Inactivation of Myosin by 2,4-Dinitrophenol and Protection by Adenosine Triphosphate and Other Phosphate Compounds*

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It has become apparent that adenosine triphosphate binds to the active site of myosin in a way which is not optimal for hydrolytic cleavage of the terminal phosphate bond. Depending on conditions, a wide variety of reagents, including 2,4-dinitrophenol, can improve the catalysis of this reaction by altering the interaction between enzyme and substrate. Concerning the way in which these modifiers affect the myosin-ATP complex, there are two general points of view which are not exclusive of each other.

First, it is often assumed that the modifier directly alters substrate binding by interacting at the active site in place of some group on the substrate. If this is true, then the chemical nature of the modifying reagent tells us something about the groups at the active site. For example, reasoning along these lines, there appear to be at least two —SH groups at the active site of myosin. This follows from observations that a number of —SH-binding reagents (2-5) activate myosin by binding up to half its —SH groups; these same compounds begin to inhibit when they mask the remaining half. There is, then, by this view at least one rate-retarding and one rate-promoting —SH at the active center; blocking the former activates whereas blocking the latter inhibits. Similar effects of other reagents have been used to implicate an amino group of lysine at the active site (to explain inhibition by 2,4,6-trinitrobenzenesulfonate) (6) and 2 bound metal ions ( inferred from the effects of reagents like inorganic pyrophosphate, which are themselves "off-site" but which contribute to the three-dimensional conformation of the enzyme-substrate complex.

At the present time these two alternatives seem to have equal weight, and it appears most likely that both kinds of action play a role in modifying the enzymic activity of myosin.

The purpose of these studies with modifiers like 2,4-dinitrophenol is to gain some understanding of the mechanism of catalysis by myosin and of the enzyme's structure which plays a central role in the contractile process of living systems. Moreover, other ATPases such as that in mitochondria show similarities to myosin; e.g. they too can be activated by p-chloromercuribenzoate and the uncoupling agent, 2,4-dinitrophenol. Thus an understanding of myosin may also help to understand the general role of ATPase sites in systems such as oxidative phosphorylation, photosynthetic phosphorylation, and active transport across cell membranes.

The work reported here bears on the general problem of modifier action in the myosin system. It began with the unexpected observation that 2,4-dinitrophenol, which markedly activates the Mg-ATPase of myosin, causes an irreversible inactivation of the enzyme in the absence of substrate.

EXPERIMENTAL PROCEDURE

Myosin was extracted from rabbit muscle by a modification of the method of Kessler and Spicer (13,14). Protein concentration was determined by Kjeldahl analysis; a factor of 6.2 was used to convert milligrams of nitrogen to milligrams of protein. The stock myosin solution was stored at 0° in 0.6 M KCl at pH 6.4. All experiments were performed within 8 days after extraction of the myosin.

ATP, ADP, and AMP were obtained as crystalline sodium salts from the Sigma Chemical Company. All other compounds were reagent grade or better. Stock solutions of 2,4-dinitrophenol were prepared by the addition of sufficient KOH to an aqueous suspension of the solid to make a solution at pH 12. This solution was then back-titrated with HCl to a pH of 7.3 to 7.4.

Enzymic activity was determined by measuring the rate of production of P1 with the iodoacetate extraction method (15).

In a typical assay, a solution containing protein, buffer, cation,
and modifier was incubated for a time in the absence of ATP; then the reaction was started by the addition of substrate. Specific conditions were: pH 7.4 to 7.5, 0.125 M Tris, 5 X 10^-3 M ATP; 0.1 M KCl; and 0.01 M MgCl₂ in a volume of 3.0 ml, at 25°. Protein and dinitrophenol concentrations varied with the experiment. The reaction was stopped by the addition of 0.3 ml of ice cold concentrated trichloroacetic acid. The tubes were centrifuged and 1 ml of supernatant solution was taken for Pi analysis.

