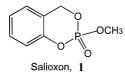# Notes

Inactivation of Protein Tyrosine Phosphatases by Salioxon

Hyeongjin Cho,[*] Junseo Park, Dongki Yang, and Seung Wook Ham[+]

Department of Chemistry, Inha University, Inchon 402-751, Korea
[+]Department of Chemistry, Chung-Ang University, Seoul 156-756, Korea
Received February 7, 2000

Protein tyrosine phosphatases (PTPases), together with protein tyrosine kinases, are important regulators of phosphotyrosine levels of various cellular proteins.[1-6] It cleaves phosphate moiety from a phosphotyrosine residue and therefore counteracts the activity of protein tyrosine kinases. Because many diseases, including cancer, are associated with the loss of control of phosphorylation levels on tyrosine of various cellular proteins,[7,8] PTPase inhibitors may possibly be an important target for therapeutic agents.

Salioxon, 1

2-Methoxy-4*H*-1,3,2-benzodioxaphosphoran-2-one (**1**), a cyclic phosphate ester, is often called salioxon and is a potent inactivator of esterases. Its thio analog, which has P−S instead of P−O, has been used as an insecticide.[9] Active site nucleophiles of some esterases react with the cyclic phosphate and the enzyme is irreversibly inactivated.[9] Because salioxon is a cyclized form of phenyl phosphate which is a substrate of PTPases, it might be envisioned that salioxon reacts with and permanently inactivates PTPases. To examine this possibility, we prepared salioxon and evaluated the inhibitory activity toward PTPases.

In initial experiments, PTPase activities were measured in the presence of 2 mM concentration of salioxon for four PTPases, including human PTP1B, yeast PTP1 (YPTP1), a *Yersinia* PTPase, YOP and the catalytic domain of human SHP-1 (SHP-1Δ). When the PTPases were preincubated with salioxon for 10 min before initiation of the enzyme reaction by addition of the substrate, p-nitrophenyl phosphate (pNPP) (method A), various levels of PTPase inhibition were observed; 70% inhibition for PTP1B and YPTP1, 40% for SHP-1Δ, and essentially no inhibition was observed for YOP. When the reactions were initiated without preincubation of the enzymes with salioxon (method B), lower levels of inhibition were observed (Table 1). Higher levels of inhibition with pretreatment of the PTPases with salioxon indicate that the inactivation is irreversible. PTP1B assay with preincubation with 2 mM salioxon for different time

*[*]To whom correspondence should be addressed. Phone: −82-32-860-7683, Fax: ·82-32-867-5604, e-mail: hcho@inha.ac.kr

Table 1. Effect of salioxon on PTPases

PTPases	PTPase activity (%)	
	With 10 min preincubation with salioxon	Without preincubation with salioxon
PTP1B	31 = 4	65 ± 4
YPTP1	31 = 6	60 ± 3
YOP	95 = 6	106 ± 7
SHPTP1Δ	59 = 5	82 ± 5

Results are expressed as means ± standard deviation for four or more independent experiments. Methods A and B were employed and salioxon concentration was 2.0 mM.

periods (2, 4, 6, 8 and 10 min) before initiation of the reaction (method A), however, gave essentially indistinguishable extent of inhibition (*ca.* 70% inhibition, data not shown) which will be discussed below.

To study further the mode of inhibition, steady-state kinetic experiments were performed with PTP1B with systematic change of inhibitor and substrate concentrations (method A). Plot of 1/V *vs.* 1/[S] according to the method of Lineweaver and Burk exhibited the pattern characteristic of simple linear noncompetitive inhibition or active site-directed irreversible inhibition as shown in Figure 1. To solve this ambiguity, we performed continuous assay (method C) and examined time-dependency of the inactivation. Figure 2
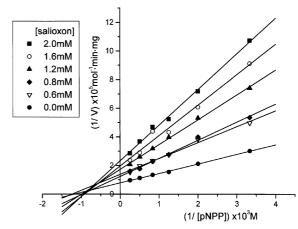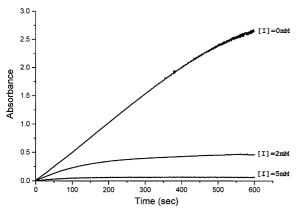

Figure 1. PTP1B activities were measured with variations of substrate and salioxon concentrations (0.3, 0.5, 0.8, 1.2, 2.0 and 4.0 mM of pNPP; 0, 0.6, 0.8, 1.2, 1.6 and 2.0 mM of salioxon) (Method B).

Figure 2. To determine the efficiency of enzyme inactivation by salioxon, we continuously monitored at 405 nm and 25 °C by a JASCO UV/Vis spectrophotometer equipped with a JASCO peltier type thermostatic cell holder (method C).

depicts the time course of the reaction in the absence of or presence of salioxon. In the absence of salioxon, a straight line was obtained from the plot of absorbance at 405 nm *vs.* time. In the presence of salioxon, the rate of p-nitrophenolate formation decreased with time. This observation, together with the result from the Lineweaver-Burk plot, provides the evidence that salioxon irreversibly inactivates PTP1B. The nucleophilic attack at the phosphorus atom or at C-4 of salioxon by the active site Cys in PTP1B, and also in other PTPases, probably results in the opening of the cyclic structure of salioxon and the formation of covalent enzyme-salioxon complex.

