Identification of an active site cysteine residue in human type I Ins(1,4,5) P_3 5-phosphatase by chemical modification and site-directed mutagenesis

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Chemical modification using thiol-directed agents and site-directed mutagenesis have been used to investigate the crucial role of an active site cysteine residue within the substrate-binding domain of human type I Ins(1,4,5) P_3 5-phosphatase. Irreversible inhibition of enzymic activity is provoked by chemical modification of the enzyme by *N*-ethylmaleimide (NEM), 5,5'-dithio-2-nitrobenzoic acid, iodoacetate and to a much smaller extent by iodoacetamide. The alkylation reaction by NEM is prevented in the presence of Ins(1,4,5) P_3 . The results indicate that NEM binds at the active site of the enzyme with a stoichiometry of 0.9 mol of NEM per mol of enzyme. A single [14 C]NEM-modified peptide was isolated after α -chymotrypsin proteolysis of the radiolabelled enzyme and reverse-phase HPLC.

Sequence analysis of the active site-labelled peptide (i.e. MNTRCPAWCD) demonstrated that Cys³⁴⁸ contained the radiolabel. Furthermore two mutant enzymes were obtained by site-directed mutagenesis of the cysteine residue to serine and alanine respectively. Both mutant enzymes had identical UV CD spectra. The two mutants (i.e. Cys³⁴⁸ → Ser and Cys³⁴⁸ → Ala) show a marked loss of enzymic activity (more than 98 % compared with the wild-type enzyme). Thus we have directly identified a reactive cysteine residue as part of the active site, i.e. the substrate-binding domain, of Ins(1,4,5) P_3 5-phosphatase. This cysteine residue is part of a sequence 10 amino acids long that is well conserved among the primary structures of inositol and phosphatidylinositol polyphosphate 5-phosphatases.

INTRODUCTION

PtdIns(4,5) P_2 represents a bifurcation point in the phosphatidylinositol and inositol phosphates signal transduction cascade in a wide variety of cell types (reviewed in [1–3]). Receptor-mediated activation of phospholipase C provokes the hydrolysis of PtdIns(4,5) P_2 into Ins(1,4,5) P_3 and 1,2-diacylglycerol. Alternatively, PtdIns(4,5) P_2 is the substrate for a specific receptor-activated 3-kinase to generate PtdIns(3,4,5) P_3 [3]. A specific 3-kinase produces Ins(1,3,4,5) P_4 from Ins(1,4,5) P_3 , both being dephosphorylated by a 5-phosphatase to produce Ins(1,3,4) P_3 and Ins(1,4) P_2 respectively ([4–6]; reviewed in [7,8]).

Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 , PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 5-phosphatase activities have been measured after expression of the 75 kDa inositol polyphosphate 5-phosphatase [9,10] in COS cells [11,12]. A protein identified by its deficiency in Lowe's oculocerebrorenal syndrome (OCRL protein) has been shown to be similar with the 75 kDa inositol polyphosphate 5-phosphatase [13]; it exhibits Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and PtdIns(4,5) P_2 5-phosphatase activities [11]. Recently a rat pre-synaptic inositol polyphosphate 5-phosphatase, called synaptojanin, has been shown to exhibit Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and PtdIns(4,5) P_2 5-phosphatase activities [14]. Type I 43 kDa Ins(1,4,5) P_3 5-phosphatase hydrolyses both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 [15], whereas PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 are not substrates [12,16]. cDNA species encoding type I Ins(1,4,5) P_3 5-phosphatase have been isolated from dog and human cDNA libraries [17–19]. A novel 150 kDa tyrosine-phosphorylated 5-phosphatase has been recently shown to hydrolyse Ins(1,3,4,5) P_4 and PtdIns(3,4,5) P_3 [20–22].

