Bovine inositol monophosphatase: enzyme–metal-ion interactions studied by pre-equilibrium fluorescence spectroscopy

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Stopped-flow fluorescence spectroscopy has been used to determine the on-rate (k_{ass}) and the off-rate (k_{diss}) for the equilibrium between inositol monophosphatase and Mg^{2+} ions. The dissociation constant (K_d) for the equilibrium calculated from these constants suggests that the ions interact at site 1 on the enzyme with a K_d typically around 450 μM , close to values determined by equilibrium studies (270–300 μM). The affinity of this site on the wild-type enzyme for Mg^{2+} ions increases as the pH is increased. This is mediated almost entirely by a change in the rate k_{diss} . A slow increase occurs in the fluorescence intensity of the pyrene-labelled enzyme after the initial, fast, increase in fluorescence caused by the binding of the Mg^{2+} ion. The rate of this change is

independent of the concentration of the metal ion, implying that it may be a structural change in the enzyme– Mg^{2+} complex. Neither the fast nor the slow change in fluorescence intensity occurs when enzyme subjected to limited proteolysis by trypsin, which removes the N-terminal 36 residues, is mixed with Mg^{2+} ions. The data suggest that interaction with Mg^{2+} ions at a high-affinity site leads to a structural change in inositol monophosphatase. The data further confirm the importance of the presence of two metal ions in the structure/function of this enzyme, and show that the binding of the metal ions is not competitive with that of H^+ ions and that the variation in K_d with pH is mediated almost totally by changes in k_{diss} .

INTRODUCTION

Inositol monophosphatase in brain tissue plays a central role in inositol metabolism [1]. The enzyme dephosphorylates inositol monophosphates (Ins1P, Ins3P and Ins4P) to release inositol, which is used to regenerate phosphatidylinositides, a key component of the phosphatidylinositol second messenger signalling pathway [2]. This activity is particularly important in brain tissue, since inositol crosses the blood–brain barrier very slowly [3] and therefore dietary inositol is effectively unavailable. In addition, although Ins1P can be synthesized in the brain from glucose 6-phosphate [4], the final dephosphorylation of inositol still requires catalysis by inositol monophosphatase. The activity of this enzyme is Mg^{2+} -dependent [5], although high concentrations of this metal cause uncompetitive inhibition [5]. The enzyme is also inhibited in an uncompetitive manner by Li^+ [6]. This inhibition may well explain the anti-manic and anti-depressive actions of this ion, since it occurs at therapeutically relevant concentrations (0.5–1.5 mM) [7,8].

Inositol monophosphatase has been isolated from a number of sources [1,4,9–15]; it has been shown to have a subunit molecular mass of approx. 30 kDa and to exist as a homodimer [1]. cDNA cloning and sequencing of the bovine [16], rat and human [17] genes has demonstrated that a very high level of sequence identity (79%) exists between the enzymes from these species.

Early kinetic analysis of the reaction catalysed by inositol monophosphatase [5] suggested that two separate Mg^{2+} ions may be responsible for the activation of the enzyme at low concentrations of the ion (up to 1 mM) and for the uncompetitive inhibition of the enzyme reaction at higher concentrations (around 10 mM). Equilibrium dialysis experiments [18] and fluorescence studies using pyrene-maleimide-labelled inositol monophosphatase [19] have shown that this enzyme has two Mg^{2+} binding sites per subunit: site 1, described by a K_d of 300 μM , and site 2, described by a K_m of 3 mM. Recent X-ray analysis studies in the presence of various ligands showed that in

fact three metal ions can be bound to the enzyme [20], although the third metal ion can be easily displaced by inorganic phosphate [20]. Spectroscopy studies using metal ions to displace the luminescent Tb^{3+} ion [21] suggest that the high-affinity site (site 1) occupied by Mg^{2+} is the same as that occupied by the Gd^{3+} ion in the original crystallographic studies [22]. Metal ions 1 and 2 are liganded primarily to acidic residues, namely Glu-70, Asp-90, Thr-95 (peptide carbonyl) and Asp-90, Asp-93, Asp-220 respectively.

