Kinetic Studies of the Inhibition of Muscle Phosphorylase Phosphatase*

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SUMMARY

Inorganic phosphate, glucose 1-phosphate, divalent metal ions, EDTA, m-propanoxym benzamidine, and AMP were found to be competitive inhibitors of the reaction catalyzed by phosphorylase phosphatase. By a comparison of kinetic studies with the substrates, phosphorylase α and the phosphorylase peptide, it was deduced that inorganic phosphate affected the reaction by binding to the catalyst, whereas glucose-1-P inhibited by binding to the substrate, phosphorylase. Inhibition by divalent metal ions and α-propanoxym benzamidine was explained by binding to a single site on phosphorylase phosphatase. This site is presumed to be the one that interacts with the arginyl function of the substrate. EDTA inhibition appears to occur at a different locus on the phosphatase. AMP changes the phosphatase reaction by binding to phosphorylase α. The phosphorylase α-AMP complex is poorly recognized, if at all, by phosphorylase phosphatase.

Enzyme-catalyzed chemical modification of enzymes is an important mechanism for the regulation of enzyme activity. The phosphorylation (1) and dephosphorylation (2) of glycogen phosphorylase catalyzed by phosphorylase kinase and phosphorylase phosphatase, respectively, is the first example of this type of mechanism. Numerous studies have shown how these interconversion reactions are coupled to physiological stimuli and the consequence of these reactions on glycogen metabolism (3). Little information is available, however, on how various factors can affect the action of an enzyme that utilizes another enzyme as a substrate.

Three general mechanisms are possible:

I. Enzymatic modification of the modifying enzyme

II. Nonenzymatic factors which affect the modifying enzyme

III. Nonenzymatic factors which affect the substrate(s) being modified

For muscle phosphorylase phosphatase, enzymic chemical modification has not been established as a means of regulation (4). To distinguish between Mechanisms II and III, we have used an alternative substrate, a phosphorylated tetradecapeptide that is obtained from a chymotryptic digestion of phosphorylase α (5). It was reasoned that modifiers that influence the phosphorylase phosphatase reaction, for example, by changing the structure of the substrate, phosphorylase α, would have little or no influence on reactions catalyzed by the tetradecapeptide as a substrate.

The phosphorylase phosphatase reaction is known to be inhibited by phosphorylated esters (6), salts (6, 7), divalent cations (7), EDTA (7), and AMP (8). The mechanism by which these modifiers influence the reaction is not known except for the case of AMP, where it has been established that the locus of action is the substrate, phosphorylase α (5). In this manuscript, the kinetics of inhibition is examined with phosphorylase α and an alternative substrate, a phosphorylated tetradecapeptide derived from phosphorylase α. Utilizing the equations developed in Reference 9, mechanisms are deduced for the action of various modifiers.

EXPERIMENTAL PROCEDURE

Materials—Phosphorylase b was prepared as described by Fischer and Krebs (10) and phosphorylase kinase by the method of Brostrom et al. (11). [γ-32P]ATP was prepared especially by the method of Glynn and Chappell (12). [α32P]Phosphorylase α was made by the procedure of Krebs (13). The enzyme preparation had a specific enzyme activity of 1800 to 2000 Cori units per mg and an activity ratio of 0.8 in the absence and presence of AMP, respectively, as measured by the procedure of Illingworth and Cori (14). Phosphorylase phosphatase was purified essentially by the procedure of Hurst et al. (15). The pooled enzyme fractions from the triethylaminoethyl column chromatography were dialyzed against deionized water, lyophilized, and stored at -15°C. Stock solutions were stored at -15°C in 50 mM Tris-acetate and 5 mM dithiothreitol, pH 7.5.

