Limited proteolysis and 'in vitro' mutagenesis of bovine brain inositol monophosphatase identifies an *N*-terminal region important for activity

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Bovine brain inositol monophosphatase is rapidly cleaved by endoprotease lys-C at a single site in the absence of SDS. Further sites are revealed only after prolonged incubation with high concentrations of protease. The initial cleavage occurs near one end of the enzyme, generating an *N*-terminally-derived 36-residue peptide, which is blocked, and a large 28 kDa fragment bearing a free *N*-terminus. The start sequence of this fragment was found to be Xaa-Ser-Pro-Ala-Asp-Leu-Val, consistent with the cDNA sequence, and Lys-36-Ser-37 was identified as the cleavage site. The activity of the cleaved enzyme was markedly decreased to 3% of that of the native enzyme, although its dimeric structure was preserved. The 36-residue peptide was not covalently associated with the large fragment after proteolytic cleavage, although the possibility of non-covalent association could not be excluded. Finally, the epitope for the inhibitory monoclonal antibody G-2A4 [Gee, Howell, Ryan & Ragan (1989) *Biochem J.* 264, 793–798] was found to lie proximal to the endoprotease lys-C cleavage site. 'In vitro' mutagenesis further mapped the epitope for monoclonal antibody G-2A4 to residues around Cys-8 of the enzyme. These results suggest that the *N*-terminal region of the enzyme is important for activity.

INTRODUCTION

Inositol monophosphatase is a key cytosolic enzyme in the inositol phospholipid signalling system, recycling the inositol moiety of its inositol phosphate substrates and catalysing the final step in the synthesis of inositol from glucose *de novo*. The enzyme has been purified from both animal and plant sources including rat [1] and bovine [2–4] brain and the pollen of *Lilium longiflorum* [5]. In all cases the enzyme appears to be dimeric, with a molecular mass of approx. 60 kDa, and is composed of two apparently identical subunits. It catalyses the hydrolysis of D- and L-Ins(1)P, D- and L-Ins(4)P, *meso*-Ins(5)P and several non-inositol-containing substrates, including 2'-AMP (for review, see [6]). The enzyme is inhibited by Li⁺ [7], which has been shown to trap the phosphoryl-enzyme intermediate, thus preventing nucleophilic attack by water [8].

Relatively little information has been reported on the detailed structure and mechanism of the enzyme. Recently we have reported the complete cDNA and deduced primary amino acid sequence of the bovine brain enzyme [9]. To now define more precisely the amino acid residues involved in the function of the enzyme we have begun to use a dual approach of protein modification together with site-directed mutagenesis. In the present paper we report on studies undertaken using both endoprotease lys-C treatment of native enzyme and 'in vitro' mutagenesis of recombinant enzyme. This approach has allowed us to identify a region which is important for activity towards the *N*-terminal end of the protein.

MATERIALS AND METHODS

Materials

Endoprotease lys-C was obtained from Boehringer, Lewes, East Sussex, U.K. L-[U-¹⁴C]Ins(1)P (55 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.).

Nitrocellulose membrane (0.45 µm) was obtained from Schleicher and Schuell, Dassel, Germany. Immobilon P membrane was obtained from Millipore, Harlow, Middlesex, U.K. Peroxidase-conjugated rabbit anti-(mouse IgG) was obtained from Dako, High Wycombe, Bucks., U.K.

Endoprotease lys-C digestions

Purified bovine brain inositol monophosphatase [2] (20 µg of protein, at least 95% pure as judged by SDS/PAGE) in 100 µl of 20 mM-Tris/HCl/150 mM-NaCl, pH 8.0, was digested with 0.1 µg of the protease at 37 °C. Samples (5 µl) were withdrawn at various times and frozen immediately in a solid CO₂/acetone bath. Samples were subsequently either analysed by SDS/PAGE or assayed for enzyme activity.

Gel filtration analysis

Samples of enzyme (either native or after digestion) in 200 µl of 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0, were chromatographed on a Superose 12 column (Pharmacia) equilibrated with the same buffer at a flow rate of 0.4 ml/min. The effluent was monitored at 280 nm. Molecular size was determined by reference to a calibration curve constructed using proteins of known molecular size.

