Arginine 343 and 350 Are Two Active Site Residues Involved in Substrate Binding by Human Type I \(\alpha\)-myo-Inositol 1,4,5-Trisphosphate 5-Phosphatase*  

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The crucial role of two reactive arginyl residues within the substrate binding domain of human Type I \(\alpha\)-myo-inositol 1,4,5-trisphosphate (Ins\(\(1,4,5\)P\(_3\)) 5-phosphatase has been investigated by chemical modification and site-directed mutagenesis. Chemical modification of the enzyme by phenylglyoxal is accompanied by irreversible inhibition of enzymic activity. Our studies demonstrate that phenylglyoxal forms an enzyme-inhibitor complex and that the modification reaction is prevented in the presence of either Ins\(\(1,4,5\)P\(_3\) \(\alpha\)-myo-inositol 1,3,4,5-tetrakisphosphate (Ins\(\(1,3,4,5\)P\(_4\)) or 2,3-bisphosphoglycerate (2,3-BPG). Direct \(^3\)HIns\(\(1,4,5\)P\(_3\) binding to the covalently modified enzyme is dramatically reduced. The stoichiometry of labeling with \(^3\)C-labeled phenylglyoxal is shown to be 2.1 mol of phenylglyoxal incorporated per mol of enzyme. A single \(^3\)C-phenylglyoxal-modified peptide is isolated following \(\alpha\)-chymotrypsin proteolysis of the radiolabeled Ins\(\(1,4,5\)P\(_3\) 5-phosphatase and reverse-phase high performance liquid chromatography (HPLC). The peptide sequence (i.e. M-N-T-R-C-P-A-W-C-D-R-I-L) corresponds to amino acids 340–352 of Ins\(\(1,4,5\)P\(_3\) 5-phosphatase. An estimate of the radioactivity of the different phenylthiohydantoin amino acid derivatives shows the modified amino acids to be Arg-343 and Arg-350. Furthermore, two mutant enzymes were obtained by site-directed mutagenesis of the two arginyl residues to alanine, and both mutant enzymes have identical UV circular dichroism (CD) spectra. The two mutants (i.e. R343A and R350A) show increased \(K_\text{m}\) values for Ins\(\(1,4,5\)P\(_3\) (10- and 15-fold, respectively) resulting in a dramatic loss in enzymic activity. In conclusion, we have directly identified two reactive arginyl residues as part of the active site of Ins\(\(1,4,5\)P\(_3\) 5-phosphatase. These results point out the crucial role for substrate recognition of a 10 amino acids-long sequence segment which is conserved among the primary structure of inositol and phosphatidylinositol polyphosphate 5-phosphatases.

In a wide variety of cell types \(\alpha\)-myo-inositol 1,4,5-trisphosphate (Ins\(\(1,4,5\)P\(_3\)) and 1,2-diacylglycerol are generated from phosphatidylinositol 4,5-bisphosphate (PtdIns\(\(4,5\)P\(_2\)) by receptor-mediated activation of phospholipase C (for review, see Refs. 1 and 2). Ins\(\(1,4,5\)P\(_3\) mobilizes intracellular calcium from internal stores generating calcium signals to control many cellular processes: smooth muscle contraction, secretion, sensory perception, neuronal signaling, and cell growth (2). Ins\(\(1,4,5\)P\(_3\) can be dephosphorylated by a 5-phosphatase to produce Ins\(\(1,4\)P\(_2\) and phosphorylated by a 3-kinase to produce Ins\(\(1,3,4,5\)P\(_4\) (3–5) (for review, see Refs. 6 and 7). Some evidence supports a role for Ins\(\(1,3,4,5\)P\(_4\) in the regulation of intracellular free calcium concentration in concert with Ins\(\(1,4,5\)P\(_3\) (4 for review, see Refs. 7 and 8). Recently, a specific Ins\(\(1,3,4,5\)P\(_4\)-binding protein has been isolated and identified as a member of the GAP1 family, suggesting a connection between phospholipase C-derived signals and a proliferative cascade involving Ras (9).

