The Properties of Particulate Phosphoprotein Phosphatase

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The very high metabolic activity of protein-bound phosphorus was first discovered by Davidson et al. (1) in P32 uptake experiments and subsequently confirmed in other laboratories. A possible explanation of these findings is that the primary step in oxidative phosphorylation results in the formation of a phosphorylated protein with subsequent transfer of the high energy phosphate to adenosine triphosphate. The existence of a high energy phosphate complex before adenosine triphosphate has been suggested by a number of investigators (see (2) for review). An enzyme, phosphoprotein phosphatase, which liberates inorganic phosphate from phosphoproteins, was first found by Harris (3) in frog eggs. Some of the properties of the enzyme as it occurs in rat (4, 5) and ox (6) tissues were subsequently reported on.

A study of this enzyme was undertaken to test the hypothesis that the activity of some uncoupling agents results from their ability to activate a mitochondrial phosphoprotein phosphatase. Although the enzyme was present in the cytoplasmic particles of liver, it was not activated by any of the uncoupling agents tested. In the course of this work, a number of additional properties of the enzyme were examined; the enzyme was found to be metal activated, and a study of the kinetics suggested a rather unusual role for the metal.

MATERIALS AND METHODS

Substrate Solution—A 5 per cent suspension of casein powder in 0.05 m NaHCO3 was brought into solution by warming to 80°. This solution was then dialyzed against 40 volumes of 2 × 10⁻³ m ethylenediaminetetraacetic acid in 0.05 m sodium maleate buffer, pH 6.4, followed by two dialyses against 40 volumes each of the maleate buffer alone.

Enzyme Preparation—Preliminary experiments indicated that mouse liver phosphoprotein phosphatase is bound to cellular particles. A stable preparation of the enzyme was therefore obtained from mitochondrial suspensions. A 15 per cent homogenate, in 0.25 m sucrose, of the livers of male Swiss mice was filtered through gauze and the resulting filtrate layered over 0.25 m sucrose and dialyzed overnight against 100 volumes of 1 m sucrose. Following centrifugation at 600 × g for 10 minutes in a horizontal yoke centrifuge, the upper layer was transferred to the No. 40 rotor of the Spinco ultracentrifuge. The pellet obtained after centrifugation at 12,000 × g for 20 minutes was washed once with 0.25 m sucrose at the same speed. The final pellet was taken up in 0.25 m sucrose and dialyzed overnight against 100 volumes of 0.01 m lactic acid. The dialyzed suspension, which contains the enzyme in soluble form, gave a negligible blank and was stable indefinitely when stored frozen. This procedure yielded a preparation having a specific activity approximately 10 times that of the original homogenate.

Enzyme Assay—A sufficient amount of the enzyme, cysteine to give a final concentration of 0.01 m, and any additions were made up to 1 m in an ice bath and the reaction started by the addition of 1 ml of substrate solution at 37°. The reaction was stopped after 20 minutes at 37° with the addition of 4 ml of 0.3 m trichloroacetic acid, plus 0.5 gm. of Dowex 50-X1 (hydrogen form). The latter served to remove phosphorylated peptides, produced during the reaction, whose presence interfered with the subsequent determination of inorganic phosphate. The reaction mixtures were filtered and the liberated inorganic phosphate determined by the method of Sumner (7).

Materials—Commercial Hammarsten quality casein (Nutritional Biochemicals Corporation) was used for the preparation of substrate solutions. Purified α- and β-casein either were prepared by the urea method of Hipp et al. (8) or were the generous gift of Dr. T. L. McMeekin. Deoxyribonucleic acid and ribonucleic acid were commercial samples of relatively low molecular weight. Samples of sulfated chitosan, Kurrol's salt (potassium polyphosphate glass), sodium polyethylene sulfonate, and sodium dextran sulfonate were gifts of Dr. W. Regelson.