Studies with a monoclonal antibody [23] raised against the enzyme have led to the α -helical N-terminal region of the protein being implicated in the catalytic function of the enzyme [23]. The epitope of the antibody is centred around Cys-8 [23], and binding gives rise to inhibition of the enzyme [23]. Furthermore, limited proteolysis studies using endoproteinase Lys-C [24] and trypsin [25] have demonstrated that the bond between Lys-36 and Ser-37 in inositol monophosphatase is highly susceptible to proteolysis. Hydrolysis of this bond leads to a concurrent loss of enzyme activity, suggesting that this region of the protein is important in the functioning of the enzyme. The susceptibility of this bond to cleavage is decreased by the presence of substrate or other ligands known to bind at the active site of the enzyme [24,25], suggesting that a conformational rearrangement occurs in this region after the ligands have bound.

In the studies reported here we describe the results of equilibrium and pre-equilibrium fluorescence spectroscopy experiments designed to probe the interactions of inositol monophosphatase with metal ions.

MATERIALS AND METHODS

Materials

All laboratory reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) Merck Ltd. (Poole, Dorset, U.K.) or Fisons Ltd. (Loughborough, U.K.).

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Preparation of inositol monophosphatase

The gene for wild-type bovine brain inositol monophosphatase was used for large-scale production of recombinant protein from a pRSET5a expression vector in *Escherichia coli* BL21(DE3) [16]. The isolation and purification of the recombinant protein has been described previously [17].

Enzyme activity

Enzyme activity was determined by the continuous fluorescence assay described by Gore et al. [26].

Protein concentration

The concentration of the enzyme was determined by the bichinonic acid method described by Smith et al. [27], using BSA as a reference.

Buffers

All solutions were prepared in AnalaR grade water, and all glassware was acid-washed and rinsed in AnalaR water. All metal ion solutions were prepared as the chlorides in 50 mM Tris/HCl, pH 8.0, as 2 M stocks. These stock solutions were then diluted in the same buffer as required. Experiments involving solutions covering a range of pH values were carried out in a three-buffer cocktail comprising 0.1 M Mes, 0.1 M Trizma and 0.033 M Caps. The pH required was obtained by addition of HCl or NaOH.

Modification of inositol monophosphatase by pyrene maleimide

A 100 mM stock solution of pyrene maleimide was prepared in DMSO and subsequently diluted to working concentrations using ethanol. In all modification procedures the concentration of ethanol present was maintained at less than 5% (v/v), which was shown not to affect enzyme activity over the time course of the experiments. Modifications were performed upon 33 μ M inositol monophosphatase in 50 mM Tris/HCl, pH 8.0, at 25 °C. Modification was monitored directly by following an increase in fluorescence of the pyrene moiety (λ^{ex} = 340 nm; λ^{em} = 380 nm) which arises upon reaction of the maleimide group.

Removal of metal ions from the enzyme

EGTA (1 mM), EDTA (1 mM) and 1,10-phenanthroline (1 mM) were added to the enzyme, which was then dialysed against 5 litres of 50 mM Trizma/HCl buffer, pH 8.0, at 4 °C, and subsequently against a minimum of 2 litres of the same buffer. The dialysed enzyme was filtered prior to use in all studies.

Stopped-flow kinetics

Rapid kinetics were analysed using an Applied Photophysics SX17 stopped-flow spectrometer equipped with fluorescence detection. The excitation wavelength of 280 nm was selected by a monochromator with a 2 nm bandwidth slit, and the emitted light was selected by use of a cut-off filter at 345 nm. Fluorescence changes were recorded by an on-line Acorn computer equipped with the manufacturer's data handling software. Data were fitted to single- and double-exponential equations. The temperature was regulated using a thermostatically controlled Techne TE-8A water bath, and experiments were performed at 20 °C unless otherwise stated. In experiments designed to determine the rate of dissociation of metal ions from a complex with the enzyme, the pre-formed complex (see below for details) was diluted 10-fold by similar buffer containing either 40 mM EDTA (final con-

centration approx. 37 mM) or various concentrations of the same metal ion.

The reaction conditions were arranged so that the ligand concentration (Mg^{2+} ions) was 16–1000-fold higher than the concentration of the enzyme, thus allowing first-order analyses to be used to derive pseudo-first-order rate constants. These, where necessary, were converted into second-order rate constants by determining the dependence of the pseudo-first-order rates on ligand concentration.

Proteolysis of pyrene-maleimide-labelled inositol monophosphatase

Inositol monophosphatase, modified by pyrene maleimide as described above, was treated with 20 μ l of 0.1 mg/ml trypsin in 50 mM Tris/HCl buffer (pH 8.0) at 25 °C. Samples were withdrawn at regular intervals and assayed for activity until at least 80% of the original activity had been lost. As a control experiment, the same concentration of labelled enzyme was incubated in the absence of trypsin; less than 3% of the activity was lost in the same time period.

