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ACTIVATION OF LOW MOLECULAR WEIGHT ACID PHOSPHATASE FROM BOVINE BRAIN BY PURINES AND GLYCEROL

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Summary

Low molecular weight acid phosphatase (orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from bovine brain is activated up to 4-fold by guanosine, guanine, adenine, adenosine, and 6-ethylmercapto-purine. Several pyrimidines and other purines were tested and did not show any activation effect. The rate enhancement induced by purines is uncompetitive and not caused by transphosphorylation to the activator. Using transphosphorylation to glycerol as a probe, it is proposed that the activator binds to one of the phosphorylated intermediates in the reaction pathway. These findings are discussed in terms of the catalytic mechanism of low molecular weight acid phosphatase.

Introduction

Acid phosphatases (orthophosphoric monoester phosphohydrolases (acid optimum), EC 3.1.3.2) from widely diverse origins can be divided in two main classes, high and low molecular weight enzymes [1]. These two classes differ with respect to intracellular distribution [1,2], sensitivity towards SH reagents [1], substrate specificity [3—7] and inhibition by several reagents [8].

High molecular weight acid phosphatase from *Fusarium moniliforme* is inhibited by adenosine, guanosine and cytidine with a kinetic pattern suggesting product inhibition [9]. The inhibition of high molecular weight acid

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† Throughout this paper acid phosphatase, unless specially stated, refers to bovine brain acid phosphatase of low molecular weight [5,8].

phosphatase from prostate by several purines and pyrimidines has been attributed to an allosteric effect [10].

In contradistinction, low molecular weight acid phosphatase from placenta has been shown to be activated by purines [4]. The mechanistic source of this activation was not identified.

As a part of a general objective of delineating the catalytic mechanism and physiological significance of acid phosphatases we have systematically studied the activation of low molecular weight acid phosphatase from bovine brain † by purines. In addition to clarifying the mechanism of purine activation the results presented here confirm and extend previous data for the catalytic pathway of this enzyme.

Materials and Methods

All ragents were analytical grade. Purines, pyrimidines and derivatives were obtained from Sigma Chem. Co. and their purity confirmed by paper chromatography. Paper chromatography of putative phosphorylated products was performed using isobutyric acid/ammonia/water (66 : 1 : 53, by vol.) for 6 h at 27° C. Deionized, twice-distilled water was used throughout.

Acid phosphatase from bovine brain was obtained using a modification of a published procedure [5] (a complete description is found in ref. 8, also to be published elsewhere). The concentrated stock solution of the enzyme (2 mg/ml specific activity 40—50 units/mg) was desalted and freed from dithiothreitol as previously described [11].

Except where indicated, assay for phosphatase activity was routinely performed using *p*-nitrophenyl phosphate (Sigma Chem. Co.) as substrate under the conditions previously described [11]. Additions to this standard system are indicated in the tables or figures.

When riboflavin phosphate was used as substrate, the liberation of P_i was followed using the modification of the Fiske-Subbarow method as previously described [12]. The extent of transphosphorylation to glycerol (Merck, Darmstadt) was estimated measuring *p*-nitrophenoxide and P_i from the same reaction tube [12].

All lines shown in the double reciprocal plots were calculated using a simple linear regression program on a Hewlett-Packard model 10 calculator.

Results

Several purines stimulate acid phosphatase (Table I). Adenine and guanosine also stimulate a partially purified preparation of low molecular weight acid phosphatase isolated from rat liver. The rate increase produced by purines is structure specific (Table I) and at the minimum, either position 2 or 6 of the purine nucleus must be substituted in order to observe a rate increase. Guanosine is a better activator than guanine (1 mM) (saturation curves with guanine were not obtained because of solubility limitations). Phosphorylation of the purine nucleoside leads to a progressive decrease in the extent of activation, resulting in a complete loss of activation with ATP and GTP. This latter phenomenon, which was not further analyzed, might be the result of a mixed

TABLE I

EFFECT OF PURINES, PYRIMIDINE, NUCLEOSIDES AND DERIVATIVES ON THE ACTIVITY OF ACID PHOSPHATASE

Compound [a]	V/V_0 [b]	Compound [a]	V/V_0 [b]
Adenine	1.57	Adenosine	1.14
Guanine	1.50	Guanosine	3.00
6-Mercaptoethyl purine	2.57	GMP	1.60
		GDP	1.20
Xanthine, hypoxanthine,		GTP	1.00
caffeine, 6-bromo-adenosine,		5'-AMP	1.14
cytosine, uracil	1.0	Cyclic AMP	1.00
Tryptophan, histidine,		ATP	1.00
imidazole	1.0	Guanosine [c]	1.57

[a] All compounds were added at final concentration of 1 mM to the standard incubation mixture using *p*-nitrophenyl phosphate (except when indicated) as substrate (see Materials and Methods).
[b] V/V_0 refers to the ratio of rates obtained in the presence or absence of modifier.
[c] Riboflavin phosphate as substrate.

activation-inhibition kinetic scheme. Rate enhancement was also observed using riboflavin phosphate as substrate (Table I), but was smaller than that observed using *p*-nitrophenyl phosphate as substrate. Pyrimidines have no effect on the rate at the concentrations tested.