RESULTS

Enzyme Concentration, Time, and pH—Reaction rate was not strictly proportional to enzyme concentration in crude tissue homogenates. However, as seen in Fig. 1, excellent proportionality was observed with partially purified preparations. The dependence of the activity on pH is shown in Fig. 2. It was not possible to determine reaction rates below pH 6 due to insolubility of the substrate.

Under the assay conditions described, the liberation of inorganic phosphate continued at a constant rate for 30 to 40 minutes and then declined. The further addition of substrate did not restore the rate, and fresh enzyme added to a spent reaction mixture was as active as the initial charge of enzyme. Hence, the decline in rate was caused by inactivation of the enzyme and not to exhaustion of the substrate or accumulation of an inhibitor. Inactivation was lessened, though not completely eliminated, when cysteine was present.

Sulfhydryl Dependence—In agreement with descriptions of the enzyme from other sources (4, 6), activity required the presence of intact enzyme sulfhydryl groups. The data of Table I show that the enzyme was activated by cysteine and inactivated by oxidation with H2O2 or treatment with p-chloromercuriphenyl sulfonate. The oxidized enzyme could be reactivated with cysteine; however, addition of the mercuriphenyl radical to the enzyme sulfhydryl was not reversible. There...
TABLE I

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmoles P/hr./mg. N</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>26.3</td>
</tr>
<tr>
<td>10^{-9} M cysteine</td>
<td>64.1</td>
</tr>
<tr>
<td>10^{-2} M pCMoS</td>
<td>0.6</td>
</tr>
<tr>
<td>10^{-2} M pCMoS + 10^{-2} M cysteine</td>
<td>0.3</td>
</tr>
<tr>
<td>10^{-3} M pCMoS</td>
<td>18.2</td>
</tr>
<tr>
<td>10^{-3} M pCMoS + 10^{-2} M cysteine</td>
<td>19.1</td>
</tr>
<tr>
<td>10^{-2} M H2O2</td>
<td>0</td>
</tr>
<tr>
<td>10^{-2} M H2O2 + 10^{-3} M cysteine</td>
<td>0.3</td>
</tr>
<tr>
<td>10^{-2} M H2O2</td>
<td>0.7</td>
</tr>
<tr>
<td>10^{-2} M H2O2 + 10^{-4} M cysteine</td>
<td>35.8</td>
</tr>
</tbody>
</table>

In addition to the above, 5 × 10^{-4} M FeSO₄ was present in all cases. pCMoS represents sodium p-chloromercuriphenyl sulfonate.

**Table 1**

Sulfhydryl dependence of phosphoprotein phosphatase

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**Fig. 1.** The proportionality of reaction rate to enzyme concentration. The reaction mixture also contained 0.005 M MnCl₂.

**Fig. 2.** The pH dependence of phosphate release. The reaction mixture also contained 0.002 M MnCl₂.

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**Fig. 3.** The dependence of reaction rate upon metal ion concentration.

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**Fig. 4.** Kinetics of activation by iron. *Kₘ* is corrected as described in the text. Reaction velocity is in arbitrary units.

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**Text**

appeared to be no specificity as to the reducing agent required, since glutathione, ascorbate, and thioglycolate all could replace cysteine in activating the enzyme.

**Metal Activation**—Although the activity of tissue homogenates was not so markedly affected by the addition of metal ions, the addition of any one of several divalent cations to partially purified enzyme preparations resulted in a considerable increase in activity. Among the ions tested, Cu++, Ni++, and Fe++ were most effective (Fig. 3). Whether Fe+++ functions as such, or after reduction to Fe++ by cysteine, is not clear. The simultaneous addition of Fe++ and Ni++ at optimal concentrations did not result in an activity higher than that in the presence of Fe++ alone. Among other ions tested, Co++ and Mn++ were much less effective, and Mg++, Zn++, Ca++, Al++, and Cr++ were inactive. In Fig. 4 is a Lineweaver-Burk plot for FeSO₄.
Inhibition, from which an apparent Michaelis constant of $1.9 \times 10^{-5}$ is obtained. When corrected for the contribution of the $K_m/S$ term of the modified rate equation (see "Discussion" for details), this value becomes $1.2 \times 10^{-4}$.

