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Chemical Modification of Porcine Brain *myo*-Inositol Monophosphate Phosphatase by N-bromosuccinimide

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***Myo*-inositol monophosphate phosphatase is a key enzyme in the phosphoinositide cell-signaling system. Incubation of *myo*-inositol monophosphate phosphatase from porcine brain with N-bromosuccinimide (NBS) resulted in a time-dependent loss of enzyme activity. The inactivation followed pseudo-first-order kinetics with the second-order rate constant of 3.8×10^3 $M^{-1}min^{-1}$. The time course of the reaction was significantly affected by the substrate *myo*-inositol-1-phosphate, which afforded complete protection against the loss of catalytic activity. Spectrophotometric studies indicated that about one oxindole group per molecule of enzyme was formed following complete loss of enzymatic activity. It is suggested that the catalytic function of *myo*-inositol monophosphate phosphatase is modulated by the binding of NBS to a specific tryptophan residue at or near the substrate binding site of the enzyme.**

Keywords: Brain, *myo*-Inositol monophosphate phosphatase, N-Bromosuccinimide, Phosphoinositide cell signaling system, Tryptophan residue.

Introduction

Myo-inositol monophosphate phosphatase (EC 3.1.3.25) is a key enzyme in the phosphoinositide cell-signaling system (Nishizuka, 1984; Abdel-Latif, 1986; Berridge *et al.*, 1989). Binding of many hormones, growth factors, and extracellular agonists to receptors stimulates the generation of the second messengers, inositol-1,4,5-triphosphate and sn-1,2-diacylglycerol, via the phosphatidylinositol cycle. A series of phosphatases and kinases (Shears, 1989) result in the recycling of inositol for subsequent resynthesis of the phosphatidylinositols. *Myo*-inositol monophosphate phosphatase catalyzes dephosphorylation of cell *myo*-inositol monophosphate to *myo*-inositol, with the exception of *myo*-inositol-2-phosphate, which is a crucial step in the mechanism of recycling. All the pathways within the inositol lipid cycle converge at this point to replenish the pool of free inositol (Mausk *et al.*, 1980).

Myo-inositol monophosphate phosphatase has been purified from a variety of sources including rat (Takimoto *et al.*, 1985), bovine (Gee *et al.*, 1988), and porcine brains (Kwon *et al.*, 1993), as well as lily pollen (Gumber *et al.*, 1984). This enzyme is considered to be the most probable target of Li^+ when lithium chloride is used to treat manic-depressive patients (Hallcher and Sherman, 1980; Sherman *et al.*, 1981). It has been reported that Li^+ inhibits *myo*-inositol monophosphate phosphatase of bovine brain at therapeutic concentrations (Hallcher and Sherman, 1980; Takimoto *et al.*, 1985) and that Li^+ decreases the concentration of free inositol and increases inositol phosphate levels in rat brain (Allison and Stewart, 1971; Allison *et al.*, 1976). Berridge *et al.* (1982) have proposed that *myo*-inositol monophosphate phophatase is the target for Li^+ in the treatment of manic-depressive illness, even though other mechanisms have been reported (Batty and Nahorski, 1987; Avissar *et al.*, 1988; Godfrey *et al.*, 1989). Recently, cDNA clones encoding the human, rat, and bovine brain enzymes had been isolated (Diehl *et al.*, 1990; McAllister *et al.*, 1992), however, regulatory and structural properties of the enzyme have not been studied sufficiently.

In the present study, we investigated the role of the tryptophan residue at the catalytic site of porcine brain *myo*-inositol monophosphate phophatase using chemical modification with the tryptophan-specific reagent N-bromosuccinimide (NBS).

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Materials and Methods

Materials Ammonium sulfate, bovine serum albumin, EDTA, β-glycerophosphate, D,L-*myo*-inositol-1-phosphate, NBS, 2-mercaptoethanol, $HClO_4$, ammonium molybdate, and trizma base were purchased from Sigma Chemical Co. (St. Louis, USA). Mono-Q, Sephacryl S-200, Superose-6, Mono-S, and Phenyl-Superose were purchased from Pharmacia/LKB Ltd. (Uppsala, Sweden). All other materials used were of the best reagent grade available. Fresh porcine brains were obtained from the Majang-dong Slaughter House (Seoul, Korea).