Western blotting

Samples of bovine brain enzyme (native or after digestion) or recombinant enzyme were electrophoresed on 12%-acrylamide resolving gels and transferred to a nitrocellulose membrane exactly as described in [10]. Briefly, blots were probed successively with monoclonal antibody G-2A4 (omitted for controls) and peroxidase-conjugated rabbit anti-(mouse IgG). Bands were visualized by addition of substrate solution [0.2 mg of 3-amino-9-ethylcarbazole/ml of 50 mM-sodium acetate/acetic acid, pH 5.0, containing 1 µl of 30% (v/v) H₂O₂/ml].

Abbreviations used: TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PCR, polymerase chain reaction.

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Preparation of samples for sequencing

Purified enzyme (2.5 mg in 10 ml of 10 mM-Tris/HCl, pH 8.0, was digested with 5 μ g of endoprotease lys-C for 2 h at room temperature and freeze-dried. Lyophilized protein (1 mg; equivalent to 33 nmol) was redissolved in 1.0 ml of 0.1 % trifluoroacetic acid (TFA) and 30 μ l (1 nmol) was applied to a Biobrene-coated glass-fibre disc which had been subjected to three cycles of the Edman degradation process to remove contaminants, and sequenced on an Applied Biosystems 477A Sequencer. The phenylhydantoin (PTH)-amino acid released at each turn was detected by its absorption at 269 nm and identified by comparison with known standards on reverse-phase chromatography.

Sequencing of peptides blotted on to an Immobilon membrane

Digested enzyme (125 μ g) was electrophoresed in a 12 % polyacrylamide gel in the presence of SDS and transferred to an Immobilon membrane. The blot was stained with Coomassie Blue and protein bands were excised and cut into small pieces (approx. 4 mm $\times$ 1.5 mm). The membrane was destained by 2 $\times$ 1 ml washes with 100 % methanol and then protein was extracted with 4 $\times$ 0.1 ml of 70 % propan-2-ol/30 % water (containing 5 % TFA). The extracts were dried down *in vacuo* and the residue was redissolved in 0.1 ml of 0.1 % TFA in water. The whole sample was applied to a Biobrene-treated disc and sequenced as described above.

'In vitro' mutagenesis of recombinant inositol monophosphatase

Both the primary amino acid sequence and the expression of recombinant bovine inositol monophosphatase in *Escherichia coli* using the T7 polymerase expression system [11] have been described in detail elsewhere [9]. *N*-Terminal deletion mutants of recombinant enzyme were constructed using polymerase chain reaction (PCR) technology. Deletion mutant Met 9 (lacking the first 8 residues) was constructed using the 5' oligonucleotide primer 5'-CCTTGGCAGGAACATATGGATTATGCA-3'. Deletion mutant Gln 18 (lacking the first 16 residues, with an initiating Met residue inserted at position 17) was constructed using the 5' oligonucleotide 5'-GCAGTAACCCTGCATATGCAAGCTGGAGAG-3'. Deletion mutant Met 34 (lacking the first 33 residues) was constructed using the 5' oligonucleotide 5'-GAAATGAATCATATGGTTAAAAGTTCTCCG-3'. The point mutant Cys-8 $\rightarrow$ Ser was constructed using the 5' oligonucleotide 5'-GGAGATATACATATGGCTGATCC-TTGGCAGGAATCCATGGAT-3', converting the Cys codon TGC to the Ser codon TCC (underlined). All 5' oligonucleotides incorporated an *Nde*I restriction site at the initiating Met position. The 3' oligonucleotide used for all PCR reactions was 5'-TGTGTGAAGCCGTCAGCTTAATCTTCATC-3', incorporating a *Hind*III restriction site after the stop codon. PCR was performed as previously described [9]. PCR products were digested with *Hind*III and *Nde*I and subcloned into *Nde*I-*Hind*III-digested pRSET5a expression vector. All constructs were verified by determining the DNA sequence of the first ~ 100 bases of the mutants. Expression of recombinant enzyme was performed exactly as described previously [9]. After induction with isopropyl β -thiogalactoside, bacteria were pelleted using a Microfuge, resuspended in 0.5 vol. of SDS/PAGE sample buffer [2] and boiled, and 15 μ l portions were analysed by SDS/gel electrophoresis and Coomassie Blue staining or Western blotting, as described above.

