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# REGULATION OF SKELETAL MUSCLE PHOSPHORYLASE PHOSPHATASE ACTIVITY

## I. KINETIC PROPERTIES OF THE ACTIVE AND INACTIVE FORMS

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SUMMARY

1. The inactivation of phosphorylase *a* phosphatase decreased the maximum velocity of the phosphorylase *a* to phosphorylase *b* conversion reaction when it was assayed at different phosphorylase *a* concentrations.

2. Maximal phosphorylase *a* phosphatase activities were found between pH 8 and 8.3. Inactivation of the phosphorylase *a* phosphatase led to a decrease in the activity in all the pH ranges tested.

3. Theophylline and caffeine stimulated the phosphorylase *a* phosphatase. The effect of these substances was exerted in the reaction assay of the enzyme.

4. ATP, ADP, AMP, GTP, UTP, CTP and pyrophosphate were found to decrease the rate of the reaction catalyzed by phosphorylase *a* phosphatase. This effect showed a striking parallelism with the capacity of these compounds to stimulate the phosphatase inactivation.

## INTRODUCTION

Muscle phosphorylase *a* phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17) catalyzes the conversion of phosphorylase *a* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) to phosphorylase *b* according to the following equation:

$$\text{phosphorylase } a \xrightarrow{H_2O} 2 \text{ phosphorylase } b + 4\ P_i$$

In this reaction the phosphate, bound to a seryl residue in each phosphorylase subunit, is hydrolyzed[1–3].

Evidence obtained in experiments carried out *in vivo* and *in vitro* indicates that the product of the phosphorylase *a* phosphatase reaction, *i.e.* phosphorylase *b*, is

almost inactive at the metabolite concentrations measured in the muscle cells[4,5]. Under these conditions, it could be expected that a stimulation in the rate of phosphorylase *a* to *b* conversion through an increase in the phosphatase activity could reduce the rate of phosphorolysis of glycogen.

The regulation of glycogen metabolism in mammalian cells has been studied in some detail. It seems that phosphorylase *b* kinase and glycogen synthetase are the two enzymes of the glycogen-metabolizing system subject to hormonal control. This supposition is supported by evidence from this and other laboratories[6–10]. Another kind of regulation was, however, demonstrated by RILEY and co-workers[11,12] in adrenal cortex. In this tissue, they found a control step at the level of phosphorylase *a* phosphatase activation.

In a preliminary report, evidence was given indicating that muscle phosphorylase phosphatase exists in two interconvertible forms[13]. In the present paper, experiments are reported which were designed to find the kinetic properties that differentiate each form of the phosphatase.

## EXPERIMENTAL PROCEDURES

Phosphorylase phosphatase activity was assayed using $^{32}$P-labeled rabbit-muscle phosphorylase *a* as substrate, following the liberation of trichloroacetic acid-soluble radioactive phosphate. The labeled phosphorylase was prepared by enzymatic phosphorylation. A mixture containing 1 ml of crystalline rabbit-muscle phosphorylase *b* (60 000 Cori units), 0.015 ml of 1 M mercaptoethanol, 0.2 ml of 1 M Tris–HCl buffer (pH 8.6), 0.1 ml of 1 M magnesium acetate, 1.2 ml of $^{32}$P-labeled ATP ($5 \cdot 10^9$ counts/min, specific activity 1 mC/mmole), and 0.2 ml of rabbit-muscle phosphorylase *b* kinase (11.4 mg protein per ml) was incubated for 20 min at 30°. The reaction was stopped by the addition of 3 ml of a solution containing 100 mM NaF, 80 mM glycerophosphate buffer (pH 6.8), 40 mM mercaptoethanol, and 20 mM EDTA. After the addition of 0.05 ml of 100 mM ATP and 0.05 ml of 1 M phosphate buffer (pH 7.0), the mixture was cooled in ice for 60 min. The crystalline labeled phosphorylase *a* was collected by centrifugation at 10 000 × *g* for 15 min. The supernatant was discarded and the precipitate was resuspended in 10 ml of a solution containing 50 mM NaF, 40 mM glycerophosphate buffer (pH 6.8), 10 mM EDTA, and 20 mM mercaptoethanol plus 0.05 ml of 100 mM ATP and 0.05 ml of 1 M phosphate buffer (pH 7.0). The mixture was shaken at 37° until the phosphorylase was dissolved and then it was left to crystallize at 0° for 60 min. After 5–8 crystallizations the precipitate of crystalline labeled phosphorylase *a* was resuspended in 5 ml of a solution containing 5 mM glycerophosphate buffer (pH 6.5), 10 mM mercaptoethanol, and 1 mM EDTA *plus* ATP and phosphate buffer as indicated above, and dialyzed against the same solution except that ATP and phosphate buffer were omitted. After 6–16 h more ATP and phosphate buffer were added into the dialysis bag and the dialysis medium was renewed. The addition of ATP and the phosphate buffer and the change of the dialysis medium were repeated 3–5 times. Finally, the enzyme was dialyzed against the same solution, omitting the addition of ATP and phosphate buffer to the dialysis bag. In a typical preparation, 3 ml of labeled phosphorylase *a* were obtained with the following characteristics: enzymatic activity, 14 000 Cori units/ml; specific activity, 5000 counts/min per Cori unit; ratio of bound to unbound radioactive phosphate (5%