It is worth to note in Figure 2 that the absorbance curve forms almost plateau in a few minutes when 2 mM salioxon was used. This observation partly explaines the aforementioned result of PTP1B assay with variations of preincubation time. PTP1B is inactivated in a few minutes and, therefore, treatment of the enzyme with salioxon longer than 2 min does not inactivate PTP1B any further. This result, however, does not explain the 30% residual activity after 10min preincubation of PTP1B with salioxon and needs to be further studied.

In this report, we described salioxon as an inactivator of PTPases. Salioxon was selective to PTP1B, YPTP1 and SHP-1Δ, and did not inactivate YOP; this selectivity remains to be explained. Even though salioxon is a week inactivator of PTPases and the exact mode of inhibition of PTP1B by salioxon needs further study, the cyclic phosphate ester structure of salioxon provides an insight for the structural motif for the design of more potent PTPase inhibitors. Modifications and derivatizations of the cyclic phosphate moiety and/or the phenyl ring might improve the potency of the inactivator and these studies are currently in progress.

Experimental Section

Materials. All chemicals and column materials were from Aldrich or Sigma. YOP PTPase was purchased from NEB. The expression plasmid for YPTP1 (pT7-7-*YPTP1*) was pre-

viously described[10] and that for pET-SHP-1Δ[11] was kindly donated by Dr. Dehua Pei (Ohio State University).

Preparation of salioxion (2-Methoxy-4*H*-1,3,2-benzo-dioxaphosphoran-2-one). Salioxon was prepared according to a described method.[12] Briefly, a mixture of 2-Hydroxy-benzyl alcohol (1.00 g) and anh. pyridine (1.3 mL) in anh. CHCl₃ (5 mL) was treated methyl dichlorophosphate (0.90 mL). The reaction completes in a few hours at room temperature and, after usual work-up, salioxon was obtained in a 50% yield. The yield was not maximized.

Enzyme expression and purification. PTPases are over-expressed in *E. coli* using the plasmids containing PTPase genes under the control of T7 promoter. PTP1B was expressed as a His-tag fusion protein and it was purified by CM-cellulose followed by Ni²⁺-affinity chromatography. YPTP1 was purified using L-histidyldiazobenzylphosphonic acid-agarose affinity column.[13,14] SHP-1Δ was purified by sequential application of ammonium sulfate precipitation, gel filtration, and phosphocellulose column chromatography.

PTPase inhibitor assay. For PTPase assay, the enzymes were diluted with enzyme dilution buffer (25 mM Hepes, 5 mM EDTA, 10 mM DTT, 1 mg/mL bovine serum albumin, pH 7.3) and, for typical 50 μL reaction, the reaction mixture contains 10 μL of 5x reaction buffer (500 mM Hepes, 25 mM EDTA, 50 mM DTT, pH 7.0), pNPP (100 mM, 5 μL), water (30 μL) and enzyme (5 μL). Except for the continuous assay, the progress of the reaction was determined for the formation of p-nitrophenolate by measuring the absorbances at 405 nm after quenching the reaction with 0.9 mL of 0.5 M NaOH. For continuous assay, the absorbance at 405 nm was monitored without addition of NaOH solution.

Method A. Salioxon (100 mM, 1 μL) was added to the mixture of 5x reaction buffer (10 μL), water (29 μL), and PTPase (5 μL) and the resulting mixture was incubated for 2-10 min before the reaction was initiated by addition of pNPP (5 μL).

Method B. The reaction was initiated by addition of PTPase (5 μL) to the fresh mixture of 5x reaction buffer (10 μL), water (29 μL), pNPP (5 μL) and salioxon (1 μL). After 2 min of incubation at 25 °C, the reaction was quenched by addition of 0.5 M NaOH.

Method C. The reaction was performed in a buffer containing 20 mM Tris, 5 mM Imidazole, and 80 mM NaCl (pH 7.9). Other components in the reaction mixture are the same as those in method B except that the reaction was carried out in a total volume of 1 mL.

References

1. Walton, K. M.; Dixon, J. E. *Annu. Rev. Biochem.* **1993**, *62*, 101.
2. Zhang, Z.-Y.; Dixon, J. E. *Adv. Enzymol.* **1994**, *68*, 1.
3. Tonks, N. K.; Neel, B. G. *Cell* **1996**, *87*, 365.

4. Zhang, Z. Y. *Curr. Top. Cell. Regul.* **1997**, *35*, 21.
5. Huijsduijnen, R. H. *Gene* **1998**, *225*, 1.
6. Burke, T. R. Jr.; Zhang, Z.-Y. *Biopolymers* **1998**, *47*, 225.
7. Hunter, T. *Cell* **1995**, *80*, 225.
8. Tonks, N. K.; Neel, B. G. *Cell* **1996**, *81*, 365.
9. Eto, M. *J. Environ. Sci. Health* **1983**, *B18*, 119.
10. Kwon, M.; Oh, M.; Han, J.; Cho, H. *J. Biochem. Mol.*

Biol. **1996**, *29*, 386.
11. Pei, D.; Neel, B. G.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1092.
12. Eto, M.; Oshima, Y. *Agric. Biol. Chem.* **1962**, *26*, 452.
13. Park, S.; Cho, H. *Bull. Korean Chem. Soc.* **1997**, *18*, 691.
14. Han, J.; Kwon, M.; Cho, H. *J. Biochem. Mol. Biol.* **1998**, *31*, 135.