Kinetic characterization of enzyme forms involved in metal ion activation and inhibition of *myo*-inositol monophosphatase

Françoise STRASSER, Patricia D. PELTON and Axel J. GANZHORN*

Marion Merrell Dow Research Institute, 16, rue d'Ankara, F-67080 Strasbourg Cédex, France

Activation and inhibition of recombinant bovine *myo*-inositol monophosphatase by metal ions was studied with two substrates, D,L-inositol 1-phosphate and 4-nitrophenyl phosphate. Mg^{2+} and Co^{2+} are essential activators of both reactions. At high concentrations, they inhibit hydrolysis of inositol 1-phosphate, but not 4-nitrophenyl phosphate. Mg^{2+} is highly selective for inositol 1-phosphate ($k_{cat.} = 26\ s^{-1}$) compared with the aromatic substrate ($k_{cat.} = 1\ s^{-1}$), and follows sigmoid activation kinetics in both cases. Co^{2+} catalyses the two reactions at similar rates ($k_{cat.} = 4\ s^{-1}$), but shows sigmoid activation only with the natural substrate. Li^+ and Ca^{2+} are uncompetitive inhibitors with respect to inositol 1-phosphate, but non-competitive with respect to 4-nitrophenyl phosphate. Both metal ions are competitive inhibitors with respect to Mg^{2+} with 4-nitrophenyl phosphate as the substrate. With inositol 1-phosphate, Ca^{2+} is competitive and Li^+

non-competitive with respect to Mg^{2+} . Multiple inhibition studies indicate that Li^+ and P_i can bind simultaneously, whereas no such complex was detected with Ca^{2+} and P_i . Several sugar phosphates were also characterized as substrates of *myo*-inositol monophosphatase. D-Ribose 5-phosphate is slowly hydrolysed ($k_{cat.} = 3\ s^{-1}$), but inhibition by Li^+ is very efficient ($K_i = 0.15\ mM$), non-competitive with respect to the substrate and competitive with respect to Mg^{2+} . Depending on the nature of the substrate, Li^+ inhibits by preferential binding to free enzyme (E), the enzyme–substrate (E·S) or the enzyme–phosphate (E· P_i) complex. Ca^{2+} , on the other hand, inhibits by binding to E and E·S, in competition with Mg^{2+} . The results are discussed in terms of a catalytic mechanism involving two metal ions.

INTRODUCTION

myo-Inositol monophosphatase (EC 3.1.3.25) plays a crucial role in the metabolism of mammalian brain inositol phosphates. This enzyme hydrolyses a number of inositol monophosphates, generated on breakdown of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, providing free inositol to replenish the neurotransmitter-sensitive pool of phosphoinositides (see refs. [1,2] for recent reviews and more references). It is uncompetitively inhibited by Li^+ [3] and may be the target of this metal ion in the treatment of manic depression [4,5]. The enzyme has therefore received considerable attention not only with respect to its structure and catalytic mechanism, but also as a target for drug design.

The interactions with metal ions are of particular interest, considering the importance of the uncompetitive inhibitor Li^+ in the treatment of affective disorders. Mg^{2+} is an essential activator of *myo*-inositol monophosphatase [3], the activation phase of which follows sigmoid kinetics [6,7]. At high concentrations, Mg^{2+} acts as an uncompetitive inhibitor, mutually exclusive with Li^+ [6]. Leech et al. [7] suggested that both Li^+ and Mg^{2+} exert their inhibitory action through binding to an enzyme–phosphate complex and also that the activator, Mg^{2+} , binds after the substrate, Ins1P. Other studies have provided evidence that Mg^{2+} is needed for the binding of phosphate as well as an inhibitory substrate analogue [8,9]. Several other metal ions also interact with *myo*-inositol monophosphatase. Ca^{2+} is a potent inhibitor, competitive with Mg^{2+} [3,10], whereas Co^{2+} can replace Mg^{2+} to some extent as an activator [3]. Zn^{2+} and Mn^{2+} also inhibit the Mg^{2+} -activated enzyme, but become activators at higher concentrations [11]. Recently, the X-ray-crystallographic

structures of the enzyme complexed to sulphate· Gd^{3+} [12], Ins1P· Gd^{3+} [13] and Mn^{2+} [14] became available. They showed at least two metal-ion-binding sites and were interpreted in terms of a catalytic mechanism that involves two essential Mg^{2+} ions, one binding before and one after the substrate [14].

Despite considerable progress with regard to the structure and catalytic mechanism of *myo*-inositol monophosphatase, the precise nature of the various inhibitory enzyme–metal complexes is still unclear. To investigate these interactions further, we have used 4-nitrophenyl phosphate, a substrate with a low commitment to catalysis, in addition to the natural substrate, Ins1P. From inhibition studies with Li^+ and Ca^{2+} , we have obtained more information on the order of substrate and metal binding, the occurrence of inhibitory complexes depending on the nature of the metal and substrate, and the affinity of metal ions for different enzyme forms.

EXPERIMENTAL

Materials

4-Nitrophenyl phosphate, $MgCl_2$ and $CaCl_2$ and most of the chemicals for molecular biology were obtained from Sigma. Sugar phosphates came from either Sigma or Fluka. $LiCl$ and $CoCl_2$ were from Aldrich, and D,L-Ins1P was purchased from Bachem. Calf spleen purine nucleoside phosphorylase was from Sigma and was purified on a Sephadex G-75 column in 25 mM Tris/HCl, pH 7.5. 2-Amino-6-mercapto-7-methylpurine ribonucleoside was prepared as described [15]. Kits for PCR reactions, DNA purification and sequencing (Sequenase) came from

* To whom correspondence should be addressed.

Perkin-Elmer, Bio 101 and US Biochemicals respectively. The *Escherichia coli* strain, JM109, was obtained as competent cells from Stratagene, and the strain BL21(DE3)pLysS was donated by Dr. Raymond Leppick. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Reagents for preparation of total RNA and agarose for gel electrophoresis were obtained from Gibco-BRL. Antibiotics and calf intestinal alkaline phosphatase came from Boehringer-Mannheim, bactotryptone and yeast extract from Difco (Detroit, MI, U.S.A.) and isopropyl β -thiogalactoside from Clontech. Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. The expression vector pT7.5 was generously given by Dr. Stan Tabor (Harvard University), Cambridge, MA, U.S.A.).

