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Bovine inositol monophosphatase

The identification of a histidine residue reactive to diethylpyrocarbonate

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The inositol monophosphatase from bovine brain is inactivated by the histidine-specific reagent diethylpyrocarbonate. Using 4 mM reagent at pH 6.5, the reaction results in the modification of 3 equivalents of histidine per polypeptide chain. The loss of activity occurs at the same rate as the slowest reacting of these residues. Site directed mutagenesis studies have been used to generate a mutated enzyme species bearing a His-217→Gln replacement and have shown that it is the modification of histidine 217 which results in the inactivation of the enzyme.

Inositol monophosphatase; Histidine; Diethylpyrocarbonate; Site-directed mutagenesis

1. INTRODUCTION

Bovine inositol monophosphatase is a key enzyme of the phosphatidylinositol signalling system [1,2] and has been implicated as the therapeutic target for lithium in the treatment of manic depression [3]. Agonists binding to receptors stimulate the generation of the second messengers, inositol-1,4,5-trisphosphate (Ins(1,4,5)*P*) and *sn*1,2-diacylglycerol, via the phosphatidylinositol cycle. A series of phosphatases and kinases [1] result in the recycling of inositol for subsequent re-synthesis of the phosphatidylinositols. Bovine inositol monophosphatase catalyses the final dephosphorylation step of inositol 1-phosphate (Ins(1)*P*), inositol 3-phosphate (Ins(3)*P*) and inositol 4-phosphate (Ins(4)*P*) to inositol and inorganic phosphate [1].

Since transport of dietary inositol across the blood–brain barrier is slow [4] there are just two main sources of the inositol to maintain the synthesis of phosphatidyl inositol. One is the de novo synthesis of inositol from glucose 6-phosphate via inositol monophosphatase [5] and the second is the dephosphorylation of Ins(1)*P*, Ins(3)*P*, or Ins(4)*P* again by inositol monophosphatase. Hence, this enzyme occupies a pivotal position in the recycling and supply of inositol in the brain.

Bovine inositol monophosphatase is a homodimer with subunits of M_r 30,055 [6]. There is an absolute requirement for Mg^{2+} for catalytic activity [7] although concentrations of this ion above 10 mM lead to uncompetitive inhibition of the enzyme [7].

To date, there is relatively little known concerning the amino acid residues important for catalysis. The modification studies of Knowles et al. [8] have demonstrated that reaction of Cys-218 with *N*-ethylmaleimide results in the loss of catalytic activity seen in this enzyme. However, use of site directed mutagenesis to replace Cys-218 by Ala showed that although substrate protected the enzyme against modification , this residue is not essential for activity suggesting that this modification inhibits the enzyme by steric effects.

More recently, studies using the histidine specific reagent, DEP, have been performed and shown that all five histidine residues of the enzyme can be modified [9]. The modification occurs in at least two identifiable phases, two equivalents reacting rapidly with no effect on activity and three more equivalents reacting at a slower rate. Although the enzyme is inhibited by DEP modification, the loss of activity occurs at the slower rate. The site of action was not identified but total loss of activity did not occur until all five histidine residues had been modified.

In this paper we combine the use of site directed mutagenesis and chemical modification techniques to propose that it is the modification of His-217 by DEP which leads to the loss of enzyme activity.

2. MATERIALS AND METHODS

2.1. *Preparation of enzyme*

The recombinant enzyme was expressed and purified by the method described for the human enzyme [10] except that the heat treatment step was carried out at 60°C.

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Abbreviations: DEP, diethylpyrocarbonate; IMPase, inositol monophosphatase.

2.2. *Assay of enzyme activity*

The enzyme was assayed using the direct fluorescence assay as described in Gore et al. [11].

2.3. *Protein estimation*

The protein concentration was estimated using the Bicinchinoic acid method of Smith et al [12].

2.4. *Determination of extinction coefficient for DEP modified histidine residues*

The extinction coefficient for DEP modified histidine depends on the environment [13], and was therefore determined in the buffer system employed in these experiments (20 mM MES, pH 6.5), by the addition of excess DEP to known concentrations of histidine. Under these conditions the extinction coefficient at 240 nm for DEP modified histidine is 2727 $M^{-1}\cdot cm^{-1}$.

2.5. *Chemical modification*

A solution of enzyme (25 μM) in 20 mM MES buffer, pH 6.5 at 25°C was incubated with 4 mM DEP taken from a stock of 1 M DEP dissolved in anhydrous ethanol. The effective concentration of the DEP solution was determined using the imidazole method [13]. The stoichiometry of the reaction was monitored by following the increase in absorbance at 240 nm (ε_{240nm} = 2727 $M^{-1}\cdot cm^{-1}$).

