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Bovine inositol monophosphatase: proteolysis and structural studies

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Bovine brain inositol monophosphatase is inactivated when trypsin catalyses the cleavage of a single peptide bond between Lys-36 and Ser-37. This proteolysis is closely followed by cleavage at two other sites in the protein between Lys-78 and Ser-79 and between Lys-156 and Ser-157 suggesting that all of these sites are exposed in the native conformation of the protein. All of these residues are predicted to lie at the ends of α helices. The most susceptible bond (Lys-36–Ser-37) is predicted to lie in a highly flexible region of the protein. Circular dichroism studies suggest that approximately 40% of the secondary structure of this protein is helical which is similar to that predicted by the algorithm of Garnier et al. [(1978) J. Mol. Biol. 120, 97–120].

Inositol monophosphatase; Proteolysis; Protein structure; Circular dichroism

1. INTRODUCTION

Inositol monophosphatase dephosphorylase Ins(1)P, Ins(3)P and Ins(4)P as well as a number of non-inositol-containing compounds [2]. The enzyme has a subunit molecular weight of 30 kDa and exists as a homodimer. Catalysis is a Mg^{2+}-dependent process although high concentrations of this metal causes inhibition of the enzyme [3]. The enzyme is also inhibited in an uncompetitive manner by Li^{+} [3], a feature which may underlie the anti-manic and anti-depressive actions of this ion [4]. The cDNA cloning and sequencing of the bovine [5], rat and human [6] genes has demonstrated a high level of sequence homology between these species (79%) and therefore fails to highlight any one particular area of the protein as being significant in the catalytic process of the enzyme. The production of a monoclonal antibody against the enzyme has led to the N-terminal region of the protein being implicated in the catalytic process [7]. The epitope of the antibody is centred around Cys-8 [8] and binding gives rise to inhibition of the enzyme [9]. Further studies which utilised the enzyme endoprotease Lys-C have demonstrated that in inositol monophosphatase there is a bond located between Lys-36 and Ser-37 highly susceptible to proteolysis [9]. The susceptibility of this bond to cleavage is decreased by the presence of substrate and other ligands known to bind at the active site of this enzyme [9]. Hydrolysis of this bond by endoprotease Lys-C leads to concurrent loss of enzyme activity suggesting that this region of the protein is important in the functioning of the enzyme. Prolonged exposure to very high concentrations of endoprotease Lys-C was shown to cleave the protein at one additional site (not identified) producing a fragment of M_r 23,000. Here we report observations made using limited proteolysis by an alternative protease, trypsin, which has enabled the identification of three other peptide regions of the enzyme with exposed target residues. These sites of cleavage are correlated with secondary structural predictions which place the target residues at ends of α-helices.

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2. MATERIALS AND METHODS

2.1. *Materials*

The growth of bacterial cultures and subsequent extraction and purification of inositol monophosphatase is described elsewhere [5]. All reagents were purchased from Sigma Chemical Co., Poole, UK.

2.2. *Enzyme activity*

Inositol monophosphatase activity was determined either by the discontinuous assay of Lanzetta et al. [10] or the fluorescent assay described by Gore et al. [11].

2.3. *Protein concentration*

The concentration of inositol monophosphatase was determined by the method of Smith et al. [12].

2.4. *Proteolysis of inositol monophosphatase*

To 1 ml of inositol monophosphatase (1 mg/ml) was added 20 μl of 0.005% trypsin in 10 mM Tris-HCl buffer (pH 8.0) and 10 mM $CaCl_2$ at 21°C. Samples were withdrawn at various times and run on a 12% SDS-PAGE gel [13].

2.5. *Electroblotting of peptides from SDS-PAGE*

Inositol monophosphatase was treated with 0.01% trypsin as described above for 30 min and the various proteolytic products separated by 12% SDS-PAGE. The gel was electroblotted onto Problott (ABI) by the method of Matsudaira [14]. The blot was then stained for 30 s using Coomassie blue and the visualised protein bands excised and identified by N-terminal sequencing.

2.6. *Protein sequencing*

The amino-terminus of each peptide was determined using an Applied Biosystems 477A sequencer with on line 120A HPLC for identification of the PTH-amino acids released at each cycle.