Enzyme purification and assay The *myo*-inositol monophosphate phosphatase from porcine brain was purified according to the procedure developed in our laboratory (Kwon *et al.*, 1993). The enzyme isolated with this procedure has a specific activity of 1.7 units/mg at 37°C and migrates as a single protein on polyacrylamide gel electrophoresis. Protein concentration was determined by the Bradford procedure with a bovine serum albumin as a standard (Bradford, 1976).

Enzyme activity was measured by colorimetric determination of released Pi (Itaya and Ui, 1966) from the hydrolysis of β-glycerophosphate and *myo*-inositol-monophosphate (Meek *et al.*, 1988). One unit of activity was defined as that amount of enzyme converting 1 μmol of substrate into product in 1 min at 37°C. Initial velocity data were fitted by a least square method to the double reciprocal transformation of Eq. (1).

$$v = V_{max}[S]/(K_m+[S]) \quad (1)$$

Spectroscopy Spectrophotometric measurements were carried out using a Kontron UVIKON 930 double-beam spectrophotometer. Fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorimeter.

Chemical modification of *myo*-inositol monophosphate phosphatase with NBS The purified enzyme was dialyzed against 20 mM Tris·HCl, pH 6.5, and then used immediately. NBS (200 mM) was freshly prepared in 20 mM Tris·HCl, pH 6.5, and kept on ice. The incubation mixture (1 ml) contained *myo*-inositol monophosphate phosphatase (5 μM), NBS (10–100 μM), and 20 mM Tris·HCl, pH 7.0. The reaction was initiated by the addition of NBS in the dark at 25°C. All solutions containing NBS were protected from photolytic destruction by covering with metal foil. At various intervals after initiation of inactivation, aliquots were withdrawn for the assay of activity.

In some experiments, the capacity of *myo*-inositol-1-phosphate to protect the enzyme against inactivation was tested by including it in the incubation mixture. If the concentration of *myo*-inositol-1-phosphate was sufficient to affect activity, separate control experiments were included to determine the appropriate value of the 100% 'zero time' activity.

The number of tryptophan residues (n) oxidized per mole of enzyme was calculated from Eq. (2), designed by Spande and Witkop (1967).

$$n = \frac{1.31 \times \Delta A_{280}}{5500 \times \text{molarity of enzyme}}, \quad (2)$$

where ΔA_{280} is the corrected optical density decreased at 280 nm; 1.31 is an empirical factor which corrects for the absorption at 280 nm of the oxidation product of tryptophan, oxindole; and 5,500 is the molar extinction coefficient for tryptophan.

Results

Inactivation of *myo*-inositol monophosphate phosphatase by NBS Incubation of *myo*-inositol monophosphate phosphatase with increasing concentrations of NBS resulted in a progressive decrease in enzyme activity (Fig. 1). The inactivation followed pseudo-first-order kinetics with NBS in the concentration range of 10–100 μM. The pseudo-first-order rate constants obtained at each NBS concentration were replotted as a function of NBS concentration (Fig. 2). The second-order rate constant for the inactivation of the enzyme by NBS was 3.8×10^3 M^{-1} min^{-1} as determined from the slope of this plot. Inactivation studies were carried out in the presence of substrate to define the sites of NBS modification. The reaction of *myo*-inositol monophophate phosphatase with NBS was prevented by incubation of the enzyme with the substrate, *myo*-inositol-1-phosphate (Table 1).

