Active site labelling of inositol 1,4,5-trisphosphate 3-kinase A by phenylglyoxal

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Chemical modification by phenylglyoxal, an arginine-specific reagent, of both native and recombinant rat brain inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] 3-kinase A was accompanied by irreversible inhibition of enzyme activity. This effect was prevented in the presence of the substrate ATP but not Ins(1,4,5)P_3. The modification reaction obeyed pseudo-first-order rate kinetics. Complete inhibition of activity corresponded to incorporation of 1.2 mol of phenylglyoxal per mol of protein. A single [^{14}C]phenylglyoxal-modified peptide was isolated following α-chymotrypsin digestion of the radiolabelled Ins(1,4,5)P_3 3-kinase and reverse-phase HPLC. ATP prevented the incorporation of radioactivity to this peptide. The peptide sequence (i.e. QWREGISSSTTL) corresponded to amino acids 315 to 326 of rat brain Ins(1,4,5)P_3 3-kinase A. An estimate of the radioactivity of the different phenylthiohydantoin amino acid derivatives showed the modified amino acid to be Arg-317. The data directly identify a reactive arginine residue as part of the ATP-binding site. Arg-317 is located within a sequence segment which is conserved among the catalytic domain of Ins(1,4,5)P_3 3-kinase isoenzymes A and B in human and rat species.

INTRODUCTION

Receptor-mediated activation of phospholipase C generates the secondary messengers inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and 1,2-diacylglycerol from phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] in a wide variety of cell types [1,2]. Ins(1,4,5)P_3 mobilizes intracellular calcium from internal stores generating calcium signals to control many cellular processes such as smooth muscle contraction, secretion, sensory perception, neuronal signalling and cell growth [2]. Ins(1,4,5)P_3 can be metabolized by a 5-phosphatase and a 3-kinase to produce Ins(1,4)P_2 and Ins(1,3,4,5)P_4 respectively [2–7]. Recently York et al. [8] reported the presence of Ins(1,4)P_2 1-phosphatase in the nucleus; they observed that overexpression of this enzyme inhibited DNA synthesis. The data suggest a potential role for Ins(1,4)P_2 in this process. 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, e.g. in extracellular calcium entry [7], although this is not universally accepted (discussed in ref. [9]). Moreover, Ins(1,3,4,5)P_4 is the precursor of a considerable number of inositol phosphates, including Ins(1,3,4,6)P_4 and Ins(1,3,4,5,6)P_5 [10]. Ins(1,4,5)P_3 3-kinase therefore occupies a crucial position in the pathway of inositol phosphate metabolism.

cDNAs encoding rat and human isoenzymes A and B of Ins(1,4,5)P_3 3-kinase have been cloned [11–15]. The catalytic domain of rat brain Ins(1,4,5)P_3 3-kinase A has been shown to correspond to the C-terminal end of the enzyme from residues 185 to 459 [16]. Recently, the essential role of two active-site residues, Lys-197 and Asp-414, was reported based on site-directed mutagenesis data [17]. These residues form part of the ATP-binding site of rat brain Ins(1,4,5)P_3 3-kinase A [17]. They correspond to invariant lysine and aspartate residues found in the catalytic domain of most protein kinases, e.g. cyclic-AMP-dependent protein kinase catalytic subunit and protein kinase C [18,19].

Arginine residues act as anionic binding sites in proteins and may thus assist in the binding of substrates or enzyme catalysis. The crucial role of arginine residues in enzyme activity, e.g. in phosphate binding, has been shown for various enzymes, such as 6-phosphofructo-1-kinase [20] and glycogen phosphorylase b [21]. Several arginine residues are present in conserved amino acid segments of the C-terminal region of both Ins(1,4,5)P_3 3-kinase isoenzymes [14]. We therefore investigated the possibility that an active-site arginine may play an essential role in substrate binding or enzyme catalysis for Ins(1,4,5)P_3 3-kinase. This was conducted by studying the effects of the arginine-specific chemical modification reagent phenylglyoxal [22]. Modification and inhibition of Ins(1,4,5)P_3 3-kinase A by phenylglyoxal was shown to be prevented by ATP and not Ins(1,4,5)P_3. We identified the essential arginine to be Arg-317. This residue is conserved among the catalytic domains of both Ins(1,4,5)P_3 3-kinase isoenzymes.

MATERIALS AND METHODS

Materials

ATP, α-chymotrypsin (tosyl-L-lysyl-chloromethane-treated), 1-hexanesulphonic acid and trifluoroacetic acid were purchased from Sigma. Phenylglyoxal monohydrate was from Janssen Chimica and [7-^{14}C]phenylglyoxal (24 mCi/mmol) was from Amersham. Acetonitrile was from Romil Chemicals. Ins(1,4,5)P_3 was from Boehringer and [2-^{3}H]Ins(1,4,5)P_3 (3.3 Ci/mmol) was from NEN–DuPont. Phosphocellulose paper P81 was from Whatman. Calmodulin–Sepharose was prepared as previously reported [23]. Scintillation cocktail Insta-Gel II Plus was from Packard. All other reagents were of the highest available grade.

