Modification of *myo*-inositol monophosphatase by the arginine-specific reagent phenylglyoxal

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myo-Inositol monophosphatase is inhibited by the arginine-specific reagent phenylglyoxal. The rate of inactivation is decreased in the presence of P_i , a competitive inhibitor of the enzyme. The effect of P_i is dependent on the presence of Mg^{2+} , but is unaffected by Li^+ , an uncompetitive inhibitor. In the absence of Mg^{2+} , the substrate, *Ins*(1)*P*, binds to the enzyme but is not converted into products, and affords only a small degree of protection against inactivation by phenylglyoxal. Li^+ had no further effect under these conditions, but in the presence of Mg^{2+} caused a marked potentiation of the protective effect of substrate alone. In the absence of substrate, Li^+ had no effect on activation by phenylglyoxal. Incorporation of ^{14}C -labelled phenylglyoxal showed that inactivation was associated with modification of a single arginine residue per monomer in the dimeric enzyme. These findings support a mechanism in which Li^+ inhibits monophosphatase by trapping a phosphorylated enzyme intermediate and preventing its hydrolysis.

INTRODUCTION

myo-Inositol monophosphatase catalyses the dephosphorylation of D-*Ins*(1)*P*, D-*Ins*(3)*P* and D-*Ins*(4)*P* [1], and is therefore responsible for the provision of inositol for phosphoinositide production from both synthesis *de novo* and degradation of inositol polyphosphates [2]. The key role played by this enzyme in the maintenance of cell signalling through phosphoinositide hydrolysis and its sensitivity to inhibition by Li^+ [1,3,4] have made the monophosphatase an attractive candidate for the site of action of Li^+ in the treatment of manic depression [5,6]. As has been widely demonstrated, Li^+ inhibits the monophosphatase uncompetitively [1,3,4] and it has been proposed recently that Li^+ binds to a phosphoryl-enzyme intermediate, thereby preventing its hydrolysis [7]. In the present paper we describe the inactivation of monophosphatase by the arginine-specific reagent phenylglyoxal [8]. We show that inactivation can be caused by modification of a single arginine residue per subunit of the enzyme. Protection against inactivation by substrate and other ligands provides support for the proposed mechanism of action of Li^+ , and identifies the arginine residue as one having an important function at the active site.

MATERIALS AND METHODS

Materials

Bovine brain inositol monophosphatase was purified by monoclonal-antibody immunoaffinity chromatography [9]. DL-*Ins*(1)*P* was prepared as described by Billington *et al.* [10]. L-[U- ^{14}C]*Ins*(1)*P* (55 mCi/mmol) and [7- ^{14}C]phenylglyoxal (18 mCi/mmol) were from Amersham International (Amersham, Bucks., U.K.).

Modification by phenylglyoxal

Monophosphatase (specific activity 7.3 units/mg of

protein at 21 °C) was dialysed against 150 mM-KCl/3 mM- $MgCl_2$ /50 mM- $NaHCO_3$ buffer, pH 7.8, and stored at –70 °C. For modification, monophosphatase (3.5 μ g of protein) was incubated in a final volume of 25 μ l containing phenylglyoxal (routinely 100 mM from a 500 mM stock in dimethyl sulphoxide), 250 mM-KCl, 5 mM-Tris, 3 mM- $MgCl_2$ and 50 mM- $NaHCO_3$, pH 7.8, and further addition (or omissions) as detailed in the Figure and Table legends. After various times at 21 °C, 4 μ l samples were diluted with 196 μ l of 'assay buffer' [250 mM-KCl/50 mM-Tris/HCl (pH 7.8)/3 mM- $MgCl_2$]. Duplicate 30 μ l portions of this were then assayed for monophosphatase activity at 21 °C by the radiochemical method described in [11].

Mg^{2+} buffering was provided by nitrilotriacetic acid [12]. In experiments lacking Li^+ , nitrilotriacetate was present at 8.5 mM, a concentration which caused no inhibition of the enzyme. $MgCl_2$ was added at concentrations between 0.15 mM and 2.35 mM to provide free Mg^{2+} concentrations of 5–100 μ M [12]. In the presence of 10 mM- Li^+ , the nitrilotriacetate concentration was increased to 10 mM to compensate for chelation of Li^+ [13].