RESULTS

Determination of the on-rate (k_{ass}) and off-rate (k_{diss}) for the complexes formed between metal ions and site 1 of the enzyme using stopped-flow fluorescence techniques

The changes in fluorescence characteristics of the pyrene moiety have been used to determine the binding constants for Mg^{2+} ions to wild-type enzyme by stopped-flow fluorescence spectroscopy. Figure 1 shows a typical increase in the fluorescence emission of

Figure 1 Rate of change of fluorescence of pyrene-labelled wild-type enzyme mixed with Mg^{2+} ions

Wild-type bovine inositol monophosphatase (3 μ M final concentration in 50 mM Tris/HCl buffer, pH 8.0), labelled stoichiometrically with pyrene maleimide, was mixed 1:1 (v/v) with 0.5 mM Mg^{2+} ions (final concentration) in the same buffer at 20 °C. The resultant increase in fluorescence from the pyrene moiety was monitored over 160 ms (λ^{ex} = 280 nm; λ^{em} > 345 nm). The trace shown represents the accumulated average of 10 separate experiments and was fitted to a double-exponential equation. The residuals, describing the closeness of fit of the data to the equation, are described by the lower plot. Inset: semi-logarithmic analysis of the reaction progress curve obtained at pH 8.0 (●), and for the same reaction mixtures at pH 9.0 (○), pH 7.3 (□) and pH 6.5 (◼). Estimates of k_{diss} were obtained by extrapolating the curves to the y-axis. Each data point shown is the average of 10 separate experiments.

Table 1 Effect of pH on the kinetic constants k_{ass} , k_{diss} and the calculated K_d for Mg^{2+} binding to inositol monophosphatase

Values of k_{ass} , k_{diss} and K_d were determined by stopped-flow fluorimetry of the pyrene-labelled wild-type enzyme at pH values between 6.5 and 9.0. Pyrene-maleimide-labelled enzyme (final concentration 3 μM) was mixed with various concentrations of Mg^{2+} ions between 80 and 400 μM (final concentrations) at 20 °C. Values of K_d were calculated using $K_d = k_{\text{diss}}/k_{\text{ass}}$.

pH	$10^{-5} \times k_{\text{ass}} (\text{M}^{-1} \cdot \text{s}^{-1})$	$k_{\text{diss}} (\text{s}^{-1})$	$K_d (\mu\text{M})$
9.0	3.3 ± 0.34	146.9 ± 13.6	441 ± 79
8.0	3.8 ± 0.21	172.3 ± 8.4	455 ± 41
7.3	4.4 ± 0.18	210.9 ± 7.2	482 ± 36
6.5	3.7 ± 0.27	360.2 ± 10.7	980 ± 123

the pyrene label at wavelengths above 345 nm obtained when 6 μM pyrene-labelled enzyme (in 50 mM Tris/HCl buffer, pH 8.0, 20 °C) was mixed with an equal volume of the same buffer containing 100 μM –10 mM Mg^{2+} ions in a stopped-flow fluorimeter. A rapid increase (12 %) in the intensity of the emitted light at 380 nm was observed, with a half-time of reaction of approx. 12 ms under these conditions. The reaction progress curves over the first 30–40 ms fitted a single-exponential equation yielding pseudo-first-order rates for k_{app} (see inset to Figure 1 and Table 1) which are dependent upon the concentration of metal ion used. Although no compensation was made for the slight increase in ionic strength caused by the incremental concentrations of Mg^{2+} ions used in these experiments (0–0.4 mM; inset to Figure 1), similar experiments performed in the presence of 50 mM KCl showed that values of k_{diss} and k_{ass} were not significantly altered by this large increase in ionic strength (increase in k_{app} of less than 2.5 %). The data collected in this manner were subsequently used to calculate the on-rate (k_{ass}) and the off-rate (k_{diss}) for the reaction at pH 8.0 and 20 °C (Table 1). The ratio of $k_{\text{diss}}/k_{\text{ass}}$ at pH 8.0 (see Table 1) for this enzyme determined from pre-equilibrium metal binding studies yields an estimate of the K_d for the equilibrium of 455 μM , which is close to, but higher than, those determined by equilibrium methods at pH 8.0 [19].