Abbreviations used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,4,5)P_3 3-kinase, inositol 1,4,5-trisphosphate 3-kinase.

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Enzyme preparation

Purification to apparent homogeneity of native rat brain Ins(1,4,5)P_3 3-kinase A was performed as described previously [24]. Recombinant rat brain Ins(1,4,5)P_3 3-kinase A was expressed as a β-galactosidase fusion product starting from pBluescript plasmid 3.5M7, which encodes the sequence region from amino acid 156 to 459 of rat brain 459-amino-acid-long Ins(1,4,5)P_3 3-kinase A [16]. A single colony of *Escherichia coli* XL1-blue host bacteria containing the plasmid was grown overnight at 37 °C in 0.5 litre of LB medium containing 50 μg/ml ampicillin. The culture was diluted to 1 litre (A of 0.6 at 600 nm) and grown for 3 h at 30 °C in the presence of 1 mM isopropyl-β-galactopyranoside. Preparation of bacterial extracts in the presence of 1% (v/v) Triton X-100 and purification of the recombinant enzyme by calmodulin–Sepharose was performed as described previously [12,16] using a 3 cm × 30 cm calmodulin–Sepharose column to obtain 2.2 mg of pure enzyme (0.88 mg/ml) starting from 1 litre of bacterial culture. When the pure recombinant enzyme was separated by SDS/PAGE, a single 34 kDa band was silver stained (data not shown) and recognized by anti-[rat brain 50 kDa Ins(1,4,5)P_3 3-kinase] antibodies [16]. The protein concentration was determined by the method of Petterson [25].

Inactivation of Ins(1,4,5)P_3 3-kinase A by phenylglyoxal

Purified native or expressed Ins(1,4,5)P_3 3-kinase A (0.1 mg/ml) was preincubated at 23 °C in a final volume of 50 μl in buffer M [50 mM Hepes/NaOH (pH 7.4), 0.1 mM EDTA, 12 mM 2-mercaptoethanol, 10 mM $MgCl_2$] containing various concentrations of phenylglyoxal (0–20 mM) for different times (0–10 min). At the indicated times of incubation, 5 μl aliquots of reaction mixture were removed, diluted 4000-fold in dilution buffer [84 mM Hepes/NaOH (pH 7.4), 1 mg/ml BSA, 12 mM 2-mercaptoethanol] and directly assayed for remaining enzyme activity. In other experiments, Ins(1,4,5)P_3 3-kinase (0.1 mg/ml) was preincubated for 10 min in buffer M containing 10 mM phenylglyoxal in the presence and absence of various concentrations of ATP (0–20 mM) or Ins(1,4,5)P_3 (0–100 μM) before remaining activity was determined. The assay mixture contained 84 mM Hepes/NaOH (pH 7.4), 20 mM $MgCl_2$, 10 mM ATP, 1 mM EGTA, 12 mM 2-mercaptoethanol, 1 mg/ml BSA, 10 μM Ins(1,4,5)P_3 and 1500 c.p.m. [^{3}H]Ins(1,4,5)P_3 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 [^{3}H]Ins(1,3,4,5)P_4 from [^{3}H]Ins(1,4,5)P_3 was achieved on Dowex columns as previously reported [24].

Stoichiometry of radiolabelling

Expressed Ins(1,4,5)P_3 3-kinase A (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 10 mM [^{14}C]phenylglyoxal. After different incubation times, aliquots (10 μl) were removed for the determination of protein-bound radioactivity by spotting on to phosphocellulose paper and precipitating in 75 mM ice-cold phosphoric acid under agitation. The paper filters were washed five times in 75 mM phosphoric acid and dried before radioactivity was estimated in the presence of 10 ml of Insta-Gel II Plus [26]. In the same experiment, aliquots (5 μl) were removed and directly assayed for remaining enzyme activity. The protective effect of ATP or Ins(1,4,5)P_3 on chemical modification was determined by incubating Ins(1,4,5)P_3 3-kinase with 10 mM [^{14}C]phenylglyoxal in the presence and absence of 10 mM ATP or 100 μM Ins(1,4,5)P_3. The proportion of labelled protein was estimated as described above.