Whenever there was a diminution of activity at high metal concentrations, this was invariably correlated with visible precipitation of the substrate.

With the exception of 1,10-phenanthroline, metal-complexing agents had little effect on the activity obtained in the absence of added metal activator. Sodium fluoride, thiosemicarbazide, sodium azide, 8-hydroxyquinoline, $\alpha,\alpha'$-dipyridyl, diethyldithiocarbamate, oxalate, and ethylenediaminetetraacetate were without effect at concentrations below 0.01 m. Inhibitions ranged from 0 to 30 per cent at concentrations above 0.01 m.

In contrast to these, phenanthroline was a powerful inhibitor, giving 50 per cent inhibition when added to reaction mixtures at $10^{-4}$ m (Fig. 5). In spite of this, prolonged dialysis of both enzyme and substrate solutions against $10^{-4}$ m phenanthroline, with subsequent removal of the phenanthroline by dialysis against buffer, did not result in any loss in activity. The ability of phenanthroline to act as an inhibitor without removing metal from the reaction components suggests that inhibition resulted from a competition by phenanthroline for a metal-containing binding site. Attempts were made, therefore, to determine whether phenanthroline inhibition is competitive with the substrate. A conventional kinetic analysis failed, since in the absence of an added metal activator linear plots of $1/v$ versus $1/s$ were not obtained in experiments in which the substrate concentration was varied (see below). However, the concentration of phenanthroline required for 50 per cent inhibition was essentially the same at substrate concentrations of from 0.1 to 2.5 per cent casein. It thus appears unlikely that casein and phenanthroline both bind to the same site.

$\alpha$- and $\beta$-Casein—The enzyme attacks both $\alpha$- and $\beta$-casein. In Fig. 6 are shown the substrate-activity curves for both $\alpha$- and $\beta$-casein with and without added FeSO$_4$. In the presence of Fe$^{++}$, values of $K_m$ = 0.40 per cent for $\alpha$ and 0.43 per cent for $\beta$ were obtained; these correspond to $9.7 \times 10^{-4}$ m and $6.8 \times 10^{-4}$ m phosphorus, respectively.

In the absence of added Fe$^{++}$, as the substrate concentration was raised, the reaction rate increased faster than is accounted for by simple enzyme-substrate complex formation. This is demonstrated by the nonlinearity of the lower curves in Fig. 6 and is particularly marked at low substrate concentrations. The explanation for this probably lies in the finding that casein solutions which had been exhaustively dialyzed against metal-complexing agents still showed a residual Fe content of 1 pg./ml. when examined by emission spectroscopy. (This corresponds to approximately 1 Fe atom per 100 casein molecules, assuming a molecular weight of 33,000.) This residual Fe can apparently serve to activate the enzyme so that, as the casein concentration was raised, the reaction proceeded more rapidly both from an

![Fig. 5. Inhibition by 1,10-phenanthroline. Control activity in the absence of added phenanthroline was 56 pmol of P/mg. of N/hour.](image)

![Fig. 6. The dependence of reaction rate on substrate concentration for $\alpha$- and $\beta$-casein. O, no added metal; 0.048 mg. of enzyme N/ml. , $5 \times 10^{-4}$ m FeSO$_4$ present; 0.012 mg. of enzyme N/ml. Substrate concentration is expressed as final per cent casein, reaction velocity in arbitrary units.](image)
increase in substrate concentration and from the activation of new enzyme molecules.