The reverse reaction was investigated directly in experiments where enzyme– Mg^{2+} complexes (formed by pre-mixing 6 μM enzyme with 300 μM Mg^{2+}) were rapidly diluted 10-fold with the same buffer containing 10 mM EDTA. The dissociation of the metal ion from its complex with the enzyme was shown to be a simple process of rate 179 s^{-1} , with no evidence of a second phase of debinding (Figure 2). To corroborate this, experiments were carried out in which enzyme– Mg^{2+} complexes (pre-formed by mixing 6 μM enzyme with different concentrations of Mg^{2+} ions) were rapidly diluted 1:1 (v/v) into buffer so that the final concentration of the free metal ions was varied. These experiments showed that both the amplitude of the signal change and the apparent rate of dissociation of the metal ion are dependent upon the concentration of the free metal ion in solution, as would be expected for a simple reversible process [28].

Identification of a second, slow, phase of enhancement of pyrene fluorescence emission

It was noted that, after the initial rapid increase in fluorescence intensity, a slower secondary increase of approx. 6 % in fluorescence emission from the pyrene label occurred (Figure 1), which could not be accounted for by mixing or other possible interference. The rate of this slow change in signal was typically around 8–12 s^{-1} between pH 6.5 and 9.0 respectively, i.e. approxi-

Figure 2 Dissociation of the enzyme– Mg^{2+} complex

Labelled wild-type enzyme (6 μM), prepared in 50 mM Tris/HCl buffer, pH 8.0, was incubated with 300 μM Mg^{2+} ions to form a metal–enzyme complex. The pre-formed complex was subsequently mixed 1:10 (v/v) with buffer containing 10 mM EDTA, and the resultant dissociation of Mg^{2+} was monitored over 100 ms. The trace represents the average of 10 experiments and was fitted to a single-exponential equation. The residuals, describing the closeness of fit of the data to the equation, are described by the lower plot, where $\pm 6.44\text{E-}3$ represents maximum deviation from the calculated curve.

mately one-tenth of the rate of the fast phase. Furthermore, the rate of this process appeared to be independent of the concentration of metal ion used, suggesting the possibility of a rate-limiting conformational change occurring after the initial binding of the metal ion. To further examine this phenomenon, pyrene-labelled wild-type enzyme was mixed stoichiometrically with a range of different Mg^{2+} concentrations at pH 8.0, 6.8 °C, and the rates and amplitudes of both phases of the fluorescence change were estimated by fitting the data to a double-exponential equation (a lower temperature was selected to increase the intensity of the fluorescence changes produced and to increase the difference in the relative rates of phases 1 and 2; see below). Figure 3 shows that the amplitudes of both the fast and slow phases of signal change elicited by Mg^{2+} binding increased with increasing metal ion concentration. Both amplitudes increased concomitantly, with approx. 60 % of the total signal change attributable to the slow phase and a 40 % contribution made by the initial fast phase of fluorescence enhancement at this temperature. On fitting the data to the Michaelis–Menten equation, the mid-points of these curves were determined to be 324 μM and 240 μM for the fast and slow fluorescence changes respectively. Saturation of both events occurred at around 1 mM Mg^{2+} . This result strongly suggests that the slower secondary phase can only occur after the initial binding of Mg^{2+} (fast phase), and therefore its extent is Mg^{2+} -dependent even though the rate does not show a correlation with metal ion concentration.

Effects of pH on the K_d for Mg^{2+} –enzyme complexes

Stopped-flow fluorescence experiments were again used to determine the effects of pH on the equilibrium between Mg^{2+} ions, enzyme and the binary complex, deriving values for k_{diss} and k_{ass} over a range of pH values from 5.0 to 9.2. Surprisingly, the data show that k_{ass} does not change significantly with pH, being typically of the order of $(3.5\text{--}4.0) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ over the entire range of pH values studied. In contrast the off-rate, k_{diss} , was

Figure 3 Amplitudes of the fast and slow phases of pyrene fluorescence enhancement elicited by the binding of Mg^{2+} ions

The figure shows the amplitudes (Amp) of the observed first phase (■; broken line) and second phase (▲; solid line) of Mg^{2+} -induced pyrene fluorescence enhancement elicited by different final concentrations of Mg^{2+} ions (200–2500 μM) at pH 8.0 and 6.8 °C. The data points shown represent averages from 10 separate experiments. The reaction progress curves were fitted to a double-exponential equation provided with the manufacturer's software in order to discriminate between the two phases. Note that at this temperature the relative amplitudes of the two phases are not the same as at 20 °C. The bars indicate S.E.M.

much more sensitive to pH, increasing by more than 2-fold as the pH was lowered from 9.0 to 6.5. It was also noted that the rate of the second, slow, change in fluorescence intensity was virtually independent of pH.