Preparative labelling of Ins(1,4,5)P_3 3-kinase A with [^{14}C]phenylglyoxal and α-chymotryptic digestion

Expressed Ins(1,4,5)P_3 3-kinase A was concentrated to 3.5 mg/ml using an Amicon Centricon 10 centrifugal concentrator. Enzyme (120 μg) was incubated at 23 °C for 30 min in a final volume of 200 μl in the presence and absence of 10 mM ATP or 100 μM Ins(1,4,5)P_3 in buffer M with 10 mM [^{14}C]phenylglyoxal. The reaction was stopped at 4 °C with 1 ml of 10% (w/v) trichloroacetic acid and left on ice for 15 min. The sample was centrifuged for 5 min at 15000 g (Eppendorf centrifuge) and the protein pellet was washed with 1 ml of acetone. After drying the pellet (SpeedVac concentrator), the protein was dissolved in 50 μl of 8 M urea/0.4 M NH_4HCO_3 (pH 8.0), diluted to 200 μl with water and digested for 8 h at 30 °C with 3 μg of α-chymotrypsin.

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

Chymotryptic fragments of [^{14}C]phenylglyoxal-labelled Ins(1,4,5)P_3 3-kinase were separated by reverse-phase HPLC on an Alltech Macrosphere 300 A C_{18} 5U column (2.1 mm × 250 mm) by eluting with a gradient of solvent A [5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid] and solvent B [95% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid] at a flow rate of 0.2 ml/min as follows: 100% (v/v) solvent A for 10 min, followed by a linear acetonitrile gradient [0–72% (v/v) solvent B over 90 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 5 μl aliquot of each peak fraction was counted with 10 ml of scintillation cocktail to estimate the radioactivity associated with each peak. The radioactive 120 μl peak fraction (corresponding to the [^{14}C]phenylglyoxal-labelled peptide of which radioactive modification was protected by ATP) was concentrated by SpeedVac to 20 μl and diluted in 0.4 ml of 5% (v/v) acetonitrile/0.1% (v/v) 1-hexanesulphonic acid. The peptide was further purified by re-injecting on to the same Alltech C_{18} column and eluting with the same gradient described above except that 0.1% (v/v) trifluoroacetic acid was replaced by 0.1% (v/v) 1-hexanesulphonic acid. A 5 μl aliquot of each peak fraction was counted for radioactivity.

Peptide microsequencing and identification of the modified arginine

The amino acid sequence of the labelled 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 were collected in the internal fraction collector and counted for radioactivity to identify the labelled amino acid residue.

RESULTS

Production in high yield of recombinant rat brain Ins(1,4,5)P_3 3-kinase A

A sample (2.2 mg) of pure enzyme was obtained after expression of clone 3.5M7 [16] as described in the Materials and methods section. Plasmid 3.5M7 encodes the sequence region from amino acid 156 to 459 of rat brain Ins(1,4,5)P_3 3-kinase A [12]. It

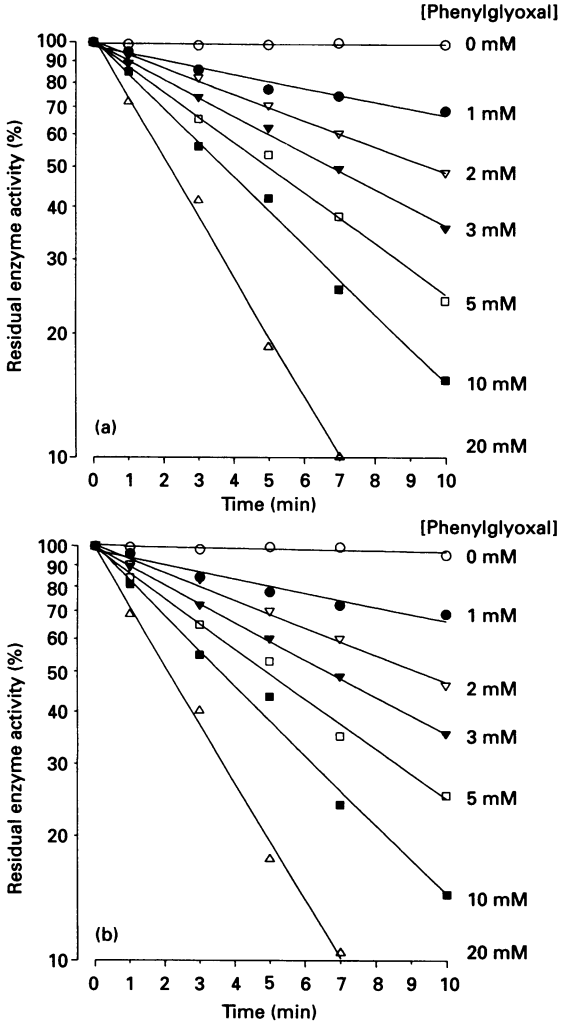

Figure 1 Time course of inactivation of Ins(1,4,5)P_3 3-kinase A by phenylglyoxal

Purified native (**a**) and expressed (**b**) rat brain Ins(1,4,5)P_3 3-kinase A (0.1 mg/ml) 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 in the Materials and methods section. Results of (**a**) and (**b**) are from one representative experiment out of four and six respectively.

