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The effects of substrates, products and other ligands on the susceptibility of inositol monophosphatase to proteolysis by endoprotease lys-C

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Inositol monophosphatase is cleaved by endoprotease lys-C at a single site (Lys^{36}–Ser^{37}). The rate of proteolysis is greatly reduced in the presence of substrate (D,L-Ins(1)P) and Mg^{2+}, and less so in the presence of P_i and Mg^{2+}, consistent with protection of the susceptible bond in the E–P or E·P_i states of the enzyme. Potentiation by Li^+ of the protection afforded by a substrate analogue, 1*S*-phosphoryloxy-2*R*,4*S*-dihydroxycyclohexane, and Mg^{2+} supports the idea that Li^+ binds to the E–P state.

Inositol monophosphatase; Lithium; Proteolysis; Phosphoenzyme

1. INTRODUCTION

Inositol monophosphatase (EC 3.1.3.25) is responsible for the production of inositol from Ins(1)P, Ins(3)P and Ins(4)P [1], and is therefore essential for the continued functioning of the phosphoinositide cycle [2]. Uncompetitive inhibition of the enzyme by lithium has been proposed as the underlying mechanism through which lithium is effective in the treatment of manic depression [1,3]. Inositol monophosphatase from bovine brain is a homodimeric enzyme [1], each monomer consisting of 277 amino acid residues [4]. Proteolysis by endoprotease lys-C causes a single cleavage after Lys^{36}, leading to a drastic loss of activity [5]. Previously, we had demonstrated that loss of activity caused by chemical modification with phenylglyoxal could be inhibited by substrate and other ligands [6]. In this paper, we show that the susceptibility of the enzyme to proteolysis with endoprotease lys-C is similarly decreased by substrate and ligands, lending support to our previous conclusions that the enzyme undergoes a conformational change to form a phosphoenzyme (E–P) complex.

2. MATERIALS AND METHODS

Bovine brain monophosphatase was expressed in *E. coli* as previously described [4] and purified by ion-exchange chromatography. Activity was measured by colorimetric determination of released P_i [1]. Digestion with endoprotease lys-C (Boehringer) was carried out at 37°C in a final vol of 20 μl containing 50 mM Tris-HCl, pH 8.0, 10 μg of monophosphatase, 5 μg of endoprotease lys-C and further additions as indicated in the text and figure legends. At intervals (see figure legends) 4 μl samples were withdrawn and frozen immediately in solid CO_2/acetone. Samples were subsequently analysed by SDS-PAGE [7] using 15% acrylamide separating gels and Coomassie blue staining. D,L-Ins(1)P and 1*S*-phosphoryloxy-2*R*,4*S*-dihydroxycyclohexane were synthesised as in [8] and [9], respectively. Protein was estimated by the method of Bradford [1]. The effect of monophosphatase ligands on endoprotease lys-C activity were measured using Chromozym PL (Boehringer) as a substrate. Each assay contained, in a volume of 1 ml, 50 mM Tris-HCl, pH 8.0, 0.1 mM Chromozym PL, 2.5 mU endoproteinase lys-C and monophosphatase ligands at concentrations indicated in the figure legends or in the text. The absorbance increase at 405 nm was measured at room temperature. Inhibition of 20% or less was found with Li^+, Na^+, Mg^{2+}, inositol and the substrate analogue. D,L-Ins(1)P inhibited activity by 37% at 0.1 mM and 80% at 1 mM, while P_i inhibited by 42% at 10 mM and was without effect at 1 mM. Li^+, Na^+ and Mg^{2+} in addition to D,L-Ins(1)P caused no further inhibition over that obtained with D,L-Ins(1)P alone.