**Differentiation from Acid Phosphatase and Cathepsin**—The possibility that the inorganic phosphate liberated from casein resulted from the cooperation of a cathepsin with acid phosphatase was ruled out on several grounds. First, enzyme activity was directly proportional to enzyme concentration. Second, there was no time lag in the appearance of inorganic phosphate, even at product concentrations as low as 3 \( \times 10^{-5} \) M. Third, the catheptic activity (measured with either hemoglobin or casein as substrates) and acid phosphatase activity (measured with β-glycerophosphate) present in the enzyme preparations were completely stable for 1 hour at 37°C in the absence of a reducing agent, while phosphoprotein phosphatase activity had a half-life of 15 minutes under the same conditions. Fourth, neither the catheptic nor acid phosphatase activities present showed a requirement for either a reducing agent or a metal activator. Fifth, as mentioned above, fluoride and oxalate at 0.01 M had no effect on phosphoprotein phosphatase although they are strong inhibitors of acid phosphatase (9, 10). And finally, inorganic phosphate was liberated several times more rapidly from casein than from β-glycerophosphate.

**Uncoupling Agents**—In line with the hypothesis that uncoupling may result from the hydrolysis of a protein phosphate formed in the primary reaction of oxidative phosphorylation, uncoupling agents were tested for their ability to activate phosphoprotein phosphatase. Compounds were tested for their ability to activate the partially purified enzyme or to effect the release of the enzyme from cytoplasmic particles. Thyroxine, tri-iodothyronine, 2,4-dinitrophenol, and Ca++, all at concentrations at and above those effective in uncoupling oxidative phosphorylation, had no effect in either test system.

**Inactive Compounds**—A number of substances have been examined for their effect on the enzyme with essentially negative results. Amino acids, which have been widely reported as stimulating various phosphatase activities, were tested. Glutamic acid, alanine, phenylalanine, and histidine were without effect. Sodium, potassium, ammonium, chloride, sulfate, succinate, cacodylate, and maleate ions were also inactive. Di-hydrocortisone was inert, nor could an activator or inhibitor be detected in normal mouse serum.

**Molybdate and Other Polyanions**—In view of the familiar ability of molybdate to form complex phosphomolybdates, the effect of ammonium molybdate on the reaction was tested. It proved to be an unusually powerful inhibitor, giving a 50 per cent decrease in reaction rate at approximately 10^{-5} M molybdenum (see Fig. 7). Due to the tendency for molybdate to form phosphomolybdates, the true concentration of molybdate ions was probably lower than this. At 5 \( \times 10^{-4} \) M Mo, where inhibition was still complete, the reaction mixture contained approximately 10 Fe++ ions, 15 casein molecules (of molecular weight 33,000), and 100 phosphate ester bonds for each atom of Mo present; hence, the only component present in sufficiently small amounts to be appreciably affected by reaction with molybdate was the enzyme itself. This reaction with molybdate was "uncompetitive" with Fe++, a type of inhibition resulting from combination of an inhibitor with an enzyme-substrate or enzyme-metal complex, but not with the free enzyme itself. This type of inhibition was first observed by Winzler (11) for the effect of azide on yeast respiration and was further analyzed by Burk (quoted in Ebersole et al. (12)). In contrast to the relation with Fe++, molybdate inhibition was noncompetitive with the substrate, implying that substrate and inhibitor molecules cannot displace each other from the enzyme surface. These results are demonstrated in Fig. 8.

To test the possibility that inhibition by molybdate was a nonspecific property of polyanions in general, several other polyanions were examined for activity. At a level of 0.5 mg./ml., deoxyribonucleic acid, ribonucleic acid, Kurrol's salt, and sodium dextran sulfonate were inactive, while sodium polyethylene sulfonate and sodium dextran sulfonate gave 50 per cent inhibition. It thus appears that while some polyanions are inhibitory there is a considerable degree of specificity involved.
The phosphoprotein phosphatase described here shows no relation to the uncoupling of oxidative phosphorylation. Whether such an enzyme is concerned is still open to question since there is one unavoidable flaw in the design of these experiments. A protein phosphate of a high-energy type would be expected to be an acid anhydride, or possibly a phosphorylated sulfhydryl, whereas the only phosphorylated proteins available for use as test substrates contain primarily ester phosphates.