trichloroacetic acid-insoluble radioactivity/5% trichloroacetic acid-soluble radioactivity), 138.

$^{32}$P-Labeled ATP was prepared according to LOWENSTEIN[14] with some modifications. Ten $\mu$moles of ADP and 10 mC of carrier-free radioactive phosphate were converted into the pyridinium salt by passing through a 0.8 × 10-cm Dowex 50 column (X4, 20–50 mesh, $H^+$ form) and adding pyridine in excess to the percolate. The mixture was dried in a rotatory evaporator and mixed with 0.45 ml of pyridine, 0.05 ml of water and 100 mg of dicyclohexylcarbodiimide in a heavy-wall glass centrifuge tube. After addition of a glass bead (3-mm diameter), the tube was plugged with a rubber stopper covered with aluminum foil and was shaken for 16 h at room temperature in a Griffin flask shaker. The reaction was stopped by the addition of 10 ml of water, and the mixture was filtered through a funnel plugged with cotton. The filtrate was concentrated on a rotatory evaporator and was then spread in a 10-cm band on washed Whatman 3 MM paper. Chromatography was carried out on ethanol–ammonium acetate at pH 7.2 (ref. 15) for 20 h. The ammonium acetate was washed out with absolute ethanol and the radioactive band which had a mobility like that of ATP was eluted with water. Usually, 60% of the radioactive phosphate was recovered as ATP.

The rabbit-muscle acid precipitate used as the source of phosphorylase *b* kinase was prepared as previously indicated[16]. Crystalline rabbit-muscle phosphorylase *b* was prepared as described by FISCHER AND KREBS[17]. The mixture for the assay of phosphorylase phosphatase contained 0.02 ml of enzyme sample (diluted in 10 mM mercaptoethanol, 5 mM EDTA, and 40 mM glycerophosphate buffer of pH 6.8) and 0.01 ml of $^{32}$P-labeled phosphorylase *a* (48 Cori units; 1000–3000 counts/min per Cori unit). Incubations were carried out at 30° for 5 min. Reactions were stopped by the addition of 1 ml of 5% trichloroacetic acid. After centrifugation at 3000 rev./min in a clinical centrifuge for 30 min, the supernatant was transferred to a glass vial containing 0.04 ml of 60% KOH. After the addition of 5 ml of BRAY solution[18] the radioactivity was measured in a liquid scintillation spectrometer. The radioactive phosphate liberated during the incubation was expressed in terms of consumed phosphorylase *a* according to the following equation:

$$\text{pmoles of phosphorylase } a \text{ consumed per unit of time} = \frac{\text{liberated counts/min}}{\text{initial counts/min}} \times \frac{\text{P}a}{t \cdot k}$$

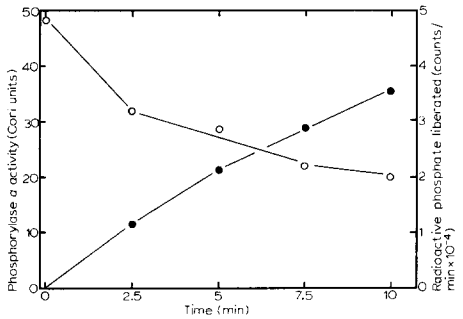
$t$, incubation time (5 min); P $a$, initial phosphorylase *a* activity in the assay mixture (48 units); $k$, Cori units per pmole of phosphorylase *a* (0.93); this value was estimated assuming a specific activity of the phosphorylase *a* of 2515 Cori units/mg (ref. 19) and a molecular weight of 370 000 (ref. 20).

