Mammalian inositol monophosphatase: the identification of residues important for the binding of Mg^{2+} and Li^+ ions using fluorescence spectroscopy and site-directed mutagenesis

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The fluorescence properties of residue Trp-219 in inositol monophosphatase are sensitive to the ionization of neighbouring groups. The pH-dependent changes in the fluorescence emission intensity and wavelength of maximum emission appear to arise as the result of two separate ionizations in the proximity of Trp-219, namely due to the ionization of His-217 and Cys-218. By studying the curve of fluorescence intensity against pH, given by the mutants Cys-218→Ala or His-217→Gln, the pK of His-217 was determined to be 7.54 and the pK of Cys-218 was estimated

to be about 8.2. These mutants have altered kinetic parameters for catalytic Mg^{2+} ions and inhibitory Mg^{2+} and Li^+ ions. The Cys-218→Ala mutant enzyme is not subject to inhibition by concentrations of Mg^{2+} ions up to 400 mM and has a specific activity of 156% of the maximum obtainable activity of the native enzyme. The His-217→Gln mutant enzyme shows reduced sensitivity to inhibition by Mg^{2+} and Li^+ ions, and has a specific activity of 110% of that obtainable for the native enzyme.

INTRODUCTION

Extracellular signals are converted into intracellular events in the process of communication between cells. One of these signal pathways utilizes the receptor-mediated action of phospholipase C on phosphatidylinositol 4,5-bisphosphate, generating the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. The latter molecule mobilizes intracellular calcium [1] and can be metabolized to generate 63 possible inositol phosphates, of which at least 25 have so far been identified *in vivo* in eukaryotic cells [2]. A number of these inositol polyphosphates have been implicated in other forms of cellular control [3]. The various inositol phosphates are metabolized by an array of kinases and phosphatases, ultimately producing Ins1P, Ins3P and Ins4P, all of which are substrates for the enzyme inositol monophosphatase (EC 3.1.3.25) which dephosphorylates them to inositol [4]. Since transfer of dietary inositol across the blood–brain barrier is slow [5], brain tissue is dependent upon inositol monophosphatase for the production of inositol by *de novo* synthesis when glucose 6-phosphate is metabolized to Ins3P [6]. In addition to the dephosphorylation of all inositol monophosphate esters (except Ins2P), the enzyme will also dephosphorylate a number of non-inositol-containing compounds, such as 2'-AMP, 2'-GMP and α -glycerophosphate [4].

Inositol monophosphatase is functionally active as a dimer of identical subunits, each of M_r 30000. It has an absolute requirement for Mg^{2+} for catalytic activity [7], but is inhibited in an uncompetitive manner by high concentration of Mg^{2+} or by Li^+ ions. It had been proposed that Li^+ can trap a phosphoenzyme intermediate [8], preventing subsequent nucleophilic attack by water, although more recently it has been proposed that Li^+ forms a complex with the enzyme and a phosphate ion after hydrolysis of the substrate [9]. The sensitivity of the enzyme to Li^+ ions has been implicated in the efficacy of lithium in the treatment of manic depression [10]. The genes coding for this

enzyme from bovine [11], human and rat [12] brain have only recently been isolated and sequenced.

Recent chemical modification studies using phenylglyoxal and diethyl pyrocarbonate have indicated that an arginine residue (unidentified) [13] and His-217 [14] are located near or at the active site of the enzyme. In addition, a reactive cysteine residue located near to the active site has recently been identified as Cys-218 [15]. These studies identified the sensitivity of the fluorescence properties of Trp-219 to modification of Cys-218 (in or near to the active site of the enzyme [15,16]) by *N*-ethylmaleimide. In this paper we demonstrate that the fluorescence intensity of this Trp residue is in fact also sensitive to the ionization of local amino acid side chains, and have used site-directed mutagenesis to identify these residues and to determine their pK values.

MATERIALS AND METHODS

Materials

The growth of bacterial cultures and subsequent extraction and purification of native and mutated inositol monophosphatase is described elsewhere [11]. All reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Enzyme activity

Inositol monophosphatase activity was determined either by the discontinuous assay of Lanzetta et al. [17] or by the radiochemical assay described by Ragan et al. [18].

Protein concentration

The concentrations of inositol monophosphatase and its mutants were determined by the method of Smith et al. [19].

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Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel and co-workers [20,21]. The plasmid used was the pRSET vector containing the gene coding for the human brain inositol monophosphatase [12].