Labelling of monophosphatase with phenylglyoxal

In labelling experiments, monophosphatase (3 μ g of protein) was incubated with 10 mM-phenylglyoxal (9 mCi/mmol) under conditions otherwise identical with the above for 60 min at 21 °C. To each 25 μ l sample, 225 μ l of ice-cold acetone was added. After 10 min on ice, the samples were centrifuged for 10 min in an Eppendorf Microfuge. The pellet was freed of phenylglyoxal that had not reacted by repeated resuspension and centrifugation in 90 % (v/v) acetone. The final pellet was solubilized in 1 % (w/v) SDS and counted for radioactivity or analysed by SDS/polyacrylamide-gel electrophoresis [14] and fluorography.

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Fig. 1. Dependence of enzyme activity on phenylglyoxal concentration

Monophosphatase activity was determined as described in the Materials and methods section in the presence of the following phenylglyoxal concentrations: Δ , 10 mM; $\blacktriangle$, 20 mM; $\circ$, 50 mM; $\bullet$, 100 mM. Rate constants (k) were determined from the initial slopes of the lines and replotted as a function of phenylglyoxal concentration (inset). Values of k are means $\pm$ S.E.M. ($n = 3$).

Kinetic analysis of monophosphatase

Partially purified monophosphatase was assayed at five substrate concentrations (0.05–1.0 mM) with five different Mg^{2+} concentrations (0.5–3 mM). Activity with each combination was assayed in duplicate. Kinetic data were analysed by using the non-linear curve-fitting programme BMDP AR [15] and functions detailed in the text or Table 1. Functions were accepted as modelling data on two criteria; the ability to account for the total variance, and a random distribution of data points around the curve. Improvement of fit between different functions was tested by using the partial F-test procedure [16].

RESULTS

Inhibition of monophosphatase by phenylglyoxal

Loss of monophosphatase activity in the presence of phenylglyoxal was pseudo-first-order for up to about 50% inactivation, but subsequently the loss of activity was faster than expected (Fig. 1). The reason for this behaviour was not determined, and pseudo-first-order rate constants (Fig. 1 inset) were calculated from the initial phase of the inactivation curve. Routinely, 100 mM-phenylglyoxal was used in subsequent experiments.

Protection by P_i

P_i inhibits monophosphatase competitively, with a K_i of 0.52 mM [1]. Inactivation of the enzyme by phenylglyoxal was considerably slowed by P_i , as shown

Fig. 2. Effect of P_i on inactivation by phenylglyoxal

Inactivation by 100 mM-phenylglyoxal was measured as described in the Materials and methods section in the presence of the indicated concentrations of P_i .

in Fig. 2. The pseudo-first-order rate constant for inactivation by 100 mM-phenylglyoxal was decreased 4.3-fold, from 0.107 min^{-1} to a minimum value of 0.025 min^{-1} , indicating decreased reactivity of the enzyme-phosphate complex with phenylglyoxal. The concentration of P_i required for 50% of the maximum decrease in rate constant was approx. 0.4 mM, similar to the K_i measured kinetically [1]. In the absence of Mg^{2+} , P_i (5 mM) had no effect on inactivation by phenylglyoxal, suggesting that binding of P_i is Mg^{2+} -dependent. Furthermore, Li^+ did not potentiate the effect of P_i , even when the latter was sub-optimal. This contrasts with its effects in the presence of substrate, as detailed in the next section.

Protection by substrate and Li^+

Protection by substrate was more difficult to demonstrate unambiguously, because of the potential problem of hydrolysis to inositol and free phosphate during incubation with phenylglyoxal. To obviate this, we looked at the effects of substrate in the absence of Mg^{2+} , since this is essential for catalytic activity [1,3,4]. Analysis of the enzyme kinetics with both $Ins(1)P$ and Mg^{2+} varying indicated little effect of Mg^{2+} on the K_m for $Ins(1)P$. Thus analysis of the data in terms of a model where K_m values for Mg^{2+} and $Ins(1)P$ were independent of whether the other ligand was bound produced as good a fit as the more general case where K_m values were not independent of the other ligand (Table 1). On the other hand, poorer fits were obtained with models in which there was compulsory order of ligand binding. Thus, in the absence of Mg^{2+} , it was possible to look at effects of substrate binding on the inactivation by phenylglyoxal free from the complication of substrate turnover. As shown in Table 2, $Ins(1)P$ in the presence of Mg^{2+} produced some degree of protection against inactivation, but this could be partly attributed to the effect

Table 1. Kinetics of monophosphatase

Rates measured at various substrate and Mg^{2+} concentrations were fitted to the models for independent binding and to more specific models in which there was compulsory order of ligand binding (results not shown). Best fits were obtained with the models shown, both of which accounted for greater than 98% of the variance. By the partial F-test, the more highly parametrized function did not give any significant improvement ($P < 0.05$). Kinetic parameters are given as means $\pm$ S.D., and are consistent with other determinations in the literature.