Spectroscopic properties of the enzyme with NBS In an effort to demonstrate that the indole chromophore of tryptophan, absorbing strongly at 280 nm, is converted on oxidation with NBS to oxindole, a much weaker chromophore at this wavelength, the enzyme (10 μM) was incubated with different concentrations of NBS (100 μM, 200 μM) at pH 6.5 for 30 min. The ultraviolet absorbance peak at 280 nm was lowered, whereas an increased absorbance was observed at 250 nm (Fig. 3). A pronounced

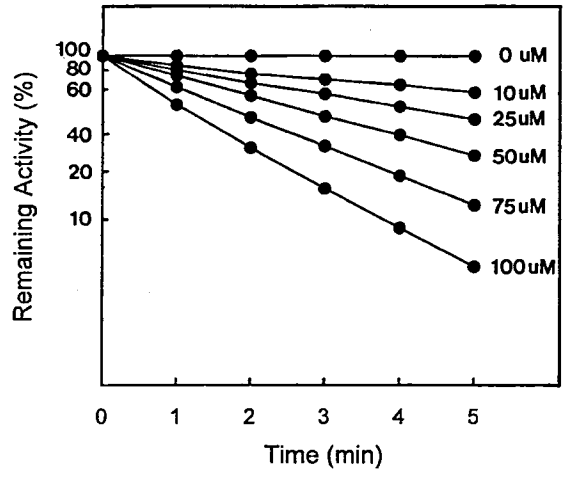

Fig. 1. Determination of the rate constant (k_{obs}) for the inactivation of myo-inositol monophosphate phosphatase at different concentrations of NBS. The enzyme (5 μM) was incubated with 10, 25, 50, 75, and 100 μM NBS in 20 mM Tris·HCl, pH 6.5 at 25°C, respectively. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity.

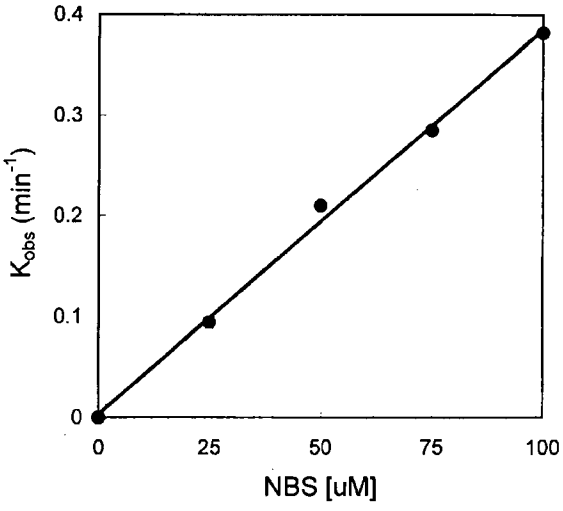

Fig. 2. Plot of apparent first-order rate constant for inactivation (k_{obs}) obtained at various concentration of NBS reagent. From this plot, the second-order rate constant (k_2) was calculated.

Table 1. Inactivation of *myo*-inositol monophosphate phosphatase by NBS at pH 6.5.

Reaction mixture	Remaining activity (%)
Enzyme (5 μM)	100
Enzyme (5 μM) + NBS (200 μM)	15
Enzyme (5 μM) + *myo*-inositol-1-phosphate (3 mM) + NBS (200 μM)	90

effect was observed on the emission spectrum of intrinsic fluorescence, characterized by a large decrease in intensity at 330–340 nm, the maximal emission wavelength that remained apparently unchanged (Fig. 4).

The values for incorporation of reagents were measured using an extinction coefficient of 5500 $M^{-1}cm^{-1}$ at 280 nm. Overall incorporation values of NBS were about 0.98 mol/enzyme monomer, indicating the masking of 1 mol tryptophan. The correlation between oxindole formation and enzyme activity is shown in Fig. 5. During the inactivation process, there was a linear relationship between oxindole formation and the loss of enzyme activity, which corresponds to a stoichiometry of 1.0 mol of oxidized tryptophan/mol enzyme, based on the decreased absorbance at 280 nm. This result indicates that a single tryptophan residue participates in the catalytic function of the enzyme.