The results reported here clearly indicate that phosphoprotein phosphatase is both sulfhydryl and metal dependent. The requirement for reduced -SH groups is in accord with the observations of Feinstein and Volk (4) and Sundararajan and Sarma (6), although not with those of Norberg (5). There have been no previous reports of metal dependence, and even several reports of inhibition by metal (4, 5). The discrepancy concerning metal activation of the enzyme is not due to the use of enzyme from different sources since, in this laboratory, the activities of crude homogenates of rat spleen and liver are also significantly enhanced by the addition of iron. It is conceivable that some commercial samples of casein contain sufficient iron to activate the enzyme fully. We have found that metal dependence is enhanced following dialysis of either enzyme or substrate; optimal enhancement follows dialysis of both. The cases of metal inhibition which have been reported probably resulted from substrate precipitation at higher metal concentrations.

In attempting to understand the nature of the reactions studied here, several facts should be borne in mind. The failure of a variety of chelating agents, including 8-hydroxyquinoline, α,α′-dipyridyl, and ethylenediaminetetraacetate, to inhibit suggests that the inhibition by phenanthroline results from a specific combination with enzyme-bound metal, rather than from the removal of any free iron in solution. This interpretation is supported by the inability of phenanthroline to remove an essential reaction component upon dialysis. The uncompetitive nature of molybdate inhibition with respect to Fe++ implies that molybdate can combine only with the enzyme-metal complex and not with free enzyme molecules. In neither of these cases was the inhibition competitive with the substrate. This failure of casein to displace inhibitor molecules combining with enzyme-bound metal makes it unlikely that substrate molecules react with enzyme-bound metal.

The simplest explanation of these findings is that the metal activator does not participate directly in the binding of substrate molecules, but rather reacts with the enzyme at another site, altering the configuration to a new and more active form. The inhibitory action of phenanthroline and molybdate results from acceptance of some of the coordination bonds of the metal ion, thus preventing a metal "bridge" from holding the enzyme in its active form; and since the metal is not at the substrate-binding site, these inhibitions are not reversed by substrate.

Inhibition of some plant acid phosphatases by molybdate has been noted previously by Courtois and Bossard (10). In those cases, with β-glycerophosphate as the substrate, it is likely that inhibition resulted from the similarity of molybdate to, or its reactivity with, phosphate groups, since acid phosphatase is not dependent on a metal for activity, and the inhibition was of the competitive type.

A reaction mechanism in which the metal serves to hold the enzyme in an active configuration rather than to bind the sub-

![Diagram](https://example.com/diagram.png)

**FIG. 9.** A diagrammatic representation of the proposed reaction mechanism; a configurational change involving rotation of a peptide chain carrying a terminal -SH was chosen only for illustrative purposes and is not meant to imply that this is, in correct detail, the configurational change involved in activation.
substrate is also of assistance in explaining the specificity of activation by metals. Metal dependent phosphatases are usually optimally activated by Mg++, Mn++, Zn++, or Co++, with Fe++ and Ni++ less effective (13). Such an order of effectiveness parallels the ability of these ions to form stable phosphate complexes. The present enzyme, however, is activated not at all by Mg++ or Zn++, weakly by Mn++ and Co++, and optimally by Fe++, Ni++, and Cu++. This latter order reflects the capacity of these ions to enter stable amino and sulfhydryl complexes (14).

These ideas concerning the configuration near the active site and the effects of various inhibitors can be represented somewhat diagrammatically as shown in Fig. 9. In this representation of the mechanism, activation and inactivation result from changes in the accessibility of the active site to approaching substrate molecules.

The direct participation of sulfhydryl groups in determining protein structure has been shown in the case of phosphorylase by Madsen and Cori (15).