Model	Equation for fitting	Kinetic parameters
$\begin{array}{c} E \xrightleftharpoons{K_A} ES \\ \parallel \qquad \parallel \\ EMg \xrightleftharpoons{K_{AB}} E \cdot Mg \cdot S \rightarrow \end{array}$	$v = \frac{V_m[S][Mg]}{K_A K_B + K_B[S] + K_{AB}[Mg] + [S][Mg]}$	$K_A = 0.18 \pm 0.05 \text{ mM}$ $K_{AB} = 0.08 \pm 0.03 \text{ mM}$ $K_B = 1.6 \pm 0.3 \text{ mM}$
$\begin{array}{c} E \xrightleftharpoons{K_A} ES \\ \parallel \qquad \parallel \\ EMg \xrightleftharpoons{K_A} E \cdot Mg \cdot S \rightarrow \end{array}$	$v = \frac{V_m[S][Mg]}{K_A K_B + K_B[S] + K_A[Mg] + [S][Mg]}$	$K_A = 0.12 \pm 0.01 \text{ mM}$ $K_B = 2.0 \pm 0.2 \text{ mM}$

Table 2. Protection against inactivation by substrate and Li^+

Inactivation by 100 mM-phenylglyoxal was as described in the Materials and methods section with the additions and omissions listed. Results are expressed as the fold decrease in the pseudo-first-order rate constant (k) for inactivation caused by various treatments, and are means $\pm$ S.E.M., ($n = 2$ or 3).

Treatment	Fold decrease in k in presence of:	
	10 mM-Ins(1) <i>P</i>	10 mM-Ins(1) <i>P</i> + 10 mM- $LiCl$
$MgCl_2$ (3 mM)	2.72 ± 0.07 (3)	4.6 ± 0.3 (3)
No added $MgCl_2$	1.60 ± 0.11 (2)	4.9 ± 0.5 (3)
EDTA (5 mM)	1.39 ± 0.07 (3)	1.35 ± 0.06 (2)

of P_i , Li^+ increased the protection further, but alone had no effect. In the presence of EDTA, Ins(1)*P* gave only a small decrease in the rate constant for inactivation, and this was not further affected by Li^+ . However, in the absence of either EDTA or added Mg^{2+} , the same small degree of protection afforded by Ins(1)*P* was greatly increased by Li^+ . Contamination of reagents with Mg^{2+} was estimated to be in the low-micromolar range in this experiment. Since substrate was near saturating, the low degree of protection afforded by Ins(1)*P* alone suggests that the reactivity of the arginine residue is not appreciably lower in the enzyme-substrate (ES) complex than in the free enzyme. The potentiating effect of Li^+ in the presence of residual Mg^{2+} , but not in its absence, is consistent with the proposal that Li^+ binds to a phosphoenzyme intermediate [7]. At low Mg^{2+} concentrations, the amount of phosphoenzyme (E-P) would be very small, but addition of Li^+ would trap most of the enzyme as an E-P· Li^+ complex, which appears to be resistant to reaction with phenylglyoxal. In the complete absence of Mg^{2+} , however, no E-P would be formed, and Li^+ would not induce formation of the E-P· Li^+ state.