A 75-kDa inositol polyphosphate 5-phosphatase was initially identified in human platelet lysates (10). cDNAs encoding the enzyme have been isolated from human cDNA libraries (11, 12). When expressed in COS cells, it shows 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 (13, 14). A protein identified due to its deficiency in the Lowe’s oculocerebrorenal syndrome has been shown to be homologous to the 75-kDa inositol polyphosphate 5-phosphatase (15). Expression of a truncated form of the protein demonstrates Ins\(\(1,4,5\)P\(_3\), Ins\(\(1,3,4,5\)P\(_4\), and PtdIns\(\(3,4,5\)P\(_3\) 5-phosphatase activities (13). In brain, Type I 43-kDa Ins\(\(1,4,5\)P\(_3\) 5-phosphatase is the major enzyme hydrolyzing the calcium-mobilizing second messenger Ins\(\(1,4,5\)P\(_3\). It hydrolyzes both Ins\(\(1,4,5\)P\(_3\) and Ins\(\(1,3,4,5\)P\(_4\) with higher affinity for Ins\(\(1,3,4,5\)P\(_4\), but lower velocity (16). PtdIns\(\(4,5\)P\(_2\), and PtdIns\(\(3,4,5\)P\(_3\) are not substrates (14, 17). cDNAs encoding Type I Ins\(\(1,4,5\)P\(_3\) 5-phosphatase have been isolated from several dog and human cDNAs libraries (18–20).

Arginyl residues are known to act as anionic binding sites in proteins and may thus assist in the binding of substrates or enzyme catalysis. Covalent and irreversible modification with amino acid specific reagents has been used successfully to identify lysyl or arginyl residues in the substrate binding domain in many enzymes, such as tyrosidine synthetase 1 (21), Ca\(^{2+}\)/ATPase (22), 6-phosphofructo-2-kinase (23), and Ins\(\(1,4,5\)P\(_3\) 3-kinase (24). In addition, two arginyl residues were shown using site-directed mutagenesis to be critical to bind the C-2-phospho group of fructose 2,6-bisphosphate in rat
liver fructose 2,6-bisphosphatase (25). We therefore investigated the possibility that active site arginines may play such a role in substrate binding for Type I Ins(1,4,5)P₃ 5-phosphatase.

In this study, modification and inactivation of human Type I Ins(1,4,5)P₃ 5-phosphatase by phenylglyoxal, an arginine-specific chemical modification reagent (see Ref. 26), was shown to be prevented by Ins(1,4,5)P₃. We identified two essential arginine-specific chemical modification reagents. PCR products were obtained separately using forward primer and mutant 5'-TGAGGAGTGGGGTCAACAGCGGCGTC-3' and 5'-GTGTGGAGCCCATCCTCATGTC-3' (the mutations are indicated in bold), respectively. Both final modified PCR fragments were cloned in wild-type plasmid ECH11 after digestion with BglII and KpnI restriction enzymes. The presence of the mutations and the absence of any undesired mutation was confirmed by sequence analysis. The two mutated plasmids were expressed in HB 101 bacteria. R334A and R350A mutant enzymes were purified on Ni-NTA agarose resin by the procedure described above.

Inactivation of Type I Ins(1,4,5)P₃ 5-phosphatase by Phenylglyoxal—Purified native and expressed Type I Ins(1,4,5)P₃ 5-phosphatase were preincubated at 23°C in a final volume of 50 μl in buffer M (50 mM Hepes/NaOH (pH 7.4), 12 mM MgCl₂, 5 mM dithiothreitol) containing various concentrations of phenylglyoxal (0–20 mM) for different times (0–20 min). At the indicated times of incubation, 5 μl aliquots of reaction mixture were removed, diluted 10,000-fold in dilution buffer (50 mM Hepes/NaOH (pH 7.4), 1 mM bovine serum albumin, 5 mM β-mercaptoethanol) and directly assayed for remaining enzyme activity. In other experiments, Ins(1,4,5)P₃ 5-phosphatase was preincubated in buffer M containing 20 mM phenylglyoxal in the presence or absence of various concentrations of Ins(1,4,5)P₃ (0–100 μM), Ins(1,3,4,5)P₄ (0–30 μM), or 2,3-BPG (0–5 mM) before activity assay. Ins(1,4,5)P₃ 5-phosphatase activity measured in the absence of MgCl₂ at 23°C was less than 10% as compared to the enzymic activity measured in the presence of 2 mM MgCl₂ at 37°C (data not shown). The assay mixture containing 20 mM Hepes/NaOH (pH 7.4), 2 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM bovine serum albumin, 10 μM Ins(1,4,5)P₃, and 1,500 cpm of [³H]Ins(1,4,5)P₃, in a final volume of 50 μl. The reaction was started by addition of enzyme and allowed to proceed for 10 min at 37°C. Separation of [³H]Ins(1,4,5)P₃ from [³H]Ins(1,4,5)P₄ was achieved on Dowex columns as previously reported (30).

