 10^{-7} M NAD^+, 50% at 10^{-5} M NAD^+, and 90% at 5×10^{-4} M NAD^+. The inactivation was stereochemically specific. Inactivation at equimolar concentrations was 95% for NAD^+, 71% for NADH, 63% for $NADP^+$, 58% for nicotinamide mononucleotide, and 61% for ribosylnicotinamide. 1-Methylnicotinamide, nicotinamide, adenosine diphosphoribose, AMP, ADP, and *d*-ribose 5-phosphate did not inactivate NAD glycohydrolase. Nicotinamide protected the enzyme from inactivation suggesting a competition by the nicotinamide for a specific NAD^+ binding site on the enzyme. The inactivation could not be reversed by temperature, pH, or dialysis, or by treatment with 1.0 M salt solutions, 2.0 or 4.0 M guanidine, SH binding, chelating, or reducing agents. These results show the presence of an uncommon form of pH-dependent enzyme inactivation by its substrate.

Nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) (NADase),[1] the enzyme that mediates the hydrolysis of NAD^+ to nicotinamide (Nam) and adenosine diphosphoribose (ADPR) (Handler and Klein, 1942) has been found in the microsomes and in the nuclei of normal and tumor cells (Waravdekar and Griffin, 1964). During a recent investigation in our laboratory into methods for the isolation and purification of NADase from nuclei of mouse Ehrlich ascites tumor cells, the NADase activity was found to decrease rapidly during incubation up to 10 min with substrate at 37° and pH 7.4, the conditions used to assess its activity (Mamaril *et al.*, 1970). These observations raised the possibility that the nuclear NADase might be inactivated during incubation with it susbstrate, NAD^+. Preliminary studies indicated the possibility of a similar phenomenon for microsomal NADase.

The present paper is concerned with establishing this similarity, investigating in some detail the conditions determining the inactivation of microsomal NADase by NAD^+ and proposing a mechanism for this inactivation.

Materials

Enzymes, substrates, and reagents were obtained from the following sources: twice-crystallized yeast alcohol dehydrogenase (EC 1.1.1.1), Worthington Biochemical Corp., Freehold, N. J.; β-NAD^+, β-$NADP^+$, β-NADH, Nam, EDTA, Tris, and *d*-ribose 5′-phosphate (d-R5P), the Sigma Chemical Co., St. Louis, Mo.; nicotinamide mononucleotide (NMN), adenosine diphosphoribose, sodium salt (ADPR), and adenosine 5′-phosphate (AMP), P-L Biochemicals, Inc., Milwaukee, Wis. Ribosylnicotinamide (NR) was prepared by incubating nicotinamide mononucleotide with purified bull seminal 5′-nucleotidase at pH 7.4 in 0.1 M Tris buffer at 37° until all the organic phosphate was split off. The 5′-nucleotidase was then inactivated by heating the reaction mixture in a boiling-water bath for 5 min. This solution was lyophilized, the dry material was dissolved in water, adjusted to a pH of 8.0 with 0.1 M sodium hydroxide, and the solution diluted withwater to yield a final concentration of 10^{-3} M NR.

Methods

Preparation of Microsomal NADase from Ehrlich Ascites Cells. Ehrlich ascites cells in 0.2 mM EDTA (pH 7.0) were allowed to swell for 10 min in 0.05 M potassium phosphate buffer (pH 7.0) and were disrupted in a 100-W, 20-kc MSE ultrasonic disintegrator (Green and Dobrjansky, 1970). Su-

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[1] Abbreviations used as follows: Nam, nicotinamide; ADPR, adenosine diphosphoribose; d-R5P, *d*-ribose 5′-phosphate; NR, ribosylnicotinamide. All other abbreviations are as listed in *Biochemistry 5*, 1455 (1966).