A [32P]tetradecapeptide incorporating the phosphorylated site was isolated after chymotryptic digestion of [γ32P]phosphorylase α by modifications of the method used by Nolan et al. (5). Approximately 6 g of phosphorylase were dissolved into 0.1 M (NH4)2CO3 to form a 1% solution. One per cent the weight of protein substrate was added of α-chymotrypsin which had been
preincubated with 5% of its weight of soybean trypsin inhibitor. The digestion mixture was incubated at 37° for 10 hours. After 6 hours, additional chymotrypsin and soybean trypsin inhibitor were added in their original proportions. After lyophilization, the material was dissolved in a small volume of water and one-tenth its volume of a pyridine acetic acid buffer (0.50 M:10.4 M) was added. The copious precipitate which formed was removed by centrifugation. The precipitate was extracted twice with small volumes of pyridine acetic acid buffer (0.02 M:1.04 M), pH 3.3, and the washings were added to the original supernatant fluid. This solution was applied to a Sephadex G-25 column equilibrated with the latter buffer. Fractions from the major radioactive peak were pooled, lyophilized, and rechromatographed on the same column. The radioactive peak was lyophilized and dissolved in a pyridine acetate buffer (0.20 M:4.8 M), adjusted to pH 1.8 with HCl, and applied to a column (0.9 X 60 cm) of Dowex 50W-X2 (200 to 400 mesh) equilibrated with the latter pH buffer and thermostatted at 37°. The peptide was eluted with a linear gradient of a concentrated pyridine acetate buffer (2.0 M:2.4 M), pH 5.0 (500 ml). The peak radioactive fractions were pooled and lyophilized. The peptide was found pure based on amino acid analysis and migration on high voltage electrophoresis.

Methods—Hydrolyses of peptides were performed in vacuo with glass-distilled acetic acid 5.7 n HCl. Samples were incubated at 108° for 20 hours in a refluxing bath of toluene. After hydrolysis, the hydrolysate was taken to dryness and dissolved in citrate buffer used for amino acid analyses. Amino acid analyses were performed on a Beckman 12013 updated analyzer. No correction was made for the degradative loss of serine during the hydrolysis period. High voltage electrophoresis was performed on Whatman No. 1MM or 3MM paper at pH 4.0 with a Gilson Model D high voltage electrophorater.

Dephosphorylation of the [32P]phosphopeptides by phosphorylase phosphatase was measured at 37° in 50 mM Tris-acetate and 5 mM dithiothreitol, pH 7.5. The sample volume varied according to the specific radioactivity of the peptide. Normally an assay of 0.15 ml was used with product formation amounting to 10 to 15% of the substrate utilized. Linear reaction rates could be demonstrated for 15 min under these conditions. The reaction was terminated by the addition of 0.5 ml of pyridine acetate buffer, pH 3.1, 10 mM in phosphate. The reaction contents were transferred into Pasteur pipettes containing approximately 1 g of Dowex 50 resin which had been equilibrated with pyridine acetate buffer, pH 3.1. There 0.5-ml washes and one 10-ml wash removed all of the "P1" which was liberated. The eluate was collected into a scintillation vial, and 15 ml of Bray's solution (16) were added prior to counting.

Dephosphorylation of [32P]phosphorylase a was measured by modification of the direct method described by Hurd et al. (15). [32P]Phosphorylase a was incubated at 37° in 50 mM Tris-acetate and 5 mM dithiothreitol, pH 7.5, with phosphorylase phosphatase. Linear reaction rates could be demonstrated for 15 min, and product formation was limited to 10 to 15% of the total radioactivity. The reaction was stopped by the addition of 0.50 ml of a solution containing 50 mg per ml of bovine serum albumin, 0.4 M β-glycerol phosphate, pH 5.8. Trichloroacetic acid solution (50%), 0.50 ml, was then added, the assays were placed on ice, and the samples were diluted to 3.50 ml with a carrier phosphate solution. After centrifugation at 4°, the supernatant fluids were decanted and 3.0 ml transferred to a scintillation vial for counting in Bray's solution (16).

Phosphorylases a and b concentrations were determined spectrophotometrically with the use of absorbance indices of 1.27 and 1.32, respectively, for a 1.0 mg per ml solution (17).

RESULTS

Mechanism of Inhibition by Inorganic Phosphate, Glucose-1-P, and Phosphorylated Esters—Inorganic phosphate inhibits the dephosphorylation of phosphorylase a. Since phosphorylase a binds phosphate, a substrate, it might be expected that the inhibition was substrate-directed. However, since the substrate of the phosphatase is a phosphate ester, it would be expected that a binding site for phosphate exists on the phosphatase so that inhibition could result from competition with the substrate-binding site. If both mechanisms operate, inhibition would be expected to be different with phosphorylase a than with the phosphorylated tetradecapeptide as the substrate. The results of Fig. 1A show that phosphate is a competitive inhibitor with respect to phosphorylase a. The Dixon plot (Fig. 1B) is linear, characteristic of simple competitive inhibition, and a K_i of 8 mM was obtained. Inhibition with respect to the phosphopeptide was also found competitive (Fig. 2) and a K_i of 6 mM was found. Since in each case the kinetics of the simple competitive type and the K_i values are similar, it would appear that the inhibition is solely enzyme-directed and phosphate binding to phosphorylase a does not alter its characteristics as a substrate.