Identification of Two Active Site Arginyl Residues—Recombination Ins(1,4,5)P₃ 5-phosphatase (1 μg) was incubated in a buffer containing 50 mM Tris/His (pH 7.5), [³H]Ins(1,4,5)P₃ (40,000 cpm), 10 mM EDTA (pH 7.5) in the presence or absence of unlabeled Ins(1,4,5)P₃ (0–75 μM), Ins(1,3,4,5)P₄ (0–30 μM), or 2,3-BPG (0–5 mM) in a total volume of 50 μl. After 2 min at 4°C, 10 μl of bovine γ-globulins (2 mg/ml), and 100 μl of 30 mM KCl and 50 mM KPO₄ pH 7.4 were added. The reaction mixture was eluted with a gradient of solvent A (5% acetonitrile, 0.1% heptafluorobutyric acid) for 15 min at 13,000 × g (Eppendorf centrifuge at 4°C). The pellets were washed twice with 100 μl of 15% 8-kDa poly(ethylene glycol), 0.075 M NaCl solution before being resuspended in 1 ml of water and 5 ml of scintillation mixture.

Steady-state Kinetics of Radiolabeling—Expressed Ins(1,4,5)P₃ 5-phosphatase (0.1 mg/ml) was incubated at 23°C in a final volume of 200 μl for different times (0–30 min) in buffer M with 20 mM [³H]phenylglyoxal. After different incubation times, aliquots (10 μl) were removed for the determination of protein-bound radioactivity by spotting onto phosphocellulose paper and precipitating in 75 mM ice-cold phosphoric acid under agitation. The paper filters were washed five times in 75 mM Tris·HCl (pH 8.0) containing 0.1% (mass/volume) trichloroacetic acid and dried for 2 min in a final volume of 15 μl. The labeled peptides were eluted in buffer A containing 100 mM imidazole to obtain 8 μg of pure enzyme per liter of initial bacteria culture. The protein concentration was determined by the method of Peterson (28). Immunodetection of the enzyme with anti-human brain Type I Ins(1,4,5)P₃ 5-phosphatase antibodies was performed using alkaline phosphatase-conjugated anti-rabbit IgG and corresponding colorimetric methods (29). When the pure recombinant enzyme was separated by SDS-polyacrylamide gel electrophoresis, a single 43-kDa band was stained by Coomassie Blue and recognized by Western blotting (data not shown). Kₘ for Ins(1,4,5)P₃ was 21 μM, which is comparable to the purified native enzyme (see Table I).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by using sequential polymerase chain reactions. Forward and reverse primers were 5'-GGCGAATGAGATCTCCGG-3' (BglII restriction site is underlined) and 5'-CTAGGTTACACCTGACTGCAAGC-3' (KpnI restriction site is underlined), respectively. In brief, original wild-type DNA (plasmid ECH11) was used as a template for a first PCR step (25 cycles). To generate mutant plasmid ECH11R334A, two PCR products were obtained separately using forward primer and mutated primer 1 (5'-TGAGGAGTGGGGTCAACAGCGGCGTC-3', with the mutations in bold), and reverse primer and mutated primer 2 (5'-GAAACACCGGCTGACCGGC-3', with the mutations in bold), respectively. Both purified PCR fragments encompassing the mutation were annealed with each other and extended by mutually primed synthesis using forward and reverse primers in a second unique PCR step (25 cycles). To generate mutant plasmid ECH11R350A, the procedure was the same, except that the two mutated primers 1 and 2 were 5'-TGAGGAGTGGGGTCAACAGCGGCGTC-3' and 5'-GTGTGGAGCCCATCCTCATGTC-3' (the mutations are indicated in bold), respectively. Both final mutated PCR fragments were cloned in wild-type plasmid ECH11 after digestion with BglII and KpnI restriction enzymes. The presence of the mutations and the absence of any undesired mutation was confirmed by sequence analysis. The two mutated plasmids were expressed in HB 101 bacteria. R334A and R350A mutant enzymes were purified on Ni-NTA agarose resin by the procedure described above.
butyric acid) and solvent B (95% acetonitrile, 0.1% heptafluorobutyric acid) 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 mixture to estimate the radioactivity associated with each peak. The radioactive 115-μl peak fraction (corresponding to the [14C]phenylglyoxal-labeled peptide of which radioactive modification was totally protected by Ins(1,4,5)P3) was concentrated by SpeedVac to 20 μl and diluted in 0.5 ml of 5% acetonitrile, 0.1% trifluoroacetic acid. The peptide was further purified onto the same Alltech C18 column and eluted with the same gradient described above except that 0.1% heptafluorobutyric acid 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 Arginines—The amino acid sequence of the labeled peptide was determined by Edman degradation using an Applied Biosystems model 477A peptide sequenator, with on-line quantification of the phenylthiohydantoin derivatives by HPLC. Thirty percent of the amino acid phenylthiohydantoin derivatives was collected in the internal fraction collector and counted for radioactivity to identify the labeled amino acid residues.