2.7. *Gel filtration*

A column of Sephadex G-75 (2.2 × 30 cm) was prepared and equilibrated in 100 mM Tris-HCl buffer (pH 8.0) with a flow rate of 1.4 ml/min. The column was calibrated using solutions of Dextran blue, pyridoxal phosphate, cytochrome *c*, trypsin inhibitor, DNase I, ovalbumin, glyceralderhyde 3-phosphate dehydrogenase, alcohol dehydrogenase and alkaline phosphatase. Inositol monophosphatase was digested with 0.005% trypsin for 10 min at 21°C followed by the addition of trypsin inhibitor to prevent further hydrolysis. This was then applied to the column for estimation of the molecular weight of the proteolysed inositol monophosphatase.

2.8. *Gel scanning*

The M_r of each peptide fragment was estimated by SDS-PAGE and the intensity of each band was estimated by integration using a Joyce Loebl Chromoscan 3 gel scanner.

2.9. *Circular dichroism studies*

The far CD spectrum of the bovine inositol monophosphatase was determined using a Jasco J600 CD spectrometer with a 0.2 mm light path. The protein concentration used was 1 mg/ml in 50 mM Tris-HCl (pH 8.0).

3. RESULTS AND DISCUSSION

Approximately 85% of the activity of inositol monophosphatase is lost in 10 min when the enzyme is incubated with 0.005% w/w trypsin at 21°C and pH 8.0. The loss in enzyme activity is accompanied by a decrease in M_r from 30,000 to 26,500 (Fig. 1). Ligand protection studies (data not shown) demonstrated that the cleavage by trypsin is prevented by the inclusion of the substrate Ins(1)P (0.6 mM) and the inhibitor LiCl (10 mM). The latter forms a stable complex with the enzyme and the phosphate group released after the hydrolytic step of the reaction [15]. Control experiments demonstrated that these ligands have no effect on the action of trypsin. These results are consistent with those of Gee et al. [9] who demonstrated the loss of enzyme activity as a result of proteolysis by endoprotease Lys-C.

The structural nature of the major proteolytic product of this first period of incubation was investigated by gel filtration chromatography. Native inositol monophosphatase was found to have a molecular weight of 48 kDa when chromatographed on a Sephadex G-75 column. This value is low when compared to the molecular weight deduced from the gene sequence but is consistent with the findings of other workers [8,16]. When proteolysed enzyme was applied to the same column, the apparent molecular weight was reduced to 44.7

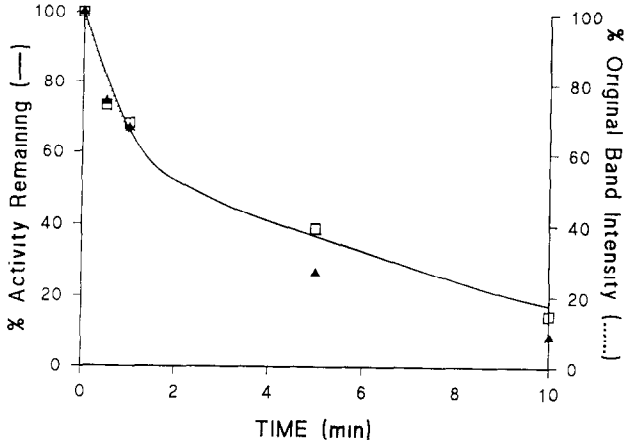

Fig. 1. The correlation of the time course of inactivation of inositol monophosphatase by trypsin with the time course of loss of the N-terminal fragment from the enzyme. 1 mg of enzyme in 1 ml of 50 mM Tris-HCl, pH 8.0 was incubated for various times with 0.005% w/w trypsin at 21°C. Aliquots were removed and assayed for activity (see section 2) and subjected to SDS-PAGE.