Little is known about the catalytic mechanism of inositol

polyphosphate 5-phosphatases. Previous studies in crude homogenates of rat brain and GH3 cells have shown that the thiol-blocking agents *p*-hydroxymercuribenzoate and disulfiram have a marked inhibitory effect on Ins(1,4,5) P_3 5-phosphatase activity [23,24]. Recently two arginine residues, Arg³⁴³ and Arg³⁵⁰, were shown to be involved in substrate binding to type I Ins(1,4,5) P_3 5-phosphatase [25]. Interestingly, a cysteine residue has been found to be conserved between the primary structure of 43 kDa Ins(1,4,5) P_3 5-phosphatase, 75 kDa inositol polyphosphate 5-phosphatase, OCRL protein, 150 kDa Ins(1,3,4,5) P_4 /PtdIns(3,4,5) P_3 5-phosphatase, but not synaptojanin (see Figure 7). With a view to developing a more detailed understanding of inositol polyphosphate 5-phosphatase structure/function relationships, we therefore investigated the possibility that an active site cysteine residue might play a role in substrate binding or catalysis for type I Ins(1,4,5) P_3 5-phosphatase. In this study, the inactivation of human type I Ins(1,4,5) P_3 5-phosphatase by a cysteine-specific chemical modification reagent was shown to involve Cys³⁴⁸. This cysteine residue is located in the active site segment ³⁴³RCPAWCDRIL³⁵², which is crucial for enzymic activity and especially for substrate binding. Site-directed mutagenesis of the cysteine residue has confirmed that it is essential for enzymic activity.

EXPERIMENTAL

Materials

α -Chymotrypsin (treated with 7-amino-1-chloro-3-L-tosylamidoheptan-2-one), heptafluorobutyric acid (HFBA), trifluoroacetic

Abbreviations used: DTNB, 5,5'-dithio-2-nitrobenzoic acid; HFBA, heptafluorobutyric acid; NEM, *N*-ethylmaleimide; Ni-NTA, nickel nitrilotriacetic acid; OCRL protein, protein deficient in Lowe's oculocerebrorenal syndrome.

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acid, 5,5'-dithio-2-nitrobenzoic acid (DTNB), iodoacetate and iodoacetamide were purchased from Sigma. NEM was from Boehringer, and [^{14}C]NEM (20 mCi/mmol) was from NEN-DuPont. Acetonitrile was from Romil Chemicals. Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 were from Boehringer. [^3H]Ins(1,4,5) P_3 (3.3 Ci/mmol) was from NEN-DuPont. Phosphocellulose paper P81 was from Whatman. The expression vector pTrcHis A was from Invitrogen. Top 10 and HB 101 strains, as well as nickel nitrilotriacetic acid (Ni-NTA)-agarose resin, were from Qiagen.

Enzyme preparation

Native bovine brain enzyme was purified as previously reported [26]. The complete 412-residue coding region of human type I Ins(1,4,5) P_3 5-phosphatase (plasmid ECH10 in [18]) was subcloned in pTrcHis A expression vector to generate plasmid ECH11. The plasmid was used to transform competent Top 10 host bacteria with the intention of inducing recombinant enzyme expression and purifying the enzyme on Ni-NTA-agarose resin as described previously [25]. A yield of 8 mg of pure enzyme per litre of initial bacterial culture was obtained. The protein concentration was determined by the method of Petterson [27]. When the pure recombinant enzyme was separated by SDS/PAGE, a single 43 kDa band was stained by Coomassie Blue and recognized by Western blotting.

Site-directed mutagenesis

Site-directed mutagenesis was performed by using sequential PCRs [25]. Forward and reverse primers were 5'-GGCAAAG-AGATCTACTCGG-3' and 5'-CTAGGGTACCACGTCAC-TGCACGACAC-3' respectively. To generate mutant plasmid ECH11Cys³⁴⁸ → Ser, two PCR products were obtained separately by using plasmid ECH11 as template with forward primer and mutated primer 1 (5'-GCGGTCACTCCAGGCTGGGCA-3'), and reverse primer and mutated primer 2 (5'-GCCTGGAGT-GACCGCATCCTC-3') respectively. Mutually primed synthesis was used for both annealed PCR fragments by using forward and reverse primers in a second unique PCR step. To generate mutant plasmid ECH11Cys³⁴⁸ → Ala, the procedure was the same, except that the two mutated primers 1 and 2 were 5'-GCGGTCAAGCCAGGCTGGGCA-3' and 5'-GCCTGGGC-TGACCGCATCCTC-3' respectively. The presence of the mutations and the absence of any undesired mutation was confirmed by nucleotide sequence analysis. Cys³⁴⁸ → Ser and Cys³⁴⁸ → Ala mutant enzymes were expressed and purified on Ni-NTA-agarose resin by the procedure described above.