Determination of Circular Dichroism 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 spectra is the result of 12 accumulated scans. Samples were concentrated using an Amicon Centricon 10 centrifugal concentrator and diluted to 1 mg/ml in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. Samples were centrifuged at 13,000 × g for 5 min to remove any precipitated protein. The A280 was measured to scale the CD data to the same protein.

**Fig. 1.** Time course of inactivation of Ins(1,4,5)P3 5-phosphatase by phenylglyoxal. Purified native (A) and expressed (B) Ins(1,4,5)P3 5-phosphatase were incubated at 23 °C (pH 7.4) in the presence of indicated concentrations of phenylglyoxal. Aliquots were removed at indicated times and assayed for residual activity as described under “Experimental Procedures.” Rate constants (k) were calculated from plots of the negative natural logarithm of residual activity versus time. The results are from one representative experiment out of three. C, The reciprocal of k was plotted against the reciprocal of the concentration of phenylglyoxal.
Identification of Two Active Site Arginyl Residues

RESULTS

Inactivation of Human Type I Ins(1,4,5)P_3 5-Phosphatase by Phenylglyoxal—The arginine-specific modifying reagent phenylglyoxal inactivated native and recombinant Ins(1,4,5)P_3 5-phosphatase in a time- and dose-dependent manner (Fig. 1). The time course of inactivation was very similar for both native (Fig. 1A) and recombinant enzyme (Fig. 1B). The linear plots of the logarithm of residual enzymic activity versus the reaction time indicate that the time-dependent decrease in activity displayed pseudo first-order kinetics (Fig. 1). This behavior could be indicative of a two-step mechanism of inactivation described in Reaction 1, where a rapid reversible binding of phenylglyoxal (I) to the enzyme (E) precedes the covalent modification to an inactive enzyme-inhibitor complex (EI*).

\[
E + I \rightarrow EI \rightarrow EI^* \\
\text{k}_1 \quad \text{k}_2 \\
\text{k}_{-1}
\]

REACTION 1

The first order reaction may be described by Equation 1 where \((V\sqrt{V_0})\) is the residual enzymic activity, \(k\) is the observed first order rate constant of inactivation, and \(t\) is the time of reaction with phenylglyoxal (33).

\[
\log(V\sqrt{V_0}) = -(k(2.303) \times t)
\]

(A Eq. 1)

A steady state treatment of the reaction described in Reaction 1 yields Equation 2 (34) where \(K_1 = k_{-1}k_2\).

\[
(1/k) = (K_1/k_2)(1/([I]) + (1/k_2)
\]

(Eq. 2)

The linearity of a secondary plot of \((1/k)\) versus \((1/[I])\) using the data from the primary plot of Fig. 1 indicated that phenylglyoxal binding takes place through the two-step mechanism of interaction described by Reaction 1 (Fig. 1C).