Cloning, expression and purification of bovine brain myo-inositol monophosphatase

Bovine brains were obtained from the local slaughterhouse and were minced, frozen in liquid nitrogen and stored at -80°C . Total cellular RNA from the cortex was purified by the guanidinium isothiocyanate method of Chirgwin et al. [16]. The published sequence of bovine inositol monophosphatase [17] was used to design the primers for cloning by reverse transcriptase PCR: 5'-GAATTCATCGATAGGAGGAATATAATATGGCTGATCCTT-3' (upstream primer), 5'-GTCGACTCTAGATTAACTTCATCATCTC-3' (downstream primer). Both primers contained added-on restriction sites (italized) for subcloning into vectors and the upstream primer included a Shine-Dalgarno sequence and spacer for prokaryotic expression (underlined). The coding sequence is in bold print. cDNA preparation and subsequent amplification reactions were performed using the protocols and reagents supplied with the RNA PCR kit. The PCR product was analysed by ethidium bromide staining on agarose-gel electrophoresis and purified by glassmilk (Gene-Clean, Bio 101) binding. The purified DNA was inserted into the *Sma*I site of the expression vector, pT7.5, and sequenced using the Sequenase kit. The DNA sequence translated into an amino acid sequence identical with the one previously published for bovine inositol monophosphatase [17]. After subcloning into the *Sma*I site of vector pT7.5, the protein was expressed in *E. coli* strain BL21(DE3)pLysS. Then 1 litre of medium containing 20 g/l bactotryptone, 10 g/l yeast extract, 5 g/l NaCl, 0.2% glycerol, 50 $\mu\text{g}/\text{ml}$ ampicillin and 20 $\mu\text{g}/\text{ml}$ chloramphenicol was inoculated with 5 ml of saturated culture and placed in a shaking incubator (225 rev./min) at 30°C . When the A_{600} reached 0.6–0.8, protein expression was induced by the addition of 0.4 mM isopropyl β -thiogalactoside. The cells were harvested 5–6 h later by centrifugation for 5 min at 4420 *g* and frozen at -20°C until needed. For purification of the expressed protein, the bacterial pellets were thawed, resuspended in 30 ml of cold 50 mM Tris/HCl (pH 7.5 at 25°C) containing 1 mM EGTA, 1 mM EDTA, 1 mM 1,10-phenanthroline, 1 mM phenylmethanesulphonyl fluoride and 0.1 M NaCl. The homogenate was sonicated in 30 s bursts with 1.5 min intervals on ice until no longer viscous followed by centrifugation for 30 min at 11 500 *g*. The supernatant was incubated for 45 min at 68°C [18]. The heat-treated material was centrifuged under the same conditions as the homogenate and dialysed against 4 litres of 20 mM Tris/HCl (pH 7.5 at 25°C) containing 0.1 mM EGTA and 1 mM 1,10-phenanthroline at 4°C . From this point, the purification protocol was essentially as previously described [19], except that the Blue Sepharose chromatography was eliminated. Yields were 60–70% and between 9 and 15 mg of protein was obtained from 1 litre of culture. A typical purification is shown

Table 1 Purification of recombinant bovine myo-inositol monophosphatase

	Protein (mg)	Activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)
Homogenate	300	360	1.2
Heat step	63	280	4.4
Q Sepharose HP	20	230	12
Phenyl-Superose	9	220	25

in Table 1. The purity was at least 90% as determined by SDS/PAGE with silver nitrate staining using the Phast System (Pharmacia). The enzyme had a specific activity of $25 \mu\text{mol}$ of phosphate released/min per mg of protein, which is identical with that previously determined for the tissue-purified enzyme in a standard assay at 37°C [20]. The concentration of protein was determined by u.v. spectroscopy with $\epsilon_{280} = 26\,200 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [19].

Enzyme assays

With *Ins1P* and D-ribose 5-phosphate, release of P_i was measured colorimetrically in a coupled spectrophotometric assay, using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine nucleoside phosphorylase [15]. The assay mixture contained 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside and 0.5 unit of nucleoside phosphorylase in the presence of substrate and metal ions, as indicated, in a total volume of 0.5 ml. The absorbance was followed at 360 nm and absorbance changes were transformed into turnover numbers using an absorption coefficient (ϵ) of $10\,600 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The amount of enzyme was adjusted to maintain a linear response during the time course of the assay. When inhibition by Ca^{2+} was measured, EGTA was omitted, and the *Ins1P* and 2-amino-6-mercapto-7-methylpurine ribonucleoside solutions were passed over a Chelex 100 column to remove contaminating Ca^{2+} and other metal ions. A similar assay system, but with xanthine oxidase as a second auxiliary enzyme [6], was used for the other sugar phosphates. With 4-nitrophenyl phosphate, formation of nitrophenolate was monitored at 400 nm ($\epsilon = 16.7 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$). All enzyme assays were performed at 37°C .

Data analysis

Initial velocities at fixed levels of activator and in the absence of inhibitors were fitted by the Michaelis-Menten equation. When Mg^{2+} or Co^{2+} were varied at a fixed concentration of substrate, data were analysed using the Hill equation [eqn. (1)]:

$$v = VA^h/(K_a^h + A^h) \quad (1)$$

$$v = VA^h[(K_a^h + A^h(1 + A/K_i))] \quad (1a)$$

where V is the maximal velocity, A the concentration of metal ion, h the Hill coefficient, and K_a the concentration of metal ion at half-maximal velocity. If necessary, a term was included to account for inhibition at higher concentrations of the metal ion [eqn. (1a)]. If both substrate and activator were varied simultaneously, best fits were obtained with eqn. (2):

$$v = VA^hB/(K_{1a}^hK_b + K_a^hB + K_bA^h + A^hB) \quad (2)$$

where A and B are the concentrations of metal ion and substrate, h is the Hill coefficient to account for the non-linear activation by metal, K_a and K_b are Michaelis constants, and K_{ia} is an inhibition constant to describe binding of the metal ion in the absence of substrate. The programs COMP, NONCOMP and UNCOMP [21] were used to analyse inhibition experiments with various concentrations of substrate. When Mg^{2+} was the varied component, the appropriate equations were modified as shown in eqn. (3) for the case of competitive and eqn. (4) for non-competitive inhibition:

$$v = VA^h[K_a^h(1 + I/K_{is}) + A^h] \quad (3)$$

$$v = VA^h/[K_a^h(1 + I/K_{is}) + A^h(1 + A/K_i)] \quad (3a)$$

$$v = VA^h/[K_a^h(1 + I/K_{is}) + A^h(1 + I/K_{ii})] \quad (4)$$

where K_{is} and K_{ii} are slope and intercept inhibition constants respectively. Eqn. (3a) contains an additional term to account for inhibition at high concentrations of Mg^{2+} and was used to analyse Ca^{2+}/Mg^{2+} inhibition patterns, when *Ins1P* was the fixed substrate.