kDa. Both of these figures are low compared to the expected M_r of 60,000 deduced from the gene sequence [5] but are in broad agreement with the data of Meek et al. [16]. However, Whiting et al. [8] showed that the molecular weight of proteolysed enzyme appeared to increase from 48 kDa (native) to 51 kDa. The latter could be due to conformational relaxation following proteolytic cleavage, thus giving rise to a larger Stokes radius. It is also possible that the low molecular weight of the protein determined by gel filtration chromatography may indicate that hydrophobic interactions between the protein and the matrix delays the elution of the enzyme resulting in an erroneously low estimate of the molecular weight. In order to test this, native and proteolysed enzyme was applied to a Gilson HPLC system equipped with a 250 × 7.5 mm TOSOH-TSK G3000SW gel permeation column in high (50 mM Tris-HCl, 200 mM NaCl, pH 8.0) or low (50 mM Tris-HCl, pH 8.0) salt conditions. Using the high salt conditions of Whiting et al. [8] the native protein eluted after 17.25 ml and the proteolysed enzyme eluted after 16.35 ml consistent with an apparent increase in size. However, under the low salt conditions the reverse situation occurred. The native protein eluted after 12.9 ml whereas the cleaved protein eluted after 13.8 ml consistent with an apparent decrease in size. These data show that both the native and the proteolysed samples interact with this matrix since both eluted earlier when in low salt conditions. The observation that the cleaved protein in particular is retained longer by the column matrix under high salt conditions may suggest that the loss of the N-terminal 36 residues of the protein exposed an additional hydrophobic surface. Both groups identify a change in molecular weight of about 3 kDa although the possible interactions with the gel matrices used make this value

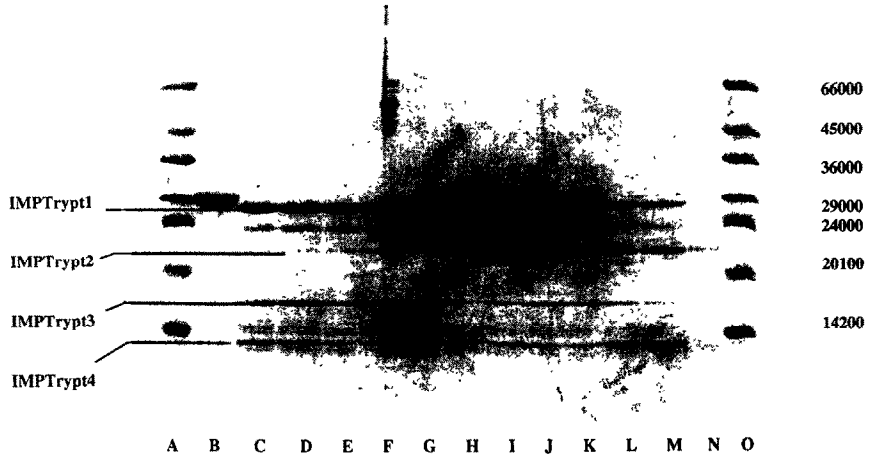

Fig. 2. Proteolytic digestion of inositol monophosphatase with trypsin produced four main peptide intermediates labelled as IMPTrypt 1–4. The band of M_r 24,000 corresponds to trypsin inhibitor which was used to terminate the reaction. Lanes (A) and (O) are M_r markers; (B) shows undigested inositol monophosphatase; (C) 1 min; (D) 2 min; (E) 5 min; (F) 10 min; (G) 20 min; (H) 30 min; (I) 45 min; (J) 60 min; (K) 90 min; (L) 120 min; (M) 150 min and (N) 24 h incubation with 0.01% w/w trypsin.

questionable. Since SDS-PAGE experiments show that all subunits are cleaved with a loss of a peptide of M_r 3,000 from each subunit the data above also suggest that the dimeric nature of the enzyme is maintained in the first proteolysed protein intermediate.