Substrate Protection against Chemically Modified by Phenylglyoxal—To further investigate the interaction of phenylglyoxal with Ins(1,4,5)P_3 5-phosphatase, protection from labeling was examined by incubating the enzyme with 20 mM phenylglyoxal in the presence of Ins(1,4,5)P_3 (0–100 \(\mu M\)) (Fig. 2). Ins(1,4,5)P_3 almost completely protected against phenylglyoxal-induced inactivation of the enzyme. The rate of inactivation decreased with increasing substrate Ins(1,4,5)P_3 concentration, reaching a limit value at approximately 70 \(\mu M\) Ins(1,4,5)P_3. Protection was also provided by the other substrate Ins(1,3,4,5)P_4, and the competitive inhibitor 2,3-BPG (data not shown). These results suggest that at least one reactive arginyl residue is involved in substrate binding.

Lack of Direct \([	ext{H}]\)Ins(1,4,5)P_3 Binding to Modified Ins(1,4,5)P_3 5-Phosphatase—Experiments showed that phenylglyoxal was covalently modified in the presence of 20 mM phenylglyoxal for 10 min at 23 \(^\circ C\). The same treatment in the absence of phenylglyoxal was used to obtain unmodified enzyme as control. Direct substrate binding to the enzyme could be observed. It is displaced by increasing concentrations of unlabeled Ins(1,4,5)P_3, 50% displacement was reached at approximately 28 \(\mu M\) Ins(1,4,5)P_3. Protection was also provided by the other substrate Ins(1,3,4,5)P_4, and the competitive inhibitor 2,3-BPG (data not shown). These results suggest that at least one reactive arginyl residue is involved in substrate binding.

Stoichiometry of Phenylglyoxal Binding to Ins(1,4,5)P_3 5-Phosphatase—Since it appeared that phenylglyoxal interacts with the active site of the enzyme, we established the stoichiometry of this covalent modification using \(^{14}C\)-radiolabeled phenylglyoxal. Fig. 4A shows a time course of phenylglyoxal incorporation in the presence or absence of 70 \(\mu M\) Ins(1,4,5)P_3. The curve showed an exponential approach to a limiting value of 2.1 arginyl residues modified in the absence of substrate. Ins(1,4,5)P_3 almost completely abolished phenylglyoxal incorporation (data not shown). The amount of incorporated phenylglyoxal in the absence of substrate at each time point was measured as a function of the residual Ins(1,4,5)P_3 5-phosphatase activity (Fig. 4B). This linear plot showed a direct correlation between the loss of enzyme activity and the incorporation of phenylglyoxal. Extrapolation of the data to 100% loss of enzyme activity indicated that activity was completely abolished when 2.1 mol of modifying...
Identification of Two Active Site Arginyl Residues

