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Vicinal Thiols Are Involved in Inositol 1,2,3,5,6-Pentakisphosphate 5-Phosphatase Activity from Fetal Calf Thymus

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Inositol 1,2,3,5,6-pentakisphosphate (Ins(1,2,3,5,6)P_5) 5-phosphatase present in fetal calf thymus has been partially purified. This enzyme was inhibited dose-dependently by different thiol modifiers like *N*-ethylmaleimide (NEM), *p*-chloromercuribenzene sulfonate (PCMBS), diamide, and phenylarsine oxide (PAO). The inhibition by PCMBS and diamide was protected by preincubation with dithiothreitol (DTT) and the phosphatase substrate, Ins(1,2,3,5,6)P_5. Diamide, a compound that specifically modifies vicinal thiol groups, also blocked the 5-phosphatase dose-dependently. Specificity of this blockade was proven by using dimercaptopropanol (DMP), a compound known to protect vicinal thiol groups. DMP prevented the enzyme from inhibition by diamide. These data suggest that vicinal thiols are involved in Ins(1,2,3,5,6)P_5 5-phosphatase activity.

Among the inositol phosphates, inositol 1,4,5-trisphosphate is well known for its Ca^{2+}-mobilising role in a huge number of different cell types [1]. However, inositol pentakisphosphate ($InsP_5$) and inositol hexakisphosphate ($InsP_6$) are present in virtually all mammalian cells at higher concentration than any other inositol polyphosphate [2-5]. Despite this, little is known about their metabolising enzymes and precise functions. For some cell types it has been shown that stimulation of cell surface receptors induced changes in the intracellular concentration of $InsP_5$ and $InsP_6$ [2, 3, 5-8]. In rat thymocytes significant alteration of $InsP_5$ and $InsP_6$ were observed during cell cycle progression indicating a role in cell cycle regulation [4]. In addition, roles of these compounds in cell differentiation [9] and as neurotransmitters have been reported [10].

In rat liver Ins(1,3,4,5,6)P_5 synthesis occurred by dephosphorylation of Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 by a 5-phosphatase and stepwise phosphorylation by an Ins(1,3,4)P_3 6-kinase and an Ins(1,3,4,6)P_4 5-kinase [11].

We have identified an Ins(1,2,3,5,6)P_5 5-phosphatase from fetal calf thymus [12]. There are some reports about the active site architecture of Ins(1,4,5)P_3 5-phosphatase [13-15] but little is known about the active site of the novel Ins(1,2,3,5,6)P_5 5-phosphatase.

In the present study we have investigated the active site of Ins(1,2,3,5,6)P_5 5-phosphatase (InsP5 5-phosphatase) using different thiols modifiers. Inactivation of this enzyme by these modifiers and protection by DTT and its substrate strongly favor the involvement of free thiols in the active center. Moreover, dimercaptopropanol (DMP) protects the enzyme from inhibition by diamide which inactivates proteins by vicinal thiol modification. We propose that vicinal thiols are involved in Ins(1,2,3,5,6)P_5-5-phosphatase activity.

MATERIALS AND METHODS

Partial purification of Ins(1,2,3,5,6)P_5 5-phosphatase. The enzyme was partially purified by the following methods:

(a) *Preparation of cytosolic extract:* 40 g of frozen fetal calf thymus was minced and a 20% (w/v) homogenate was prepared with a turrax-mixer (10 times for 5 sec on ice) in ice-cold extraction buffer (110 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 20 mM HEPES, 2 mM EGTA, and 1 mM dithiotreitol (DTT), pH 7.5) containing a mixture of protease inhibitors (50 μM leupeptin, 1 μM pepstatin and 50 μM antipain). The homogenate was centrifuged at 105000×g for 30 min at 4°C. The clear supernatant was collected without the fatty layer on top of it.

