Cloning and Expression of Bovine Brain Inositol Monophosphatase

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Inositol monophosphatase is a key enzyme of the inositol phosphate second messenger signaling pathway. It is responsible for the provision of inositol required for the synthesis of phosphatidylinositol and polyphosphoinositides and has been implicated as the pharmacological target for lithium action in brain. Using oligonucleotide probes based on partial amino acid sequence data for the bovine brain enzyme, several overlapping cDNA clones of 2-3 kilobases in length have been isolated. All contain an open reading frame encoding a 277-amino acid protein. No significant sequence homology was found with any known protein. The open reading frame was inserted into a bacterial expression vector in order to confirm the presumed identity of the protein. The expressed protein reacted with an anti-inositol monophosphatase monoclonal antibody. In addition, the protein was enzymatically active and indistinguishable from the bovine brain enzyme with respect to Km values for substrate and Li+ sensitivity of inositol 1-phosphate hydrolysis.

The enzyme myo-inositol monophosphatase is a key component of the phosphoinositide cell signaling system. It hydrolyzes D-inositol 1-phosphate (for review, see Majerus et al., 1988), D-inositol 3-phosphate, and D-inositol 4-phosphate and is responsible for the provision of inositol required for the synthesis of phosphatidylinositol and polyphosphoinositides. Its role in brain is particularly important since plasma inositol is required for synthesis of phosphatidylinositol and polyphosphoinositides. Its release of [3H]inositol from [3H]inositol phosphate as described (Gee et al., 1988). Enzyme activity was determined by measuring release of [14C]inositol from [14C]inositol phosphate as described previously (Ragan et al., 1988). One unit of enzyme activity represents 1 pmol of substrate hydrolyzed per min at 37 °C. Protein concentrations were determined by the method of Bradford (1976).

Materials and Methods

Inositol Monophosphatase Purification and Enzyme Assays

The enzyme was purified from bovine brain as described previously (Gee et al., 1988). Enzyme activity was determined by measuring release of [14C]inositol from [14C]inositol phosphate as described previously (Ragan et al., 1988). One unit of enzyme activity represents 1 pmol of substrate hydrolyzed per min at 37 °C. Protein concentrations were determined by the method of Bradford (1976).

Protein Sequencing

Purified inositol monophosphatase was treated with CNBr in formic acid for 12 h followed by lyophilization (Spiess et al., 1979). Individual peptides were isolated by chromatography on a Vydac C4 high pressure liquid chromatography column, eluted with a 10-70% acetonitrile gradient in 20 mM trifluoroacetic acid. Both the intact protein and the CNBr-derived peptides were subjected to gas phase sequencing using an Applied Biosystems gas phase sequenator (Hewick et al., 1981).

cDNA Cloning

Total cellular RNA was isolated from bovine brain cortex by the guanidinium isothiocyanate-CsCl method (Chirgwin et al., 1979) followed by oligo(dT) cellulose chromatography (Maniatis et al., 1982). cDNA was synthesized by a modification of the procedure of Gubler and Hoffman (1983) as described (Dixon et al., 1988). The EcoRI-digested cDNA was size-fractionated to be greater than 1 kilobase in size by agarose-gel electrophoresis and ligated to λgt10 arms. The cDNA library (109 recombinants) was screened unamplified on Escherichia coli strain LE392 as described (Dixon et al., 1988). Oligonucleotide probes corresponding to the derived peptide sequences were synthesized using an Applied Biosystems model 380 DNA synthesizer. Overlapping complementary oligonucleotides were labeled using Kleen DNA polymerase and all four [32P]deoxynucleotide triphosphates. Other oligonucleotides were labeled with T4 polynucleotide kinase and [32P]ATP; insert fragments were labeled by nick translation (Maniatis et al., 1982). Hybridization and washing conditions were as described (Dixon et al., 1988). Standard recombinant DNA procedures were used for the analysis of clones (Maniatis et al., 1982). Both strands of the sequence were determined in their entirety by the dideoxy chain termination method (Sanger et al., 1977; Hattori and Sakaki, 1983).
Expression of Recombinant Bovine Inositol Monophosphatase in Bacteria