Identification of the Sites of Ins(1,4,5)P₃ 5-Phosphatase Modification—To determine the sites of modification by phenylglyoxal, the enzyme was radiolabeled with 10 mM [¹⁴C]phenylglyoxal in the presence and absence of 70 mM Ins(1,4,5)P₃ and extensively digested with α-chymotrypsin. Fig. 5, A and B, show the separation of the resulting peptides by reverse-phase HPLC after labeling in the absence of Ins(1,4,5)P₃ and the amount of radioactivity contained within each peak. The appearance of the HPLC profile suggested that complete digestion by the protease was obtained (Fig. 5A). After labeling in the absence of Ins(1,4,5)P₃, a single major radioactive peak was observed with a retention time of 48.2 min (Fig. 5B). Since two arginyl residues were covalently modified, we supposed that this single peptide contained both modified residues. In the presence of 70 mM Ins(1,4,5)P₃, the HPLC profile was identical to the profile shown in Fig. 5A (data not shown) but the extent of [¹⁴C]phenylglyoxal incorporation was greatly reduced (at least 16-fold) (Fig. 5C). Preparation of peptide fraction suitable for sequence analysis required an additional HPLC purification using a different ion pairing agent. The radioactive fraction that was protected by Ins(1,4,5)P₃ gave only one major radioactive peak upon rechromatography (Fig. 6). The sites of modification of the radioactive peptide were elucidated by automated gas phase Edman degradation sequencing. Fig. 7 shows the observed amino acid sequence, the radioactivity present in the first 13 cycles, and the yield quantified for each cycle. The major radioactivity appeared at cycles 4 and 11. Some carryover of radioactivity was observed into the two subsequent cycles.

Fig. 4. Relationship between incorporation of phenylglyoxal protected by Ins(1,4,5)P₃ and enzyme inactivation. A, Ins(1,4,5)P₃ 5-phosphatase was incubated at 23°C and pH 7.4 for various times (0-30 min) with 20 mM [¹⁴C]phenylglyoxal in the absence (filled squares) or presence (open triangles) of 70 μM Ins(1,4,5)P₃. Incorporation of radioactive phenylglyoxal was estimated as described under “Experimental Procedures.” B, Ins(1,4,5)P₃ 5-phosphatase was incubated as described in A in the absence of any substrate. Residual activity and stoichiometry of labeling were determined as described previously.

Fig. 5. Reverse-phase HPLC profile of labeled Ins(1,4,5)P₃ 5-phosphatase digested by α-chymotrypsin. A, Ins(1,4,5)P₃ 5-phosphatase was incubated in the presence of 10 mM [¹⁴C]phenylglyoxal at 23°C (pH 7.4) for 30 min and digested by α-chymotrypsin. Resulting peptides were separated on a C₁₈ reverse-phase HPLC column using a gradient of acetonitrile in 0.1% heptfluorobutyric acid. An arrowhead indicates the position of the major radioactive peak. B, 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 labeling was performed in the presence of 70 μM Ins(1,4,5)P₃. Ordinate represents the radioactivity detected in each peak of the HPLC profile, which was identical to the profile shown in A as mentioned under “Results.”

Identification of the Sites of Ins(1,4,5)P₃ 5-Phosphatase Modification—To determine the sites of modification by phenylglyoxal, the enzyme was radiolabeled with 10 mM [¹⁴C]phenylglyoxal in the presence and absence of 70 μM Ins(1,4,5)P₃ and extensively digested with α-chymotrypsin. Fig. 5, A and B, show the separation of the resulting peptides by reverse-phase HPLC after labeling in the absence of Ins(1,4,5)P₃ and the amount of radioactivity contained within each peak. The appearance of the HPLC profile suggested that complete digestion by the protease was obtained (Fig. 5A). After labeling in the absence of Ins(1,4,5)P₃, a single major radioactive peak was observed with a retention time of 48.2 min (Fig. 5B). Since two arginyl residues were covalently modified, we supposed that this single peptide contained both modified residues. In the presence of 70 μM Ins(1,4,5)P₃, the HPLC profile was identical to the profile shown in Fig. 5A (data not shown) but the extent of [¹⁴C]phenylglyoxal incorporation was greatly reduced (at least 16-fold) (Fig. 5C). Preparation of peptide fraction suitable for sequence analysis required an additional HPLC purification using a different ion pairing agent. The radioactive fraction that was protected by Ins(1,4,5)P₃ gave only one major radioactive peak upon rechromatography (Fig. 6). The sites of modification of the radioactive peptide were elucidated by automated gas phase Edman degradation sequencing. Fig. 7 shows the observed amino acid sequence, the radioactivity present in the first 13 cycles, and the yield quantified for each cycle. The major radioactivity appeared at cycles 4 and 11. Some carryover of radioactivity was observed into the two subsequent cycles.
fractions, which was probably due to incomplete cleavage of the modified residues in cycles 4 and 11. Comparison of the obtained microsequence (M-N-T-R/-P-A-W/-D-R-I-L) with the predicted protein sequence of human brain Type I Ins(1,4,5)P3 5-phosphatase (12) showed that this sequence corresponded to amino acids 340–352 and that [14C]phenylglyoxal was covalently attached to Arg-343 and Arg-350. The last residue of the peptide is a leucine residue in spite of the fact this peptide is a chymotryptic digest; the most likely explanation is that low specificity protease activity cleaved at leucine under extensive digestion. Moreover, the yield of phenylthiohydantoin-arginines in the amino acid analyzer was much lower than for the other amino acids in the sequence, consistent with their chemical modification by phenylglyoxal (Fig. 7).

