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Binding Subsites in the Active Site of Zn^{2+}-Glycerophosphocholine Cholinephosphodiesterase

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Abstract: The properties of binding sites in the active site of Zn^{2+}-glycerophosphocholine cholinephosphodiesterase were examined using substrates and inhibitors of the enzyme. Phosphodiesterase hydrolyzed *p*-nitrophenylphosphocholine, *p*-aminophenylphosphocholine, and glycerophosphocholine, but did not hydrolyze either acylated glycerophosphocholine or bis (*p*-nitrophenyl)phosphate, suggesting a size limitation for interaction with a glyceryl moiety-binding subsite. The hydrolysis of *p*-nitrophenylphosphocholine was competitively inhibited by glycerophosphocholine and *p*-aminophenylphosphocholine, while glycerophosphoethanolamine was a weak inhibitor. The enzyme was also inhibited by choline, but not by ethanolamine. Thiocholine, a much more potent inhibitor than choline, was more inhibitory than cysteamine, suggesting a strict specificity of an anionic subsite adjacent to a Zn^{2+} subsite. Of all oxyanions tested, the tellurite ion was found to strongly inhibit the enzyme by binding to a Zn^{2+} subsite. The inhibitory role of tellurite was synergistically enhanced by tetraalkylammonium salts, but not by glycerol. Deactivation of the enzyme by diethylpyrocarbonate was partially protected by choline, but not by glycerophosphate. It is suggested that the active site of phosphodiesterase contains three binding subsites.
Key words: active site, glycerophosphocholine, phosphodiesterase.

Zn^{2+}-glycerophosphocholine (GPC) cholinephosphodiesterase (EC 3.1.4.38) is a GPC phosphodiesterase which catalyzes the conversion of GPC to glycerol and phosphocholine, which might be reused for the synthesis of phosphatidylcholine (Kanfer and McCartney, 1989). Interestingly, *p*-nitrophenylphosphocholine (*p*-NPPC) is an artificial chromogenic substrate for Zn^{2+}-GPC cholinephosphodiesterase under alkaline pH conditions (Sok and Kim, 1992a). Phosphodiesterase was purified from mouse brain membrane, and was composed of two forms having different molecular weights.

A Zn^{2+}-requiring GPC cholinephosphodiesterase, which was enriched in myelin membranes (Kanfer and McCartney, 1989), is markedly decreased in multiple sclerosis (Janzen *et al.*, 1990). However, there are no reports concerning regulation of phosphodiesterase activity, except the observation that the enzyme is inactivated by EDTA (Kanfer and McCartney, 1989).

A selective inhibitor of Zn^{2+}-GPC cholinephosphodiesterase would be useful to elucidate the cellular role of the enzyme, which is supposed to play an important role in the homeostasis of phospholipid metabolism in brain membranes, especially myelin. The inhibitor could be used to distinguish the enzyme from other GPC phosphodiesterases.

Zn^{2+}-metallohydrolases interact with either divalent cation chelators or thiols, such as DTT or thio-carboxylates (Martinez *et al.*, 1992; Orning *et al.*, 1991). It was suggested that thiol compounds containing a group showing good affinity toward a binding site of the enzyme correspond to an effective inhibitor of Zn^{2+}-metallopeptidases (Cushman *et al.*, 1977). However, there are no extensive studies of the binding site of the enzyme. A Zn^{2+}-GPC cholinephosphodiesterase was suggested to be competitively inhibited by tellurous ions (Te^{4+}) (Sok and Kim, 1992b). The inhibitory effect of tellurium compound was significantly enhanced in the presence of tetramethylammonium chloride. However, the binding site for inhibition was not investigated. Therefore, there has been a need to understand the binding sites of the enzyme. A model of the active site would be useful for the design of a selective inhibitor of the enzyme.

It is proposed that the active site of Zn^{2+}-GPC cholinephosphodiesterase contains an anionic binding subsite and a neutrally-charged binding subsite for a glyceryl moiety in addition to a Zn^{2+}-subsite.