Fig. 3 illustrates kinetic data of the inhibition by glucose-1-P. It can be seen that the inhibition conforms to that of partial competitive inhibition when phosphorylase a is used as the substrate. Surprisingly, when the phosphopeptide was utilized as a substrate, no inhibition was observed by glucose-1-P even at concentrations several-fold greater than that necessary to produce significant inhibition with the native substrate. The kinetic data support an inhibition mechanism whereby the binding of glucose-1-P to phosphorylase a results in the formation of an S1 complex which is less tightly bound to the phosphatase when compared to phosphorylase a alone but yet is converted to product at the same rate (Mechanism 1-C) (9). Other phosphate esters such as β-glycerol-P and serine phosphate were inhibitory at much higher concentrations than glucose-1-P with phosphorylase a as substrate. Glycerol-P caused mixed inhibition and nonlinear convex Dixon plots. With the phosphotetradecapep-

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

**Fig. 1.** Kinetics of phosphate inhibition of the dephosphorylation of phosphorylase a by phosphorylase phosphatase. A, Line-Weaver-Burk plot with respect to [32P]phosphorylase a at 0 (●), 5 (●), 10 (■), and 15 (▲) mM phosphate. B, Dixon plot with respect to phosphate of data from A at phosphorylase a concentrations of 1.0 (●), 0.33 (○), 0.20 (■), 0.14 (▲) and 0.042 (●) mg per ml.
Mechanism of Inhibition by Divalent Metal Ions—The inhibition of phosphorylase phosphatase by divalent metal ions was demonstrated by Keller and Cori (7). The type of inhibition was not determined. Fig. 4 shows that inhibition by Mg²⁺ or Mn²⁺ is competitive with respect to phosphorylase a. The convex character of the Dixon plot suggests that another locus of inhibition exists. One site could be on phosphorylase phosphatase and a second on phosphorylase a, as it has been demonstrated that phosphorylase contains a metal-binding site (18). To simplify the interpretation, experiments were also done with the phosphopeptide substrate. Fig. 5 shows that Mg²⁺ gives simple competitive kinetics with respect to the peptide substrate. A value of approximately 40 mM was obtained for $K_i$. Due to the complex nature of inhibition kinetics by Mg²⁺ ion when phosphorylase a is utilized as a substrate, a meaningful $K_i$ cannot be obtained directly from the data. Yet, inspection of the inhibition shows that Mg²⁺ concentrations which inhibit the dephosphorylation are nearly one order of magnitude less than those necessary to inhibit the dephosphorylation of the peptide. The results can be rationalized if Mg²⁺ binding to phosphorylase phosphatase causes inhibition with a $K_i$ of 40 mM and Mg²⁺ binding to phosphorylase causes inhibition with a $K_i$ of approximately 4 mM. This is consistent with the predictions of the inhibition mechanism II-C or II-D described in Reference 9.

It is well known that many phosphatases contain a metal ion at their active center. It has not been demonstrated that phosphorylase phosphatase contains a divalent cation but yet inhibition by metal ions could be explained by a competition for...
this site with some unknown essential metal ion. Also EDTA inhibition (7) might be explained by removal of an essential cation, but some experiments suggest that inhibition by EDTA is not a property of its metal-binding capacity (7). Fig. 6 shows that EDTA is a simple competitive inhibitor with respect to the phosphopeptide substrate. To ascertain whether inhibition by EDTA and divalent metal ions could be explained by competition for this alternative substrate was approximately one order of magnitude may be bound to the substrate. A direct approach of using magnesium ion and EDTA in inhibition studies could not be undertaken, however, because of the formation of magnesium-EDTA complexes.

The competitive inhibitor, m-propoxybenzamidine (Fig. 7), was used in experiments with divalent metal ions and in experiments with EDTA. Yonetani and Theorell (19) demonstrated a method to distinguish whether inhibition by EDTA is a simple competitive inhibitor with respect to the phosphopeptide substrate. To ascertain whether inhibition by EDTA and divalent metal ions could be explained by competition for a single site on the enzyme, kinetic studies were carried out in the presence of two competitive inhibitors. A direct approach of using magnesium ion and EDTA in inhibition studies could not be undertaken, however, because of the formation of magnesium-EDTA complexes.