Slope and intercept inhibition constants have to be corrected for the presence of the activator, Mg^{2+} , when it is binding to the same enzyme form as the inhibitor. Correction factors were determined from Mg^{2+} activation constants (K_a), when binding to enzyme-substrate complexes was being considered (intercept inhibition constants). When slope inhibition constants were being corrected, the affinity constant of the enzyme for Mg^{2+} , $K_d = 0.3$ mM [9], was used. Multiple inhibition experiments with two inhibitors were analysed using eqn. (5) [22], where v_0 is the velocity in the absence of inhibition, K_i and K_j are inhibition constants for inhibitors I and J respectively and β a constant to account for any interaction between the two inhibitors:

$$v = v_0/(1 + I/K_i + J/K_j + IJ/\beta K_i K_j) \quad (5)$$

Eqn. (6) was applied when inhibition was mutually exclusive:

$$v = v_0/(1 + I/K_i + J/K_j) \quad (6)$$

Curve fitting was carried out using the programs SIGMAPLOT (Jandel Scientific) or ENZFITTER (Biosoft).

RESULTS

4-Nitrophenyl phosphate as a substrate of *myo*-inositol monophosphatase

The activity of our *myo*-inositol monophosphatase preparations with 4-nitrophenyl phosphate is not due to a contaminating phosphatase activity. Even though a crude extract of untransformed bacterial cells also hydrolyses 4-nitrophenyl phosphate, presumably the result of non-specific *E. coli* alkaline phosphatase, this activity is completely lost during the heat-treatment step. In contrast, 4-nitrophenyl phosphatase activity (total activity about 5% of that obtained in the standard assay with 2-glycerophosphate) remains after heat treatment of cells transformed with the *myo*-inositol monophosphatase gene. In addition, the recombinant enzyme used in this study has the same kinetic properties with respect to this substrate as the previously characterized enzyme from bovine brain. As with the natural substrate, Mg^{2+} is an essential cofactor and activation follows sigmoid kinetics. Li^+ , Ca^{2+} , F^- , *Ins1P* and hydroxy-[4-(5,6,7,8-tetrahydronaphthalen-1-yloxy)-phenyl]methyl phosphonic acid, a selective inositol phosphatase inhibitor [23], inhibit the reaction. Similar solvent isotope effects have been found with *Ins1P*, 2-glycerophosphate and 4-nitrophenyl phosphate [6]. These observations rule out the possibility that hydrolysis of 4-nitro-

Figure 1 Activation of *myo*-inositol monophosphatase by metal ions

Initial velocities were measured at different concentrations of Mg^{2+} (○, ●) or Co^{2+} (△, ▲) in the presence of 0.5 mM *Ins1P* (○, △) or 10 mM 4-nitrophenyl phosphate (●, ▲) at pH 7.5 and 37 °C. Enzyme concentrations were 6 nM (○), 60 nM (●), 15 nM (△) and 16 nM (▲). The y-axis corresponds to the change in A_{360}/min (for *Ins1P*) or A_{400}/min (for 4-nitrophenyl phosphate). The curves are calculated using eqn. (1) or (1a) and the values in Table 2.

Table 2 Apparent kinetic constants for *myo*-inositol monophosphatase at pH 7.5 and 37 °C

k_{cat} (catalytic-centre activity), K_a (the concentration of metal ion at half-maximal velocity), h (the Hill coefficient) and K_i (the metal inhibition constants) were determined by varying the metal ion concentration over a wide range at 0.5 mM *Ins1P* or 10 mM 4-nitrophenyl phosphate and fitting the data by eqn. (1) or (1a). Standard errors were less than 25% in all cases, but 40% for K_i (Co^{2+}) with 4-nitrophenyl phosphate. Catalytic-centre activities were calculated on the basis of protein concentration from the u.v. spectrum [19]. K_m values were obtained at 1 mM Mg^{2+} and 0.2 mM Co^{2+} (metal ion concentration at maximal velocity) for *Ins1P*, and 10 or 4 mM Mg^{2+} or Co^{2+} for 4-nitrophenyl phosphate.

Activator	k_{cat} (s^{-1})	K_a (mM)	K_m (mM)	h	K_i (mM)
<i>Ins1P</i>					
Mg^{2+}	26	0.2	0.1	1.8	5.2
Co^{2+}	4.4	0.05	0.4	2.5	1.3
4-Nitrophenyl phosphate					
Mg^{2+}	1.1	2.8	1.9	2.2	—
Co^{2+}	4.6	0.7	1.1	1.1	(27)

phenyl phosphate is catalysed by another phosphatase that is not removed by our purification protocol.

4-Nitrophenyl phosphate has previously been shown to be a weak substrate ($k_{cat}/K_m = 0.2\%$ of *Ins1P*) of *myo*-inositol monophosphatase from bovine brain [6,24], and our results with the recombinant bovine enzyme are in agreement with this finding (Figure 1 and Table 2). Activation by Mg^{2+} in the presence of saturating 4-nitrophenyl phosphate follows sigmoid kinetics with a Hill coefficient close to 2, similar to that for the natural substrate. Unlike the situation with *Ins1P*, no inhibition by the metal ion occurs up to 10 mM. Co^{2+} , another known metal activator of *myo*-inositol monophosphatase [3], shows the same overall characteristics with *Ins1P* as Mg^{2+} , including sigmoid kinetics at low and inhibition at high concentration of the metal (Figure 1 and Table 2). However, it appears to bind more tightly, but activate less efficiently than Mg^{2+} . With 4-nitrophenyl phosphate, on the other hand, a 4 times higher rate is observed than with Mg^{2+} (Table 2). No apparent co-operativity

Figure 2 Double-reciprocal plots of initial velocities of *myo*-inositol monophosphatase with Mg^{2+} and 4-nitrophenyl phosphate at pH 7.5 and 37 °C

v has units of $\Delta A_{400}/\text{min}$ and corresponds to an enzyme concentration of 24 μM . Concentrations of 4-nitrophenyl phosphate in (a) were 0.7 ($\circ$), 0.9 ($\bullet$), 1.2 ($\square$), 1.6 ($\blacksquare$), 2.8 ($\triangle$) and 8 ($\blacktriangle$) mM. Concentrations of Mg^{2+} in (b) were 1 ($\circ$), 2 ($\bullet$), 3 ($\square$) and 10 ($\blacksquare$) mM. (Data at 2.5 and 4 mM Mg^{2+} were omitted for clarity.) The lines are from an overall fit of the data by eqn. (2). The derived kinetic constants are $K_a = 3.4 \pm 0.2$ mM, $K_b = 2.1 \pm 0.1$ mM, $K_c = 1.9 \pm 0.1$ mM, $V = 26 \pm 1$ and $h = 2.2 \pm 0.2$, where a refers to Mg^{2+} and b to substrate.