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Experimental Procedures

Materials

Tetramethylammonium chloride, tetraethylammonium chloride, and tetrabutylammonium hydrogensulfate were purchased from Aldrich Chemical Co. (Milwaukee, USA). Choline and carnitine were obtained from Merck Chemical Co. (Darmstadt, Germany) and Calbiochem Chemical Co. (San Diego, USA), respectively. Other chemicals were supplied by Sigma Chemical Co. (St. Louis, USA). Thiocholine was prepared by alkaline hydrolysis of acetylthiocholine chloride in 0.1 M NaOH, or reduction of dithio(trimethylammonium ethanolamine by sodium borohydride, and was quantified according to the method of Ellman *et al.* (1961).

Enzyme assay

Zn^{2+}-GPC cholinephosphodiesterase activity was determined by measuring the amount of *p*-nitrophenol released during the hydrolysis of *p*-NPPC, as described by Sok and Kim (1992). Absorbance was measured on a Gilford 250 spectrophotometer at 410 nm (ε_{410}= 1.35×10^4 $M^{-1}\cdot cm^{-1}$). Unless otherwise noted, assays were conducted in 1 ml of 0.1 M glycine buffer, pH 10, containing 150 μM *p*-NPPC at 25℃. One unit of enzyme activity was defined as one mol of *p*-nitrophenol produced per h.

Preparation of purified GPC cholinephosphodiesterase

Phosphodiesterase was solubilized from brain membranes by microbial phospholipase-C, as described by Sok and Kim (1992a), and sequentially purified by chromatography on Concanavalin A-Sepharose and CM-Sephadex columns. The major fraction (65%) of total phosphodiesterase activity bound to CM-Sephadex was further purified on a Sephadex G-150 column. The purified enzyme, with specific activities of 270 μmol/mg·h in the hydrolysis of *p*-NPPC, and 320 μmol/mg·h in the hydrolysis of glycerophosphocholine, behaved like a homogeneous component during gel electrophoresis. GPC cholinephosphodiesterase activity and *p*-NPPC phosphodiesterase activity were found to comigrate in Sephadex G 150 gel chromatography (Sok and Kim, 1992).

Inhibition of phosphodiesterase

Kinetic analyses were performed as described in Experimental procedures. Values were expressed as an average of two or three analyses. Unless otherwise noted, a reversible inhibition was performed by incubating the enzyme at 20℃ with the respective compound in 0.1 M glycine buffer, pH 10, and the remaining activity was measured spectrophotometrically as described above. For inactivation study, the enzyme was preincubated with each inhibitor at 30℃ in 0.05 M phosphate buffer, pH 7.4, and at indicated times aliquots were added to the assay mixture for measurement of remaining enzyme activity. Inactivation by modifying reagents was performed in 50 mM Tris buffer, pH 7.0 containing 0.5 M NaCl and 0.2 M α-methylmannoside.