**DISCUSSION**

In this study, we aimed to identify active site residues interacting with the substrate for human Type I InsP3 5-phosphatase. This was investigated in a first step without any assumption concerning the localization of reactive arginyl residues by covalent chemical modification with phenylglyoxal, and in a second step by site-directed mutagenesis of the previously identified arginyl residues.

Inactivation kinetics of the enzyme by phenylglyoxal-induced chemical modification are identical for native and expressed protein. Our results indicate that the amount of phenylglyoxal labeling paralleled the loss in enzyme activity and that the labeling involves two residues. The peptide mapping of the protein, which had been labeled with radioactive phenylglyoxal, enabled us to find a peptide which was preferentially labeled in the absence of substrate. We have identified two reactive arginyl residues within the active site of Ins(1,4,5)P3 5-phosphatase, i.e. Arg-343 and Arg-350 (Fig. 9). The identification of both reactive amino acids is supported by two lines of evidence. First, the phenylthiohydantoin residues in these cycles was particularly low. The covalent modification results in both an inactivation of the enzyme and a drastic decrease in Ins(1,4,5)P3 binding. This strongly suggests that the Ins(1,4,5)P3 binding domain of human Type I Ins(1,4,5)P3 5-phosphatase is the target of the chemical modification. The role of Arg-343 and Arg-350 in substrate binding to the enzyme is also supported by the catalytic properties of the two mutants where Arg-343 and Arg-350 have been mu-
Identification of Two Active Site Arginyl Residues

The signals are corrected to a comparable A_{280} for each enzyme preparation. Secondary structure estimates consistent with these data are helix, 27.0 ± 0.7%; β, 25.8 ± 2.7%; turn, 28.4 ± 1.3%; and random, 18.7 ± 1.5%.

Table I

| Enzyme forms     | K_m (μM) | V_{max} (μmol/min/mg) |
|------------------|----------|-----------------------|
| Wild-type        | 21 ± 3   | 191 ± 6               |
| R343A            | 205 ± 5  | 31 ± 2                |
| R350A            | 320 ± 9  | 237 ± 6               |

43 kDa 5-phosphatase

43 kDa 5-phosphatase refers to the amino acid sequence of human Type I Ins(1,4,5)P_3 5-phosphatase (19). 75 kDa 5-phosphatase refers to the amino acid sequence of human insulin polyphosphate 5-phosphatase (11). OCR protein refers to the amino acid sequence of human Lowe’s syndrome PtdIns(4,5)P_2 5-phosphatase gene open reading frame (15). The 10-amino acid-long sequence segment is well conserved between insulin and phosphatidylinositol polyphosphate 5-phosphatase sequences. It includes R-343 and R-350 (double underlined) which were covalently labeled with [14C]phenylglyoxal.

The similarity in the induction yields and circular dichroism spectra between the mutant and the wild-type enzymes indicate that mutation of these arginyl residues did not affect the gross secondary structure of the enzymes. However, the R343A and R350A mutants display a higher K_m for Ins(1,4,5)P_3 as compared to the wild-type enzyme (10- and 15-fold, respectively). The dramatic decrease in affinity for Ins(1,4,5)P_3 for both the R343A and R350A mutant enzymes indicates that both Arg-343 and Arg-350 are involved in binding Ins(1,4,5)P_3. Although both mutants have decreased affinities for substrate, they exhibit distinct effects on the V_{max} for Ins(1,4,5)P_3 5-phosphatase. The more important change in V_{max} for R343A mutant enzyme suggest that Arg-343 may also be involved in Ins(1,4,5)P_3 5-phosphatase catalysis. Taking together, our data clearly indicate for the first time that Arg-343 and Arg-350 are two reactive residues involved in Ins(1,4,5)P_3 binding by human Type I Ins(1,4,5)P_3 5-phosphatase.