Since acid phosphatases are known to transfer phosphate from *p*-nitrophenyl phosphate to a series of acceptors [12—14], the rate increase caused by purines and especially nucleosides could potentially arise from transphosphorylation. Chromatography of the reaction products of scaled-up reaction mixtures demonstrated no change in the activator nor appearance of phosphorylated products (in the case of guanosine, adenosine or adenine) after total *p*-nitrophenyl phosphate hydrolysis. Moreover, the *p*-nitrophenol/P_i ratio obtained when *p*-nitrophenyl phosphate is hydrolyzed in the presence of acid phosphatase and guanosine is 1.0 (Table II), thus confirming the absence of

TABLE II

EFFECT OF GUANOSINE ON THE TRANSPHOSPHORYLATION TO GLYCEROL IN ACID PHOSPHATASE

The standard assay system using *p*-nitrophenyl phosphate was employed. V/V_0 refers to the ratio of rates obtained in the presence and absence of the additions indicated in this Table. The *p*-nitrophenol/P_i ratio was measured as indicated in Materials and Methods, and is the average of at least two data points ± standard deviation.

Additions to assay system	*p*-Nitrophenol/P_i	V/V_0
None	1.00 ± 0.01	(1.0)
2.2 mM guanosine	1.02 ± 0.01	3.6
0.29 M glycerol	1.51 ± 0.13	1.3
0.29 M glycerol + 0.27 mM guanosine	1.32 ± 0.03	1.6
0.29 M glycerol + 0.55 mM guanosine	1.22 ± 0.05	2.0
0.29 M glycerol + 1.1 mM guanosine	1.16 ± 0.03	2.7
0.58 M glycerol	1.91 ± 0.13	1.8
0.58 M glycerol + 0.27 mM guanosine	1.74 ± 0.04	2.0
0.58 M glycerol + 1.1 mM guanosine	1.41 ± 0.01	2.4
0.58 M glycerol + 2.2 mM guanosine	1.25 ± 0.01	3.2

measureable transphosphorylation. Transient formation of phosphorylation products is also unlikely since they are known to be poor substrates for acid phosphatase [5].

Activation by adenine and guanosine is hyerpbolic (Fig. 1). The concentrations which result in half of the maximum activation are 1 and 2 mM for adenine and guanosine, respectively. Both adenine and guanosine produce a parallel kinetic pattern in Lineweaver-Burk plots (Figs. 2A and 2B), but the intercepts (or the reciprocals) are not linear with activator concentration. It has been shown, in particular in the case of inhibition, that this kinetic pattern precludes the binding of the activator of the free enzyme [15,16]. In order to ascertain that guanosine does not bind significantly to free acid phosphatase, various experimental protocols were executed. The thermal inactivation rate and the rate of inactivation of acid phosphatase by dithiobis *p*-nitrobenzoic acid have been shown to diminish markedly when an active site ligand is added to acid phosphatase [11,12,17]. Guanosine, on the other hand, does not alter the rate of either inactivation process. Moreover, we obtained no evidence for guanosine binding to the free enzyme using differential absorption spectrophotometry, equilibrium gel filtration [18] with [^{3}H]guanosine, or the effect of guanosine on the intrinsic fluorescence [8] of acid phosphatase. Taken together, this negative evidence strongly supports our contention that the pattern of kinetic activation of acid phosphatase by guanosine indeed excludes binding of the activator to the free enzyme.

Transphosphorylation to external acceptor was used in order to define the site of activator binding in the catalytic pathway. Glycerol serves as an efficient acceptor for transphosphorylation by acid phosphatase (Table II). The presence of glycerol phosphate in the reaction was qualitatively demonstrated by thin-layer chromatography of the reaction products on poly(ethylenimine) cellulose plates.