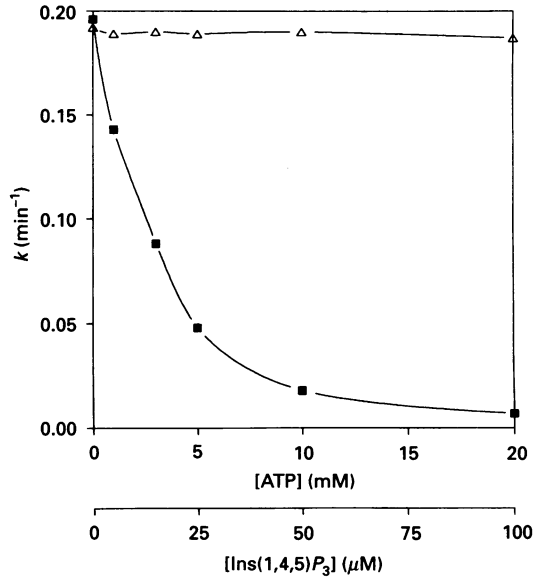

Figure 2 Protection against phenylglyoxal inactivation by ATP

Ins(1,4,5)P_3 3-kinase A (0.1 mg/ml) was incubated at pH 7.4 and 23 °C with 10 mM phenylglyoxal in the presence of 0–20 mM ATP (■) or 0–100 μM Ins(1,4,5)P_3 (△). 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. The experiment was performed five times.

contains the complete catalytic domain and the calmodulin-binding site [16]. The purified recombinant Ins(1,4,5)P_3 3-kinase activity was stimulated 2- to 3-fold by calcium/calmodulin; $V_{max.}$ was 1.9 μmol/min per mg of protein and K_m for Ins(1,4,5)P_3 was 8 μM (at 1 mM ATP) (data not shown). The data are very similar to values obtained for the purified native enzyme where stimulation by calcium/calmodulin was 2- to 3-fold, $V_{max.}$ was 1.5 μmol/min per mg of protein and K_m for Ins(1,4,5)P_3 was 11 μM (at 1 mM ATP) [24].

Inactivation of rat brain Ins(1,4,5)P_3 3-kinase A by phenylglyoxal

Native and recombinant Ins(1,4,5)P_3 3-kinase A were inactivated in a time- and dose-dependent manner by the arginine-specific modifying reagent phenylglyoxal. The time course of inactivation was very similar for both native (Figure 1a) and recombinant enzyme (Figure 1b). The time-dependent decrease in activity displayed pseudo-first-order kinetics. This is apparent from the linear plots of the logarithm of residual enzymic activity versus the reaction time (Figure 1). This behaviour is indicative of a two-step mechanism of inactivation described in eqn. (1), where a rapid reversible binding of phenylglyoxal (I) to the enzyme (E) precedes the covalent modification to an inactive enzyme–inhibitor complex (EI*).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \quad (1)$$

The first-order reaction may be described by eqn. (2) where (V/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 [27].

$$\log(V/V_0) = -(k/2.303) \times t \quad (2)$$

To investigate the interaction of phenylglyoxal with Ins(1,4,5)P_3 3-kinase further, protection from labelling was examined by incubating native or expressed Ins(1,4,5)P_3 3-kinase A with 10 mM phenylglyoxal in the presence of ATP (0–20 mM) or Ins(1,4,5)P_3 (0–100 μM) (Figure 2). ATP almost completely protected against phenylglyoxal-induced inactivation of Ins(1,4,5)P_3 3-kinase. The rate of inactivation decreased with increasing substrate ATP concentration, reaching a limit value at approximately 10 mM ATP. In contrast, no protection was offered by Ins(1,4,5)P_3 against phenylglyoxal-induced inactivation of Ins(1,4,5)P_3 3-kinase A. This result strongly suggests that a reactive arginine residue is involved in ATP binding by Ins(1,4,5)P_3 3-kinase A.