The competitive inhibitor, m-propoxybenzamidine (Fig. 7), was used in experiments with divalent metal ions and in experiments with EDTA. Yonetani and Theorell (19) demonstrated a method to distinguish whether two competitive inhibitors acted at the same, adjacent, or independent sites. From the rate equations a plot of 1/v versus $I_1$ at different levels of $I_2$ will have a constant slope when $I_1$ and $I_2$ bind at the same site. If $I_1$ and $I_2$ bind at different sites, the slope will vary as a function of $I_2$.

![Fig. 6. Kinetics of EDTA inhibition of phosphorylase phosphatase. A, Lineweaver-Burk plot with respect to [32P]tetradecapeptide at 0 (●), 5 (▲), 10 (■), and 25 (▲) mM EDTA. B, Dixon plot with respect to EDTA of data from A at 0.67 (●), 0.22 (▲), 0.13 (■), and 0.095 (▲) mM [32P]tetradecapeptide.](image)

At different levels of $I_2$ will have a constant slope when $I_1$ and $I_2$ bind at the same site. If $I_1$ and $I_2$ bind at different sites, the slope will vary as a function of $I_2$.

![Fig. 7. Kinetics of m-propoxybenzamidine inhibition of phosphorylase phosphatase. A, Lineweaver-Burk plot with respect to [32P]tetradecapeptide at 0 (●), 1.0 (▲), 2.5 (■), and 5.0 (▲) mM. B, Dixon plot with respect to the substituted benzamidine of data from A at 0.67 (●), 0.22 (▲), 0.13 (■), and 0.095 (▲) mM [32P]tetradecapeptide.](image)

At different levels of $I_2$ will have a constant slope when $I_1$ and $I_2$ bind at the same site. If $I_1$ and $I_2$ bind at different sites, the slope will vary as a function of $I_2$.

For these studies shown in Fig. 9 were undertaken. The inhibition conforms to the simple competitive case. These data suggest that inhibition by AMP operates by the formation of an SI complex that cannot be bound by the phosphatase which corresponds to Case I-A described in the "Appendix" (9). Confirmation for this mechanism was provided by the use of another alternative substrate, phosphorylase a, which had the AMP site irreversibly occupied by an adenine derivative (20) (Fig. 10). This substrate, whose properties resemble those of a phosphorylase AMP complex (21), was dephosphorylated very poorly in comparison to native phosphorylase a. Kinetic studies demonstrated that a $K_m$ value for this alternative substrate was approximately one order of magnitude lower than the native enzyme.

$^1$AMP concentrations are expressed as total amounts, although at the two highest concentrations of phosphorylase, a significant amount may be bound to the substrate.
that the mechanism of AMP inhibition involves the production of a tetramer which is unable to combine with the phosphatase and become dephosphorylated. Yet a considerable amount of evidence argues against this interpretation. Wang and Graves (24) and de Vincenzi and Hedrick (25) have shown that AMP promotes the dissociation of tetrameric a to dimers. It was suggested by de Barby et al. (26) that AMP inhibition could not involve tetramer formation, since the nucleotide caused dissociation of a 2-methylanilino-naphthalene-6-sulfonate phosphorylase a complex and inhibited the dephosphorylation of liver phosphorylase a which is dimeric. Bailey and Whelan (27) have demonstrated that phosphorylase a in the presence of AMP at 35 °C sediments solely as a dimeric species. The kinetics we have obtained suggests that binding of AMP to dimeric phosphorylase a produces a dimeric species which is much less tightly bound, if it is bound at all, by phosphorylase phosphatase. The $K_2$ obtained for this model (9) should be equivalent to the $K_d$ for AMP binding to phosphorylase a. The $K_2$ that was obtained ranged from 5 to 10 μM and is in agreement with $K_d$ values obtained from kinetic studies at the same temperature (28).

The inhibition of phosphorylase phosphatase by divalent metal ions and EDTA is suggested in this work to be due to binding at different sites on phosphorylase phosphatase. First, divalent metal ions were found to compete with m-propoxybenzamidine. Earlier studies (29) showed that arginine and lysine ethyl esters were competitive inhibitors with respect to phosphorylase a. This result, along with the effect of removal of arginyl residues from peptide substrates (2, 5), suggests that phosphorylase phosphatase has an absolute requirement for a positively charged function on the substrate for binding and catalysis. We feel that the most direct way to interpret the competitive kinetics between metal ions and m-propoxybenzamidine is for an interaction at a site on phosphorylase phosphatase that normally accommodates an arginyl residue. EDTA and m-propoxybenzamidine do not compete with each other and suggest that EDTA inhibition is due to some different kind of interaction. From our experiments and others with EDTA and other chelating agents (7), there is no convincing evidence which suggests that phosphorylase phosphatase contains an enzyme-bound metal that is essential for catalysis. Our kinetic analyses were done with the phosphopeptide substrate, as more complex kinetics might be expected with phosphorylase a due to binding of the inhibitors to both the substrate and the enzyme. Thus, in addition to the use of an alternative substrate in elucidating metabolic control, it has advantages for mechanistic studies.