The oligonucleotides 5' TGCAACATCGAAGCAGTGAAT 3' (Trp-219→Phe), 5' GTCAATGATGAATGTGGGGTT 3' (Trp-87→Phe), 5' GCATTCCTGGAAAGGATCAGC 3' (Trp-5→Phe), 5' GGAATTCACGCCTGGGATGTT 3' (Cys-218→Ala) and 5' ATCCCAGCATTGAATTCCCAT 3' (His-217→Gln) were synthesized on an Applied Biosystems model 380 DNA synthesizer and used to generate the respective mutants. The sequence of each mutant was determined by the dideoxy chain termination method [22].

Fluorescence measurements

Protein fluorescence was determined using a Perkin-Elmer 650S fluorescence detector. The determination of protein fluorescence over the pH range 6.4–8.8 was achieved using three buffering systems. Between pH 6.4 and 7.0 the buffer used was 10 mM Mes; between 6.8 and 8.2 the buffer used was 10 mM Hepes; and between 8.0 and 8.8 the buffer used was 10 mM Tricine. In all cases the buffer concentration was 10 mM. In each experiment 10 μ M enzyme was incubated in 1 ml of buffer of known pH for 5 min at 25 °C and the protein fluorescence was determined ($\lambda_{ex.}$ = 292 nm; $\lambda_{em.}$ = 334 nm).

RESULTS AND DISCUSSION

Fluorescence intensity changes with pH are due to the change in environment of Trp-219

The pH-dependent fluorescence of inositol monophosphatase was studied between pH 6.4 and 8.8 (Figure 1). The emission intensity first increases as the pH is raised to 7.7 and then decreases as the pH approaches 8.8. This bell-shaped curve is not obtained when the same experiment is carried out using a

Figure 1 pH-dependent changes in the protein fluorescence of native inositol monophosphatase and of the mutant Trp-219→Phe enzyme

The variation of protein fluorescence with pH is shown for human native inositol monophosphatase (A) and for a mutated enzyme where Trp-219 has been replaced by Phe (B). In each case 10 μ M enzyme was incubated for 5 min in 10 mM buffer at the required pH at 25 °C, and the fluorescence was determined using an excitation wavelength of 292 nm and an emission wavelength of 334 nm. The results shown are the averages of three experiments.

functionally active mutated protein bearing a Trp-219→Phe replacement, suggesting that the source of the pH-sensitive fluorescence is Trp-219. This conclusion has been confirmed using another mutated protein which has had both Trp-5 and Trp-87 replaced by Phe, leaving Trp-219 as the sole indole fluorophore in the protein. In this latter protein the overall intensity of fluorescence is lower but the sensitivity to pH remains the same as that of the native enzyme (results not shown).

The fluorescence spectra presented in Figure 2 are those of native and mutated enzymes bearing a Gln substitution for His-217 or an Ala substitution for Cys-218, in solution at pH 6.7 and 8.8. The native enzyme is more fluorescent at pH 6.7 than at pH 8.8; however, it is clear from Table 1 and from the other spectra in Figure 2 that the substitution of either His-217 (by Gln) or Cys-218 (by Ala) results in a large change in fluorescence emission intensity from these proteins at the same pH values. Both mutations lead to an overall increase in the quantum yield of fluorescence, the absorption spectra of all three proteins being identical, showing that the presence of either ionizable group near to Trp-219 quenches its fluorescence. Whereas the spectra of the native protein show few differences at pH 6.7 or 8.8, both mutated proteins fluoresce in a pH-dependent manner both in terms of fluorescence intensity and in the position of the emission maximum (Table 1).

The Cys-218→Ala mutated enzyme fluoresces more intensely than the native enzyme at either pH. The difference spectra shown in Figure 3 (Cys-218→Ala minus native), and the data in Table 1, show that at pH 6.7 the emission intensity of the native protein is approximately 4% lower than that of the mutated protein, and that at pH 8.8 this difference has increased to approx. 69%. This large difference in intensity at pH 8.8 arises from the fact that an increase in pH causes reciprocal changes in the fluorescence intensity and illustrates that the absence of the thiol group leads to an overall increase in the fluorescence intensity of the protein at either pH. In addition, the presence of the His-217 residue in the absence of Cys-218 causes the fluorescence intensity of the protein to be markedly more sensitive to pH than that of the native protein.

The His-217→Gln mutated enzyme also fluoresces more intensely than the native protein at either pH. However, the difference between the fluorescence of this protein and that of the native protein at low pH is greater than for the Cys-218→Ala protein (Figure 3), being increased by 37% at pH 6.7, although the difference is lower at pH 8.8 (14.2%). The effect of pH on the fluorescence intensity of this mutant is parallel to that of the native protein, i.e. the intensity of both proteins is lower at pH 8.8 than at 6.7. From these data it is apparent that there is a strong fluorescence quenching effect occurring in the native protein that is mediated by the presence of His-217.