2.6. *Site directed mutagenesis*

Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel et al. [14,15]. The plasmid used was the pRSET vector containing the gene coding for the bovine brain inositol monophosphatase [16]. The oligonucleotide 5'ATCCCAGCATTGGATCCCAT (His-217→Gln) was synthesised on an Applied Biosystems model 380 DNA synthesiser and used in the mutagenesis procedure to generate the mutant. The sequence of the mutant was determined by the dideoxy chain termination method of Sanger et al. [17].

2.7. *Gel permeation studies*

The integrity of the dimeric nature of the enzyme after modification with DEP was determined by use of a Gilson HPLC system equipped with a 250 mm×7.5 mm TOSOH-TSK G3000SW gel permeation column equilibrated 20 mM MES pH 6.5.

3. RESULTS AND DISCUSSION

Preliminary studies using DEP to inactivate IMPase showed that the use of a lower concentration of the reagent than that used by Pelton and Ganzhorn [9] permits inactivation of the enzyme without total modification of all five histidine residues. For example, Fig. 1 shows that when IMPase is incubated with 4 mM DEP at pH 6.5 it is approximately 63% inactivated with a second order rate of 14.1 ± 1.6 (n=3) $M^{-1}\cdot min^{-1}$ (Table I). Analysis of the stoichiometry of modification monitored by the change in absorption at 240 nm (ε_{240nm} = 2727 $M^{-1}\cdot cm^{-1}$, see section 2) show that under these conditions three equivalents of histidine per subunit are modified (Table I). The data analysis was carried out as described in Dominici et al. [18] and compensates for the hydrolysis of the reagent during the incubation with enzyme. Two equivalents react with a second order rate of approximately 224 ± 17 $M^{-1}\cdot min^{-1}$ and these reactions have no effect on enzyme activity. A third equivalent reacts at a slower rate of 19.4 ± 1.8 $M^{-1}\cdot min^{-1}$ which is approximately the same rate as that of loss of activity. In the additional presence of 2 mM Ins(1)P,

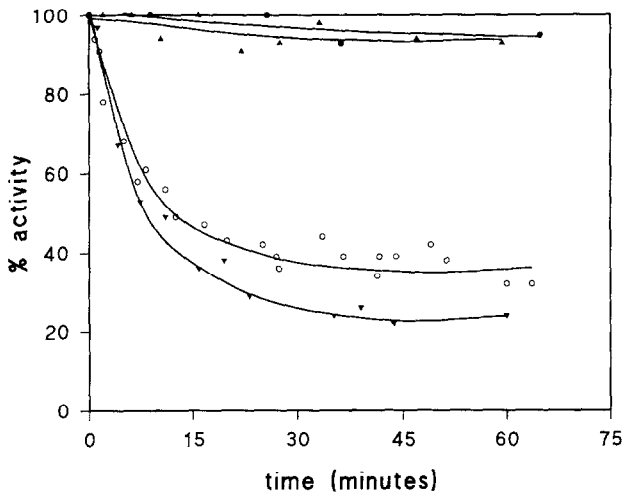

Fig 1. Inactivation of bovine inositol monophosphatase by DEP. 1.5 ml of enzyme (M_r 30,055) in 20 mM MES, pH 6.5 was incubated at 25°C with 4 mM DEP and 50 l samples taken periodically and assayed for activity by a fluorescent assay [9]. The curves shown represent incubations of DEP with native IMPase (○) or mutated enzymes C218A (▼), H217Q (●) and IMPase +2 mM Ins(1)P, 20 mM $MgCl_2$, 100 mM LiCl (▲).

100 mM Li^+ and 20 mM Mg^{2+} (as the chloride) the enzyme was not inactivated by DEP (Fig. 1) and only the 2 fast reacting equivalents of histidine were modified per subunit. These data show that the slowest reacting of the three sites modified under these conditions may be protected by the presence of substrate and Li^+ and may therefore be at or near the active site of the enzyme. The enzyme is a dimer of identical subunits and gel-filtration experiments (see section 2) have confirmed that this quaternary arrangement is not perturbed by the modification of these three residues per subunit.