A rate equation which correctly predicts the kinetics observed with an inhibitor like molybdate is generated by assuming that the following reactions occur:

\[ E + M \xrightleftharpoons[k_2]{k_1} EM \]  
\[ EM + S \xrightarrow[k_4]{k_3} EMS + \text{Product} \]  
\[ EM + I \xrightarrow[k_6]{k_4} EMI \text{ (inactive)} \]  
\[ EMS + I \xrightarrow[k_6]{k_4} EMSI \text{ (inactive)} \]

where \( E \) is enzyme concentration, \( M \) the concentration of an activating metal ion, \( S \) the concentration of substrate, and \( I \) the concentration of an inhibitor. A consideration of the steady state rate equations yields:

\[ v = \frac{V_{max}}{1 + K_i'/M + K_a/M + 1/K_I} \]

where \( K_a = \frac{k_2}{k_1}, K_i = \frac{k_4 + k_5}{k_3}, K_I = \frac{k_2}{k_9}, \text{ and } K_i' = \frac{k_6}{k_9}. \)

Omission of reaction (4) would make the inhibition competitive with Fe++. If an additional reaction \( E + I \xrightarrow{EI} \text{ (inactive)} \) occurred, the inhibition would be noncompetitive with Fe++. The mechanisms postulated are further supported by the experimental result that \( K_I = K_i' \). This equality results from the finding that the addition of molybdate in the presence of a high metal concentration increases the slope and intercept of the metal activation curves (Fig. 3). This inhibition at high metal concentrations is due, as mentioned previously, to the secondary formation of insoluble metal caseinates.

One difficulty with this formulation of inhibitor action should be pointed out. It is assumed that phenanthroline, as well as molybdate, reacts with an enzyme site containing reversibly bound metal. However, exposure of the enzyme to large amounts of phenanthroline, followed by dialysis to remove the phenanthroline, did not effect a reduction in enzyme activity measured in the absence of an added metal activator. This, and the failure of other chelating agents to inhibit, would be explained if a small fraction of enzyme molecules contained firmly bound iron. Unfortunately, there is no other evidence for such a heterogeneity of metal-binding sites.

One point of some significance should be noted. It is customary to view the part played by metals in enzyme catalysis as promoting the formation of the enzyme substrate complex. This is usually written as a double complex in which some of the coordination bonds of the metal are accepted by the enzyme and some by the substrate. In the cases of the decarboxylases (14), peptidases (18), reactions participated in by pyridoxal (17), and alcohol dehydrogenase (18), where the role of the metal has been investigated in some detail, this concept has been confirmed. The results with phosphoprotein phosphatase serve, however, to demonstrate that metal activation is not restricted to mechanisms involving substrate binding. Instead, as seems to be the case here, the metal may serve to hold the enzyme in an active configuration. Such a mechanism holds certain advantages as a regulatory device in vivo in the control of metabolic rates since it can, in principle, control any enzymatic reaction and is not restricted to those cases where the substrate is capable of forming strong metal chelates.

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SUMMARY

1. The preparation and properties of partially purified mouse liver phosphoprotein phosphatase are described. The enzyme attacks both \( \alpha \) - and \( \beta \)-casein and has a pH optimum below 6.
2. Enzyme activity was not the result of a summation of catheptic and acid phosphatase activities.
3. Uncoupling agents had no effect on either the release of the enzyme from cytoplasmic particles or the activity of the free enzyme.
4. Activity was both sulfhydryl and metal dependent. Iron, nickel, and copper were the most effective metal ions tested, with cobalt and manganese less active. A number of other metal ions had negligible effects. The enzyme was inhibited by p-chloromercuri phenyl sulfonate, \( \mathrm{HgCl}_2 \), and 1,10-phenanthroline, but not by some other metal-complexing agents.
5. Ammonium molybdate was a powerful inhibitor of the enzyme, giving 50 per cent inhibition at \( 10^{-4} \) M Mo. This inhibition was noncompetitive with the substrate and uncompetitive with the metal activator.
6. These findings are best explained by assuming a mechanism in which the metal ion holds the enzyme in an active configuration, but does not participate directly in substrate-binding. Inhibition results from complexing of the metal ion by other molecules, or by blocking enzyme sulfhydryl and preventing it from complexing the metal.
REFERENCES

7. Sumner, J. B., Science, 100, 413 (1944).
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