Stoichiometry of phenylglyoxal binding to Ins(1,4,5)P_3 3-kinase A

Since it appeared that phenylglyoxal interacts with the active site of the enzyme, we established the stoichiometry of this covalent

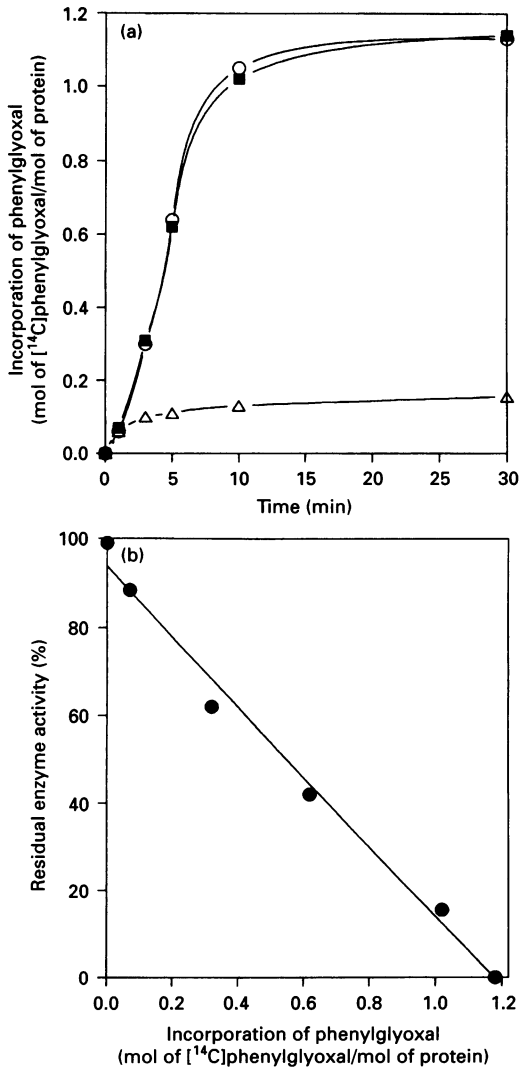

Figure 3 Relationship between incorporation of phenylglyoxal protected by ATP and enzyme inactivation

(**a**) Ins(1,4,5)P_3 3-kinase A (0.1 mg/ml) was incubated at 23 °C and pH 7.4 for various times (0–30 min) with 10 mM [^{14}C]phenylglyoxal in the absence of any substrate (■) and in the presence of 10 mM ATP (△) or 100 μM Ins(1,4,5)P_3 (○). Incorporation of radioactive phenylglyoxal was estimated as described in the Materials and methods section. Protein concentration was determined according to the method of Petterson [25]. The experiment was performed six times. (**b**) Ins(1,4,5)P_3 3-kinase A was incubated as described in (**a**) in the absence of any substrate. Residual activity and stoichiometry of labelling were determined as previously described. The experiment was performed four times.

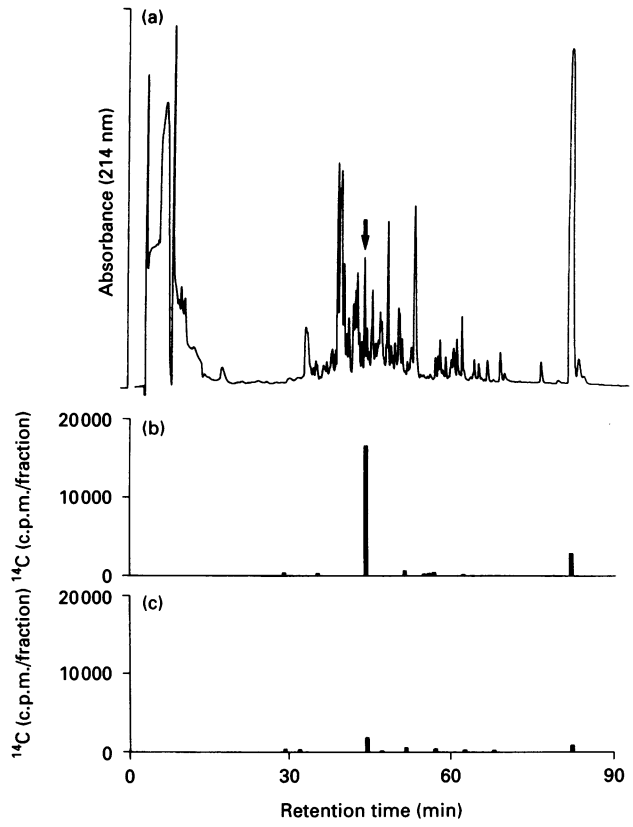

Figure 4 Reverse-phase HPLC profile of labelled Ins(1,4,5)P_3 3-kinase A α-chymotrypsin digestion

(**a**) Ins(1,4,5)P_3 3-kinase A (120 μg) was incubated in the presence of 10 mM [^{14}C]phenylglyoxal at 23 °C (pH 7.4) for 30 min and digested by α-chymotrypsin. Resulting peptides were separated on a C_{18} reverse-phase HPLC column using a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. An arrow indicates the position of the major radioactive peak. (**b**) The ordinate represents the radioactivity detected in each peak of the profile shown in (**a**). Results are from one representative experiment out of five. (**c**) Ins(1,4,5)P_3 3-kinase A (120 μg) was incubated as described in (**a**) except that labelling was performed in the presence of 10 mM ATP. The ordinate represents the radioactivity detected in each peak of the HPLC profile, which was identical to the profile shown in (**a**) as mentioned in the Results section. Results are from one representative experiment out of five.