From the data in Table 1, the reciprocal effect of pH on the fluorescence intensity of these mutated proteins may be seen. At low pH the Cys-218→Ala mutant has a higher fluorescence whereas His-217→Gln has a lower fluorescence than at pH 8.8. The possibility that these changes in spectral properties arise from a change in the state of ionization of these two residues was examined by closely monitoring the fluorescence properties of the native and mutated proteins at various pH values between 6.4 and 8.8.

The pH-dependent change between pH 6.4 and 8.0 is due to His-217

We first considered the initial part of the curve describing changes in fluorescence intensity of the native protein with pH,

Figure 2 Fluorescence emission spectra of native inositol monophosphatase and of the mutants His-217→Gln and Cys-218→Ala

The fluorescence emission spectra are shown at (a) pH 6.7 and (b) pH 8.8 for the native (A), His-217→Gln (B) and Cys-218→Ala (C) inositol monophosphatases. In each experiment, 10 μ M enzyme was incubated with 10 mM buffer for 5 min at 25 °C and the spectrum was measured using an excitation wavelength of 280 nm.

Table 1 Fluorescence intensities and emission maxima of native and mutated enzymes at pH 6.7 and 8.8

H217Q, His-217→Gln mutant; C218A, Cys-218→Ala mutant.

Enzyme	Fluorescence (%)		$\lambda_{em. max.}$	
	pH 6.7	pH 8.8	pH 6.7	pH 8.8
Native	100	84	336	337
H217Q	137	96	336	336
C218A	104	142	335	337

which shows an increase in fluorescence between pH 6.7 and 7.7 with an apparent mid-point at pH 7.3. This could indicate that an ionizing histidine residue perturbs the fluorescence intensity of Trp-219. As shown in Table 1, the replacement of His-217 by Gln gives rise to an increase in protein fluorescence at both high and low pH. This therefore implies that this residue is sufficiently close to Trp-219 and that a change in its ionization may perturb the environment of this fluorophore. As can be seen in Figure 4,

Figure 3 Difference fluorescence spectra (mutated minus native) for the mutated proteins at pH 6.7 and 8.8

The spectra shown were generated by subtraction of the fluorescence emission spectrum of the native enzyme from that of the mutated enzymes His-217→Gln ($\square$) and Cys-218→Ala ($\circ$) at pH 6.7 (----) and pH 8.8 (—).

Figure 4 pH-dependent changes in the protein fluorescence of the His-217→Gln and Cys-218→Ala mutant inositol monophosphatases

The change in protein fluorescence with pH is shown for the enzymes with His-217→Gln (A) or Cys-218→Ala (B) substitutions. In each case, 10 μ M enzyme was incubated for 5 min in the appropriate buffer at 25 °C and the fluorescence was determined using an excitation wavelength of 292 nm and an emission wavelength of 334 nm. The results shown are the average of three experiments.

when the His-217→Gln protein was subjected to changes in pH there was little change in the fluorescence intensity between pH 6.4 and 7.2, but decreases were observed at higher pH values. Estimates of the mid-point of the curve (the enzyme is unstable at pH values above 9.0) show that the pK of the group responsible is about 8.2. The observation that the fluorescence emission intensity of His-217→Gln is constant between pH 6.4 and 7.2, whereas that of the native protein shows an increase, points to His-217 as the residue responsible for the initial increase in the fluorescence of the native protein seen in Figure 1. This sigmoidal effect upon the protein's fluorescence is dependent upon the ionization of a group with a pK of approx. 8.2 (in the absence of the His residue).

The pH-dependent change between pH 7.7 and 8.8 is due to Cys-218

Using the same rationale as above, attempts were directed at the determination of the pK of His-217 by the use of a protein bearing a Cys-218→Ala replacement, thus eliminating one component from the ionization pattern of the native protein. In an experiment where the pH of a solution of Cys-218→Ala protein

was changed over the same range as above, a fluorescence intensity increase occurred between pH 6.8 and 8.2, with a mid-point at 7.5. This shows the same trend as the titration curve of the native enzyme between these pH values, but rather than showing a decrease in fluorescence between pH 7.7 and 8.8, the fluorescence of the mutant protein continued to rise before levelling out (Figure 4). It would therefore seem probable that Cys-218 has a pK of approx. 8.2 and is responsible for the fluorescence decrease seen in the native protein at high pH, and that His-217 has a pK of 7.5 in the absence of the Cys residue.