Discussion

Myo-inositol monophosphate phosphatase is one of the key enzymes in the regeneration of inositol in the phosphoinositide-related signal transduction system (Berridge and Irvine, 1989; Majerus, 1992) and, therefore, may play an important role in cell growth and proliferation. In addition, decreased production of *myo*-inositol has been the cornerstone for the "inositol depletion hypothesis" to explain the therapeutic mechanism of action of lithium ion in manic-depressive disorder (Baraban *et al.*, 1989; Berridge *et al.*, 1989). In view of its potential importance as a drug target, we have investigated the catalytic and structural characteristics of *myo*-inositol monophosphate phosphatase isolated from porcine brain.

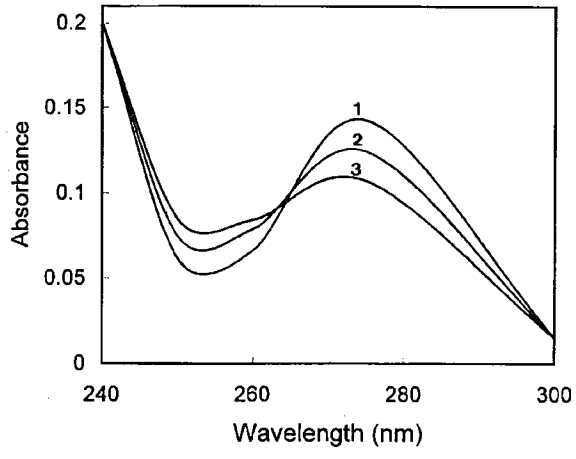

Fig. 3. Absorption spectra of native and modified *myo*-inositol monophosphate phosphatase. The enzyme (10 μM) was incubated without NBS (1) or with 100 μM NBS (2) or 200 μM NBS (3) in 20 mM Tris·HCl buffer, pH 6.5, at 25°C. The reaction mixture was dialyzed against the same buffer, and the absorption spectra were determined as described in Materials and Methods.

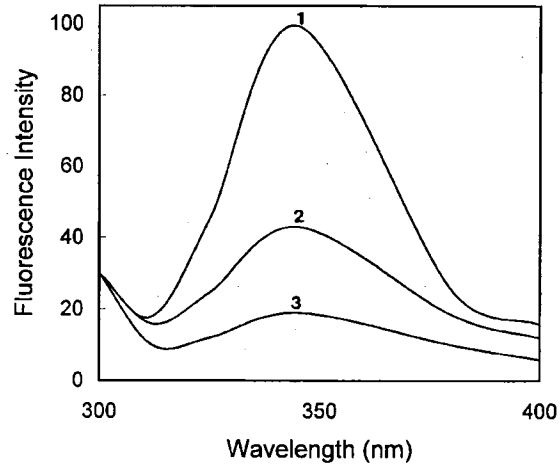

Fig. 4. Fluorescence emission spectra of native and modified *myo*-inositol monophosphate phosphatase. The enzyme (2 μM) was incubated without NBS (1) or with 100 μM NBS (2) or 200 μM NBS (3) in 20 mM Tris·HCl, pH 6.5 at 25°C. (Excitation wavelength: 280 nm)

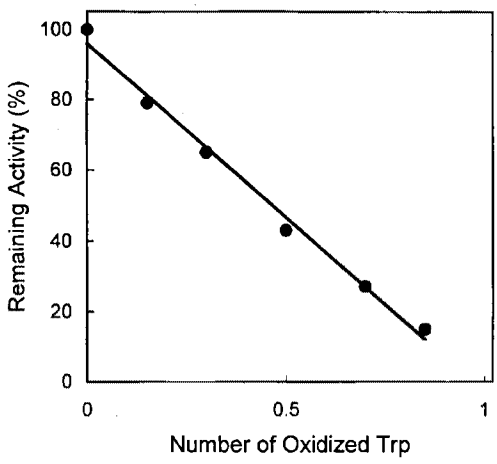

Fig. 5. Quantitative relationship between oxidized tryptophan residues and *myo*-inositol monophosphate phosphatase activity. *Myo*-inositol monophosphate phosphatase (5 μM) was incubated with 200 μM NBS in 20 mM Tris·HCl, pH 6.5 at 25°C. At various times, aliquots were withdrawn and diluted with an assay mixture to assay the enzyme activity or with the same buffer to measure the induced spectral change, as described in Materials and Methods.