Different effects of inorganic phosphate and glucose-1-P were reported. Although phosphorylase a binds phosphate, its influence upon the structure is dependent upon certain conditions. Orthophosphate promotes an allosteric transition enhancing AMP binding to phosphorylase a in glycerophosphate buffer. However, this effect is not very significant unless glycogen is present, whereas in imidazole buffer the promotion is significant whether glycogen is present or absent (30). Our data suggest that inhibition of the phosphatase reaction by phosphate can be attributed solely to its effect on the phosphatase, since inhibition studies utilizing the native and alternative substrate yield simple competitive kinetics with similar inhibition constants. This result need not be interpreted as evidence that phosphate does not affect the structure of phosphorylase a in Tris buffer, only that a conformational change that may occur is ineffective in altering the structure of the substrate to dephosphorylation. Glucose-1-P has no effect on dephosphorylation of the phosphopeptide, but it does influence the reaction with phosphorylase a.

**DISCUSSION**

The substrate for phosphorylase phosphatase, phosphorylase a, can exist as a dimer or a tetramer (22), and inhibition caused by a substrate-directed effect could be due to a change in conformation, equilibrium position, or both. To simplify interpretation of the inhibition data, all experiments were carried out under conditions of buffer (Tris-acetate), temperature (37°C), and pH (7.5) where phosphorylase a showed one peak with a $\delta_{20, w}$ corresponding to the dimeric species.3

Bot and Döös have suggested that the tetrameric form of phosphorylase a is unable to be dephosphorylated by phosphorylase phosphatase (23). On this basis, it could be argued that the mechanism of AMP inhibition involves the production of a tetramer which is unable to combine with the phosphatase and become dephosphorylated. Yet a considerable amount of evidence argues against this interpretation. Wang and Graves (24) and de Vincenzi and Hedrick (25) have shown that AMP promotes the dissociation of tetrameric a to dimers. It was suggested by de Barby et al. (26) that AMP inhibition could not involve tetramer formation, since the nucleotide caused dissociation of a 2-methylanilino-naphthalene-6-sulfonate phosphorylase a complex and inhibited the dephosphorylation of liver phosphorylase a which is dimeric. Bailey and Whelan (27) have demonstrated that phosphorylase a in the presence of AMP at 35°C sediments solely as a dimeric species. The kinetics we have obtained suggests that binding of AMP to dimeric phosphorylase a produces a dimeric species which is much less tightly bound, if it is bound at all, by phosphorylase phosphatase. The $K_2$ obtained for this model (9) should be equivalent to the $K_d$ for AMP binding to phosphorylase a. The $K_2$ that was obtained ranged from 5 to 10 μM and is in agreement with $K_d$ values obtained from kinetic studies at the same temperature (28).

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Different effects of inorganic phosphate and glucose-1-P were reported. Although phosphorylase a binds phosphate, its influence upon the structure is dependent upon certain conditions. Orthophosphate promotes an allosteric transition enhancing AMP binding to phosphorylase a in glycerophosphate buffer. However, this effect is not very significant unless glycogen is present, whereas in imidazole buffer the promotion is significant whether glycogen is present or absent (30). Our data suggest that inhibition of the phosphatase reaction by phosphate can be attributed solely to its effect on the phosphatase, since inhibition studies utilizing the native and alternative substrate yield simple competitive kinetics with similar inhibition constants. This result need not be interpreted as evidence that phosphate does not affect the structure of phosphorylase a in Tris buffer, only that a conformational change that may occur is ineffective in altering the structure of the substrate to dephosphorylation. Glucose-1-P has no effect on dephosphorylation of the phosphopeptide, but it does influence the reaction with phosphorylase a.
We interpret the kinetic data to be related to the formation on an SI complex which is poorly bound to the phosphatase. The lack of inhibition of glucose-1-P at the concentrations tested with the peptide substrate in comparison with phosphate could be explained by a steric effect.

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