Other methods

SDS/PAGE was performed using 12 % acrylamide resolving gels (3 % stacking) using the Laemmli buffer system [12]. Proteins were visualized by staining with Coomassie Blue. Protein was

determined by the method of Bradford [13], with BSA as standard. Inositol monophosphatase activity was measured by a radiochemical assay using [14 C]Ins(1)*P* as the tracer, as described previously [14]. Bovine brain inositol monophosphatase was purified by immunoaffinity chromatography as described previously [10].

RESULTS AND DISCUSSION

Endoprotease lys-C cleavage of inositol monophosphatase

Inositol monophosphatase (subunit molecular mass 30 kDa) was rapidly cleaved by endoprotease lys-C in the absence of SDS, generating a 28 kDa fragment plus peptide material amounting to approx. 2 kDa. Fig. 1(a) shows a time course for the production of the 28 kDa fragment. Cleavage was found to be associated with a marked loss of enzyme activity, down to a minimum of 3 % of the original activity (Fig. 1b). After prolonged exposure to very high concentrations of protease, cleavage at a second site occurred, generating a 23 kDa fragment which was devoid of activity (results not shown). Preliminary studies on the kinetic properties of the 28 kDa fragment suggest a marked decrease in $V_{\max}$ compared with the uncleaved enzyme, but no significant alteration in K_m .

Identification of the initial cleavage site

Inositol monophosphatase is known to be blocked at the *N*-terminus [9]. The enzyme was therefore digested with 0.2 % (w/w) endoprotease lys-C, and sequencing of the whole mixture was undertaken without further purification. The sequence of the only peptide generated with a free *N*-terminus began Xaa-Ser-

Fig. 1. Time course for the digestion of inositol monophosphatase by endoprotease lys-C

Bovine brain inositol monophosphatase was digested with endoprotease lys-C as indicated in the Materials and methods section. Samples were removed at various times and analysed in one of two ways. (a) SDS/PAGE. The molecular masses of proteolytic fragments were determined by reference to markers of 94, 43, 30, 20 and 14 kDa. (b) Enzyme activity. Samples were assayed by the radiochemical method. Values are expressed relative to that of undigested enzyme (100 %).

Fig. 2. Immunoblotting of inositol monophosphatase and its 28 kDa proteolytic fragment using monoclonal antibody G-2A4

Samples of bovine brain inositol monophosphatase (2 μ g) were incubated with or without 0.1 μ g of endoprotease lys-C in 20 μ l of 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0, for 1 h at 37 $^{\circ}$ C. Samples were then electrophoresed in a 12% SDS/polyacrylamide gel and either stained for protein with Coomassie Blue (tracks 1–3) or transferred on to a nitrocellulose membrane which was then probed successively with monoclonal antibody G-2A4 and then peroxidase-conjugated rabbit anti-(mouse IgG). Bands on lanes 4 and 5 were visualized by the peroxidase reaction. Lanes were loaded with samples as follows: lane 1, marker proteins; lanes 2 and 4, undigested enzyme; lanes 3 and 5, digested enzyme.

Pro-Ala-Asp-Leu-Tyr. The initial yield of sequencing was 79.4% and the repetitive yield was 96.8%. Direct sequencing of the large 28 kDa fragment isolated on an Immobilon membrane yielded the same initial sequence, indicating that the cleavage site must be located towards the *N*-terminal end of the protein. The exact cleavage site was subsequently confirmed as Lys-36–Ser-37 when the cDNA sequence became available [9].

Molecular size of the cleaved enzyme

The molecular mass of native inositol monophosphatase was found to be 48 kDa, similar to that reported previously [2]. Endoprotease lys-C-cleaved enzyme chromatographed as a 51 kDa species, suggesting that the dimeric structure of the enzyme had been preserved. The slight but reproducible increase

in Stokes' radius for the cleaved enzyme may be indicative of a subtle conformational change. We have been unable to show conclusively that the small fragment was no longer associated with the 28 kDa fragment. However, the fragment did not appear to be covalently associated via an S–S bridge through Cys-8, since the electrophoretic behaviours of the cleaved and uncleaved enzyme were unchanged by omission of β -mercaptoethanol in SDS/polyacrylamide gels (results not shown).