Inactivation of type I Ins(1,4,5) P_3 5-phosphatase by NEM, DTNB, iodoacetate and iodoacetamide

Purified native and bacterially expressed type I Ins(1,4,5) P_3 5-phosphatase were preincubated at 20 °C in a final volume of 50 μl in modification buffer containing 50 mM Hepes/NaOH, pH 7.5, and 1 mM EDTA with 10 mM DTNB, iodoacetate or iodoacetamide for 20 min, or in the presence of various concentrations of NEM (0–10 mM) for different periods (0–20 min). At the indicated times during incubation, 5 μl aliquots of reaction mixture were removed, diluted 1000-fold in dilution buffer [50 mM Hepes/NaOH (pH 7.5)/1 mg/ml BSA] and assayed directly for remaining enzyme activity. In other experiments, Ins(1,4,5) P_3 5-phosphatase was preincubated in modification buffer containing 10 mM NEM in the presence or absence of various concentrations of Ins(1,4,5) P_3 (0–100 μM) and Ins(1,3,4,5) P_4 (0–30 μM) before activity assay. Ins(1,4,5) P_3 5-

phosphatase activity measured in the absence of MgCl_2 at 20 °C was less than 10 % of the activity measured in the presence of 2 mM MgCl_2 at 37 °C (results not shown). The enzymic activity assay was conducted as previously reported [23].

Stoichiometry of radiolabelling

Expressed Ins(1,4,5) P_3 5-phosphatase (0.1 mg/ml) was incubated at 20 °C in a final volume of 200 μl for different times (0–30 min) in modification buffer with 10 mM [^{14}C]NEM. After different incubation times, 10 μl aliquots were removed for the determination of protein-bound radioactivity by spotting on phosphocellulose paper and precipitating in ice-cold 75 mM phosphoric acid with agitation [28]. In the same experiment, 5 μl aliquots were removed, diluted and assayed directly for remaining enzyme activity. The protective effect of Ins(1,4,5) P_3 or Ins(1,3,4,5) P_4 on chemical modification was determined by incubating Ins(1,4,5) P_3 5-phosphatase with 10 mM [^{14}C]NEM in the presence or absence of 60 μM Ins(1,4,5) P_3 or 30 μM Ins(1,3,4,5) P_4 . The proportion of labelled protein was estimated as described above.

Preparative labelling of type I Ins(1,4,5) P_3 5-phosphatase with [^{14}C]NEM, and digestion with α -chymotrypsin

Enzyme (150 μg) was incubated at 20 °C for 30 min in a final volume of 200 μl in the presence or absence of 60 μM Ins(1,4,5) P_3 in modification buffer with 10 mM [^{14}C]NEM. The reaction was stopped at 4 °C with 1 ml of 10 % (w/v) trichloroacetic acid and left on ice for 45 min. Each sample was centrifuged for 15 min at 13000 g at 4 °C (Eppendorf centrifuge) and washed with 1 ml of acetone. After dissolution of the dried protein pellet in 50 μl of 8 M urea/0.4 M NH_4HCO_3 (pH 8.0) and dilution to 200 μl with water, digestion in the presence of 3.5 μg of α -chymotrypsin was performed for 12 h at 30 °C.

Isolation of [^{14}C]NEM-labelled peptide by reverse-phase HPLC

Chymotryptic fragments of radiolabelled Ins(1,4,5) P_3 5-phosphatase were separated by reverse-phase HPLC on an Alltech Macrosphere 300 A C18 5U column (2.1 mm $\times$ 250 mm) by elution with a gradient of solvent A (5 % acetonitrile/0.1 % HFBA) and solvent B (95 % acetonitrile/0.1 % HFBA) at a flow rate of 0.2 ml/min as follows: 100 % solvent A for 10 min, followed by a linear acetonitrile gradient (0–72 % solvent B over 85 min) and finally 100 % solvent B for 5 min. The elution was followed by measuring the absorbance at 214 nm with an Applied Biosystems 1000S Diode Array detector, and each peak was collected separately. A 3 μl aliquot of each peak fraction was counted with 10 ml of scintillation cocktail to estimate the radioactivity associated with each peak. The radioactive 90 μl peak fraction (corresponding to the [^{14}C]NEM-labelled peptide whose radioactive modification was totally protected by Ins(1,4,5) P_3) was further purified on the same Alltech C18 column and eluted with the same gradient described above except that 0.1 % HFBA was replaced by 0.1 % trifluoroacetic acid. A 3 μl aliquot of each peak fraction was counted for radioactivity.