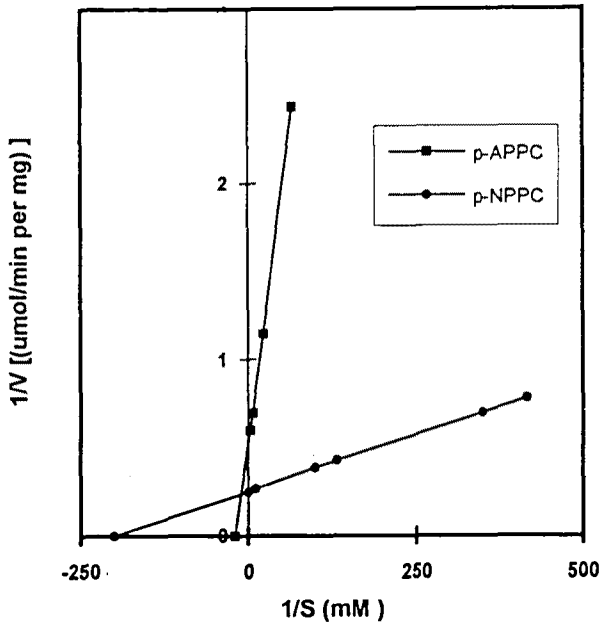

Fig. 1. Double-reciprocal plot of the velocity (v) of the phosphodiesterase-catalyzed hydrolysis of *p*-NPPC or *p*-APPC vs. substrate concentrations (S). The concentration range of substrate was 0.0025 to 0.15 mM for *p*-NPPC and 0.016 to 1 mM for *p*-APPC.

Results and Discussion

Earlier, it had been observed that Zn^{2+}- glycerophosphocholine (GPC) phosphodiesterase expressed a strict substrate specificity (Kanfer *et al.*, 1989). Therefore, it was supposed that there may be binding subsites for a glyceryl moiety and a trimethylammonium group as well as a Zn^{2+} subsite in the active site of Zn^{2+}-GPC cholinephosphodiesterase.

The properties of binding subsites in the active site of Zn^{2+}-GPC cholinephosphodiesterase were investigated using substrates and inhibitors of the enzyme. When phosphodiesterase was incubated with various phosphodiesters containing a phosphocholine moiety, it was found (Fig. 1) that the enzyme, which used glycerophosphocholine (K_m, 48 μM) as a native substrate, hydrolyzed not only *p*-nitrophenylphosphocholine (*p*-NPPC) but also *p*-aminophenylphosphocholine (*p*-APPC), indicating a broad substrate specificity for a glyceryl moiety-binding subsite. As might be expected from the

Table 1. The inhibition of Zn^{2+}-GPC cholinephosphodiesterase by phosphodiesters.

Compound	(mM)	Inhibition, %
dipalmitoyl phosphatidylcholine	1	<3
palmitoyl lysophosphatidylcholine	1	<3
sphingosyl phosphorylcholine	1	<3
dipropionyl phosphatidylcholine	0.6	23.5± 3.6
glycerophosphorylcholine	0.2	8.6± 2.0
p-aminophenylphosphorylcholine	0.2	20.6± 4.6

The phosphodiesterase was incubated with 150 μM *p*-NPPC in 0.1M glycine buffer, pH 10 containing the appropriate ester, and assayed as described in Materials and Methods section. The inhibition degree was expressed as the percentage of total.

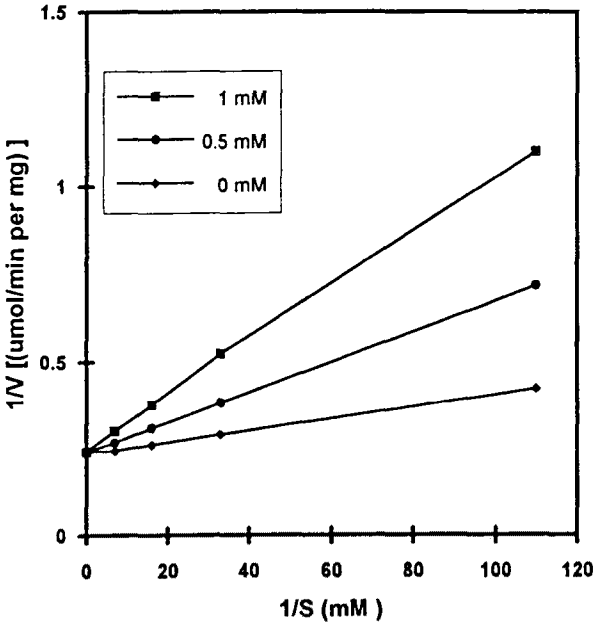

Fig. 2. Double reciprocal plots for the inhibition of the phosphodiesterase by caproyl lysophosphatidylcholine. Hydrolysis of *p*-NPPC (25~150 μM) by the phosphodiesterase (40 milliunits) was measured at 25℃ in either the presence or absence of caproyl lysophosphatidylcholine in 0.1 M glycine buffer, pH 10 as described in Materials and Methods section.