Pigeon breast muscle phosphorylase phosphatase was prepared as described in the following paper[21].

## RESULTS

### *Conversion of $^{32}$P-labeled phosphorylase a to phosphorylase b*

The conversion of phosphorylase *a* to phosphorylase *b* may be followed either by the disappearance of phosphorylase *a* activity or by the release of radioactive inorganic phosphate. As can be seen in Fig. 1, there is a good correlation between the



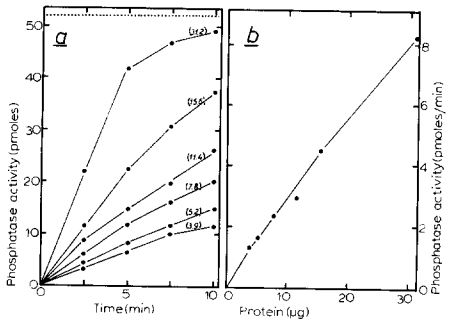




Fig. 1. Correlation between the change of enzymatic activity and the liberation of trichloroacetic acid-soluble radioactive phosphate during the incubation of a pigeon breast muscle phosphorylase phosphatase with $^{32}$P-labeled phosphorylase *a*. A "crude preparation" was obtained from a pigeon breast muscle homogenate that was not incubated at 37°. The enzyme was diluted in 40 mM glycerophosphate buffer (pH 6.8), containing 5 mM EDTA and 10 mM mercaptoethanol, to give a concentration of 0.78 mg protein/ml. Incubations were carried out as described under EXPERIMENTAL PROCEDURES in the presence of 1.25 mM theophylline. Reactions were stopped by the addition either of 1 ml of 5% trichloroacetic acid (radioactive assay) or 2.5 ml of 40 mM glycerophosphate buffer (pH 6.8), containing 50 mM NaF, 10 mM EDTA and 20 mM mercaptoethanol (enzymatic assay). Radioactivity (●—●) was counted on the trichloroacetic acid supernatant as described under EXPERIMENTAL PROCEDURES. Phosphorylase *a* assays (○—○) were carried out in the absence of AMP with the method of CORI *et al.*[19].

Fig. 2. Relation between time, enzyme concentration and phosphorylase phosphatase activity of a crude preparation of pigeon breast muscle. *a*. Time-course of the reactions carried out in assay mixtures containing the µg of protein indicated in numbers in parentheses. *b*. Dependence on enzyme concentration of the phosphatase activity. Enzymatic activities corresponding to the incubation carried out for 5 min were plotted as a function of the µg of protein in the phosphatase assay. Other conditions were as indicated in Fig. 1.

radioactive phosphate liberated and the decrease of phosphorylase *a* activity in the standard assay. Fig. 2 shows that the release of radioactivity from the $^{32}$P-labeled phosphorylase *a* is proportional to the incubation time and to the concentration of the enzyme.

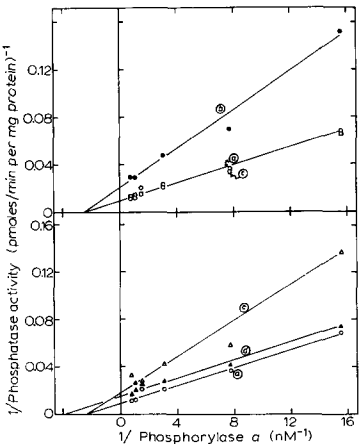
*Properties of the $^{32}$P-labeled phosphorylase a to phosphorylase b conversion*

*Substrate dependence*

Fig. 3 shows the reciprocal plots of the initial velocities of the reaction catalyzed by muscle phosphorylase phosphatase, against $^{32}$P-labeled phosphorylase *a* concentration. The inactivation of phosphorylase phosphatase decreased the maximum velocity; no appreciable change in the apparent $K_m$ for $^{32}$P-labeled phosphorylase *a* was observed (Curves *a* and *b*). At pH 6.8, in the presence of 0.45 mM theophylline, the value for the apparent Michaelis constant was $0.25 \cdot 10^{-6}$ M of $^{32}$P-labeled phosphorylase *a* (230 Cori units/ml). When the inactivated phosphatase was reactivated, an enzymatic preparation was obtained which behaved as the initial partially active phosphatase (Curve *c*).