RESULTS AND DISCUSSION

In the ordinary test for the effects of 2,4-dinitrophenol on the enzymic activity of myosin, the modifier and the substrate are added to the enzyme at the same time. When this is done, a plot of dinitrophenol concentration against Mg-ATPase activity gives the typical activation curve, A, seen in Fig. 1. However, when dinitrophenol is incubated with myosin for a time before the addition of ATP (e.g. Curve B, Fig. 1), some permanent loss of activity occurs. The rate curves for ATP hydrolysis are linear regardless of prior treatment of the enzyme with dinitrophenol (e.g. Fig. 2), showing that ATP stops but does not reverse the inactivating process. This ability of the substrate to stop inactivation but not reverse it allowed us to study conveniently the kinetics of inactivation by incubating the enzyme with dinitrophenol for varying time periods under different conditions, stopping the process by the addition of substrate, and measuring the enzymic activity which remained. Since the activating and inactivating effects are demonstrated at the same concentrations of dinitrophenol, we conclude that the substrate protects the enzyme against the killing action of dinitrophenol but allows dinitrophenol-myosin interactions which lead to increased activity.

Order of Inactivation Reaction with Respect to Time—A plot of the log of ATPase activity against time of dinitrophenol inactivation gives a straight line down to about 15% of the starting rate (Fig. 3). This indicates that the inactivation reaction approximates first order kinetics with respect to time at any fixed concentration of dinitrophenol. However, as shown in Fig. 4,

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Activation of myosin Mg-ATPase by 2,4-dinitrophenol. Conditions: 2 mg of myosin per ml; 0.125 M Tris, pH 7.4; 0.1 M KCl; 0.01 M MgCl₂; 25°. The reaction was started by adding ATP (final concentration, 0.005 M). In Curve A, dinitrophenol was added with ATP. In Curve B, the dinitrophenol was added 10 minutes before the addition of substrate. In this and subsequent figures, DNP denotes 2,4-dinitrophenol.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Rate curves of ATP hydrolysis by myosin in 0.03 M 2,4-dinitrophenol. Conditions were the same as for Fig. 1. In Curve A, dinitrophenol was added with ATP to start the reaction. In Curve B, the dinitrophenol was added 10 minutes before the addition of substrate. The linearity of Curve B and its smaller slope show that the addition of ATP stopped but did not reverse the inactivation which occurred in its absence.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** First order plot of inactivation by 2,4-dinitrophenol. General conditions were the same as given in Fig. 1. The reaction solution without substrate was incubated for the times shown on the abscissa. Then ATP was added to start the reaction. The logarithms of the assayed rates are plotted on the ordinate. The linearity of the lower curve fits Equation 9.

The enzymic activity is not completely lost; instead, it is reduced to a low residual level. Moreover, the rate of reduction to this residual activity varies markedly with dinitrophenol concentration.

To explain these facts, we assume that $E_a$, the total active enzyme at any time $t$, is in equilibrium with dinitrophenol so that there exist one or more different dinitrophenol-enzyme complexes. At least one of these, in an amount $E_a$, is susceptible to inactivation. Since the system is in equilibrium, the amount of this labile enzyme at any time will be a constant fraction of $E_a$:

$$E_a = kE_a$$

(1)
MINUTES OF INCUBATION WITH DNP

FIG. 4. Time dependence of 2,4-dinitrophenol inactivation. Conditions and procedure are given in Fig. 3.

where k is some function of dinitrophenol concentration. The rate of formation of residual enzyme, $E_r$, is then

$$\frac{dE_r}{dt} = -\frac{dE_a}{dt} = k_iE_a = k_i k E_a = k'E_a$$

where $k_i$ is the first order rate constant for inactivation of $E_a$. Integration gives the value of $E_a$ at any time after the start of incubation with dinitrophenol

$$E_a = E_a^0 e^{-k't}$$

where $E_a^0$ is the initial amount of active enzyme. Once ATP is added, the inactivation stops and the observed hydrolytic activity, $V$, is equal to an average rate constant for hydrolysis, $k_a$, multiplied by the amount of active enzyme, $E_a$, plus hydrolysis contributed by the residual species ($E_r \times E_a$).

With no incubation before the addition of ATP, $t = 0$, the rate of hydrolysis is

$$V^0 = k_a E_a^0$$

At long times of incubation, after virtually all of the enzyme has been irreversibly modified, the final residual rate is

$$V_\infty = k E_a$$

After any time of incubation with dinitrophenol the rate of hydrolysis is

$$V = k_a E_a + k E_r$$

Then, since $E_r = E_a^0 - E_a$, we can substitute and obtain

$$V - k_a E_a = k E_a + k E_a^0$$

Transposing and substituting from Equations 3, 4, and 5,

$$V - V_\infty = (k_a - k)E_a = (k_a - k)E_a^0 e^{-k't} = (V^0 - V_\infty) e^{-k't}$$

so that taking logarithms gives the general first order rate equation where $k'$ in this case is a function of dinitrophenol concentration

$$\ln (V - V_\infty) = \ln (V^0 - V_\infty) - k't$$

Fig. 3 shows that a linear relationship is obtained when the log of $(V - V_\infty)$ is plotted against $t$. Since the final residual rate, $V_\infty$, is small compared to the early values of $V$, a plot of log $V$ against time is linear during the loss of a major portion of the activity, and the initial slope of such a plot gives a good approximation to the value of $k'$.