To establish conditions for the Li^+ effect more pre-

Fig. 3. Effects of substrates, Li^+ and Mg^{2+} on inactivation by phenylglyoxal

Inactivation by 100 mM-phenylglyoxal was carried out at the indicated free Mg^{2+} concentrations as described in the Materials and methods section. Where present, substrate [Ins(1)*P*] and Li^+ were each 10 mM; ■, no substrate or Li^+ ; ●, substrate alone; ○, substrate + Li^+ .

cisely, similar experiments were performed in the presence of nitrilotriacetate, to provide a Mg^{2+} -buffering system [12]. As shown in Fig. 3, the degree of protection afforded by substrate alone was not substantially changed over the Mg^{2+} concentration range from zero to 30 μM , but thereafter increased. Estimates of the degree to which this was due to the presence of P_i are complicated not only by the simultaneous presence of substrate (which competes with P_i) but also by the unknown concentration-dependence of P_i binding on Mg^{2+} and the progressive decrease in activity owing to inactivation by phenylglyoxal. However, despite the uncertainty in the calculation, it was possible to show that the amount of

E·P_i complex present was unlikely to cause any measurable degree of protection in experiments with Mg²⁺ concentrations of ≤ 50 μM. Thus the protection afforded by substrate at low Mg²⁺ concentration can be safely attributed to ES formation.

Li⁺ increased the protection afforded by substrate in a manner which was dependent on Mg²⁺. Under the conditions used, half-maximal protection was obtained with 13 μM free Mg²⁺. At very high Mg²⁺ concentration, the rate constant for inactivation in the presence of substrate and Li⁺ was the same as that for the E·P_i complex. Thus the E·P_i and and E·P·Li complexes are similarly resistant to phenylglyoxal.

Stoichiometry of phenylglyoxal derivative formation

Monophosphatase was modified with [³H]phenylglyoxal under conditions which led to approx. 80% inactivation of the enzyme. Under these conditions, 4.60 ± 0.47 (S.E.M., *n* = 3) mol of phenylglyoxal was incorporated per mol of monophosphatase (*M_r* 60000). Since two molecules of phenylglyoxal react with one arginine residue, and monophosphatase is a homodimer, these results show that 1.15 ± 0.12 arginine residues per monomer were modified under these conditions. In the presence of 10 mM-Ins(1)*P* and 0.4 M-LiCl, inactivation was decreased to approx. 25% and labelling was decreased to 0.46 ± 0.04 (*n* = 2) arginine residue per monomer. Thus inactivation is caused by reaction with a single arginine residue per monomer, and clearly this residue reacts preferentially with phenylglyoxal. At longer times of incubation with phenylglyoxal or with higher concentrations, much greater incorporation could be achieved. The labelling experiments were also confirmed by SDS/polyacrylamide-gel electrophoresis and fluorography. Labelling of the *M_r*-30000 monophosphatase subunit was substantially prevented by the combination of substrate and Li⁺ or phosphate. Labelling of a mixture of marker proteins was unaffected, showing that the effects of monophosphatase ligands are specific.

The stoichiometry of modification may explain the finding that inactivation did not follow pseudo-first-order kinetics (Fig. 1). If modification of one subunit of the dimer results in only partial loss of activity (e.g. 50%) but increases the reactivity of the residue in the other subunit, then plots with a downward curvature would be expected. Indeed, calculation shows that only a modest increase (less than 2-fold) in reaction rate is sufficient to produce a marked curvature.

DISCUSSION

The results of this study are consistent with the kinetic mechanism proposed by Shute *et al.* [7] and shown in Scheme 1. Mg²⁺ is essential for formation of

phosphoenzyme (E-P), but not for substrate binding. On the other hand, Mg²⁺ seems to be more strictly required for binding of P_i. Li⁺ traps the E-P complex in a form which is resistant to hydrolysis, thereby producing uncompetitive inhibition [1,3,4]. The inability of Li⁺ to potentiate the protective effect of P_i shows that the step E-P → E·P_i is irreversible, and moreover proves that Li⁺ does not inhibit the enzyme by binding to the E·P_i complex. This possibility was consistent with previous data [1,3,4], but not favoured by Shute *et al.* [7].

The arginine residue modified by phenylglyoxal is protected in the E-P/E·P·Li⁺ and E·P_i states, but only to a small extent in the ES state. The simplest interpretation would be that the arginyl residue is involved in ionic-bond formation with an oxygen atom of the phosphate residue. Formation of phosphoarginine cannot be ruled out, but is not favoured by precedent, other phosphatases using histidine, serine or aspartic acid as the reactive residue (see, e.g., [17,18]). Of particular interest is the relative failure of substrate to protect the arginine residue against inactivation. This suggests that either the arginine residue is not involved in substrate binding, or, more likely, that the active-site structure changes as a result of phosphoenzyme formation restricting access of the reagent. This would be consistent with the inability of inositol to inhibit activity even at very high concentrations [7].

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