*pH curves*

The pH-dependence of the phosphatase reaction is shown in Fig. 4. In the presence of 0.45 mM theophylline, no major differences were evident between an active phosphatase preparation and an inactivated-reactivated enzyme. Maximum activities were found between pH 8.0 and 8.3 (Curves *a* and *b*). Inactivation of the



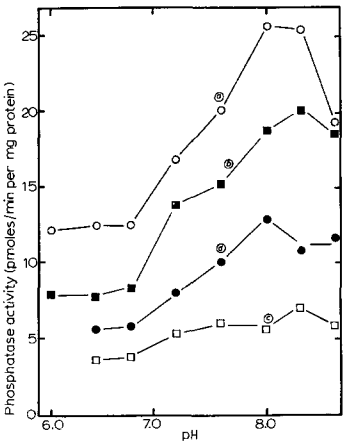




Fig. 3. Effect of phosphorylase *a* concentration on the activity of muscle phosphorylase phosphatase. In the experiments corresponding to Curves *a*, *d* and *e*, the enzyme was a crude preparation obtained from a homogenate that was not incubated at 37° (active enzyme). In Curve *b* the enzyme was a crude preparation obtained from a homogenate treated for 40 min at 37° (inactive enzyme). This enzyme preparation (0.25 ml) was further incubated with 10 mM mercaptoethanol, 6.7 mM theophylline, 2.5 mM ATP–$MgCl_2$, and 5 mM phosphocreatine–$MgCl_2$ for 5 min at 37° in a total volume of 0.3 ml. The reaction was stopped by the addition of 2.7 ml of a solution containing 40 mM glycerophosphate buffer (pH 6.8), 5 mM EDTA and 10 mM mercaptoethanol (reactivated enzyme). Curve *c* represents the experiment carried out with that enzyme. Before the assay all the enzymatic preparations were diluted in the glycerophosphate–EDTA–mercaptoethanol solution, and aliquots of these dilutions were again passed through Sephadex G-25 columns equilibrated with 10 mM NaCl containing 5 mM EDTA and 10 mM mercaptoethanol. After that, the pH of the samples was adjusted to 6.8. Incubations for phosphatase activity were carried out as indicated under EXPERIMENTAL PROCEDURES except that the total volume was 0.04 ml with the following additions: Curves *a*, *b* and *c*, 0.45 mM theophylline; Curve *e*, 0.45 mM theophylline *plus* 2.5 mM ATP; and Curve *d*, no additions.

Fig. 4. Phosphatase activity as a function of the pH. Curves *a* and *d*, active enzyme; Curve *c*, inactive enzyme; and Curve *b*, reactivated enzyme. The phosphatase assay mixtures were made up containing a final concentration of 50 mM glycerophosphate and 50 mM Tris buffer at the indicated pH. Incubations were carried out in the presence (Curves *a*, *b* and *c*) or absence (Curve *d*) of 0.45 mM theophylline. Other conditions were as those described in Fig. 3.

phosphatase led to a decrease in the activity at all the pH ranges tested (Curve *c*).

*Effect of theophylline*

In order to inhibit cyclic phosphodiesterase activity in the phosphorylase phosphatase preparation, theophylline was added as a standard component of the activation and inactivation reaction mixtures. It can be seen in Fig. 5 of this paper and Table I (Expts. I and II) of the following one[21], that theophylline stimulated the phosphatase and that the effect of this substance was exerted in the reaction assay of the enzyme.