Location of the epitope for the inhibitory monoclonal antibody G-2A4

(i) **Endoprotease-lys-C treated enzyme.** In view of the apparent importance of the *N*-terminal region of the protein for enzyme activity, we investigated whether or not the epitope for the inhibitory monoclonal antibody G-2A4 [10] was located in the same region. Western blots of cleaved and uncleaved enzyme were probed with the monoclonal antibody (Fig. 2). A complete loss of antibody reactivity with the 28 kDa fragment was observed, suggesting that the epitope for the antibody either encompasses the endoprotease lys-C cleavage site or lies proximal to it within the small *N*-terminally derived fragment.

(ii) **Deletion mutants of recombinant inositol monophosphatase.** The epitope for monoclonal antibody G-2A4 was initially mapped using deletion mutants of recombinant enzyme. Three *N*-terminal deletion mutants were constructed, Met 9, Gln 18 and Met 34 (Fig. 3a). Western blots of the three deletion mutants, wild-type recombinant enzyme and native brain enzyme demonstrated binding of monoclonal antibody G-2A4 to only the undeleted enzyme (Fig. 3b), suggesting that the epitope for G-2A4 lies within residues 3–13 (since epitopes are composed of 4–6 amino acid residues) of the inositol monophosphatase. Point mutagenesis of inositol monophosphatase (Cys-8 $\rightarrow$ Ser) almost abolished the binding of monoclonal antibody G-2A4 to the enzyme on Western blots (Fig. 4). Thus the epitope of monoclonal antibody G-2A4 has been fine-mapped to amino acid residues around Cys-8. The K_m of the Cys-8 point mutant was essentially unchanged from that of the wild-type enzyme (0.20 mM versus 0.22 mM), suggesting that Cys-8 itself is probably not an important residue at the active site of the enzyme. Additionally, because Cys-8 lies within the epitope for monoclonal antibody G-2A4, it is very unlikely that this residue is involved in disulphide bridging with another Cys residue in the enzyme.

Fig. 3. Mapping of the epitope for monoclonal antibody G-2A4 using inositol monophosphatase deletion mutants

Samples of purified brain enzyme and bacterial whole-cell lysates containing recombinant enzyme were electrophoresed in a 12% polyacrylamide gel in the presence of SDS and then stained with Coomassie Blue (a) or Western blotted (b) and probed successively with monoclonal antibody G-2A4 and then peroxidase-conjugated rabbit anti-(mouse IgG). Lane 1, purified brain enzyme (0.2 μ g); lane 2, deletion mutant Met 34; lane 3, deletion mutant Gln 18; lane 4, deletion mutant Met 9; lane 5, wild-type recombinant enzyme.

Fig. 4. Mapping of the epitope for monoclonal antibody G-2A4 to residues around Cys-8

Samples of purified brain enzyme and bacterial whole cell lysates containing recombinant enzyme were electrophoresed in a 12% polyacrylamide gel in the presence of SDS and then either stained for protein with Coomassie Blue (a) or Western blotted and then probed with monoclonal antibody G-2A4 and then with peroxidase conjugated rabbit anti-(mouse IgG) (b). Lane 1, wild-type; lane 2, Cys 8 mutant; lane 3, brain enzyme.

Conclusions

In the present study we have identified a region close to the *N*-terminus of inositol monophosphatase which appears to be important for enzyme activity. Binding of monoclonal antibody G-2A4 to its linear epitope around Cys-8 of the protein blocks enzyme activity by up to 83%. In addition, cleavage of the polypeptide backbone by endoprotease lys-C at Lys-36-Ser-37 drastically decreased activity. This suggests that the *N*-terminus of the enzyme is critically involved in enzyme activity. This could

be through residues involved at the active site of the enzyme or through a conformational change in the *N*-terminal group (prevented by binding of monoclonal antibody G-2A4) during substrate binding and/or catalysis which is critical for enzyme activity.

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