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(b) *$(NH_4)_2SO_4$ precipitation and desalting:* The clear supernatant was treated with 60% $(NH_4)_2SO_4$ at 4°C with continuous mixing for 15 min. Precipitated proteins were centrifuged at 15000×g for 20 min and the supernatant containing enzyme activity was collected. This supernatant was desalted through dialysis overnight against 10 mM Tris-HCl, pH 7.5 (50 times excess volume of the supernatant).

(c) *Q-Sepharose FF chromatography:* The dialysed supernatant (300 ml) was loaded onto a column containing Q-Sepharose FF (Pharmacia Biotech, Freiburg, Germany; column dimensions: 8.6×5 cm, 150 ml gel volume) at a flow rate of 2 ml/min. The enzyme activity present in the flowthrough was collected on ice.

(d) *Hydroxylapatite (HA) column chromatography:* For further purification the Q-Sepharose flowthrough was loaded onto a HA column (HA-Ultrogel 1000, Sigma Deisenhofen, Germany; column dimensions: 7.6×5 cm, 176 ml gel volume) at a flow rate of 1 ml/min. The column was washed with two column volumes of wash buffer (20 mM HEPES, 110 mM KCl; pH 7.5). Finally, the enzyme was eluted at 1 ml/min applying a linear gradient from 0 to 500 mM phosphate, pH 7.5. Fractions enriched in activity were collected and stored at −80°C. High phosphate was eliminated by passing the enzyme fraction through a Hitrap desalting column (Hitrap G-25, 5 ml, Pharmacia Biotech, Freiburg, Germany) and the enzyme was eluted in assay buffer (20 mM HEPES, 110 KCl; pH 7.5) before kinetic studies.

Ins(1,2,3,5,6)P_5-5-phosphatase assay.

(a) *Enzyme reaction:* In a total volume of 200 μl assay buffer (the same as the extraction buffer) the partially purified enzyme (84-100 μg of protein) was incubated in the presence of Ins(1,2,3,5,6)P_5 (45 μM) for 100 min at 37°C with continuous shaking. The reaction was stopped by adding 150 μl of ice-cold 10% perchloric acid and 50 μl of 0.2 M EDTA. In case of thiol and substrate protection studies, thiols compounds and substrate were added 15 min before the inhibitor. For dose-response studies, inhibitors were added just before the addition of substrate.

(b) *Preparation of samples for HPLC:* After the additions of perchloric acid and EDTA, samples were kept on ice for 30 min to extract the soluble inositol phosphates. Precipitated protein was removed by centrifugation at 8000×g for 10 min and the supernatants were titrated to pH 4-5 by KOH (7M). The samples were kept again on ice for 30 min and after that centrifuged at 8000×g for 10 min. The supernatants were collected and made up to 2.05 ml with double distilled water before injection into the HPLC.

(c) *Detection of inositol phosphates by metal dye-detection HPLC:* The product after enzyme reaction, Ins(1,2,3,6)P_4, was determined with an HPLC system based on the post column complexometric metal dye technique as described earlier [16]. The separation was carried out using a MonoQ column (10/5; Pharmacia Biotech, Freiburg, Germany), an HPLC pump (Pharmacia, model 2248) and a low pressure mixture with controller (Pharmacia, model LCC 2252). The dye was pumped by a second HPLC pump and mixed with the column eluate in a ratio of 1:2 by a T-junction and a 500 μl knitted coil. The detection of inositol phosphates was done by an UV/VIS-monitor (Pharmacia, model VWM 2141) adjusted to 546 nm wavelength. Samples were injected using the autosampler model 2157 (Pharmacia, Freiburg, Germany). After each HPLC-run the monitor cell and the knitted coil were washed automatically with 25% HCl/ethanol (15:85) by using a motor valve (Pharmacia, model IMV 7). The composition of the buffers was: (A) 15 μM YCl_3, 0.2 mM HCl; (B) 15 μM YCl_3, 500 mM HCl; dye solution (C) 300 μM 2-(4-pyridylazo)resorcinol, 1.6 M triethanolamine pH 9.1. The following upward concave gradient at a flow rate of 1.2 ml/min was used: 0 min, 0 % B; 21 sec, 5 % B; 3.21 min, 10 % B; 6.21 min, 15 % B; 9.21 min, 30 % B; 12.21 min, 45 % B; 15.21 min, 55 % B; 16.21 min, 60 % B; 16.51 min, 100 % B; 18.51 min, 100 % B; 19 min, 0 % B; 19.50 min, 0 % B. For an accurate quantification, calibration with standard inositol phosphates was carried out. We used a mixture of inositol polyphosphates, made by alkaline hydrolysis of phytic acid, which had been analysed by nmr spectroscopy [17].