Some insight into the nature of the phosphatase activation by theophylline was obtained by comparing the activity of the enzyme measured at different concentrations of $^{32}P$-labeled phosphorylase *a* in the presence and absence of theophylline. Fig. 3 (Curves *a* and *d*) shows that theophylline increases both the maximum velocity and the apparent Michaelis constant of the enzyme for $^{32}P$-labeled phosphorylase *a* in such a manner that the reciprocal plots of the experiments carried out without the modifier were roughly parallel. On the other hand, activation by theophylline was

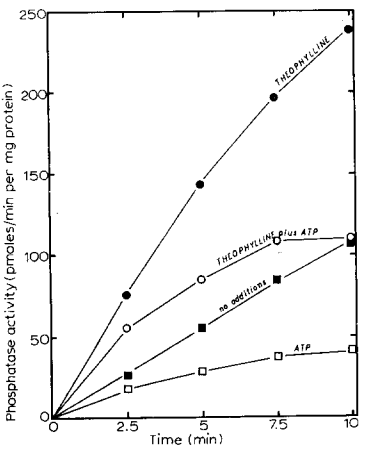
TABLE I

EFFECT OF DIFFERENT NUCLEOTIDES AND OTHER METABOLITES ON THE ACTIVITY OF MUSCLE PHOSPHORYLASE PHOSPHATASE

An "$(NH_4)_2SO_4$ preparation" was obtained from a homogenate that was not incubated at 37°. The enzyme was diluted in 40 mM glycerophosphate buffer (pH 6.8) containing 5 mM EDTA and 10 mM mercaptoethanol and was assayed in the presence of 0.45 mM theophylline. In addition, the assay mixtures contained the indicated nucleotides and metabolites at the final concentration of 5 mM. Phosphorylase phosphatase activity was assayed as described under EXPERIMENTAL PROCEDURES.

| *Additions* | *Activity (pmoles/min per mg protein)* |
|---|---|
| None | 53 |
| ATP | 2 |
| ADP | 0 |
| AMP | 1.3 |
| UTP | 7 |
| CTP | 9.4 |
| GTP | 2.7 |
| Carbamyl phosphate | 33 |
| Creatine phosphate | 38 |
| Pyrophosphate | 2.4 |
| Phosphate | 39 |

evident at all the pH's tested in the range between 6.1 and 8.6 (Fig. 4, Curves *a* and *d*). Activation of the phosphatase was also observed with caffeine. As can be seen in Fig. 6 both methylxanthines stimulated the enzyme to a similar extent. The optimum effect was found at 10 mM.



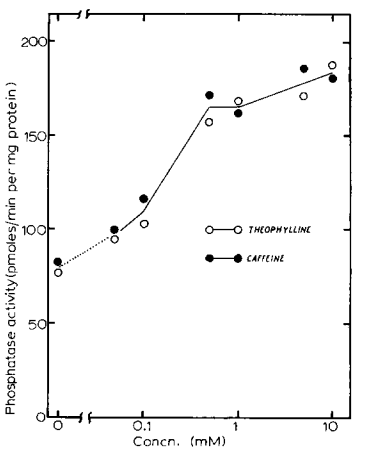




Fig. 5. Effect of ATP and/or theophylline in the time-course of the phosphatase reaction. The final concentration of ATP and/of theophylline was 3.1 and 1.25, respectively. Other conditions were as those described in Fig. 1.

Fig. 6. Effect of different concentrations of theophylline or caffeine on the activity of muscle phosphorylase phosphatase. The reaction mixture for the phosphatase assay contained the indicated concentrations of theophylline or caffeine. Other conditions were as those indicated in Fig. 4.

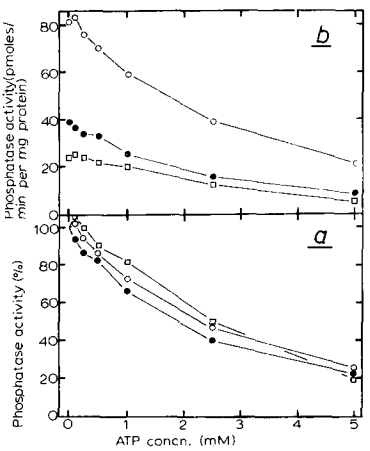
*Effect of ATP and other modifiers*

Several substances were found to decrease the rate of the reaction catalyzed by phosphorylase phosphatase. At 5 mM ATP, ADP, AMP, GTP, UTP, CTP and pyrophosphate were all effective. $P_i$, carbamyl phosphate and creatine phosphate also decreased the rate of the reaction but only slightly (Table I).