Peptide microsequencing and identification of the modified cysteine

The amino acid sequence of the labelled peptide was determined by Edman degradation with an Applied Biosystems model 477A peptide sequencer, with on-line quantification of the phenylthiohydantoin derivatives by HPLC. The amino acid

phenylthiohydantoin derivatives were collected in the internal fraction collector and counted for radioactivity to identify the labelled amino acid residues.

Determination of CD spectra

All spectra were collected in a Jasco 710 spectropolarimeter in a 0.01 cm cell at 20 °C. Scans were collected with a bandwidth of 1 nm, a sensitivity of 10 millidegrees and a time response of 0.125 s. For each enzyme preparation, the final spectrum is the result of 12 accumulated scans. Samples were concentrated with an Amicon Centricon 10 centrifugal concentrator and diluted to 1 mg/ml in 20 mM Tris/HCl (pH 8.0)/100 mM NaCl/10 % (v/v) glycerol. Samples were centrifuged at 13 000 *g* for 5 min at room temperature to remove any precipitated protein. A_{280} was measured to scale the CD data to the same protein concentrations.

RESULTS

Inactivation of human type I Ins(1,4,5) P_3 5-phosphatase by thiol-directed reagents

A marked decrease in Ins(1,4,5) P_3 5-phosphatase activity was provoked by NEM, DTNB or iodoacetate, but not iodoacetamide (Table 1). In particular, the cysteine-modifying reagent NEM inactivated the recombinant enzyme in a time- and dose-dependent manner (Figure 1). The time course of inactivation was very similar for both native and recombinant enzymes (results not shown). The linear plots of the logarithm of residual enzymic activity against the reaction time indicate that the time-dependent decrease in activity displayed pseudo-first-order kinetics (Figure 1). The first-order reaction can be described by the following equation, where (V/V_0) is the residual enzymic activity, k is the observed first-order rate constant of inactivation and t is the reaction time with NEM [29]:

$$\log(V/V_0) = -(k/2.303)t$$

The linearity of a secondary plot of $1/k$ against $1/[NEM]$ with the data from the primary plot of Figure 1 indicated that NEM binding takes place through a two-step mechanism of inactivation, where a rapid reversible binding of NEM to the enzyme precedes the covalent modification to an inactive enzyme-inhibitor complex [28].

Protection by substrate against chemical modification by NEM

To investigate further the interaction of NEM with Ins(1,4,5) P_3 5-phosphatase, protection from labelling was examined by incu-

Table 1 Inactivation of type I Ins(1,4,5) P_3 5-phosphatase by various thiol-directed reagents

The enzyme was preincubated at 20 °C for 20 min with each reagent at 10 mM before the activity was assayed. The assay was at 10 μ M Ins(1,4,5) P_3 . Residual activity values are means $\pm$ S.D. for three experiments.

Reagent	Residual enzymic activity (%)
None	100 $\pm$ 4
NEM	0
DTNB	5 $\pm$ 2
Iodoacetate	0
Iodoacetamide	85 $\pm$ 5

Figure 1 Time course of inactivation of Ins(1,4,5) P_3 5-phosphatase by NEM

Purified expressed Ins(1,4,5) P_3 5-phosphatase was preincubated at 20 °C (pH 7.5) in the presence of indicated concentrations of NEM (0–10 mM) before assay. Aliquots were removed at indicated times (0–20 min) and assayed for residual activity as described in the Experimental section. Rate constants (k) were calculated from plots of the negative natural logarithm of residual activity against time. The results are from one representative experiment out of three.

bating the enzyme with 10 mM NEM in the presence of Ins(1,4,5) P_3 (0–100 μ M) (Figure 2). Ins(1,4,5) P_3 almost completely protected the enzyme against NEM-induced inactivation. The rate of inactivation decreased with increasing substrate Ins(1,4,5) P_3 concentration, reaching a maximum at approx. 60 μ M Ins(1,4,5) P_3 . Protection was also provided by the other substrate Ins(1,3,4,5) P_4 (results not shown).

Figure 2 Protection by substrate Ins(1,4,5) P_3 against inactivation by NEM

Ins(1,4,5) P_3 5-phosphatase was incubated at pH 7.5 and 20 °C in the presence of 10 mM NEM and different concentrations of Ins(1,4,5) P_3 (0–100 μ M). Aliquots were withdrawn at intervals and assayed for enzymic activity. The inactivation rate constants were calculated from these data as described in the Results section.