Construction of Expression Vector—The T7 polymerase bacterial expression system (Rosenberg et al., 1987) was used. The coding region of the bovine inositol monophosphatase cDNA was reconstructed to contain an NdeI site at the start codon and a HindIII site at the stop codon using polymerase chain reaction methodology (Saiki et al., 1988). Oligonucleotides 5'-CGCCGCCCTCATATGGCTGATCCTTTGAGG-3' and 5'-TGTGTAAGCCGTCAAGCTTAATCTTCATC-3' were synthesized on an Applied Biosystems 380B instrument and purified using oligonucleotide purification cartridges (Applied Biosystems). Inositol monophosphatase cDNA (100 ng), in Bluescript SK- (Stratagene), was linearized with KpnI and subjected to polymerase chain reaction under standard conditions (Saiki et al., 1988). Denaturation was performed at 94 °C for 2 min, the annealing at 55 °C for 2 min, and the polymerization at 72 °C for 6 min. 20 cycles were performed, with the last polymerization step lasting 12 min. The polymerase chain reaction products were extracted with phenol/chloroform, ethanol-precipitated and digested with NdeI and HindIII, and the 837-base pair product of polymerase chain reaction subsequently purified by agarose gel electrophoresis. The T7 polymerase expression system used, pRSETa (Schoepfer et al., 1990) was derived from the pET3a vector (Rosenberg et al., 1987). The 837-base pair NdeI-HindIII inositol monophosphatase polymerase chain reaction product was cloned into NdeI-HindIII-digested pRSETa, transformed into E. coli strain DH5α competent cells. Positive clones were identified by colony hybridization and characterized by detailed restriction mapping. For subsequent expression studies, the expression vector was transformed into competent BL21 (DE3) cells.

Analysis of Recombinant Inositol Monophosphatase

E. coli strain BL21-DE3 bacteria, containing the inositol monophosphatase expression construct, were grown in LB medium containing 20 μg/ml ampicillin to an A660 of 0.9 by rapid shaking at 37 °C. The mother, isopropyl-β-D-galactoside (IPTG), was then added to a final concentration of 0.4 mM, and shaking continued for 4 more hours. The cells were then pelleted, resuspended in half the original culture volume of SDS-PAGE sample buffer (Gee et al., 1988), boiled for 2 min, repelleted in a microcentrifuge, and aliquots (15 μl) of the supernatant analyzed by SDS-PAGE and Coomassie Blue staining (Gee et al., 1988). Additionally, Western blot analysis of recombinant protein was performed using a monoclonal antibody prepared to bovine inositol monophosphatase as described previously (Gee et al., 1989). For assay of enzyme activity, bacteria were pelleted in a microcentrifuge, and then resuspended in half the original culture volume of 25 mM Tris HCl, pH 7.6, 50 mM NaCl, containing 1 mg/ml lysozyme. After a 5-min incubation the cells were sonicated (1 min, using a Ultrasonics Ltd. Soniprep). Triton X-100 was then added to a final concentration of 0.5%, incubated for a further 15 min, and then pelleted in a microcentrifuge to remove insoluble material. The insoluble material was resuspended in the same buffer by extensive vortexing. Both the soluble and insoluble fractions were then assayed for inositol monophosphatase activity as described above.

RESULTS AND DISCUSSION

Cloning of Bovine Brain Inositol Monophosphatase—The inositol monophosphatase from bovine brain was purified to homogeneity as previously described (Gee et al., 1988). When the intact protein was subjected to amino acid sequence analysis the amino terminus was found to be blocked. The protein was then reacted with CNBr which released several peptide fragments. The amino termini of three of these peptides were determined and are indicated in Fig. 1B. Oligonucleotide probes were designed corresponding to each of the peptide coding sequences. These oligonucleotides were used as probes for bovine brain cDNA libraries. A large number of clones were detected which hybridized to several of the probes. Several of the clones were analyzed and found to have inserts of 2-3 kilobases with overlapping restriction maps. The coding

The abbreviations used are: IPTG, isopropyl-β-D-galactoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fig. 1. A, nucleotide sequencing strategy and restriction map of bovine brain inositol monophosphatase cDNA clones. The locations of several restriction sites on the bovine brain inositol monophosphatase cDNA are indicated. The limits of the map correspond to the sizes of the longest clones with the coding region indicated by the box. B, sequence of bovine brain inositol monophosphatase. The nucleotide sequence surrounding the open reading frame of the cDNA is shown. The amino acid sequence is given using the single-letter code. The numbering is given beginning with the first base of the initiator methionine as base 1 (numbered at left of sequence line). The amino acids are numbered along the right of the sequence line. Amino acid sequences derived from purified inositol monophosphatase protein are indicated by underlining.