modification using ^{14}C-radiolabelled phenylglyoxal as described under the Materials and methods section. Figure 3(a) shows a time course of phenylglyoxal incorporation in the presence and absence of 10 mM ATP or 100 μM Ins(1,4,5)P_3. The curve showed an exponential approach to a limiting value of 1.2 arginine residues modified in the absence of substrate. The sigmoidal-like appearance of the curve is most probably due to the experimental error in the points at short times of incubation with [^{14}C]phenylglyoxal. ATP almost completely abolished phenylglyoxal incorporation, whereas Ins(1,4,5)P_3 had no effect (Figure 3a). 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 3-kinase activity (Figure 3b). 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 1.2 mol of modifying reagent/mol of Ins(1,4,5)P_3 3-kinase A had been incorporated.

Identification of the site of Ins(1,4,5)P_3 3-kinase A modification

To determine the site of modification by phenylglyoxal within Ins(1,4,5)P_3 3-kinase A, the enzyme was radiolabelled with 10 mM [^{14}C]phenylglyoxal in the presence and absence of 10 mM ATP or 100 μM Ins(1,4,5)P_3 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 ATP and the amount of radioactivity contained within each peak. The appearance of the HPLC profile suggested that complete digestion by the protease was obtained (Figure 4a). After labelling in the absence of ATP, a single major radioactive peak was observed with a retention time of 42.7 min (Figure 4b). In the presence of 10 mM ATP, the HPLC profile

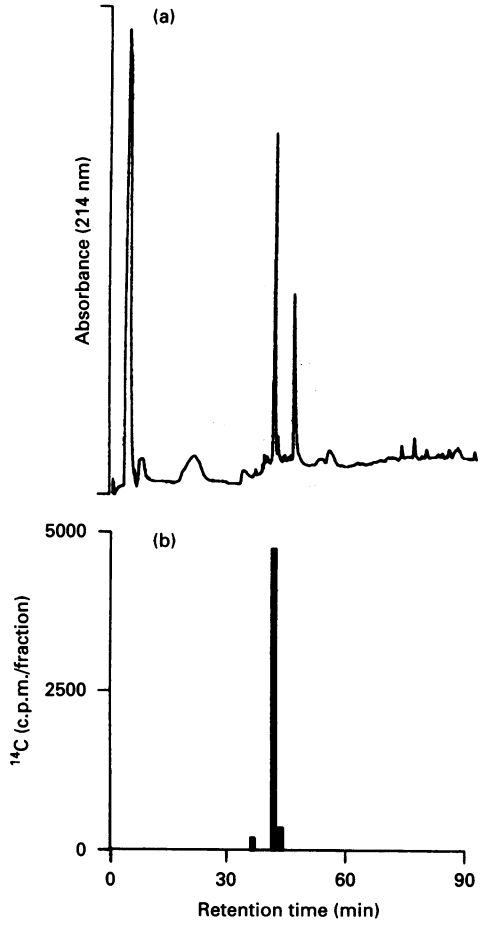

Figure 5 Purification of the labelled peptide by reverse-phase HPLC

(**a**) Profile after separation of the peptide indicated by an arrow in the HPLC profile shown in Figure 4(a) (after labelling in the absence of any substrate), using a gradient of acetonitrile in 0.1% (v/v) 1-hexanesulphonic acid. (**b**) The ordinate represents the radioactivity detected in each peak of the profile.

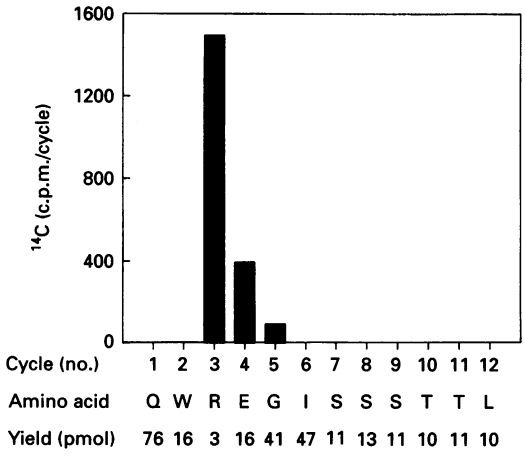

Figure 6 Amino acid sequence determination of the ^{14}C-radiolabelled peptide

Histogram showing the radioactivity released during each cycle of Edman degradation of the labelled peptide. The lower lines indicate the experimental sequence (12 amino acids) which corresponds to residues 315 to 326 of rat brain Ins(1,4,5)P_3 3-kinase A [12] and the yield quantified at each cycle by the protein sequenator.