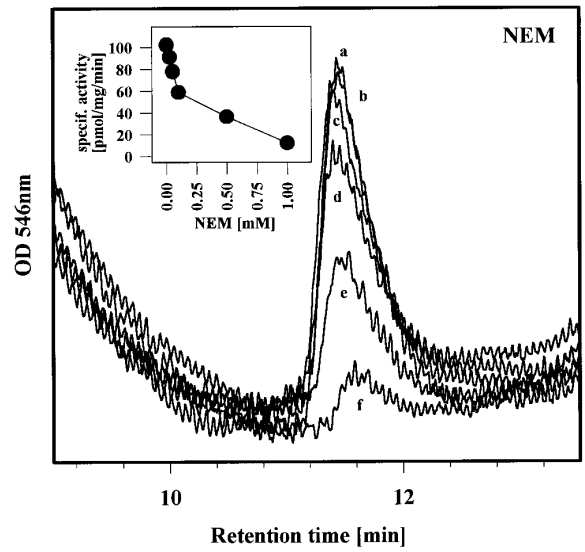

FIG. 1. Effect of N-ethylmaleimide (NEM) on Ins(1,2,3,5,6)P_5 5-phosphatase activity. Partially purified InsP_5 5-phosphatase (84-100 μg) was incubated for 100 min at 37°C with continuous shaking in the presence of Ins(1,2,3,5,6)P_5 (45 μM) and various concentrations of NEM in a final volume of 220 μl of assay buffer (20 mM HEPES, 110 mM KCl; pH 7.5). A selected region from the HPLC profile showing the product peak (Ins(1,2,3,6)P_4) is displayed as an overlay plot from 6 different samples obtained as follows: a, control; b, plus NEM (25 μM); c, plus NEM (50 μM); d, plus NEM (100 μM); e, plus NEM (500 μM); f, plus NEM (1 mM). The inset demonstrates the dose-response relationship.

Materials. Enantiomerically pure Ins(1,2,3,5,6)P_5 was synthesized by Rudolf et al. [18]. All other chemicals were from Sigma.

RESULTS AND DISCUSSION

To obtain information whether InsP_5 5-phosphatase contains any free thiol in its active site we treated the enzyme with different thiol modifiers. N-ethylmaleimide (NEM) dose dependently inhibited InsP_5- 5-phosphatase activity with an IC_{50} value of about 500 μM (Fig. 1). This indicated that this enzyme contains thiol group(s) involved in its activity. To confirm this result we selected another thiol modifier, p-chloromercuribenzene sulfonate (PCMBS). Interestingly, PCMBS was much more potent in inhibiting the activity (Fig. 2). At a concentration of 50 μM it nearly completely inhibited the enzyme. Probably the access of PCMBS to interact with free thiol group was better as for NEM and thereby made it more potent than NEM. The results indicated that thiol group(s) is/are essential for activity of the Ins$P_5$5-phosphatase.