Figure 3 Relationship between NEM incorporation and enzyme inactivation

(A) Ins(1,4,5)P₃ 5-phosphatase was incubated at 20 °C for various times (0–30 min) with 10 mM [¹⁴C]NEM in the absence (■) or presence (△) of 60 μM Ins(1,4,5)P₃. Incorporation of radioactive NEM was estimated as described in the Experimental section. (B) Ins(1,4,5)P₃ 5-phosphatase was incubated as described in (A) in the absence of substrate. Residual activity and stoichiometry of labelling were determined as previously described.

Stoichiometry of the binding of NEM to Ins(1,4,5)P₃ 5-phosphatase

We established the stoichiometry of this covalent modification by using [¹⁴C]NEM. Figure 3(A) shows a time course of NEM incorporation in the presence or absence of 60 μM Ins(1,4,5)P₃. The curve showed an exponential approach to a limiting value of 0.9 cysteine residue modified in the absence of substrate. Ins(1,4,5)P₃ almost completely abolished NEM incorporation (Figure 3A). Ins(1,3,4,5)P₄ also protected against NEM incorporation (results not shown). The amount of NEM incorporated in the absence of substrate at each time point was measured as a function of the residual Ins(1,4,5)P₃ 5-phosphatase activity (Figure 3B). This linear plot showed a direct correlation between the loss of enzyme activity and the incorporation of NEM. Extrapolation of the data to 100 % loss of enzyme activity indicated that activity was completely abolished when 0.9 mol of modifying reagent/mol of Ins(1,4,5)P₃ 5-phosphatase had been incorporated.

Identification of the site of Ins(1,4,5)P₃ 5-phosphatase modification

The enzyme was radiolabelled with 10 mM [¹⁴C]NEM in the presence or absence of 60 μM Ins(1,4,5)P₃ and extensively digested with α-chymotrypsin. Figures 4(A) and 4(B) show the separation of the resulting peptides by reverse-phase HPLC after labelling in the absence of Ins(1,4,5)P₃, and the amount of radioactivity within each peak. After labelling in the absence of Ins(1,4,5)P₃, a single major radioactive peak was observed with a retention time of 45.3 min (Figure 4B). In the presence of 60 μM Ins(1,4,5)P₃, the HPLC profile was identical with the profile shown in Figure 4A (results not shown) but the extent of

Figure 4 Isolation of NEM-modified peptide in Ins(1,4,5)P₃ 5-phosphatase by reverse-phase HPLC

(A) Ins(1,4,5)P₃ 5-phosphatase was incubated in the presence of 10 mM [¹⁴C]NEM at 20 °C and pH 7.5 for 30 min, and then digested by α-chymotrypsin. The resulting peptides were separated by reverse-phase HPLC with a gradient of acetonitrile in 0.1 % HFBA. The arrowhead indicates the position of the major radioactive peak. (B) The ordinate represents the radioactivity detected in each peak of profile shown in (A). (C) Ins(1,4,5)P₃ 5-phosphatase was incubated as described in (A), except that labelling was performed in the presence of 60 μM Ins(1,4,5)P₃. The ordinate represents the radioactivity detected in each peak of the HPLC profile, which was identical with that shown in (A), as mentioned in the Results section.

[¹⁴C]NEM incorporation was greatly decreased (to one-fourteenth or less) (Figure 4C). Preparation of a peptide fraction suitable for sequence analysis required an additional HPLC purification with a different ion pairing agent. The radioactive fraction that was protected by Ins(1,4,5)P₃ gave only one major radioactive peak on re-chromatography (result not shown). The site of modification of the radioactive peptide was elucidated by automated gas-phase Edman degradation sequencing. Figure 5 shows the observed amino acid sequence, the radioactivity present in the first 10 cycles and the yield quantified for each cycle. The major radioactivity appeared at cycle 9. Some carry-over of radioactivity was observed into the subsequent fraction, which was probably due to incomplete cleavage of the modified residue in cycle 9. Comparison of the obtained microsequence (MNTRXPAXD) with the predicted protein sequence of human brain type I Ins(1,4,5)P₃ 5-phosphatase [18] showed that this sequence corresponded to residues 340–349 and that [¹⁴C]NEM was covalently attached to Cys³⁴⁸ (Figure 5). Cys³⁴⁴ did not contain any radioactivity, showing that this residue does not participate in covalent-bond formation with NEM. The last residue of the peptide is an aspartic residue, in spite of the fact this peptide is a chymotryptic digest: the most likely explanation is that low-specificity protease activity cleaved at aspartic acid under extensive digestion.