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3. RESULTS

Fig. 1 shows that in the absence of ligands, the monophosphatase subunit of M_r 30000 was rapidly cleaved to a fragment of M_r 28000 by endoprotease lys-C. In contrast, in the presence of substrate (1 mM) and Mg^{2+} (3 mM), degradation was almost completely prevented. The decrease in rate was estimated as several hundred-fold. Both Mg^{2+} and substrate alone caused a more modest decrease in the rate of proteolysis, approximately 4-fold. For the substrate this effect could be wholly attributed to inhibition of the activity of the protease (80% at 1 mM D,L-Ins(1)P). Since previous work had demonstrated that substrate can bind to the enzyme in the absence of Mg^{2+} [6], the inability of substrate alone to afford any protection suggests that

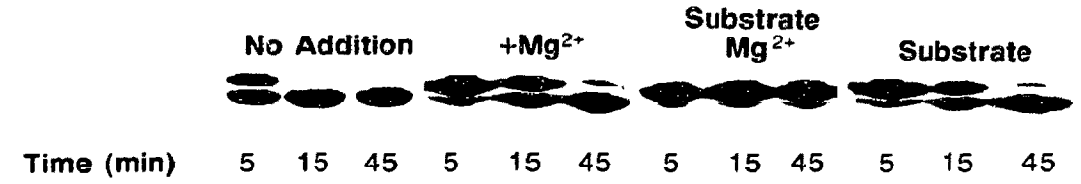

Fig. 1. Protection against proteolysis by substrate (1 mM D,L-Ins(1)P) and Mg^{2+} (3 mM).

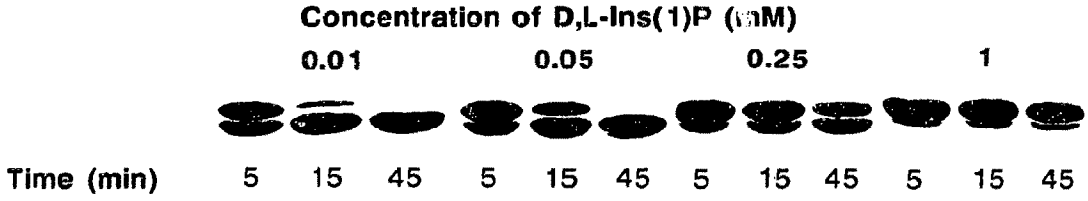

Fig. 2. Concentration dependence of effect of substrate on rate of proteolysis. Mg^{2+} (3 mM) and Li^+ (25 mM) were present throughout.

the enzyme substrate (ES) complex is not the protected species. However, it is not clear from this experiment whether protection is a property of phosphoenzyme or non-covalent enzyme product complexes.

Lithium (25 mM) alone had no effect on the rate of proteolysis and did not alter the degree of protection afforded by Mg^{2+}, substrate or both under the conditions of Fig. 1 (results not shown). Since Li^+ inhibits substrate dephosphorylation, this demonstrates that complete protection can be achieved in the absence of product formation. In the presence of Li^+ to inhibit enzyme activity, protection was dependent on substrate concentration (Fig. 2). The concentration affording 50% of the maximum degree of protection was approximately 0.1 mM, close to the K_m value for D,L-Ins(1)P. Correction for inhibition of the protease by D,L-Ins(1)P made very little difference to either the degree of protection or the concentration dependence.

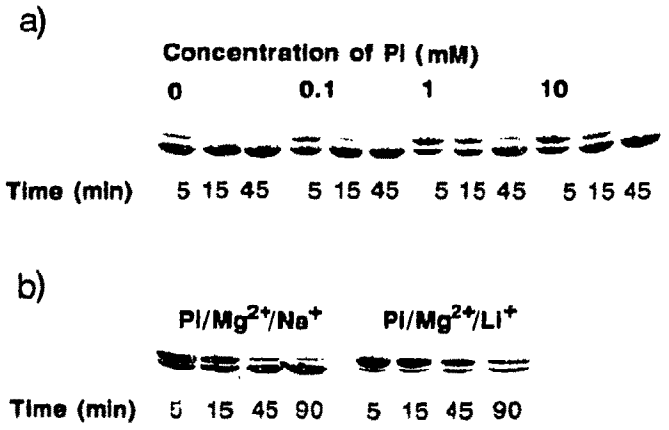

Fig. 3. Protection against proteolysis by P_i. In (a) Mg^{2+} (3 mM) was present. In (b) Mg^{2+} (3 mM) and either 25 mM Li^+ or 25 mM Na^+ were present.