Chemical modification of a tryptophan residue in brain *myo*-inositol monophosphate phosphatase provides information about the microenvironmental structure of its active site, its contribution to intrinsic fluorescence, and its role in enzyme catalysis. Oxidation of the tryptophan indole moiety into oxindole by NBS is particularly interesting since the additional steric hindrance of the substituted group is very limited, contrary to other chemical modifiers, and the modified residue becomes totally nonfluorescent (Imoto *et al.*, 1971).

Tryptophan residues may be rapidly (Ohnishi *et al.*, 1989) and specifically modified at acidic pH, which increases both reactivity and selectivity (Spande and Witkop, 1967). NBS at 200 μM inhibits the reaction catalyzed by *myo*-inositol monophosphate phosphatase. The second-order rate constant for inactivation was $3.8 \times 10^3 M^{-1}min^{-1}$ and stoichiometry of the inactivation of the enzyme with NBS showed that one tryptophan residue participates in the catalytic function.

Tryptophan residues are generally considered to play an important role in stabilizing protein conformation by virtue of the hydrophobic characteristics of the indole ring, but the residues are usually not involved in protonation-deprotonation or the charge relay system (Nishihira *et al.*, 1992). The observed loss of enzyme activity with NBS modification may be due either to the oxidation of an essential tryptophan residue or to extensive conformational change in the enzyme. If a conformational change occurred due to the modification, then both large changes in fluorescene and a shift in the emission maximum should be observed. According to the results of absorption and emission spectra, the tryptophan residue of the enzyme that reacted with NBS was oxidized with the linear decrease in absorbance at 280 nm and simultaneous increase in absorbance at 250 nm, which might be attributed to the retention of the oxindole derivative (Spande and Witkop, 1967). The fluorescent intensity was almost completely quenched without any change in the emission maximum, indicating that the modification did cause an extensive alteration in the structure of *myo*-inositol monophosphate phosphatase.

The nature of the inhibitory effect exerted by NBS was studied in detail. The possibility that NBS inhibition is the result of the reaction of essential tryptophan residues critically connected with catalysis was investigated by performing inhibition studies in the presence and absence of the substrate *myo*-inositol-1-phosphate (Table 1). Nearly complete protection was afforded by *myo*-inositol-1-phosphate. The fact that *myo*-inositol-1-phosphate protects the enzyme from inactivation, but the phosphate ion does not (data not shown) suggests that either the substrate induces a conformation not accessible by the Pi-enzyme complex or that the site of modification by NBS is protected by the inositol moiety.

The results of our study raise some questions about the role of the reactive tryptophan residue in the enzyme function. Two distinct possibilities emerge. Either the tryptophan residue is essential for catalysis, ie. it participates in some catalytic events, or blocking of the reactive tryptophan residue triggers a conformational change which affects the catalytic site domain. The first interpretation seems to be consistent with the protective effect exerted by *myo*-inositol-1-phosphate, but binding of *myo*-inositol-1-phosphate may also result in the stabilization of protein conformations which are no longer accessible to the tryptophanyl-attacking reagent, NBS.

In summary, NBS inactivates porcine brain *myo*-inositol monophosphate phosphatase by conversion of the indole chromophore of the tryptophan residue in the active site to oxindole and thereby inducing conformational changes of the enzyme structure. Although it is generally accepted that tryptophan residues are usually located on the hydrophobic pocket in protein molecules, our chemical modification results show that only one tryptophan residue has a high reactivity with NBS.

The analysis of isolated peptides labeled with NBS was not established in this work. The absence of the primary sequence and X-ray crystallographic structural data make it difficult to discuss whether the modified residue is located within the active site or in a region whose conformation is altered upon the interaction of the enzyme with substrate and inhibitor.

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