Figure 3 Inhibition of *myo*-inositol monophosphatase by metal ions with respect to Mg^{2+} or 4-nitrophenyl phosphate

v is $\Delta A_{400}/\text{min}$. (a) Inhibition by Ca^{2+} [0 ($\circ$), 0.5 ($\bullet$), 1 ($\square$) and 2 ($\blacksquare$) mM] with respect to Mg^{2+} at 10 mM 4-nitrophenyl phosphate (96 nM enzyme). The curves were calculated by a fit of eqn. (3), with $K_a = 2.1 \pm 0.1$ mM, $V = 0.12 \pm 0.01$, $h = 2.0 \pm 0.1$ and $K_{is} = 0.6 \pm 0.1$ mM. (b) Inhibition by Li^+ [0 ($\circ$), 10 ($\bullet$), 20 ($\square$), 30 ($\blacksquare$) and 50 ($\triangle$) mM] with respect to Mg^{2+} at 10 mM 4-nitrophenyl phosphate (43 nM enzyme). The curves were calculated by a fit of eqn. (3), with $K_a = 4.6 \pm 0.4$ mM, $V = 0.07 \pm 0.01$, $h = 1.4 \pm 0.1$, $K_{is} = 5.6 \pm 1.0$ mM. (c) Inhibition by Ca^{2+} [0 ($\circ$), 1 ($\bullet$), 3 ($\square$), 5 ($\blacksquare$), and 7 ($\triangle$) mM] with respect to 4-nitrophenyl phosphate at 10 mM Mg^{2+} (80 nM enzyme). (d) Inhibition by Li^+ [0 ($\circ$), 5 ($\bullet$), 10 ($\square$), and 15 ($\blacksquare$) mM] with respect to 4-nitrophenyl phosphate at 3 mM Mg^{2+} (150 nM enzyme). The lines for (c) and (d) are from a fit with the program NONCOMP. The derived constants are $K_{is}(\text{Ca}^{2+}) = 6.2 \pm 1.0$ mM, $K_{ii}(\text{Ca}^{2+}) = 3.7 \pm 0.4$ mM, $K_{is}(\text{Li}^+) = 11 \pm 2$ mM, and $K_{ii}(\text{Li}^+) = 16 \pm 4$ mM. K_m was 2.8 ± 0.2 mM in both experiments. V was 0.13 ± 0.01 for (c) and 0.10 ± 0.01 for (d).

was detected, but a small inhibitory effect may occur at higher concentrations.

As previously reported for *Ins1P* [6,7], double-reciprocal plots

of v against concentration of 4-nitrophenyl phosphate at fixed levels of Mg^{2+} are linear (Figure 2b), indicating simple Michaelis-Menten kinetics, but plots of $1/v$ against $1/[\text{Mg}^{2+}]$ (Figure 2a)

Figure 4 Inhibition of *myo*-inositol monophosphatase by Ca^{2+} with respect to Mg^{2+} or Ins1P

(a) Inhibition by Ca^{2+} [0 (○), 10 (●), 20 (□), 30 (■) and 40 (△) μM] with respect to Ins1P at 0.5 mM Mg^{2+} , 6 nM enzyme. The lines are from a fit with the program UNCOMP with $V = 0.07 \pm 0.01$ ($k_{\text{cat}} = 20 \text{ s}^{-1}$), $K_m = 0.14 \pm 0.01 \text{ mM}$, and $K_i = 10 \pm 1 \mu\text{M}$. (b) Inhibition by Ca^{2+} [0 (○), 10 (●), 20 (□), and 40 (■) μM] with respect to Mg^{2+} at 0.5 mM Ins1P . The lines are from a fit with eqn. (3a) with $V = 0.07 \pm 0.01$, $K_a = 0.24 \pm 0.02 \text{ mM}$, K_i (for Mg^{2+} inhibition) = $4.9 \pm 0.6 \text{ mM}$, and K_{is} (for Ca^{2+} inhibition) = $3.7 \pm 0.7 \mu\text{M}$. v has units of $\Delta A_{360}/\text{min}$.

are non-linear at all substrate concentrations. The results in Figure 2 rule out a rapid equilibrium ordered mechanism for substrate and Mg^{2+} binding, as $1/v$ intercepts corresponding to

saturation substrate or metal ion are different. With Ins1P , an equilibrium ordered pattern, indicating substrate binding before Mg^{2+} , occurred at pH 6.5, where catalysis is slow and the substrate K_i increases [7]. With 4-nitrophenyl phosphate, the rate of the catalysis and the affinity for the enzyme at pH 7.5 are even lower than for Ins1P at pH 6.5. It therefore seems reasonable to assume equilibrium binding and a low commitment to catalysis for this substrate. Consequently, if binding were strictly ordered, one of the double-reciprocal plots in Figure 2 should show a common $1/v$ intercept [25]. Data were analysed using the kinetic equation for a sequential two-substrate mechanism, modified to allow for the apparent co-operativity of metal activation [eqn. (2)]. The derived kinetic constants (see the legend to Figure 2) are in good agreement with the apparent values in Table 2, which shows that the simplified analysis is valid at least for determining K_m and k_{cat} values. As with the Hill equation, eqn. (2) is only an approximation that allows mathematical treatment of the experimental data. K_{ia} and K_a are constants that correspond to half-saturating metal concentration in the absence and presence of substrate, but are not to be taken as affinity constants with respect to a defined enzyme form.

Inhibition by phosphate and metal ions

Double-reciprocal plots versus Mg^{2+} at different fixed levels of Ca^{2+} or Li^+ and 4-nitrophenyl phosphate as the substrate are shown in Figures 3(a) and 3(b). Data points were fitted separately to eqn. (1) to determine apparent maximal velocities at each concentration of inhibitor and compare them by t test. No significant differences were found, indicating that Ca^{2+} and Li^+ are competitive inhibitors with respect to Mg^{2+} . An overall fit, using eqn. (3) for competitive inhibition is shown in Figure 3. Both metal ions were non-competitive with respect to 4-nitrophenyl phosphate (Figures 3c and 3d).