The decomposition rate of *p*-nitrophenyl phosphate increases when glycerol is added to the acid phosphatase catalyzed reaction (Table II, Fig. 3). In con-

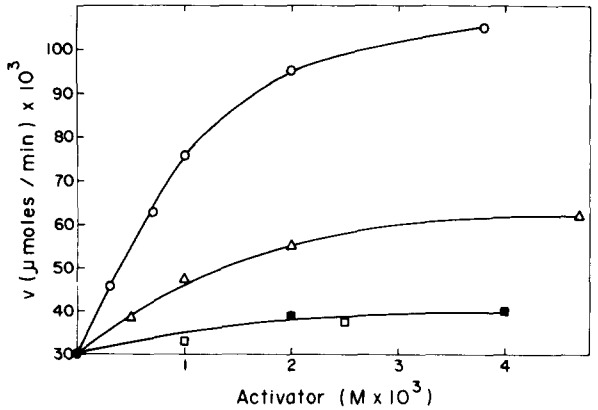


Fig. 1. Effect of guanosine (○), adenine (△), AMP (□) and adenosine (■) on the rate of hydrolysis of *p*-nitrophenyl phosphate. Conditions are indicated in Materials and Methods. 1 μg of enzyme was used per assay.

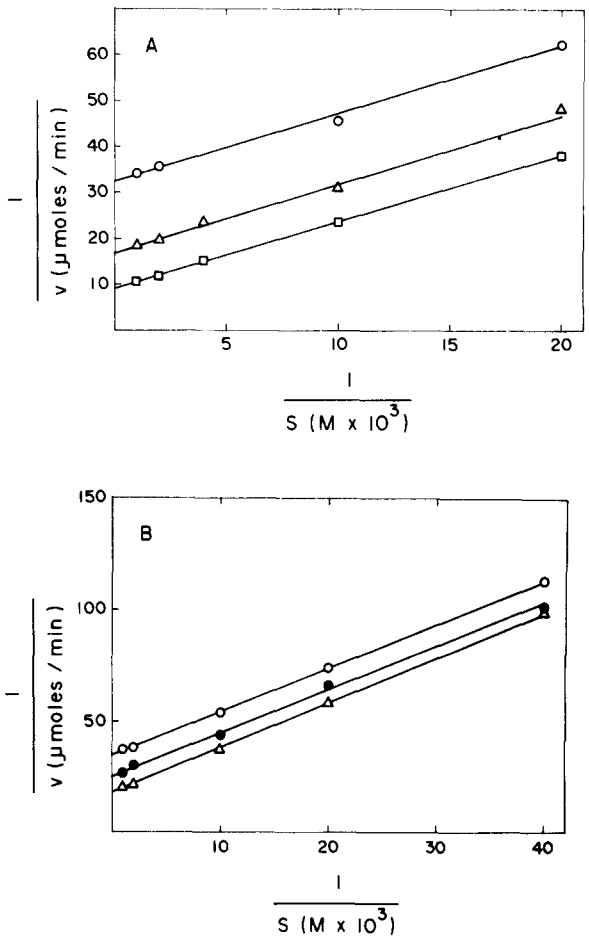


Fig. 2. Reciprocal plot of the effect of guanosine and adenine on the rate of *p*-nitrophenyl phosphate hydrolysis. (A) Guanosine concentrations: ○, none; △, 0.63 mM; □, 2.5 mM. (B) Adenine concentrations: ○, none; ●, 2.4 mM; △, 5 mM. 0.8 μg of enzyme per assay.

trast to the effect of purines, the rate increase produced by glycerol can be essentially accounted for on the basis of transphosphorylation (Table II). Conformational sensitive parameters, such as the thermal inactivation rate or the dithiobis *p*-nitrobenzoate inactivation rate [11,12,17], do not change at the concentrations of glycerol used in the kinetic experiments. Thus, the activation by glycerol is due purely to a more efficient attack on the phosphoryl enzyme (relative to water).

As in the case of purines, the activation pattern is parallel (Fig. 3). This pattern can be accomodated within a minimum scheme that has been previously used for this [12] and other [19] phosphatases.

$$E + ROP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot ROP \xrightarrow[ROH]{k_3} E'P \begin{cases} \xrightarrow{k_4 H_2O} P_i \\ \xrightarrow{k_5 R'OH} R'OP \end{cases}$$

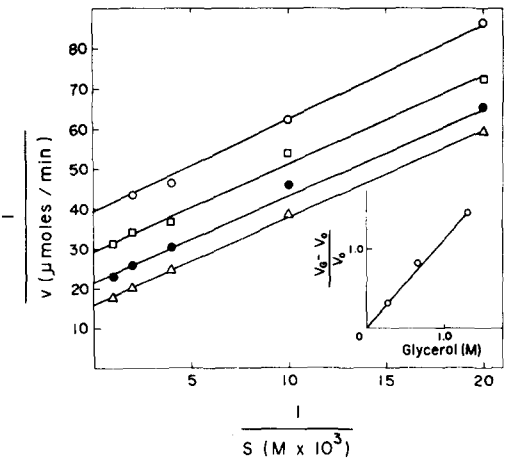


Fig. 3. Reciprocal plot of the effect of glycerol on the rate of *p*-nitrophenyl phosphate hydrolysis. Glycerol concentrations: ○, none; □, 0.277 M; ●, 0.665 M; △, 1.33 M. Inset shows the relative increase in $(V_G - V_0)/V_0$ against glycerol. V_G represents the maximum velocity in the presence of glycerol. 0.8 μg of enzyme per assay.