Order of Inactivation Reaction with Respect to 2,4-Dinitrophenol Concentration—The apparent first order rate constant of Equation 9 depends upon the concentration of dinitrophenol. We may express this by the following equation,

$$k' = k (\text{dinitrophenol})^n$$

where $n$ is a number equal to the average order of the reaction with respect to concentration of dinitrophenol. Taking the logarithm of both sides leads to

$$\log k' = \log k + n \log (\text{dinitrophenol})$$

Therefore, the order of the reaction can be experimentally estimated by determining $k'$ at a number of different concentrations of 2,4-dinitrophenol. A plot of log $k'$ against log (dinitrophenol) will have a slope equal to $n$. Fig. 5 shows the experimental results of Table I plotted in this way. For convenience we have used the reciprocal of the half-time rather than $k'$. This introduces a constant which has no effect on the slope. The points fit a straight line with a slope equal to 3, indicating that an average of at least 3 molecules of dinitrophenol bind to 1 molecule of myosin when inactivation occurs. There may be more than 3

![Graph showing the relationship between logarithm of moles of dinitrophenol/liter and logarithm of half-time, with a slope of 3 indicating the average order of reaction with respect to dinitrophenol concentration.]

**TABLE I**

Half-time for inactivation at different 2,4-dinitrophenol concentrations

<table>
<thead>
<tr>
<th>2,4-Dinitrophenol concentration</th>
<th>Estimated half-time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 M ATP, 0.01 M Mg++, 0.125 M Tris, pH 7.4, 25°C</td>
<td></td>
</tr>
<tr>
<td>0.015</td>
<td>128.0</td>
</tr>
<tr>
<td>0.025</td>
<td>30.0</td>
</tr>
<tr>
<td>0.030</td>
<td>18.0</td>
</tr>
<tr>
<td>0.035</td>
<td>12.0</td>
</tr>
<tr>
<td>0.040</td>
<td>8.0</td>
</tr>
<tr>
<td>0.045</td>
<td>5.0</td>
</tr>
<tr>
<td>0.050</td>
<td>4.1</td>
</tr>
</tbody>
</table>
molecules required, since any site saturated over the range of 
dinitrophenol concentration studied would not be apparent.

Specificity for Protection against Inactivation by 2,4-Dinitro-
phenol—In addition to ATP, other phosphate compounds pro-
tect the enzyme against inactivation by dinitrophenol. Fig. 6
shows the inactivation of myosin by 0.04 M dinitrophenol with
and without various phosphate compounds at 0.005 M. The
compounds fall into two distinct groups. Those containing a
diphosphate group (PP), namely, ADP, tripolyphosphate, and
PPi itself, offer nearly complete protection, whereas AMP and
P1 protect only slightly. It is apparent from this that the
purine ring is of little importance for protection; in fact, if any-
thing the groupings in addition to diphosphate only diminish the
effectiveness. This is borne out by the quantitative protection
studies which follow. It is pertinent to note here that these
curves are all normalized to give a relative rate of 100 at zero
time. Actually, all of these phosphate compounds inhibit the
rate of ATP hydrolysis in dinitrophenol. At 0.005 M, those
which protect well show the most marked inhibitory action
whereas those which protect poorly also inhibit very little. As
discussed in a later section, this relationship between the action
of these compounds as inhibitors and protecting agents is consist-
ent with the idea that in both actions they replace ATP from a
part of the active site.

Quantitative Estimates of Protection—If we assume that the
binding of the pyrophosphate group (PP) responsible for protec-
tion occurs at one site on the enzyme and is in equilibrium, we
may express this as follows,

$$ E = E_0 + \frac{K}{1 + \frac{(PP)}{K}} $$

where $E_0$, as before, is the total amount of active enzyme at any
time, $E$ is the amount of active enzyme not complexed with the
protecting agent, and $K$ is the dissociation constant of the en-
zyme-PP complex.