Subtraction of the spectrum of the mutated protein (Trp-219→Phe) from that of the native protein results in a difference spectrum with a maximum at 331 nm. This is equivalent, therefore, to the loss of emission from the fluorescence spectrum of the native protein due to the substitution of Trp-219 by Phe, assuming that no conformational rearrangement occurs on replacement of this residue with Phe. This wavelength of maximum emission is typical of a Trp residue in a hydrophobic environment, and may underlie the sensitivity of this residue to the ionization of the neighbouring amino acids. The difference spectrum (Cys-218→Ala minus native; Figure 3) shows that the maximum difference in emission intensity occurs at 328 nm at pH 6.7 and at 338 nm at pH 8.8. This difference spectrum reflects the quenching of the fluorescence of the native enzyme due to the presence of Cys-218, which clearly quenches more at the higher pH. The difference spectrum has a red-shifted emission maximum at pH 8.8, suggesting that the ionized side-chain increases the polarity of the environment around Trp-219.

The difference spectra generated by subtraction of the native emission spectrum from that of the His-217→Gln mutant at each pH describe the quenching effect of His-217 on Trp-219. The difference emission maximum is red-shifted as would be expected when the mutated residue is charged, i.e. at pH 8.8 the maximum occurs at 328 nm whereas at pH 6.7 the maximum occurs at 338 nm. Comparison of the four difference spectra shows that the presence of the charged thiol group has a larger quenching effect on the fluorescence of Trp-219 than the presence of the charged imidazole group, although the uncharged imidazole quenches more than the uncharged thiol side-chain. This latter observation may well indicate that there is an interaction between His-217 and Trp-219 at both pH values, which may be the cause of an increase in the pK of the His residue. Such imidazole–aromatic interactions have been shown to result in an increase in the pK of the imidazole side-chain, and these interactions occur more frequently than by chance alone [23].

Work by Ganzhorn and Chanal [24] has suggested that two groups with pK values of around 6.5 and 8.9 are involved in the process of Li^+ inhibition of this enzyme, and the other group, with a pK of 8.3, is related to the Mg^{2+} inhibition which occurs at high concentrations of the metal ion. Studies with the mutants Cys-218→Ala and His-217→Gln have shown that the binding of both of these ions is perturbed by the loss of either group.

The inhibition by Mg^{2+} or Li^+ is manifest in the presence of P_i by the formation of an enzyme· P_i ·metal complex. However, recent studies [25] have shown that P_i is unable to bind to the enzyme in the absence of Mg^{2+} , thus precluding any experimental observation of the effects of P_i alone on the two ionizations described above.

Attempts to measure the K_m for Mg^{2+} ions are complicated by the sigmoidal binding curves noted for low concentrations of this metal ion and the inhibition noted with higher concentrations [24]. However, the data of Figure 5 clearly demonstrate that both mutant proteins His-217→Gln and Cys-218→Ala have lower affinities for Mg^{2+} ions than the native protein. This conclusion is derived from several features of the curves. First, whereas

Figure 5 Relative activities of native (▲), His-217→Gln (■) and Cys-218→Ala (●) inositol monophosphatases at various concentrations of Mg^{2+}

The enzyme activity was determined by the method of Ragan et al. [18] using $10 \times$ the determined K_m of Ins1P and various concentrations of $MgCl_2$ or NaCl (control). The assays were carried out in 50 mM Tris/HCl, pH 8.0, containing 250 mM KCl and 1 mg/ml BSA, at 37 °C.

Table 2 Effects of the mutations His-217→Gln and Cys-218→Ala on the K_m for Ins1P, the apparent K_m (K_m^{app}) for Mg^{2+} , the IC_{50} for Mg^{2+} and the K_i for Li^+

Numbers in parentheses indicate numbers of independent measurements.

	Native enzyme	His-217→Gln	Cys-218→Ala
K_m for Ins1P (μM)	39 ± 7 (3)	50 ± 9 (2)	120 ± 8 (3)
K_m^{app} for Mg^{2+} (mM)	0.6 ± 0.14 (2)	2.5 ± 0.4 (2)	7.0 ± 0.8 (2)
IC_{50} for Mg^{2+} (mM)	12 ± 0.7 (2)	120 ± 7 (2)	No inhibition
K_i for Li^+ (μM)	140 ± 12 (3)	4460 ± 190 (2)	1620 ± 94 (2)