The reagent used in these modification experiments is not always specific for histidinyl residues, sometimes being reactive to cysteinyl, tyrosyl and lysyl side chains [13]. Previous studies have demonstrated that Cys-218 lies at or close to the active site and that modification of this group by *N*-ethylmaleimide causes loss of enzyme activity although mutagenesis studies subsequently demonstrated that this residue is not essential for catalytic activity. In order to eliminate the possibility of DEP reacting at this site, a mutant of IMPase, in which Cys-218 was replaced with Ala, was reacted with the reagent. This mutated enzyme was still inactivated at approximately the same rate as the native enzyme (Table I) and approximately 3 equivalents of histidine were modified per subunit, the same as for wild-type enzyme. It can be concluded therefore that it is not the modification of Cys-218 by DEP that inactivates the enzyme. Furthermore the addition of 1 M hydroxylamine to the modified and inactivated enzyme led to the regain of all control activity indicating that histidinyl residues had been modified by the DEP.

However, the close proximity of His-217 to Cys-218 in the protein sequence suggests that modification of this residue by DEP might result in the inactivation of the enzyme. This possibility was investigated by reacting another mutant of IMPase bearing a His-217→Gln replacement with DEP. This mutated enzyme was only inactivated by 6% (Fig. 1) by the reagent at a rate (0.23 ± 0.08 $M^{-1} \cdot min^{-1}$) which is approximately 100-fold slower than that of the native protein. Significantly only two equivalents of histidine per subunit were modified (Table I) whether substrate and Li^+ ions were or were not present. Thus it can be inferred that the slowest reacting of the three sites of modification by DEP is the one associated with loss of activity and that this site is His-217 in the native enzyme. This in turn implies that His-217 is located at or near the active site, a realistic possibility since Cys-218 has been suggested by other experiments to be similarly located.

Ganzhorn and Chanal [19] reported kinetic results which show that catalytic activity of this enzyme depends upon the deprotonation of two groups of pK_a 6.5 and Pelton and Ganzhorn [9] suggested that either or both of these may be due to His residues. The reagent DEP reacts with the unprotonated side chains of histidine and therefore the rate of reaction will depend upon the accessibility of the site to be modified and to the pK_a of the group. Previous studies on the enzyme from human brain have shown that the pK_a of His-217 is higher than would be expected (approximately at pH 7.5) [20] and might therefore be relatively unreactive to the reagent [13] and hence have a slower rate of modification as found in the above experiments.

Further comparison of the sequence of the enzyme from human, bovine and rat brain [10] show that His-150 is unlikely to be catalytically important since it is not conserved in the human or rat enzymes which have a Gln replacement. Previous labelling studies using [^{3}H]-iodoacetic acid [8] have shown that the only histidine residue modified under the conditions used was His-65 and no loss of activity occurred. However, this observation may suggest that His-65 is very exposed or reactive and may therefore be one of the fast reacting sites with DEP.

Stoichiometry experiments showed that when 3 equivalents are modified in the native enzyme the loss of activity is only approximately 63–69% (Table I). Whereas treatment of the native and His-217→Gln mutant enzymes by higher concentrations of DEP (29 mM) results in modification of 4.8 ± 0.2 and 4.0 ± 0.2 equivalents of histidine per subunit, respectively, we noted little further loss of activity than occurred when only 3 and 2 equivalents (respectively) were modified (see Table I). These observations are in contrast to those of Pelton and Ganzhorn [9].

Finally, previous results of experiments using the human enzyme [20] have shown that like the Cys-218 Ala mutant, the His-217→Gln mutated enzyme is fully active with the same K^m for substrate as the native enzyme but with an apparent K_m for Mg^{2+} approximately 4-fold higher than that of the native enzyme [20]. The latter data, together with the inactivation studies described above, suggest that the His-217 residue lies at or near to the active site of the enzyme but is not involved in the catalytic mechanism. This conclusion is supported by similar results we obtained using *N*-ethylmaleimide to modify Cys-218 [8]. In these studies it was also found that modification of the target residue did not result in full inactivation of the enzyme.

Table I

Modification of Inositol monophosphatase by diethylpyrocarbonate
The table shows the rates of inactivation of inositol monophosphatase enzymes by DEP in the presence or absence of substrate and Li^+ ions together with the number of equivalents of histidine modified per subunit.

Enzyme	% Active loss	K_{inact} $M^{-1} \cdot min^{-1}$	No. of equivalents modified
Native (n=3)	63.3 ± 0.6	14.1 ± 1.6	2 95 ± 0 24
Native (n=4)	64.9 ± 9.2	–	4.8 ± 0.2
Native + Ins(1) P + Li^+ (n=2)	7 ± 3.5	0.23 ± 0.08	1.90 ± 0 20
His-217→Gln (n=2)	6 ± 1.4	0.27 ± 0.12	1.85 ± 0.26
His-217→Gln (n=4)	5 ± 4.1	–	4.0 ± 0.2
Cys-218→Ala (n=2)	69 ± 8.5	14.1 ± 0.5	2.81 ± 0.2

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