By increasing either the concentration of trypsin or by prolonging the duration of exposure to the protease, native inositol monophosphatase undergoes slow but complete digestion under non denaturing conditions unlike the situation when endoprotease Lys-C is used. This process appears to occur in a very ordered manner and several major peptide intermediates are formed under the conditions used (see section 2). These peptides appear to be produced by hydrolysis at only a few of the possible 27 tryptic sites in inositol monophosphatase. It therefore seems probable that these sites are relatively accessible to trypsin in the native tertiary structure of the protein, thus generating several major intermediates before being digested to their constituent tryptic peptides. Fig. 2 shows the time course of tryptic degradation of inositol monophosphatase. The disappearance of the 30 kDa native inositol monophosphatase band is complete within one minute under the conditions used. This initial site of cleavage is shortly followed by cleavage predominantly at three other sites. These major protein fragments produced are stable for up to 2 h, though after 24 h only a small amount of a single band remains of apparent M_r 20,500. The N-terminal sequence of each of the proteolytic products was identified by electroblotting onto Problott membrane (ABI) with subsequent sequencing as described in section 2. The main product (IMPTrypt 1, see Table I) with an apparent M_r 26,540 was identified as being produced by proteolysis between Lys-36 and Ser-37 as was noted from previous experiments with endoprotease Lys-C [8]. The other three peptides have N-terminal sequences as described in Table I. The peptides IMPTrypt 3 and IMPTrypt 4 appear to be products which share a common peptide bond cleavage. These two peptides are produced by tryptic action between Lys-156 and Ser-157 of the protein already truncated by cleavage between Lys-36 and Ser-37. The fourth peptide (IMPTrypt 2) is the product of proteolysis between Lys-78 and Ser-79. However, the M_r of IMPTrypt 2 estimated from its electrophoretic mobility suggests that some truncation has occurred at a site near to the C-terminus. Its molecular weight would suggest that a suitable truncation site to give rise to such a peptide would be between Lys-256 and Thr-257.

When extended proteolysis is performed in the presence of Ins(1)P, Mg^{2+} and Li^+ hydrolysis of the bond between Lys-36 and Ser-37 occurs at a much slower rate as expected for the ligand protected enzyme. However, the secondary sites of proteolysis are still hydrolysed in the protected protein. This suggests that proteolysis of these sites does not arise as a result of denaturation caused by the primary hydrolysis at the peptide bond between Lys-36 and Ser-37.

Table I

Shows the four main tryptic products of inositol monophosphatase generated upon incubation of the enzyme with 0.01% trypsin as described in section 2. The table shows the N-terminal sequence of each peptide and its estimated molecular weight

Peptide	N-terminal sequence	M_r measured	Predicted fragment	M_r calculated
IMPTrypt1	SSPAD	26,540	37–277	25,976
IMPTrypt2	SILTD	20,700	79–256	19,781
IMPTrypt3	SSPAD	15,140	37–156	13,112
IMPTrypt4	SLLVT	11,520	157–277	12,864

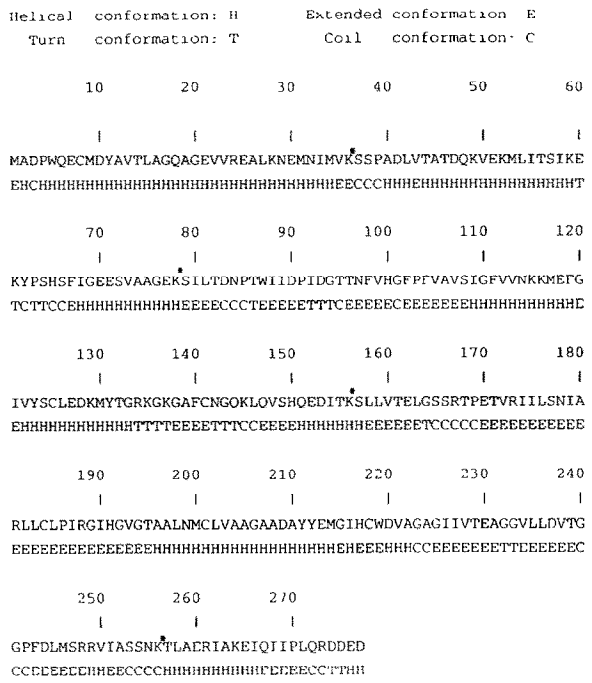

Fig. 3. The predicted secondary structure of inositol monophosphatase using the method of Garnier et al. [1].