Now in this case the rate of loss of active enzyme is still first
order but is proportional to $E$ rather than to $E_0$ as in Equation
2, so that we have

$$ \frac{dE}{dt} = k'E = \frac{k'E_0}{1 + \frac{(PP)}{K}} $$

Then, in a manner completely analogous to that for deriving
Equation 9, it can be shown that

$$ \ln (V - V_\infty) = \ln (V_0 - V_\infty) - \frac{k't}{1 + \frac{(PP)}{K}} $$

Rearranging,

$$ t = \ln \left( \frac{V_0 - V_\infty}{V - V_\infty} \right) \left( \frac{1}{k'} + \frac{(PP)}{KK'} \right) $$

and the half-time is

$$ t_1/2 = \frac{\ln 2}{k'} + \frac{\ln 2}{kK} $$

This is a useful equation for testing the assumptions of this
simple scheme. A plot of experimentally determined half-times
against concentration of protecting agent should be linear at a

![Fig. 6. Specificity for protection against inactivation by 2,4-
dinitrophenol. General conditions are given in Table I; 0.04 M
dinitrophenol was present during incubation and assay. Each of
the phosphate compounds was added at the same time as dinitro-
phenol to give a final concentration of 0.005 M. Then, after
periods of time given on the abscissa, ATP was added to start the
reaction. The logarithms of the relative rates of hydrolysis are
given on the ordinate.](http://www.jbc.org/)

![Fig. 7. Protection by inorganic pyrophosphate against inacti-
vation by 2,4-dinitrophenol. General conditions are those given
in Fig. 1. Half-times of inactivation were obtained from first
order plots such as those shown in Fig. 3, where the general pro-
cedure for incubation and assay is given. The data are plotted
according to Equation 16. The intercepts on the abscissa give a
dissociation constant of $2 \times 10^{-6}$ M for the myosin-pyro-
phosphate complex.](http://www.jbc.org/)
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DNP = 0.04 M

FIG. 8. Protection by ADP against inactivation by 2,4-dinitrophenol. Conditions and procedure were the same as for Fig. 7, but with indicated concentrations of ADP used to protect against 0.04 M dinitrophenol. The dissociation constant for the myosin-ADP complex, as indicated by the intercept on the abscissa, is $2 \times 10^{-6}$ M.

FIG. 9. Protection by tripolyphosphate against inactivation by 2,4-dinitrophenol. Conditions and procedure were the same as for Fig. 7, but with indicated concentrations of tripolyphosphate used to protect against 0.04 M dinitrophenol. The dissociation constant for the myosin-tripolyphosphate complex, as indicated by the intercept on the abscissa, is approximately $2 \times 10^{-6}$ M.

Protecting Agents as Competitive Inhibitors—Pyrophosphate and ADP appear to competitively inhibit 2,4-dinitrophenol modified Mg-ATPase of myosin, as shown in Figs. 10 and 11. On this kind of plot, the intersection of the lines occurs at a concentration of inhibitor equal to the dissociation constant of the inhibitor-enzyme complex. Because the concentrations of substrate used in these experiments were much higher than the dissociation constants of the inhibitors, these points appear to be at zero concentration and cannot be estimated. The ordinary assay for Pi is not sensitive enough to make accurate measurements of rate at concentrations of ATP less than $10^{-4}$ M, where they would have to be done to determine these constants. Nevertheless, the results show that the true dissociation constants for PP, and ADP are low and might well be the same as those obtained from the protection studies. Moreover, the much greater effectiveness of PP, compared to ADP, as an inhibitor agrees with its greater ability to protect. The strong inhibition by PP, is in sharp contrast to its extremely weak action on native Mg-ATPase. All the data on the action of these agents as competitive inhibitors of the dinitrophenol-modified system are consistent with the view that ATP and other phosphate compounds which protect against inactivation by dinitrophenol do so by binding to some group which is also involved in ATP hydrolysis.