approx. 0.6 mM Mg^{2+} permits half-maximal activity in the native enzyme, higher concentrations (2.5 and 7 mM) are required for similar activity in the mutated enzymes His-217→Gln and Cys-218→Ala respectively. Secondly, marked inhibition of the native enzyme occurs at higher concentrations of the metal, with 50% inhibition (IC_{50}) being achieved by 12 mM Mg^{2+} (Table 2). This sensitivity to Mg^{2+} is once again decreased in the case of the mutated proteins; the IC_{50} is 120 mM for the His-217→Gln enzyme, whereas no inhibition at all was evident for the Cys-218→Ala enzyme up to concentrations of Mg^{2+} as high as 400 mM. All experiments were compared with control samples containing an equivalent ionic strength of NaCl. The latter enzyme therefore has the highest specific activity of all three proteins, with 156% of the activity of the native enzyme. These marked changes in the effects of Mg^{2+} on the activity of the enzyme are not paralleled by quantitatively similar changes in the affinity for the substrate Ins1P (Table 2). This is in agreement with the reaction mechanism recently described by Leech et al. [9] in which the binding processes of substrate and Mg^{2+} are independent and random-ordered.

Previous studies [24] have analysed the sigmoidicity of Mg^{2+} binding to the bovine enzyme, and have shown that under similar conditions the Hill coefficient for the catalytic Mg^{2+} binding is 1.84. In the experiments described above, the Hill coefficients

were 1.9 and 2.0 for the mutated enzymes His-217→Gln and Cys-218→Ala respectively. The data in Figure 5 demonstrate that the human enzymes respond in a similar manner to this ion as the bovine enzyme and that the amino acid replacements have not perturbed the interactions between the catalytic sites. Ganzhorn and Chanal [24] interpreted kinetic data to propose that the activity and inhibition of the enzyme due to Mg^{2+} could best be explained by a model involving two separate binding sites for the Mg^{2+} ions, the inhibition of the enzyme by Mg^{2+} ions being caused by their binding at the same site as that occupied by inhibitory Li^+ ions. Recent rapid equilibrium dialysis studies [25] have suggested that there are two separate metal-binding sites per subunit. The higher-affinity site can be occupied by a Mg^{2+} ion, whose presence is essential for P_i binding. A concentration of 10 mM Li^+ was found to be ineffective in supporting P_i binding and did not potentiate or inhibit the binding of P_i in the presence of Mg^{2+} under the conditions used. Therefore, if Li^+ is able to form and stabilize an enzyme· P_i complex, the additional presence of Mg^{2+} is necessary, i.e. the complex formed is enzyme· Mg^{2+} · P_i · Li^+ , and therefore the enzyme would require two metal-binding sites, the second of which binds the inhibitory Mg^{2+} or Li^+ ions.

The inhibitory effects of Li^+ were investigated using native enzyme or the mutated enzymes His-217→Gln and Cys-218→Ala. Table 2 shows that there is an increase in the K_i for Li^+ in both mutants, from 140 μ M (native) to 4460 μ M in His-217→Gln and 1620 μ M in Cys-218→Ala. These increases in K_i are 32- and 11.5-fold respectively. Significantly, the latter increase in K_i for Li^+ is the same as the increase in K_m^{app} for Mg^{2+} ions in this mutant, highlighting the possibility that Li^+ binds at the same site as the catalytic Mg^{2+} , i.e. the catalytic site and the inhibitory site are the same. Such a model necessitates the dissociation of the catalytic Mg^{2+} , after hydrolysis has occurred, to permit the binding of Li^+ in agreement with the mechanism proposed by Leech et al. [9]. In view of experiments recently reported [25], the catalytic/inhibitory ion would bind to an enzyme intermediate already bearing a Mg^{2+} ion (shown to be essential for P_i binding in the reverse direction). If the catalytic and inhibitory sites are the same, then failure of the catalytic Mg^{2+} to dissociate (at high concentrations) would lead to inhibition by this ion.

The data presented support the possibility that Cys-218 is the residue of pK 8.3 proposed by the kinetic studies of Ganzhorn and Chanal [24]. The higher specific activities of the mutated enzymes compared with the native enzyme (Cys-218→Ala, 156 %; His-217→Gln, 110 %) arise from the decreased sensitivity of these mutants to inhibition by Mg^{2+} ions.

A recent report [26] has identified structural similarities between inositol monophosphatase and fructose 1,6-bisphosphatase, including a conserved metal-binding motif. In the latter enzyme, this motif has been shown to bind two metal ions at sites

in close proximity [27]. The possibility exists that the equivalent motif in inositol monophosphatase also binds two metal ions. If the catalytic and inhibitory sites are the same, then the amino acid replacements described here might be expected to affect both kinetic parameters; if the sites are not the same, then the amino acid substitutions His-217→Gln and Cys-218→Ala are located such that they affect both sites.

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