The greater susceptibility to proteolysis of all these peptide bonds is probably due to them being more exposed in the proteins tertiary structure and therefore available to the protease. Such expectations are supported by structural predictions. Analysing the protein sequence of bovine inositol monophosphatase for potential antigenic regions by searching for regions of high flexibility [17,18] revealed that three out of the four proteolytic sites are predicted to be antigenic sites and therefore of high flexibility. Of these three sites, the region encompassing residues Val-35 to Asp-41 is most strongly predicted to be flexible. The other predicted sites are the regions between Ala-252 and Leu-258 and between His-150 and Ser-157.

It is interesting that all cleavages occur between lysine–serine bonds (i.e. Lys-36–Ser-37, Lys-78–Ser-79, Lys-156–Ser-157). Serine is a very weak helix former [1,19] and the cleavage sites identified lie at the carboxy ends of predicted α-helical motifs as shown in Fig. 3. Since the molecular weight of peptide IMPTrypt 2 suggests that it has undergone proteolysis at the carboxyl-terminal end there must be a susceptible proteolytic site available to trypsin. This site is possibly between Lys-256 and Thr-257 which is predicted to be at the amino-end of an α-helix. All of the cleavage sites available to trypsin but not endoprotease Lys-C involve a lysyl residue which is also the target residue for endoprotease Lys-C. Failure of the latter to cleave the protein at 3 of these sites may reflect a greater steric hindrance to the approach of this protease.

The amino-terminal region of inositol monophosphatase is strongly predicted to be α-helical in nature by the algorithm of Garnier et al. [1]. The sequence of this region of the protein may be represented in the form of a helical wheel, amphipathic in nature. It is interesting to note that Cys-8 resides on the hydrophobic face of this helix (an expected location for this amino acid in such a structure [20]). Such an observation is consistent with our previous findings [21] in which the cysteine residues of inositol monophosphatase where chemically modified by DTNB, *N*-ethylmaleimide and iodoacetic acid. In these studies only the cysteine residues at positions 141, 184 and 218 were available in the native conformation of the protein for modification by the above reagents. This observation also supports the gel filtration studies described above which suggest that loss of the amino-terminal peptide from the protein exposes an additional hydrophobic surface.

We have estimated the helical content of the protein from its circular dichroic spectrum in the far UV range to be between 185 and 260 nm (see Fig. 4). The spectrum shows a narrow shoulder on the negative peak of ellipticity at 205–210 nm and a wide negative elliptical band at 212–224 nm. Such a spectrum in this region is indicative of helix–helix interactions or some helical distortion (Dr. A. Drake, personal communication). Analysis of the data of Fig. 4 by the methods of Fasman [22] and Provencher and Glockner [23] suggest a helical content of 40% and 34%, respectively. These estimates are reasonably similar to that (47%) obtained using the structural prediction algorithm of Garnier et al. [1] which calculates the possible helical content of a protein without consideration of tertiary or quaternary structural interactions.

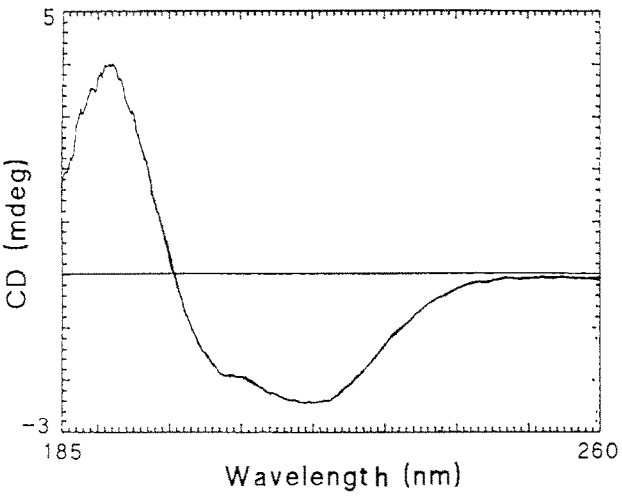

Fig. 4. The Circular Dichroic spectrum of a solution of inositol monophosphatase between 185 nm and 260 nm. The enzyme was dissolved (1 mg/ml) in 10 mM Tris-HCl buffer pH 8.0.

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