was identical with the profile shown in Figure 4(a), but the extent of [^{14}C]phenylglyoxal incorporation was greatly reduced (at least 10-fold) (Figure 4c). The same experiment done in the presence of 100 μM Ins(1,4,5)P_3 showed that the HPLC profile and radioactive phenylglyoxal incorporation obtained in the absence of substrate (Figures 4a and 4b) were not affected by the presence of Ins(1,4,5)P_3 (data not shown). Preparation of peptide fraction suitable for sequence analysis required an additional HPLC purification using a different ion pairing agent (see the Materials and methods section). The radioactive fraction that was protected by ATP gave only one major radioactive peak upon re-chromatography (Figure 5). The site of modification of the radioactive peptide was elucidated by automated gas-phase Edman degradation sequencing. Figure 6 shows the observed amino acid sequence, the radioactivity present in the first 12 cycles and the yield quantified for each cycle. The major radioactivity appeared at cycle 3. Some carry-over of radioactivity was observed into the two subsequent fractions, which was probably due to incomplete cleavage of the modified residue in cycle 3. Comparison of the obtained microsequence (QWREGISSSTTL) with the predicted protein sequence of rat brain Ins(1,4,5)P_3 3-kinase A [12] showed that this sequence corresponded to amino acids 315 to 326 of rat brain Ins(1,4,5)P_3 3-kinase A and that the [^{14}C]phenylglyoxal was covalently attached to Arg-317 of the enzyme. The first residue of the labelled peptide is preceded by a methionine residue in the predicted sequence [12] and the last residue of the peptide is a leucine residue in spite of the fact that this peptide is a chymotryptic digest: the most likely explanation is that low-specificity protease activity cleaved at methionine and leucine under extensive digestion. Moreover, the yield of phenylthiohydantoin-arginine in the amino acid analyser was much lower than for the other amino acids in the sequence, consistent with its chemical modification by phenylglyoxal.

DISCUSSION

Previous studies of deletion mutants of rat brain Ins(1,4,5)P_3 3-kinase A indicated that a maximum of 275 amino acids in the C-terminal region of the enzyme (from residues 185 to 459) is sufficient for the construction of a catalytically active domain ([16]; see Figure 7). Alignment of the amino acid sequences of human and rat Ins(1,4,5)P_3 3-kinase isoenzymes A and B reveals the presence of conserved segments in the C-terminal part [14], suggesting their involvement in enzyme function. Two residues, Lys-197 and Asp-414, have been shown by site-directed mutagenesis to play a role in ATP binding and so to be necessary for Ins(1,4,5)P_3 3-kinase activity ([17]; see Figure 7).

The general occurrence of positively charged amino acid side chains (e.g. lysine, arginine) within the nucleotide binding site of various proteins reflects their importance in binding or catalysis [18,19,28]. A glycine-rich sequence containing an invariant basic lysine ($GX_{4-8}GKS/T$) was found in many purine nucleotide-binding proteins such as F_1-ATPase, adenylate kinase, the p21^{ras} protein, myosin and most protein kinases [18,19,29–34]: this conserved sequence has been proposed as a motif diagnostic of the nucleotide phosphate-binding site [30,31,35]. This glycine-rich motif is not found in the active site of Ins(1,4,5)P_3 3-kinase isoforms [17], glucokinase [36] or 6-phosphofructo-1-kinase [20].

X-ray crystallography data contributed to showing that lysine and arginine residues are involved in anionic substrate binding in several enzymes such as F_1-ATPase [37] and 6-phosphofructo-1-

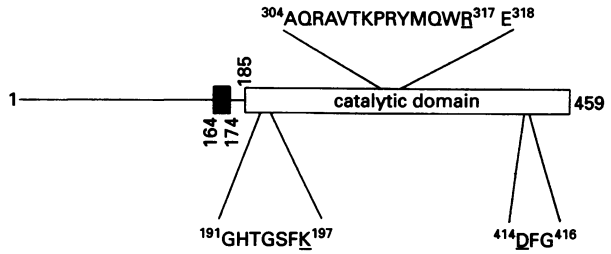

Figure 7 Location of [^{14}C]phenylglyoxal-labelled arginine residue of rat brain Ins(1,4,5)P_3 3-kinase A

The numbering of amino acid residues and domain structure was referenced to the amino acid sequence of rat brain Ins(1,4,5)P_3 3-kinase A [12]. Location of the catalytic domain and the calmodulin-binding site (filled box) has been reported [16]. Identification of Lys-197 and Asp-414 as part of the ATP-binding domain was performed by site-directed mutagenesis [17]. The sequence segment (Ala-304 to Glu-318) is conserved between Ins(1,4,5)P_3 3-kinase isoenzyme sequences in rat and human species: it includes Arg-317 (R^{317}) which was covalently labelled with [^{14}C]phenylglyoxal.

kinase [20]. Covalent and irreversible modification with amino acid-specific reagents has also been used successfully to identify lysine or arginine residues in the substrate binding site in many enzymes, such as Ca^{2+}/ATPase [38], phosphomannose isomerase [39] and tyrocidine synthetase 1 [40]. So, in the present work, we investigated the potential role of charged residues (i.e. arginine) specifically involved in substrate binding among the catalytic domain of Ins(1,4,5)P_3 3-kinase. The guanidinium group of arginine may be selectively modified by condensation with a dicarbonyl compound, phenylglyoxal [22].