Inhibition by Ca^{2+} versus Ins1P is shown in Figure 4(a). The data were best fitted by the equation for uncompetitive inhibition and, even though a small slope effect is seen at the highest Ca^{2+} concentration, the non-competitive fit gave no improvement on standard errors for V , K_m and K_{ii} , but more than 50% error for K_{is} (the slope inhibition constant). Owing to the non-linear activation at low and inhibition at high concentrations of Mg^{2+} , the interpretation of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ inhibition pattern (Figure 4b) is less straightforward. When the curves are analysed

Figure 5 Yonetani–Theorell plots for mutual inhibition of *myo*-inositol monophosphatase by phosphate and metal ions

(a) Inhibition by phosphate and 0 (○), 5 (●), 10 (△) and 20 (▲) mM Li^+ at 10 mM 4-nitrophenyl phosphate and 5 mM Mg^{2+} (22 nM enzyme). The lines are from a fit of eqn. (5), with K_i (for Li^+) = $11.0 \pm 0.3 \text{ mM}$, K_i (for phosphate) = $0.12 \pm 0.01 \text{ mM}$ and $\beta = 0.61 \pm 0.04$. (b) Inhibition by phosphate and 0 (○), 1 (●), 2 (△) and 3 (▲) mM Ca^{2+} at 10 mM 4-nitrophenyl phosphate and 5 mM Mg^{2+} (22 nM enzyme). The lines are from a fit with eqn. (6), with K_i (for Ca^{2+}) = $1.9 \pm 0.1 \text{ mM}$ and K_i (for phosphate) = $0.12 \pm 0.01 \text{ mM}$.

Table 3 Kinetic constants for *myo*-inositol phosphatase substrates at pH 7.5 and 37 °C

k_{cat} , K_a and $K_{i,\text{Mg}}$ were determined by varying the metal ion concentration in the presence of saturating substrate and fitting the data by eqn. (1) or (1a). Hill coefficients varied between 1.7 and 2.3. K_m values were obtained at saturating or optimal (non-inhibiting) concentrations of Mg^{2+} . Inhibition by Li^+ was determined at a Mg^{2+} concentration of about $5 \times K_a$ at saturating concentrations of substrate; initial velocities were fitted with $v = V/(1 + K_{i,\text{app}})$, where v is the rate in the presence and V in the absence of inhibitor.

Substrate	k_{cat} (s^{-1})	K_a (mM)	K_m (mM)	$K_{i,\text{Mg}}$ (mM)	$K_{i,\text{Li(app.)}}$ (mM)
Ins1P	26	0.2	0.1	5.2	0.6
Glycerol 2-phosphate	16	0.4	0.3	9.4	4.5
Glycerol 1-phosphate	9	1.0	1.0	18	4.9
D- α -Galactose 1-phosphate	24	0.5	0.4	4.3	1.7
D- α -Glucose 1-phosphate	14	1.1	1.2	18	8.1
D- β -Glucose 1-phosphate	13	0.7	0.6	9.0	11
D-Glucose 6-phosphate	3	2.2	1.0	—	3.7
D-Ribose 5-phosphate	3	1.0	0.8	—	1.0
D-Fructose 6-phosphate	2	1.3	0.3	—	9.1
D-Mannose 6-phosphate	1	1.2	0.3	—	3.8

separately using eqn. (1a), maximum velocities at different Ca^{2+} concentrations are not significantly different from each other, but K_a increases, indicating competitive inhibition. At high, inhibiting, concentrations of Mg^{2+} , Ca^{2+} inhibition disappears (Figure 4b), again suggesting that binding of the two metals is mutually exclusive and involves the same or overlapping sites (but not necessarily the same enzyme forms). Inhibition by Ca^{2+} is therefore competitive with respect to Mg^{2+} and uncompetitive with respect to Ins1P. Li^+ was a non-competitive inhibitor with respect to Mg^{2+} and uncompetitive with respect to Ins1P (results not shown), in agreement with previously reported results [3,7].

P_i was a competitive inhibitor with respect to 4-nitrophenyl phosphate (results not shown). The K_i of 0.040 ± 0.001 mM agrees well with the value of 0.05 mM, determined by equilibrium dialysis [8] at a similar concentration of Mg^{2+} (8 mM compared with 10 mM in our experiment). Multiple inhibition experiments at fixed concentrations of 4-nitrophenyl phosphate and Mg^{2+} were undertaken to test for the formation of $\text{E} \cdot \text{P}_i \cdot \text{Li}^+$ and $\text{E} \cdot \text{P}_i \cdot \text{Ca}^{2+}$ complexes. The results were plotted by the method of Yonetani and Theorell [26]. All the lines for different fixed concentrations of Li^+ and various concentrations of phosphate intersect at a common point above the horizontal axis (Figure 5a). An interaction constant $\beta = 0.6$ was determined between phosphate and metal ion, indicating that an inhibitory $\text{E} \cdot \text{P}_i \cdot \text{Li}^+$ complex is formed [22]. With Ca^{2+} , the lines appear parallel (Figure 5b), and the quantitative analysis suggested an infinite value for β . Obviously, Ca^{2+} does not inhibit by binding to an $\text{E} \cdot \text{P}_i$ complex.

Specificity of metal inhibition

A series of phosphate esters, in particular sugar phosphates, have been characterized as substrates of *myo*-inositol monophosphatase. Their kinetic constants as well as their influence on the inhibition by Li^+ and high concentrations of Mg^{2+} have been compared, as summarized in Table 3. Previous reports [6,7] suggested that Mg^{2+} and Li^+ are most efficient as inhibitors with substrates that are rapidly hydrolysed. The more extensive list now shows that this is not always the case. Li^+ inhibition with D-ribose 5-phosphate, a relatively weak substrate, is almost as potent as with Ins1P and ten times more efficient than with glucose 1-phosphate. Inhibition by Li^+ with this substrate was therefore analysed in more detail. As shown in Figure 6, inhibition was competitive with respect to Mg^{2+} and non-competitive with respect to substrate. The 'uncompetitive' (intercept) component

Figure 6 Inhibition of *myo*-inositol monophosphatase by Li^+ with respect to Mg^{2+} or D-ribose 5-phosphate