In a subsequent set of experiments, the enzyme or enzyme- Mg^{2+} complex was maintained at pH 8.0 and rapidly mixed with a 10-fold excess of buffer containing 40 mM EDTA

Figure 4 Effect of pH on the rate of debinding of Mg^{2+} from pyrene-labelled wild-type enzyme

Solutions of enzyme (60 μM) pre-mixed with 300 μM Mg^{2+} ions in 10 mM Tris/HCl buffer, pH 8.0, were rapidly mixed (1:10, v/v) with 200 mM Tris/HCl buffer of various initial pH values containing 40 mM EDTA at 20 °C. The final pH at the point of mixing was estimated from initial buffer titration experiments carried out on large volumes. The data shown represent the averaged k_{diss} values from eight separate experiments.

Figure 5 Arrhenius plots of the rates of the two signal changes elicited by mixing Mg^{2+} ions with pyrene-labelled wild-type enzyme at various temperatures

Enzyme, prepared in 50 mM Tris/HCl buffer, pH 8.0, and labelled stoichiometrically with pyrene maleimide as described in the text, was mixed with 300 μM Mg^{2+} ions (final concentration) in an SX17 stopped-flow spectrophotometer at a range of temperatures between 10 and 30 °C. Ten separate experiments performed at each selected temperature were accumulated into averages and analysed according to a double-exponential equation provided with the manufacturer's software to identify the two rates at each given temperature. The diagram shows the Arrhenius plots of the fast phase (○; upper plot) and the slower phase (□; lower plot) of the fluorescence increase, where $1/T \cdot A$ is K^{-1} and k is the rate [fast phase ($\text{M}^{-1} \cdot \text{s}^{-1}$) or slow phase (s^{-1})] of change in fluorescence.

and adjusted such that the final pH after mixing could be varied between pH 5.0 and 9.2 (see the Materials and methods section). In this way the period of exposure of the enzyme to low or high pH was minimized. The rapid dissociation of the metal from the complex was monitored, and the resultant values of k_{diss} are plotted against the final pH in Figure 4. In accordance with data in Table 1, k_{diss} increases significantly at pH values below 7.0.

Determination of the activation energies of the binding processes

The activation energies of the processes reflected by the fast and slow fluorescence changes were determined by measuring the rates of the signal changes obtained by mixing the enzyme with 300 μM Mg^{2+} ions over the temperature range 10–30 °C at pH 8.0. Figure 5 shows the Arrhenius plots obtained for the phases of the signal change at each temperature investigated. The data obtained supported the conclusion that the two phases are rate-limited by different processes. The rate of the fast fluorescence change, dependent on Mg^{2+} ion concentration, increases approx. 2.01 ± 0.12 -fold for every 10 °C rise in temperature, typical of chemical reactions with an activation energy E_a of approx. 48 $\text{kJ} \cdot \text{mol}^{-1}$. The rate of the slow change in fluorescence was more sensitive to temperature (see Figure 5), with a measured Q_{10} of 2.73 ± 0.18 between 15 and 25 °C, thus yielding an activation energy of 80 $\text{kJ} \cdot \text{mol}^{-1}$.

Effect of limited proteolysis by trypsin on both phases of the fluorescence change

A sample of the pyrene-labelled enzyme was subjected to partial proteolysis by trypsin, previously shown to cleave specifically between Lys-36 and Ser-37 [25]. Such treatment leads to the loss of a 36-amino-acid peptide from the N-terminal end of the

protein, resulting in complete loss of enzyme activity [25]. Stopped-flow fluorescence studies using these enzyme species showed that neither the fast nor the slow change in fluorescence intensity occurred from the pyrene moiety on mixing with Mg^{2+} ions.

DISCUSSION

The fluorescence intensity of the bound pyrene moiety of inositol monophosphatase is sensitive to metal binding and has permitted the K_d for metal ions at site 1 to be determined [19]. The 21.5 % increase in pyrene fluorescence elicited by the binding of Mg^{2+} ions is comparable with the total increase of 18 % monitored by stopped-flow spectrophotometry (12 % attributable to the fast phase and 6 % to the slow phase under the conditions used). In the studies reported in the present paper we have used the pyrene-labelled enzyme to determine the rate constants k_{ass} and k_{diss} for the binding equilibrium between the enzyme and Mg^{2+} ions.