region and part of the untranslated region of one of these clones was sequenced (Fig. 1). The sequence of the coding region, encoding an open reading frame of 277 amino acids, is shown in Fig. 1B. The predicted amino acid sequence contains all of the peptide sequences obtained from the purified protein. Analysis of the predicted protein sequence reveals that it is moderately hydrophilic with no unusual regions of hydrophobic character. No consensus sequences were identified for phosphorylation, Ca2+ binding sites, ATP binding sites, or any other recognizable regulatory sequence. Searches of the protein data base did not reveal significant homology with any known protein. Short regions of homology were noted between inositol monophosphatase and some ATPases, but no known protein. Thus, inositol monophosphatase appears to be a novel enzyme. It will be interesting to determine if this enzyme has similarity to other enzymes in the phosphatidylinositol metabolizing pathway. There is no consensus sequence available for Li+ binding, so this site will have to be determined directly.

Expression of Inositol Monophosphatase cDNA in Bacteria—To confirm that the cDNA coded for functional bovine inositol monophosphatase, the open reading frame was in-
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inserted into a T7 polymerase bacterial expression vector, prSET5a. Bacterial cells containing the expression vector were analyzed by both SDS-PAGE and Coomassie Blue staining (A) or Western blotting (B), using monoclonal antibodies G-2A prepared to bovine inositol monophosphatase. Lane 1, inositol monophosphatase (0.2 µg) purified from bovine brain; lane 2, bacteria containing the inositol monophosphatase expression vector grown without induction; lane 3, bacteria containing the inositol monophosphatase expression vector induced for 4 h with 0.4 mM IPTG; lane 4, bacteria without the expression vector grown without induction; lane 5, bacteria without the expression vector induced for 4 h with 0.4 mM IPTG. M.W., relative molecular weight.

**Table I**

<table>
<thead>
<tr>
<th>Fraction assayed</th>
<th>Insoluble fraction</th>
<th>Soluble fraction</th>
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</thead>
<tbody>
<tr>
<td>Activity (mU/ml)</td>
<td>570</td>
<td>1170</td>
</tr>
<tr>
<td>Specific activity (mU/mg)</td>
<td>356</td>
<td>650</td>
</tr>
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**Fig. 2.** Expression of recombinant bovine inositol monophosphatase. Bacteria (BL21-DE3) with or without the inositol monophosphatase expression vector were grown and induced with IPTG as described under "Materials and Methods." Total cellular protein was then analyzed by SDS-PAGE and Coomassie Blue staining (A) or Western blotting (B), using monoclonal antibodies G-2A prepared to bovine inositol monophosphatase. Lane 1, inositol monophosphatase (0.2 µg) purified from bovine brain; lane 2, bacteria containing the inositol monophosphatase expression vector grown without induction; lane 3, bacteria containing the inositol monophosphatase expression vector induced for 4 h with 0.4 mM IPTG; lane 4, bacteria without the expression vector grown without induction; lane 5, bacteria without the expression vector induced for 4 h with 0.4 mM IPTG. M.W., relative molecular weight.

Additionally, the specific activity of the inositol monophosphatase in the soluble fraction does not correct for the presence of 1 mg of lysozyme/ml. After correcting for the presence of lysozyme, the specific activity of inositol monophosphatase in the soluble fraction of induced bacteria was 1460 milliunits/mg, approximately 25% pure, assuming a specific activity of 5700 milliunits/mg for pure enzyme (Gee et al., 1988). The enzyme in the soluble fraction was further characterized. Hydrolysis of 0.1 mM inositol 1-phosphate was inhibited by Li+ with an IC50 of 2.0 mM. A superimposable inhibition curve was obtained if crude brain supernatant was used instead of bacterial extract. In addition, the Km for inositol 1-phosphate for the recombinant enzyme was 0.11 mM, similar to that reported for the bovine brain enzyme (Gee et al., 1988). Thus, the recombinant and brain enzymes appear to be identical in all respects. In this context, there are no known post-translational modifications of the native enzyme which might result in a different behaviour, compared to the recombinant enzyme. The availability of recombinant inositol monophosphatase will allow future studies using mutagenesis and chemical modification techniques to further define the structure and function of this novel enzyme.


**REFERENCES**


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