(a) Inhibition by Li^+ [0 ($\circ$), 0.5 ($\bullet$), 1 ($\square$) and 1.5 ($\blacksquare$) mM] with respect to D-ribose 5-phosphate at 1 mM Mg^{2+} (87 nM enzyme). The lines are from a fit with the program NONCOMP with $V = 0.10 \pm 0.01$ ($k_{\text{cat}} = 1.8 \text{ s}^{-1}$), $K_m = 1.7 \pm 0.1$ mM, $K_s = 3.2 \pm 0.9$ mM and $K_{i,\text{Li}} = 0.39 \pm 0.02$ mM. (b) Inhibition by Li^+ [0 ($\circ$), 1 ($\bullet$), 2 ($\square$), 3 ($\blacksquare$) and 5 ($\triangle$) mM] with respect to Mg^{2+} at 4 mM D-ribose 5-phosphate (35 nM enzyme). The lines are from a fit with the program COMP with $V = 0.09 \pm 0.01$ ($k_{\text{cat}} = 4 \text{ s}^{-1}$), $K_a = 0.9 \pm 0.1$ mM and $K_s = 0.12 \pm 0.01$ mM. Owing to relatively high concentrations of Mg^{2+} non-linearity of activation is not observed in this experiment.

was ten times greater than the slope effect, quite unlike the situation with 4-nitrophenyl phosphate, where slope and intercept effects were similar. Inhibition by Mg^{2+} , on the other

hand, was less efficient than with any of the faster substrates ($K_{i,app} > 30$ mM).

DISCUSSION

Accumulating evidence suggests that at least two Mg^{2+} ions are involved in the catalytic mechanism of *myo*-inositol monophosphatase. One Mg^{2+} (M1 site) binds to the free enzyme [27], is required for phosphate binding [8,9] and may activate a water molecule for nucleophilic attack [13]. The second (M2 site) binds to the enzyme–substrate complex and facilitates ester cleavage [7,28]. X-ray-crystallographic and modelling studies, as well as activity-titration curves with Zn^{2+} and Mn^{2+} , clearly support the two-metal mechanism [11–14]. It was suggested that M1 corresponds to the Gd^{3+} -binding site seen in the X-ray structure of enzyme–sulphate and enzyme–substrate complexes [12,13], whereas M2 can be occupied by Li^+ , causing uncompetitive inhibition [14,28]. Also, M2, but not M1, may interact with substrate hydroxy groups that are involved in binding and/or catalysis [28]. A kinetic mechanism was proposed [11], in which only the second Mg^{2+} has to bind and dissociate during the catalytic cycle (Scheme 1). In this study, we characterize the enzyme forms that are kinetically important for activation and inhibition by metal ions. We also show that the particular enzyme form involved depends on the nature of the substrate as well as the metal.

Ca^{2+} is a competitive inhibitor with respect to Mg^{2+} with inositol phosphate and with 4-nitrophenyl phosphate as the substrate. It may therefore be considered an inhibitory analogue of Mg^{2+} and can be used in dead-end inhibition studies to delineate the order of substrate and Mg^{2+} binding [29]. Consequently, the uncompetitive pattern found for Ca^{2+} inhibition with respect to *Ins1P* indicates an ordered mechanism with substrate binding before the catalytic Mg^{2+} ion, or the inhibitor, Ca^{2+} . This confirms the mechanism proposed by Leech et al. [7] based on initial-velocity data in the absence of inhibitory metal ions. With 4-nitrophenyl phosphate on the other hand, the initial-velocity patterns and the non-competitive inhibition by Ca^{2+} argue against any preferred order of binding. This means that under certain conditions an $E \cdot Mg^{2+} \cdot Mg^{2+}$ (and perhaps $E \cdot Mg^{2+} \cdot Ca^{2+}$) complex may occur (lower pathway in Scheme 1).

Obviously, a hydroxy group of the inositol ring, not present in the aromatic substrate, participates in the formation of a high-affinity Ca^{2+} -binding site (Table 4). Similar conclusions for the analogous Mg^{2+} -binding site have been drawn on the basis of

substrate-specificity studies [6,28]. It is not identical with a high-affinity Ca^{2+} site, detected by fluorescence studies with pyrene/maleimide-labelled enzyme [9]. In this case, metal ion binding to the free enzyme had been studied, whereas in our studies inhibition by Ca^{2+} depends on the presence of the substrate. In fact, Ca^{2+} binding to free enzyme and uncompetitive inhibition by the same metal ion are again clear evidence for the existence of two metal-ion-binding sites.

On a molecular basis, we suggest that the inhibitory site, seen with *Ins1P*, corresponds to M2, and an $E \cdot Mg^{2+} \cdot S \cdot Ca^{2+}$ complex is formed (Scheme 1). The multiple inhibition studies indicate that, unlike Li^+ , Ca^{2+} has no enhanced affinity for an $E \cdot Mg^{2+} \cdot P_i$ complex. With 4-nitrophenyl phosphate, one inhibitory site may still be M2, forming $E \cdot Mg^{2+} \cdot S \cdot Ca^{2+}$ and giving rise to the intercept effect in Figure 3. The slope effect may then be attributed to Ca^{2+} binding to E forming $E \cdot Ca^{2+}$, in competition with the first Mg^{2+} (M1) to bind. The corrected K_i value (0.2 mM; Table 4), describing the interaction with free enzyme, is in good agreement with the K_d value from a titration experiment using terbium fluorescence [30]. However, it is about 20-fold higher than the value reported by Greasley et al. [9]. Perhaps the kinetic value really reflects the formation of $E \cdot Mg^{2+} \cdot Ca^{2+}$, rather than $E \cdot Ca^{2+}$, which would explain the lower affinity.

An inhibitory $E \cdot Mg^{2+} \cdot S \cdot Li^+$ complex was also suggested for the uncompetitive inhibition of Li^+ with respect to *Ins1P*, based on the observation of greatly differing Li^+ K_i values in the presence of different substrates [6,24]. More recent studies [7] have provided evidence that Li^+ prevents the dissociation of the product, P_i , by taking the place of the second Mg^{2+} in the $E \cdot Mg^{2+} \cdot P_i \cdot Mg^{2+}$ complex to give $E \cdot Mg^{2+} \cdot P_i \cdot Li^+$ (Scheme 1). This is in agreement with the non-competitive inhibition with respect to the activator Mg^{2+} and the observation that Li^+ and Mg^{2+} are mutually exclusive uncompetitive inhibitors versus *Ins1P* [6]. The K_i value of Li^+ inhibition would then be an apparent constant depending on the affinity for the $E \cdot Mg^{2+} \cdot P_i$ complex, but also on the steady-state concentration of $E \cdot Mg^{2+} \cdot P_i$ compared with other enzyme forms [7]. The dissociation rate for phosphate giving $E \cdot Mg^{2+}$ is at least $28\ s^{-1}$, the catalytic-centre activity for the recombinant bovine enzyme with *Ins1P*, and of course does not depend on the nature of the substrate. The catalytic-centre activity with 4-nitrophenyl phosphate is only $1\ s^{-1}$, which means that a step other than phosphate release, most likely ester hydrolysis, must be entirely rate-limiting with this substrate and $E \cdot Mg^{2+} \cdot P_i$ does not accumulate. Consequently, there should be no inhibition by Mg^{2+} with this substrate, as

Scheme 1 Proposed mode of metal ion binding to *myo*-inositol monophosphatase

Ester hydrolysis and dissociation of the alcohol are combined in one step.