Purification and CD analysis of Cys³⁴⁸ → Ser and Cys³⁴⁸ → Ala mutant enzymes

Wild-type and mutant enzymes were induced at 30 °C in a pTrcHis A expression system as described in the Experimental

Figure 5 Amino acid sequence determination of the covalently modified peptide

Histogram showing the radioactivity released during each cycle of Edman degradation of the labelled peptide. The lower lines indicate the experimental sequence (10 amino acids) that corresponds to residues 340–349 (i.e. MNTCPAWCD) of human brain type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase [18] and the yield quantified at each cycle by the protein sequenator. A dash indicates that no amino acid was detectable (i.e. modified phenylthiohydantoin-cysteine).

section. After purification on Ni-NTA–agarose resin, mutant and wild-type enzymes appeared homogeneous on Coomassie Blue-stained SDS gels: a unique 43 kDa band was stained (result not shown). Secondary-structure analysis by CD revealed no change in the overall structure of the mutants when compared with the wild-type enzyme (Figure 6). The spectra of the unmodified enzyme and the inactive enzyme after modification by NEM were also identical (results not shown).

Effect on $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity of the mutation of Cys^{348} to serine or to alanine

As shown in Table 2, the enzymic activities of the $\text{Cys}^{348} \rightarrow \text{Ser}$ and $\text{Cys}^{348} \rightarrow \text{Ala}$ mutants were less than 2% of the wild-type

Figure 6 CD spectra of wild-type, $\text{Cys}^{348} \rightarrow \text{Ser}$ and $\text{Cys}^{348} \rightarrow \text{Ala}$ $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatases

CD signal is plotted against wavelength for the wild-type and the two mutants $\text{Cys}^{348} \rightarrow \text{Ser}$ and $\text{Cys}^{348} \rightarrow \text{Ala}$: ■, wild-type; ○, $\text{Cys}^{348} \rightarrow \text{Ser}$; ▽, $\text{Cys}^{348} \rightarrow \text{Ala}$. The signals are corrected to a comparable A_{280} for each enzyme preparation.

Table 2 Comparison of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activities between purified wild-type, $\text{Cys}^{348} \rightarrow \text{Ser}$ and $\text{Cys}^{348} \rightarrow \text{Ala}$ enzymes

An enzymic activity of 100% corresponds to 155 $\mu\text{mol}/\text{min}$ per mg of protein. The assay was at 10 μM $\text{Ins}(1,4,5)\text{P}_3$.

Enzyme form	Enzymic activity (%)
Wild-type	100
$\text{Cys}^{348} \rightarrow \text{Ser}$	< 2
$\text{Cys}^{348} \rightarrow \text{Ala}$	< 2

activity. The wild-type enzyme had an activity of 155 $\mu\text{mol}/\text{min}$ per mg of protein. Moreover, the direct binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to the enzyme was markedly reduced for the NEM-modified enzyme and the two mutant enzymes compared with the wild-type enzyme (result not shown).

DISCUSSION

In this study we wished to identify one or several reactive cysteine residues in the catalytic domain of human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase by covalent chemical modification with thiol-directed reagents and by site-directed mutagenesis of the previously identified residues.

Reagents that selectively react with thiol residues, i.e. *p*-hydroxymercuribenzoate, have a drastic effect on native type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase [23]. Our results show that NEM, DTNB and iodoacetate can completely inhibit the enzymic activity of native or recombinant type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase. However, iodoacetamide had little effect on enzymic activity. The selective nature of this interaction with iodoacetate compared with iodoacetamide suggests that the former might interact specifically at or near the active site of the enzyme. The amount of labelling with radioactive NEM paralleled the loss in enzyme activity, and the labelling involved a unique residue. The enzyme was protected against inactivation by covalent modification when the reaction was performed in the presence of substrate. The peptide mapping of the $[^{14}\text{C}]\text{NEM}$ -labelled protein enabled us to find a peptide that was preferentially labelled in the absence of substrate. These results strongly suggest that the $\text{Ins}(1,4,5)\text{P}_3$ -binding domain of human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase is the target of the chemical modification. We identified the reactive cysteine residue within the active site of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase as Cys^{348} (Figure 7). The Edman degradation cycle corresponding to Cys^{348} contained the radioactivity. The catalytic properties of the two mutants where Cys^{348} had been mutated to serine and alanine support the role of this cysteine residue in substrate binding. The similarity in the induction yields and CD spectra between the mutant and the wild-type enzymes indicate that mutation of Cys^{348} did not affect the gross secondary structure of the enzymes. However, the $\text{Cys}^{348} \rightarrow \text{Ser}$ and $\text{Cys}^{348} \rightarrow \text{Ala}$ mutants displayed a marked decrease in enzymic activity compared with the wild-type enzyme (less than 2% residual activity). The drastic loss in $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity for both mutant enzymes could indicate that Cys^{348} is also necessary for enzyme catalysis. Taken together, our chemical modification and site-directed mutagenesis results clearly indicate for the first time that Cys^{348} is a reactive residue forming part of an active site peptide involved in $\text{Ins}(1,4,5)\text{P}_3$ binding by human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase.