The recent crystallographic studies of Bone et al. [20] and the modelling, kinetic and mutagenesis work of Pollack et al. [21] suggest that the Mg^{2+} ion at site 1 activates a water molecule for nucleophilic attack of the substrate and that a second Mg^{2+} ion at site 2 may stabilize the oxyanion of the leaving group (inositol), thus emphasizing the existence of two distinct sites for this metal ion. Furthermore, indirect evidence for two sites has been provided by modelling studies based on the metal binding regions of fructose 1,6-bisphosphatase [29,30], and recently a common core structure obtained by superimposition of a sequence motif from both metal binding sites has been identified [31].

The slow increase in fluorescence (Figure 1) following rapid binding of Mg^{2+} ions could be caused either by a further increase in the pyrene fluorescence emission intensity or by an increase in affinity for the metal ions. In the latter situation, the second phase of metal binding would, under the conditions used, be rate-limited by a change in the structure of the initial enzyme– Mg^{2+} complex rather than by the concentration of metal ions. The studies described here show that the pre-equilibrium K_d of the wild-type enzyme for Mg^{2+} ions is approx. 200 μM higher than that obtained by equilibrium measurements of the K_d at values near to pH 8.0 where the enzyme is maximally active [1,5]. The elevated values of K_d determined by the stopped-flow experiments suggest that the binding of the metal ion to the enzyme is followed by a subtle structural alteration in the microenvironment of site 1, such that the affinity of this site for Mg^{2+} ions increases after initial binding. Pre-equilibrium values of k_{diss} , k_{ass} and K_d were also obtained at various pH values between 6.5 and 9.0 (Table 1). The trends observed for the changes in both k_{diss} (increasing as the pH is lowered) and k_{ass} (almost independent of pH) with changing pH support a model in which the pH affects the structure of the protein and it is this that causes k_{diss} to vary with pH. For a metal binding site containing acidic side chains which ligate bivalent cations, the binding of the cation involved would be expected to compete directly with protons for the carboxylate anions. In such a situation, k_{ass} would be decreased by an increase in the effective protonation of the groups, whereas k_{diss} would not change significantly as the pH is lowered. Conversely, however, our results show that it is k_{diss} that varies with pH and that the binding of Mg^{2+} ions (k_{ass}) is not proton-limited. Since the increase in fluorescence intensity caused by metal binding follows first-order kinetics, the apparent rate constant (k_{app}) at any concentration of Mg^{2+} ions is given by:

$$k_{\text{app}} = k_{\text{ass}}(\text{Mg}^{2+}) + k_{\text{diss}}$$

The data in the inset to Figure 1 and Table 1 show that k_{diss}

Figure 6 Schematic representation of the reaction cycle incorporating the two forms of the enzyme, E and E*

The reaction pathway described by Pollack et al. [21] has been modified to include the different conformational states of the enzyme. For details, see the Discussion section.

contributes more to k_{app} than does k_{ass} , particularly at low concentrations of metal ions, resulting in the high value of K_d .

Values of k_{diss} were obtained from two different experimental approaches. The first method was to examine the effect of the concentration of metal ions on the apparent rate of reaction, which yielded a value of 172.3 s^{-1} at pH 8.0 (see Table 1). The second method was to directly observe the debinding of the metal ion upon mixing enzyme– Mg^{2+} complexes with EDTA. The reaction profile in Figure 2 shows that at pH 8.0 a value of 179 s^{-1} was determined. The debinding process was shown to be first order, which would be expected for the simple dissociation of a binary complex or for a conformational change in the protein (e.g. $\text{E}^* \cdot \text{Mg}^{2+}$ to $\text{E} \cdot \text{Mg}^{2+}$; see Figure 6 and below) which might rate-limit the dissociation. However, the latter possibility is improbable since the rate of dissociation of the complex was shown to be dependent upon the concentration of free Mg^{2+} ions.

The direct determinations of k_{diss} values over a range of pH conditions were carried out in experiments involving the rapid mixing of pre-formed enzyme– Mg^{2+} complexes with 40 mM EDTA between pH 5.0 and pH 9.2 (Figure 4). This protocol enabled the problem of enzyme instability at pH values below 6.0 to be partially overcome, permitting the highest possible value of the pK of the dependence of k_{diss} on pH to be estimated as 5.8. This is close to the expected value for acidic side-chains, corroborating crystallographic studies [20], which have indicated that Glu-70 and/or Asp-90 are ligands for the metal ion.