By chemical modification, a phenylglyoxal-reactive arginine residue was identified within the ATP-binding site of rat brain Ins(1,4,5)P_3 3-kinase A. This residue was not involved in the binding of Ins(1,4,5)P_3. The covalent modification resulted in the inactivation of the enzyme activity; inactivation kinetics were identical for native and expressed truncated enzyme, suggesting that the active site of the enzyme was the target of the chemical modification. The similar values of $V_{max.}$ between purified rat brain and bacterially expressed Ins(1,4,5)P_3 3-kinase confirm the native conformation of the recombinant enzyme, and the equivalence of labelling and inactivation data derived for both rat brain and bacterially expressed enzyme. The recombinant enzyme was therefore produced in high yield and used to identify the modified arginine residue. The results described here show that the amount of phenylglyoxal labelling paralleled the loss in enzyme activity and that the labelling involves a single residue. A single reactive peptide has been labelled with radioactive phenylglyoxal in the absence of substrate. Labelling of this peptide was dramatically reduced in the presence of ATP but not in the presence of Ins(1,4,5)P_3. Further purification of this peptide was performed and its sequence was determined to correspond to amino acids 315 to 326 of rat brain Ins(1,4,5)P_3 3-kinase A. Radioactivity was associated with the third cycle of Edman degradation, indicating that phenylglyoxal specifically modified Arg-317 of Ins(1,4,5)P_3 3-kinase A (Figure 7), inactivating the enzyme, which is consistent with the role of this residue in ATP binding.

It is interesting to note that this arginine residue [Arg-317 in rat brain Ins(1,4,5)P_3 3-kinase A sequence] takes part in a sequence segment, ^{304}AQRAVTKPRYMQW**R**E^{318} (active-site arginine is indicated in bold), which is conserved between all sequences currently available for Ins(1,4,5)P_3 3-kinase {rat and human Ins(1,4,5)P_3 3-kinase A and B; [11–15]}. The same applies for Lys-197 and Asp-414 {using the amino acid numbering of rat brain Ins(1,4,5)P_3 3-kinase A sequence; [12]}, suggesting that Ins(1,4,5)P_3 3-kinase isoenzymes present a common ATP-binding domain, at least considering these three residues (see Figure 7). It would be of interest to investigate the effect of phenylglyoxal on the activity of other kinases involved in the phosphorylation of inositol phosphate molecules, e.g. the recently reported Ins(1,4,5)P_3 6-kinase in yeast [41], since these enzymes may present structural similarities.

The primary function of the ATP-binding domain is to anchor the nucleoside and the non-transferable phosphates α and β. This most probably occurs through locking the adenine base in a hydrophobic pocket and binding the phosphates α and β with positively charged residues. Arg-317 of rat brain Ins(1,4,5)P_3 3-kinase A could play an active role in this last interaction as shown in enzymes for which the three-dimensional structure is known, such as 6-phosphofructo-1-kinase [20] and F_1-ATPase [37]. A detailed understanding of the active site of Ins(1,4,5)P_3 3-kinase and the spatial relationship between the localized arginine residue and ATP will emerge from X-ray crystallographic analysis.

As recently demonstrated by Fry et al. [42], the ATP-binding domain may represent an important pharmacological target for the development of enzyme inhibitors: they reported a new inhibitor of the epidermal growth factor receptor tyrosine kinase of high potency and specificity. Inhibition appeared to be competitive towards ATP. The structural study of this binding site in closely related enzymes or isoenzymes, such as Ins(1,4,5)P_3 3-kinase isoforms, may lead to the discovery of discriminating inhibitors.

This research was supported by grants from the FRSM, Boehringer Ingelheim and the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Federal Service for Science, Technology and Culture. We thank Drs. J. E. Dumont, G. Vassart, J.-M. Boeynaems, M. Rider and L. Carpenter for helpful discussions. We are also grateful to Dr. A. Hepburn for help in the preparation of our manuscript. D.C. and V.V. are fellows of Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA). The scientific responsibility is assumed by its authors.

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Received 6 January 1995/29 March 1995; accepted 7 April 1995