As shown in Fig. 3a, P_i can also give rise to protection, although not as marked as with substrate. The protection by P_i was absolutely dependent on Mg^{2+} and was half-maximal at concentrations between 0.1 mM and 1 mM, similar to the K_i value of 0.5 mM [1]. Inositol, on the other hand, had no effect under any conditions, consistent with the inability of inositol to inhibit the enzyme [11].

Compared with a control containing Na^+, protection by P_i/Mg^{2+} was slightly increased by Li^+ (Fig. 3b). However, demonstrating potentiation by Li^+ of protection by substrate/Mg^{2+} is complicated by substrate dephosphorylation in the absence of Li^+ and the high degree of protection afforded by D,L-Ins(1)P and Mg^{2+} (Fig. 1). We, therefore, used a substrate analogue, 1*S*-phosphoryloxy-2*R*,4*S*-dihydroxycyclohexane, a potent competitive inhibitor of the enzyme (K_i approx. 1 μM, [9]). The analogue is also a very weak substrate with a rate of hydrolysis 2.7% of that of 1 mM D,L-Ins(1)P. As shown in Fig. 4, 1*S*-phosphoryloxy-2*R*,4*S*-dihydroxycyclohexane and Mg^{2+} afforded protection greater than that seen with Mg^{2+} alone and this was considerably potentiated by Li^+.

4. DISCUSSION

These results are reminiscent of those obtained using phenylglyoxal to modify the enzyme [6]. It was concluded that modification of a single arginyl residue was protected in the E-P and E · P_i states to the same extent. Formation of the ES complex afforded only a very small degree of protection. In the present study, ES complex formation afforded no protection against endoprotease lys-C cleavage and the E · P_i complex was only partially protected. Complete protection by substrate is probably therefore a result of E-P formation. With D,L-Ins(1)P and Mg^{2+}, under the conditions

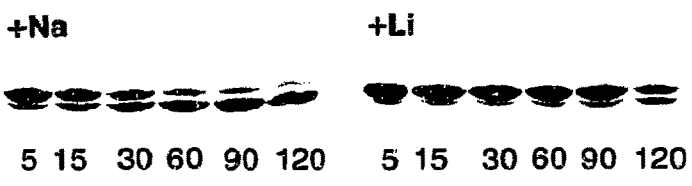

Fig. 4. Potentiation by Li^+ of the protective effect on proteolysis of 1*S*-phosphoryloxy-2*R*,4*S*-dihydroxycyclohexane (25 μM). Li^+ (25 mM) or Na^+ (25 mM) were present as indicated.

used, protection was almost total, suggesting that nearly all of the enzyme was in the E-P state. With the substrate analogue, however, protection was only partial except in the presence of Li^+. This can be explained by formation of a lower steady-state level of E-P with the substrate analogue alone. Addition of Li traps the enzyme as an E-P·Li complex (e.g. [6]) which is presumably also protected against proteolysis. The small potentiation by Li^+ of protection by P_i may suggest that phosphoenzyme can be formed by reversal of the normal reaction pathway from $E \cdot P_i$ as well as from ES.

In a simple ping-pong mechanism in which hydrolysis of E-P occurs after loss of the product, inositol, the rate constant for E-P hydrolysis will be independent of the nature of the substrate [11]. Thus, the overall velocity of substrate hydrolysis will be proportional to the extent of E-P formation. It is therefore surprising that the substrate analogue used in this study should give rise to quite a high degree of protection against proteolysis (i.e. appreciable E-P formation) even though the rate of hydrolysis of the analogue was extremely low. This suggests that the rate constant of E-P hydrolysis might also be lower with the substrate analogue. This could be explained by release of the first product (inositol) after, rather than before, hydrolysis of the E-P bond.

Finally, protection against proteolysis strongly suggests that the N-terminal region undergoes a conformational change during the catalytic cycle. This would explain the observation that a monoclonal antibody directed towards the N-terminus is inhibitory [5] since binding of the antibody would prevent the necessary structural transition.

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