To address the question whether this enzyme contained any vicinal thiols in exhibiting activity we chose another set of compounds e.g. phenylarsine oxide (PAO) and diamide. These compounds are known to modify vicinal dithiols in proteins. PAO also dose-dependently inhibited the enzyme activity (Fig. 3). At a

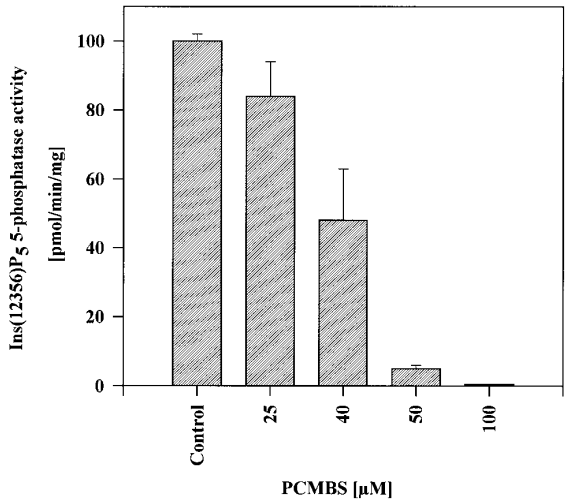

FIG. 2. Effect of p-chloromercuribenzene sulfonate (PCMBS) on Ins(1,2,3,5,6)P_5 5-phosphatase activity. Assay conditions were the same as for Fig. 1. Instead of NEM graded concentrations of PCMBS were used as indicated. Values presented are mean±SD (n= 3-4).

concentration of 2.5 mM maximum inhibition was observed. To confirm this result we treated the enzyme with the second vicinal thiols modifier, diamide. Interestingly, diamide also inhibited the enzyme in a concentration-dependent manner. Moreover, it was more

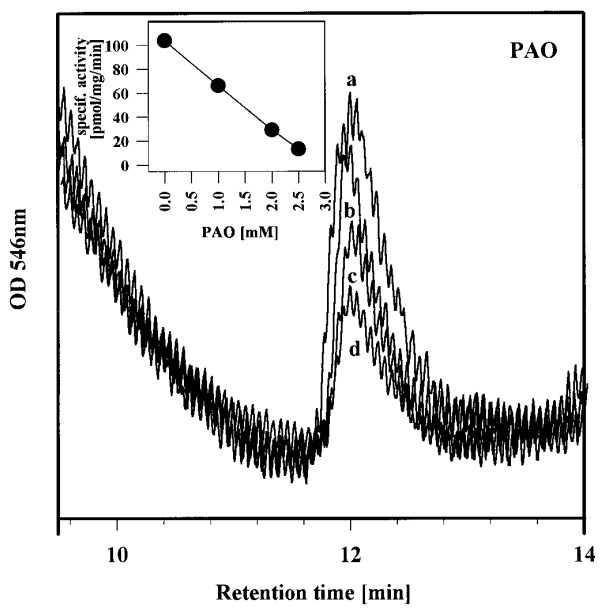

FIG. 3. Effect of phenylarsine oxide (PAO) on Ins(1,2,3,5,6)P_5 5-phosphatase activity. Assay conditions were the same as mentioned in Fig. 1. The enzyme was treated with graded concentrations of PAO. A selected region from the HPLC profile showing the product peak (Ins(1,2,3,6)P_4) is displayed as an overlay plot from 4 different samples obtained as follows: a, control; b, plus PAO (1 mM); c, plus PAO (2 mM); d, plus PAO (2.5 mM). The inset demonstrates the dose-response relationship.