A lysine-rich Ins(1,3,4,5)P_4-binding motif (R/K-R/K-T-K-X-R/K-R/K-T) has been identified in synaptotagmin I (35). Moreover, a polybasic motif has also been proposed to be necessary for PtdIns(4,5)P_2 binding (R-K-X-X-K-X-R/K-R/K) and allosteric Ins(1,4,5)P_3 binding to actin-binding proteins and phospholipase C-δ, respectively (36, 37). The pleckstrin homology domain is a noncatalytic protein module of approximately 120 amino acids which present the ability to bind PtdIns(4,5)P_2 and Ins(1,4,5)P_3 (38). The specific function of the pleckstrin homology domain has not yet been elucidated. However, none of those motives are found in the amino acid sequence of Type I Ins(1,4,5)P_3 5-phosphatase nor in other Ins(1,4,5)P_3 binding proteins such as Ins(1,4,5)P_3 3-kinases and Ins(1,4,5)P_3 receptor/calcium channels. The primary function of the Ins(1,4,5)P_3 binding domain is supposed to anchor the inositol cycle and the three phosphates in position 1, 4, and 5. This probably occurs through locking the inositol cycle in a hydrophobic pocket and binding the phosphates with positively charged residues. Arg-343 and Arg-350 of Type I Ins(1,4,5)P_3 5-phosphatase could play an active role in this last interaction. Indeed, the use of arginine to stabilize the binding of the phosphate moieties of the substrate is quite a common occurrence in proteins for which the three-dimensional structure is known, such as 6-phosphofructo-1-kinase (39), glycogen phosphorylase b (40), and fructose 1,6-bisphosphatase. An arginyl residue of fructose 1,6-bisphosphatase belongs to the active site where the 6-phosphate group is bound: this basic residue is conserved in inositol monophosphatase sequence, which shares a very similar secondary structure topology with fructose 1,6-bisphosphatase (41).

The alignment of human insulin and phosphatidylinositol polyphosphate 5-phosphatase amino acid sequences and location of [14C]phenylglyoxal-labeled arginyl residues in human Type I Ins(1,4,5)P_3 5-phosphatase. 43 kDa 5-phosphatase refers to the amino acid sequence of human Type I Ins(1,4,5)P_3 5-phosphatase (19). 75 kDa 5-phosphatase refers to the amino acid sequence of human insulin polyphosphate 5-phosphatase (11). OCR protein refers to the amino acid sequence of human Lowe’s syndrome PtdIns(4,5)P_2 5-phosphatase gene open reading frame (15). The 10-amino acid-long sequence segment is well conserved between insulin and phosphatidylinositol polyphosphate 5-phosphatase sequences. It includes R-343 and R-350 (double underlined) which were covalently labeled with [14C]phenylglyoxal. Amino acids that are conserved between the three sequences are indicated in bold. Amino acids are represented in the one-letter code.
substrates, i.e. Ins(1,4,5)P$_3$, Ins(1,3,4,5)P$_4$, PtdIns(4,5)P$_2$ or PtdIns(3,4,5)P$_3$. Each presenting an inositol cycle with at least two phospho groups in position 4 and 5 of the inositol ring. We suppose that other amino acid regions in Type I Ins(1,4,5)P$_3$ 5-phosphatase, probably also located in the carboxy-terminal half of the protein, may serve together with residues 343–352 to form a more precise conformation which is critical for the catalysis. This 10-amino acid-long peptide, which is critical for substrate binding in Type I Ins(1,4,5)P$_3$ 5-phosphatase, could represent a diagnostic motif for this family of 5-phosphatases.

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