It is interesting to note that Cys^{348} (in the human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase sequence) identified in this study forms part of a C-terminal sequence segment, i.e.

43 kDa 5-phosphatase	343	R-C-P-A-W- C -D-R-I-L	352
75 kDa 5-phosphatase	547	R-A-P-A-W- C -D-R-I-L	556
OCRL protein	570	R-V-P-A-W- C -D-R-I-L	579
SHIP	669	N-L-P-S-W- C -D-R-V-L	678
synaptojanin	800	R-T-P-A-W-T-D-R-V-L	809

Figure 7 Alignment of inositol and phosphatidylinositol polyphosphate 5-phosphatase amino acid sequences and location of the [^{14}C]NEM-labelled cysteine residue in human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase

The 43 kDa 5-phosphatase refers to the amino acid sequence of human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase [18]; 75 kDa 5-phosphatase refers to the amino acid sequence of human inositol polyphosphate 5-phosphatase [9]; OCRL protein refers to the amino acid sequence of human Lowe's syndrome $\text{PtdIns}(4,5)\text{P}_2$ 5-phosphatase gene open reading frame [13]; SHIP refers to the amino acid sequence of mouse or human 150 kDa $\text{Ins}(1,3,4,5)\text{P}_2/\text{PtdIns}(3,4,5)\text{P}_3$ 5-phosphatase [20–22]; synaptojanin refers to the amino acid sequence of rat 145 kDa presynaptic inositol polyphosphate 5-phosphatase [14]. The 10-residue sequence is well conserved between inositol and phosphatidylinositol polyphosphate 5-phosphatase sequences. It includes Cys^{348} [in human type I $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase sequence, double underlined], which is conserved in all sequences (cysteine residue in bold) except synaptojanin. The two reactive arginine residues, R^{343} and R^{350} (underlined), are also indicated [25].

$^{343}\text{RCPAWCDRIL}^{352}$ (the cysteine residue at the active site is indicated in bold), which is well conserved between sequences corresponding to inositol and phosphatidylinositol polyphosphate 5-phosphatases (Figure 7). Our results make evident the proximity of the highly reactive Cys^{348} towards the recently identified Arg^{343} and Arg^{350} within this active site peptide (Figure 7). The close proximity of crucial active site residues within the primary structure of the enzyme has also been shown in another phosphatase family, the protein tyrosine phosphatases (reviewed in [30]). In this last case, the alignment of their amino acid sequences revealed the conservation of the I/VHCXAGXGRS/TG motif. Chemical modification and site-directed mutagenesis results permitted us to show that this motif is an active site signature peptide segment, harbouring a catalytic nucleophilic cysteine residue (indicated in bold in the motif sequence). It would be of interest to investigate the effect of thiol-directed reagents on the enzymic activity of other 5-phosphatases involved in the dephosphorylation of inositol and phosphatidylinositol polyphosphate molecules because these enzymes might present structural similarities. It is intriguing to note that the recently isolated synaptojanin does not have this reactive cysteine residue within its 5-phosphatase domain [14]. However, no precise kinetic data have been reported to show affinity or the V_{max} ratio between $\text{PtdIns}(4,5)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ as substrates. It is possible that synaptojanin is instead a phosphatidylinositol polyphosphate 5-phosphatase with very little (although technically easily detectable) $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity. The presence of a well-conserved active site peptide within the primary structure of the five characterized inositol and phosphatidylinositol polyphosphate 5-phosphatases suggests that this family of enzymes possesses some common features in the mechanism of substrate binding and/or catalysis. Moreover

it may contribute to diagnose the existence of novel members of this growing family of 5-phosphatases.

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