Using the steady state assumption, the rate expression for this scheme is:

$$\frac{1}{v} = \frac{K_1}{V} \cdot \frac{1}{[S]} + \frac{1}{V}\left(1 + \frac{1}{K_2}\right) \qquad (1)$$

where

$$K_1 = \frac{k_2 + k_3}{k_1} \quad \text{and} \quad K_2 = \frac{k_5[G] + k_4}{k_3}$$

Eqn. 1 predicts that the intercept of the reciprocal plot should be modified by glycerol but that the slope should remain constant.

This expression assumes, explicitly, that glycerol, even if bound to either free or phosphoryl enzyme, exerts no appreciable kinetic influence and can be considered, as water, as a nucleophile attacking the phosphoryl enzyme.

The fact that the rate of substrate utilization increases upon addition of glycerol must be interpreted as meaning that $k_3 > k_4$. Using this assumption it can be shown that

$$\frac{V_G - V_0}{V_0} = \frac{k_5}{k_4}[G]$$

where V_G is the maximum velocity obtained in the presence of glycerol and V_0 is the maximum velocity obtained, under the same conditions, in the absence of added glycerol.

As shown in the inset of Fig. 3, this expression indeed holds giving a value of 1.1 for k_5/k_4. Since k_4 includes the water concentration, glycerol is at least 60 times more efficient than water for attack at the phosphoryl enzyme.

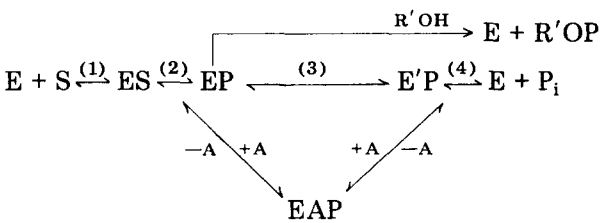
The similarity of the rate enhancement patterns of glycerol and guanosine suggests that the sites [20] for glycerol attack and guanosine binding are related. The extent of transphosphorylation should thus diminish upon

addition of guanosine to the reaction mixture in the presence of glycerol, with little or no effect on the reaction rate. Indeed, the addition of increasing concentrations of guanosine does diminish the transphosphorylation to glycerol, leading to almost complete inhibition of transphosphorylation at low glycerol concentrations (Table II). In this context, the effect of glycerol and guanosine can be considered competitive.

Discussion

The activation of low molecular weight acid phosphatases from bovine brain, rat liver and placenta [4] by purines is, apparently, general for this type of acid phosphtases and specific with respect to the structure of the activator. The variation in the extent of activation by changes in the purine nucleus and by phosphorylation of the nucleoside, together with the kinetic data, suggest the existence of a specific site on the enzyme capable of binding the purine. This binding, occurring after the formation of the enzyme substrate complex, results in an enhanced catalytic activity. It is difficult to assign a biological role to this activation in view of the fact that the (possible) physiological significance of cytoplasmic, low molecular weight acid phosphatases is yet poorly understood [1,8].

It has previously been shown [12], and confirmed herre, that at pH 5.0, phosphorylation of acid phosphatase can not be rate limiting. From the analysis of the kinetic patterns of purine and glycerol activation, it can be concluded that neither activator binds to the free enzyme. In the presence of substrate, however, activation by purines shows binding, saturation and specificity, characteristic of the presence of a binding site. On the other hand activation by transphosphorylation gives no evidence for appreciable glycerol binding. Moreover the addition of purines causes a decrease in the transphosphorylation to glycerol. All of these results can be accomodated by the following reaction scheme:



in which the isomerization of the phosphoryl enzyme (step (3)) is rate limiting using *p*-nitrophenyl phosphate as substrate. This reaction scheme is consistent with previous data for this [12] and other acid phosphatases [21]. Preliminary data obtained at higher pH suggest that there may be a pH dependent change in the rate limiting step (from (3) to (4)) and at higher pH, as expected from the scheme above, glycerol still activates but purines inhibit the reaction. These results represent a clear cut case in which a ligand, bound to the enzyme in the reaction sequence prior to the rate determining step, produces an activating effect. This is currently under investigation.

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