The effect of ATP was studied in more detail. As can be seen in Fig. 3 (Curve *e*), in the presence of 0.45 mM theophylline, the nucleotide decreases the maximum velocity of the reaction, when the enzyme was assayed at high concentrations of $^{32}$P-labeled phosphorylase *a*. No appreciable change in the apparent Michaelis constant for $^{32}$P-labeled phosphorylase *a* was observed. The ATP effect seemed to be independent of the degree of inactivation of the phosphatase preparation (Fig. 7a) and increased with the concentration of this metabolite (Fig. 7b).

Some facts indicate the complex nature of the ATP effect. Indeed, the rate of the phosphatase reaction carried out in the presence of the metabolite declines sharply with the incubation time (Fig. 5). This suggests that in the presence of ATP and in the conditions of the assay, dilute preparations of phosphorylase phosphatase can be converted to the inactive form in a time-dependent reaction.

On the other hand the experiments shown in Table I and Fig. 7 of this paper and Table IV and Fig. 7 of the following one[21], clearly evidence a striking parallelism between the capacity of a given compound to stimulate the phosphatase inactivation and the corresponding capacity of the same compound to decrease the rate of the phosphatase reaction. A comparison between these two effects is shown in Fig. 8.



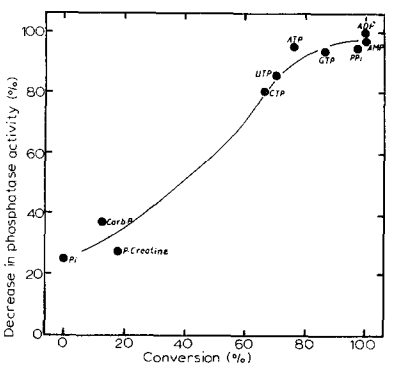




Fig. 7. Effect of different concentrations of ATP on the activity of muscle phosphorylase phosphatase. The experiment was carried out with crude preparations obtained from a homogenate that was not incubated at 37° (○—○) or homogenates that were incubated 20 min (●—●) or 40 min (□—□) at 37°, respectively. The reaction mixtures for the phosphatase assay contained the indicated concentrations of ATP *plus* 0.45 mM theophylline. Other conditions were as those indicated in Fig. 3.

Fig. 8. Correlation between the ability of a given metabolite to decrease the rate of the phosphatase activity in the assay mixture and the capacity of the same metabolite to stimulate the conversion of the active to the inactive form of the enzyme. The results of the experiments showed in Table I of this paper and Table IV of the following one[21] were normalized and used in the construction of the curve. Both experiments were carried out with the same enzymatic preparation.

## DISCUSSION

The main difference between the active and inactive forms of pigeon breast muscle phosphorylase phosphatase appears to be the maximal velocity measured at saturating levels of its substrate, phosphorylase *a*. No changes were found in the affinity for phosphorylase *a* after the activation or the inactivation of the phosphatase. If these conversions have some physiological significance *in vivo*, it seems evident that phosphatase regulation might operate through the change of the maximum velocity, since physiologically the enzyme appears to work at a concentration of phosphorylase *a* above the half saturation point. Indeed, pigeon breast muscle contains about 3000 Cori units per g of wet tissue, calculated as total phosphorylase activity, and the value for the apparent Michaelis constant for phosphorylase *a* (as substrate of the phosphatase) was about 230–380 Cori units/ml ($0.25 \cdot 10^{-6}$–$0.40 \cdot 10^{-6}$ M). This value is about 10 times lower than that reported by HURD *et al.*[24] for a 2000-fold purified phosphorylase phosphatase preparation from rabbit muscle.

Several substances have been found to decrease the phosphatase-catalyzed reaction. An inhibitory effect of ATP and AMP on phosphatase activity was previously reported by HURD *et al.*[24]. The evidence obtained in this paper and in the following one indicates that the effect of these metabolites could be due to a time-dependent inactivation of the enzyme during the assay of the phosphatase.

Another interesting property of skeletal muscle phosphorylase phosphatase is the activation by methylxanthines. The results obtained on this point corroborate the observation of WOSILAIT AND SUTHERLAND[22] concerning the liver enzyme and these results expose the possibility that a metabolite modulation of phosphatase activity may play some role in the control *in vivo* of the phosphatase activity. However, efforts to demonstrate a metabolite activation of the phosphatase in the standard assay were unsuccessful. Under these conditions, the reported activation by glucose[23] and glucose 6-phosphate[24] could not be reproduced.

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