Table 4 Kinetic constants of metal ion binding to *myo*-inositol monophosphatase

C, competitive; NC, non-competitive; UC, uncompetitive.

Metal	Varied substrate or activator	Inhibitor pattern	Enzyme form	K
Ca^{2+}	4-Nitrophenyl phosphate*	NC	E	0.2 mM‡
			$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	0.8 mM§
	Mg^{2+} †	C	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	0.6 mM
	Ins1P*	UC	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	3 μM §
Li^+	Mg^{2+} †	C	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	4 μM
	4-Nitrophenyl phosphate*	NC	E	1.0 mM‡
			$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	6.5 mM§
	Mg^{2+} †	C	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	5.6 mM
	Ins1P	UC	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i$	0.6 mM¶
	Mg^{2+} †	NC	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i$	0.4 mM¶
	D-Ribose 5-phosphate*	NC	E	0.7 mM‡
			$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	0.2 mM§
	Mg^{2+} †	C	$\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$	0.1 mM

* At a fixed concentration of Mg^{2+} , as described in the legends to Figures 3, 4 and 6.

† At 10 mM 4-nitrophenyl phosphate, 0.5 mM Ins1P or 4 mM D-ribose 5-phosphate.

‡ Calculated from slope inhibition constants $K_{is} = K(1 + [\text{Mg}^{2+}]/K_d)$, with $K_d = 0.3$ mM [9].§ Calculated from intercept inhibition constants $K_i = K(1 + [\text{Mg}^{2+}]/K_d)$, with $K_d = 0.2$ mM for Ins1P, 2.8 mM for 4-nitrophenyl phosphate, and 1 mM for D-ribose 5-phosphate (Tables 2 and 3).|| K_{is} values from fitting data with eqn. 3 or 3a.¶ Intercept inhibition constants from the uncompetitive pattern versus Ins1P [6] and the non-competitive pattern versus Mg^{2+} (this study).

binding of Mg^{2+} to E, $\text{E} \cdot \text{Mg}^{2+}$ or $\text{E} \cdot \text{Mg}^{2+} \cdot \text{S}$ of course corresponds to its function as an activator. Indeed, it was reported that no inhibition occurs up to 100 mM Mg^{2+} [6]. With Li^+ , on the other hand, inhibition still occurs, but becomes competitive with respect to Mg^{2+} and non-competitive with respect to substrate (Figure 3). This may simply be explained by Li^+ binding to free enzyme and to the enzyme-substrate complex to form $\text{E} \cdot \text{Li}^+$ (and/or $\text{E} \cdot \text{Mg}^{2+} \cdot \text{Li}^+$), and $\text{E} \cdot \text{Mg}^{2+} \cdot \text{S} \cdot \text{Li}^+$, in competition with Mg^{2+} . It should be noted that apparent (slope or intercept) inhibition constants must be corrected for the presence of Mg^{2+} , if the activator competes for the same enzyme form. As with Ca^{2+} , the corrected K_i values in the absence of substrate therefore depend on whether $\text{E} \cdot \text{Li}^+$ or $\text{E} \cdot \text{Mg}^{2+} \cdot \text{Li}^+$ is formed. Assuming $\text{E} \cdot \text{Li}^+$ as the more likely case and a dissociation constant of 0.3 mM for $\text{E} \cdot \text{Mg}^{2+}$ [9], Li^+ has an affinity of about 1 mM for the free enzyme (Table 4). This is in good agreement with previous data, showing a non-competitive component for Li^+ inhibition with respect to Ins1P above 5 mM of the metal ion at 3 mM Mg^{2+} [7]. Li^+ binding to inositol phosphatase has also been demonstrated by fluorescence studies with pyrene/maleimide-labelled enzyme [9], but the K_d value of 105 mM indicates a different binding site. As binding to $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i$ is no longer kinetically significant with 4-nitrophenyl phosphate, the pattern with respect to the activator, Mg^{2+} , becomes competitive, instead of non-competitive as with Ins1P. Of course, as soon as external P_i is added to the reaction mixture, as in Figure 5, the $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i \cdot \text{Li}^+$ complex again becomes evident.

Leech et al. [7] have also shown the existence of an $\text{E} \cdot \text{Li}^+$ complex in the presence of Ins1P, but have ruled out inhibitory $\text{E} \cdot \text{S} \cdot \text{Li}^+$ complexes. If Li^+ could only inhibit by binding to free enzyme or the $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i$ complex, then the efficiency of Li^+ inhibition with different substrates should correlate with k_{cat} . [7]. Table 3 shows that for some substrates, such a correlation does not exist. In particular, hydrolysis of ribose 5-phosphate, with a turnover number almost as small as for 4-nitrophenyl phosphate, is efficiently inhibited by Li^+ . As expected, the competitive pattern of Li^+ inhibition with respect to Mg^{2+} shows that the

$\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i \cdot \text{Li}^+$ complex is not kinetically important. The non-competitive pattern of inhibition with respect to the substrate is clearly dominated by the intercept effect, suggesting that, even though binding to the free enzyme is still evident, the most important inhibitory complex is $\text{E} \cdot \text{Mg}^{2+} \cdot \text{S} \cdot \text{Li}^+$. At least in this case, Li^+ appears to act by preventing substrate hydrolysis or, less likely, dissociation of ribose. Interestingly, Pollack et al. [27] have reported mutant enzymes with very low turnover numbers, but only slightly affected affinities for Li^+ . This result also argues for the existence of inhibitory $\text{E} \cdot \text{S} \cdot \text{Li}^+$ complexes. In the case of Mg^{2+} , on the other hand, there is no indication from Table 3 that inhibitory complexes other than $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i \cdot \text{Mg}^{2+}$ exist.