General Conclusion—The specific findings reported in this paper may be outlined as follows. (a) Concentrations of 2,4-dinitrophenol which increase the Mg-ATPase activity of myosin from 2- to 10-fold are able to inactivate the enzyme irreversibly when the modified active site is not protected by ATP. (b) The entire substrate structure is not required for protection against inactivation by dinitrophenol. Other compounds containing a diphosphate group (PP) offer good protection. In fact, PP, protects 10 times better than ADP. (c) Inactivation by dinitrophenol involves the binding of at least 3 molecules of dinitrophenol per active unit of myosin. The process appears to be prevented by the binding of a single group, PP, to the active site. (d) Activation by dinitrophenol in the presence of ATP also involves more than one myosin-dinitrophenol interaction. That is, the curve of dinitrophenol activation (Fig. 1A) is complex and does not fit...
the kinetics for a single interaction. (e) Whereas PP\textsubscript{i} is a very poor inhibitor of the unmodified relatively slow Mg-ATPase of native myosin, it is a potent inhibitor of the dinitrophenol-activated system, inhibiting the rate by 90\% at a concentration only one-sixth that of ATP. ADP, which protects only one-tenth as well as PP\textsubscript{i} against dinitrophenol inactivation, inhibits only about one-twentieth as well.

From these observations and a body of literature, some of which is cited below, we conclude generally that at least three and probably more interactions between 2,4-dinitrophenol and myosin can occur to alter protein conformation at the active site and thereby change the orientation of substrate. These interactions change every parameter of the hydrolytic reaction: substrate specificity, metal dependence (6), pH (7), and temperature dependence (2). At 25°, pH 7.4, 0.01 M magnesium, and 0.04 M dinitrophenol, when the modified active site is protected by a diphosphate group, the proteins are relatively stable. But without the binding of this diphosphate group certain of the dinitrophenol interactions lead to more extensive structural alterations which cause a progressive irreversible loss of enzymic activity. Whether PP\textsubscript{i} prevents one or more interactions between myosin and dinitrophenol or, instead, stabilizes the active site despite these interactions cannot be decided at present. In any event it appears that a single PP\textsubscript{i} has a profound effect on the reactivity or stability of the protein toward dinitrophenol, or both.

With native myosin under the conditions given above, the adenine ring of ATP and the magnesium ion contribute to a very strong interaction between enzyme and substrate (16, 18). This tight binding is associated with a very slow rate of hydrolysis, a typical Arrhenius dependence of activity on temperature (12), and a virtual indifference to PP\textsubscript{i} and ADP, which apparently compete very poorly for binding at the active site. Dinitrophenol modifies the interaction between enzyme and ATP, i.e. alters its binding orientation, in such a way as to weaken the influence of the ring (16), increase the rate of hydrolysis at 25°, and make the complex susceptible to a reversible temperature-induced change in conformation (12); in all, a situation is produced which more closely resembles the unmodified hydrolysis of ITP (7, 12, 16, 17). At the dinitrophenol-modified site, in sharp contrast to the native site, the relative binding of ATP, ADP, PPP\textsubscript{i}, and PP\textsubscript{i} is in a direction which favors the smaller molecules. Thus PP\textsubscript{i} becomes the best inhibitor of ATP hydrolysis, and the best protecting agent against inactivation by dinitrophenol.

The similar actions of 2,4-dinitrophenol on mitochondrial and myosin ATPase indicate a general ability of this reagent to alter protein structure and thereby distort the normal function of ATP-binding sites. In muscular contraction, as in oxidative phosphorylation, the ATP-binding sites of myosin do not normally allow the uncoupled release of energy by hydrolysis. 2,4-Dinitrophenol modifies the active site of myosin in a way which allows an attack by water. With the natural modifier, actin, the hydrolysis of ATP proceeds rapidly but here it is coupled in some unknown way to contraction.

**SUMMARY**

Concentrations of 2,4-dinitrophenol (0.01 to 0.05 M) which markedly activate the magnesium-modulated adenosine triphosphate activity of myosin (2- to 10-fold increase) cause an irreversible loss of enzymic activity when ATP is not present to protect the active site. Other compounds can substitute for ATP as protecting agents, the only requirement being a pyrophosphate group. Adenosine monophosphate and inorganic orthophosphate are relatively ineffective. Inorganic pyrophosphate is the most effective and appears to bind to the dinitrophenol-modified active site 10 times more tightly than adenosine diphosphate.

The inactivation process involves the binding of at least 3 molecules of dinitrophenol to 1 active unit of myosin. It is proposed that dinitrophenol alters the structure of the flexible active site. When a single diphosphate group protects the active center, then hydrolysis rate is increased by the effects of dinitrophenol. When this group is absent, then the dinitrophenol-myosin interactions lead to more extensive irreversible structural changes with a loss of enzymic function.

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Inactivation of Myosin by 2,4-Dinitrophenol and Protection by Adenosine Triphosphate and Other Phosphate Compounds
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