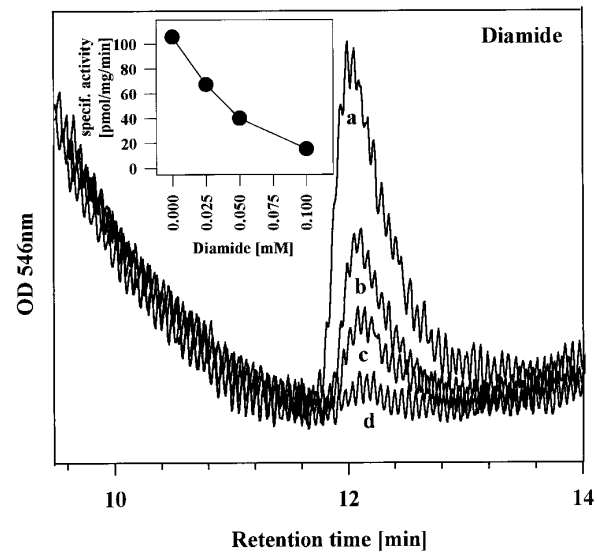

FIG. 4. Effect of diamide on Ins(1,2,3,5,6)P_5 5-phosphatase activity. Assay conditions were the same as in Fig. 1. The enzyme was treated with increasing concentrations of diamide. A selected region from the HPLC profile showing the product peak (Ins(1,2,3,6)P_4) is displayed as an overlay plot from 4 different samples obtained as follows: a, control; b, plus diamide (25 μM); c, plus diamide (50 μM); d, plus dimide (100 μM). The inset demonstrates the dose-response relationship.

effective because it inhibited 50% of the activity at a concentration of 25 μM and 85% at 100 μM (Fig. 4). Therefore it is evident that vicinal thiols are involved in $InsP_5$ 5-phosphatase activity. To see how far this inhibition was specific we did studies in the presence of the thiol compounds DTT and dimercaptopropanol (DMP) known to protect from inhibition by PCMBS or diamide, respectively. Henceforth, we used PCMBS (50 μM) and diamide (100 μM) for their inhibitory power.

TABLE 1

Protection of Inhibition of $InsP_5$ 5-Phosphatase by Thiol Compounds

	Ins(1,2,3,5,6)P_5 5-phosphatase activity [pmol/min/mg]
Control	100 ± 10
Control + PCMBS (50 μM)	5 ± 1
Control + Diamide (100 μM)	15 ± 5
Control + DTT (1 mM) + PCMBS (50 μM)	90 ± 6
Control + DTT (1 mM) + Diamide (100 μM)	78 ± 8
Control + DMP (2 mM) + Diamide (100 μM)	82 ± 15

Note. The enzyme (84 μg protein) was incubated in assay buffer in the presence or absence of different thiol compounds for 15 min before the addition of PCMBS (50 μM) or diamide (100 μM). Then Ins(1,2,3,5,6)P_5 (45 μM) was added and incubated further 100 min at 37°C. Values are presented as mean ± SD (n = 3).

Prior incorporation of DTT (1 mM) protected almost absolutely against inhibition by PCMBS (Tab. 1). PCMBS inhibition was also antagonized to the same degree in the presence of β-mercaptoethanol (result not shown). DTT also protected from inhibition by diamide (Tab. 1). Finally, we used DMP, a typical vicinal thiols protector to investigate whether this compound can protect from inhibition by diamide. DMP offered 82% protection against diamide inhibition (Tab. 1) indicating the involvement of vicinal dithiols in the active center of $InsP_5$-5-phosphatase.

To test whether the substrate Ins(1,2,3,5,6)P_5 itself can protect the enzyme from inhibition by PCMBS or diamide, we incubated the enzyme with varying concentrations of substrate and subsequently added the inhibitor. At an equimolar concentration of PCMBS, Ins(1,2,3,5,6)P_5 protected PCMBS inhibition almost completely when compared with the control (data not shown). In contrast, inhibition persisted when the substrate concentrations were kept lower than PCMBS (data not shown). In case of diamide equimolar concentration of the substrate also protected the enzyme completely (data not shown).

In conclusion we present evidence that Ins(1,2,3,5,6)P_5 5-phosphatase contains vicinal thiol groups in its active site. In addition, the complete protection at equimolar concentration of the substrate Ins(1,2,3,5,6)P_5 against both inhibition by PCMBS and diamide indicates that the substrate binding site and the inhibitor interacting site are the same.

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