Several other factors allude to the probability of the occurrence of a conformational change in the enzyme after Mg^{2+} has bound, i.e. $\text{E} \cdot \text{Mg}^{2+}$ to $\text{E}^* \cdot \text{Mg}^{2+}$ (Figure 6). The slow-phase signal change observed after the initial binding of Mg^{2+} is independent of the Mg^{2+} concentration (Figure 3), as would be expected for a conformational rearrangement. Curves describing the amplitude of each of the two phases with increasing Mg^{2+} concentration have approximately the same mid-point as would be expected if only those enzyme molecules in an $\text{E} \cdot \text{Mg}^{2+}$ complex were involved in the second phase. The mid-point of the fast phase (approx. 320 μM) is slightly higher than that of the slow phase (240 μM), which approximates to the value of the K_d at equilibrium for $\text{E}^* \cdot \text{Mg}^{2+}$ interactions at pH 8.0 (272 μM). Finally, the activation energy E_a (80 $\text{kJ} \cdot \text{mol}^{-1}$) of the process described by the second phase is much larger than would be expected for a simple chemical reaction, and is probably indicative of a structural transition in the protein. During the experiments aimed at the determination of the activation energies of the two processes, it was noted that the relative contribution of the slow phase to the overall fluorescence change increased

with decreasing temperature. The reason for this is not yet clear, but it could reflect the influence of temperature on the equilibrium between E and E*, or more likely E·Mg²⁺ and E*·Mg²⁺ (Figure 6).

A comparison of the crystallographic data [20] for the apo- and metallo-enzyme species shows that a peptide region (residues 70–75) containing Glu-70 (site 1) moves approximately 3 Å (0.3 nm) on metal binding. It then interacts with residues 35–39, which are located in a peptide region (residues 29–46) which lies around the base of the metal binding site and is more disordered in the apo form. This may well be part of the structural change monitored by the fluorescence studies described above. Finally, CD studies in the near-UV region [32] and in the far-UV region [33] suggest that significant structural transitions are induced by the presence of Mg²⁺ ions.

Figure 6 summarizes the proposed interactions between inositol monophosphatase and Mg²⁺ ions at site 1. The initial binding of Mg²⁺ to the enzyme (*k*₁) in its ground state (E) induces the enzyme to undergo a conformational change to E* via a process that occurs at a rate of approx. 15 s⁻¹ (*k*₂). The substrate Ins1P and a second Mg²⁺ ion bind to E*·Mg²⁺ and the catalytic process occurs with the dissociation of inositol, the second Mg²⁺ ion and finally P_i to release E*·Mg²⁺ [21]. Since the affinity of site 1 for Mg²⁺ ions is relatively high, the ions would almost certainly remain attached to the enzyme after each successive cycle of dephosphorylation. The binary complexes (E·Mg²⁺ and E*·Mg²⁺) dissociate at rates *k*₋₁ and *k*₋₃ respectively, and the E* form of the enzyme would revert to E at rate *k*₋₄. Computer simulation curves were performed on an IBM-compatible PC using the programme of Zhang et al. [34]. The simulation of the reaction progress curves for Mg²⁺ binding showed that such traces could only be approached if the following conditions prevail. (1) Mg²⁺ ions bind very poorly to the E* form of the enzyme, e.g. *k*₃ is approx. 1 × 10³ M⁻¹·s⁻¹ and *k*₋₃ is approx. 180 s⁻¹. (2) *k*₄ is very low compared with *k*₋₄ (e.g. 1:4000), i.e. the equilibrium between E and E* strongly favours E in the absence of Mg²⁺. (3) The ratio of the rate *k*₂ to *k*₋₂ should be greater than 10:1 for the second phase to occur. Since *k*₂ has been determined as approx. 15 s⁻¹, then *k*₋₂ would be about 1 s⁻¹. Since the rate of dissociation of the metal ion from the complex E*·Mg²⁺ is not rate-limited by a reversal of the structural change (*k*₋₂), then it follows that the dissociation of the metal ion occurs directly from E*·Mg²⁺, and *k*_{diss} (the off-rate; 180 s⁻¹) must be *k*₋₃ in the above scheme.

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