We conclude that at least three inhibitory complexes, $\text{E} \cdot \text{Li}^+$ (and/or $\text{E} \cdot \text{Mg}^{2+} \cdot \text{Li}^+$), $\text{E} \cdot \text{Mg}^{2+} \cdot \text{S} \cdot \text{Li}^+$ and $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i \cdot \text{Li}^+$ (Scheme 1), can be formed between *myo*-inositol monophosphatase and Li^+ ion. The nature of the substrate and the concentration ranges used in a given experiment will determine which of these complexes are the predominant ones. With Ins1P, the $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i \cdot \text{Li}^+$ complex appears to dominate, but with 4-nitrophenyl phosphate and ribose 5-phosphate in the absence of added phosphate, $\text{E} \cdot \text{Li}^+$ and $\text{E} \cdot \text{Mg}^{2+} \cdot \text{S} \cdot \text{Li}^+$ respectively are more important. As shown in Scheme 1, Ca^{2+} , unlike Li^+ , has no enhanced affinity for the $\text{E} \cdot \text{Mg}^{2+} \cdot \text{P}_i$ complex, but exerts its inhibitory effect solely by competition with the activator Mg^{2+} .

Ester hydrolysis is an important rate-limiting step with both Ins1P and 4-nitrophenyl phosphate, when Mg^{2+} is the activator [6]. Co^{2+} accelerates the rate of reaction with 4-nitrophenyl phosphate by about 4-fold compared with Mg^{2+} . It may be that the acceleration of ester hydrolysis is even much higher than that, but another step in the enzyme mechanism, such as phosphate release, becomes rate-limiting. The fact that the rate of Ins1P hydrolysis is reduced by about 7-fold and becomes identical with that observed with 4-nitrophenyl phosphate supports this hypothesis. Interestingly, Co^{2+} activation is non-linear in the presence of Ins1P, but follows Michaelis-Menten kinetics with 4-nitrophenyl phosphate as the substrate. The non-linearity of cofactor activation of *myo*-inositol monophosphatase is most

likely due to the requirement for two metal ions [8,14,28]. 4-Nitrophenyl phosphate lacks the hydroxy group that was shown to enhance catalysis with D-Ins1P as the substrate [31], probably by positioning the second Mg^{2+} ion [6,28]. With 4-nitrophenyl phosphate, stabilization of the leaving group by the second metal ion may be less important, as the aromatic system is able to delocalize the developing negative charge. One Co^{2+} (but not one Mg^{2+}), acting in M1, may therefore be enough to accelerate ester hydrolysis to a point where another step in the catalytic mechanism becomes rate-limiting and the non-linearity of activation disappears. A similar result was reported with Mg^{2+} and a very efficient substrate, Ins4P [7].

The present study has shown that binding of metal ions to *myo*-inositol monophosphatase involves different enzyme forms, depending on the nature of the substrate and the metal, and we have determined some of the kinetic constants that describe those interactions. More X-ray-crystallographic structures with substrates, inhibitors and metal cofactors, as well as other biophysical studies, will certainly aid a more detailed interpretation of the kinetic data.

We thank C. Fort for help with enzyme purification and S. Piettre for the synthesis of hydroxy-[4-(5,6,7,8-tetrahydronaphthalen-1-yloxy)phenyl]methyl phosphonic acid and 2-amino-6-mercapto-7-methylpurine ribonucleoside.

REFERENCES

- Gani, D., Downes, C. P., Batty, I. and Bramham, J. (1993) *Biochim. Biophys. Acta* **1177**, 253–269
- Parthasarathy, L., Vadnal, R. E., Parthasarathy, R. and Devi, C. S. S. (1994) *Life Sci.* **54**, 1127–1142
- Hallcher, L. M. and Sherman, W. R. (1980) *J. Biol. Chem.* **255**, 10896–10901
- Berridge, M. J., Downes, C. P. and Hanley, M. R. (1989) *Cell* **59**, 411–419
- Nahorski, S. R., Ragan, C. I. and Challiss, R. A. J. (1991) *Trends Pharmacol. Sci.* **12**, 297–303
- Ganzhorn, A. J. and Chanal, M.-C. (1990) *Biochemistry* **29**, 6065–6071
- Leech, A. P., Baker, G. R., Shute, J. K., Cohen, M. A. and Gani, D. (1993) *Eur. J. Biochem.* **212**, 693–704
- Greasley, P. J. and Gore, M. G. (1993) *FEBS Lett.* **331**, 114–118
- Greasley, P. J., Hunt, L. G. and Gore, M. G. (1994) *Eur. J. Biochem.* **222**, 453–460
- Gee, N. S., Ragan, C. I., Watling, K. J. et al. (1988) *Biochem. J.* **249**, 883–889
- Pollack, S. J., Atack, J. R., Knowles, M. R. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5766–5770
- Bone, R., Springer, J. P. and Atack, J. R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10031–10035
- Bone, R., Frank, L., Springer, J. P. et al. (1994) *Biochemistry* **33**, 9460–9467
- Bone, R., Frank, L., Springer, J. P. and Atack, J. R. (1994) *Biochemistry* **33**, 9468–9476
- Webb, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4884–4887
- Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Diehl, R. E., Whiting, P., Potter, J. et al. (1990) *J. Biol. Chem.* **265**, 5946–5949
- McAllister, G., Whiting, P., Hammond, E. A. et al. (1992) *Biochem. J.* **284**, 749–754
- Ganzhorn, A. J., Vincendon, P. and Pelton, J. T. (1993) *Biochim. Biophys. Acta* **1161**, 303–310
- Pelton, P. D. and Ganzhorn, A. J. (1992) *J. Biol. Chem.* **267**, 5916–5920
- Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138
- Northrop, D. B. and Cleland, W. W. (1974) *J. Biol. Chem.* **249**, 2928–2931
- MacLeod, A. M., Baker, R., Hudson, M. et al. (1992) *Med. Chem. Res.* **2**, 96–101
- Attwood, P. V., Duce, J. B. and Chanal, M.-C. (1988) *Biochem. J.* **253**, 387–394
- Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley and Sons, New York
- Yonetani, T. and Theorell, H. (1964) *Arch. Biochem. Biophys.* **106**, 243–251
- Pollack, S. J., Knowles, M. R., Atack, J. R. et al. (1993) *Eur. J. Biochem.* **217**, 281–287
- Cole, A. G. and Gani, D. (1994) *J. Chem. Soc. Chem. Commun.*, 1139–1141
- Fromm H. J. (1975) *Initial Rate Enzyme Kinetics*, pp. 94–100, Springer-Verlag, Berlin
- Kwok, F., Wang, X. and Churchich, J. E. (1994) *Arch. Biochem. Biophys.* **313**, 274–279
- Baker, R., Kulagowski, J. J., Billington, D. C., Leeson, P. D., Lennon, I. C. and Liverton, N. (1989) *J. Chem. Soc. Chem. Commun.*, 1383–1385