electronic effect of substituents on the aromatic ring, *p*-nitrophenylphosphocholine containing an electron-withdrawing substituent was hydrolyzed two times more effectively than *p*-aminophenylphosphocholine which possesses an electron-donating group. The K_m value of *p*-nitrophenylphosphocholine (5.5 μM) was approximately 11 times smaller than the value of *p*-aminophenylphosphocholine (60 μM), suggesting that the charge of the amino group may not be favorable for the glyceryl moiety-binding subsite. Sphingosylphosphocholine, dipropionyl phosphatidylcholine, palmitoyl lysophosphatidylcholine, and β-acetyl-γ-arachidonyl phosphatidylcholine are not attacked by the enzyme. These results

Table 2. K_i value of the respective compound.

Compound	K_i value, mM
carnitine	0.66
betaine	>10
acetylcholine	>10
choline	1.2
thiocholine	0.0026
cysteamine	0.039

The K_i value was obtained from double reciprocal plots as described in Fig. 2.

Table 3. The inhibition of Zn^{2+}-GPC cholinephosphodiesterase by oxyanions.

Oxyanion	(mM)	Remaining activity, %
selenite	10	96.0± 3.0
sulfite	10	>97.5
tellurite	0.015	55.0± 1.0
tellurate	10	75.5± 2.2

The phosphodiesterase was incubated with 150 μM *p*-NPPC in 0.1 M glycine buffer, pH 10, containing each oxyanion.

indicate that the glyceryl moiety-binding subsite is a neutrally-charged domain of a limited size. Thus, the properties of a glyceryl moiety-binding subsite are in contrast to the strict structural specificity reported for the anionic subsite for trimethylammonium groups (Kanfer *et al.*, 1989). Hydrolysis of glycerophosphoethanolamine by phosphodiesterase was only 5% of the hydrolysis of glycerophosphocholine, and bis (*p*-nitrophenyl) phosphate was not hydrolyzed by the enzyme.

The inhibitory effect of phosphodiesters on the enzymatic hydrolysis of *p*-NPPC was examined. As shown in Table 1, phosphodiesters such as dipalmitoyl phosphatidylcholine, palmitoyl lysophosphatidylcholine, and sphingosylphosphocholine showed no significant inhibition of *p*-NPPC hydrolysis. Caproyl lysophoshatidylcholine was found to inhibit enzyme activity in a competitive manner with a K_i value of 190 μM, as shown in Fig. 2. Dipropionyl phosphatidylcholine showed a competitive inhibition with a K_i value of 83 μM. Of phosphodiesters containing no acyl moiety, glycerophosphocholine and *p*-aminophenylphosphocholine exerted an inhibitory effect on the hydrolysis of *p*-NPPC in a competitive manner with K_i values of 50 μM and 29 μM, respectively. Glycerophosphoethanolamine, with a K_i value of 400 μM, was a much weaker inhibitor, and bis (*p*-nitrophenyl) phosphate at 1 mM exhibited no significant inhibition of phosphodiesterase. These results, which suggest a structural requirement for inhibitors

Table 4. The protection by Zn^{2+}-GPC cholinephosphodiesterase inhibitors against the inactivation of the phosphodiesterase by EDTA.

Compound	(mM)	Remaining activity, %
EDTA only		6.1
+glycerophosphocholine	1	>95.0
+glycerophosphate	2	19.0± 1.4
+choline	2	39.5± 0.7
+tellurite	0.0015	91.0± 3.0

The phosphodiesterase was preincubated at 30℃ with 1 mM EDTA for 10 min in the presence of appropriate inhibitor in 1 ml of 0.1 M glycine buffer, pH 10. The remaining phosphodiesterase activity was expressed as the percentage among control activity obtained in the absence of EDTA. Values were indicated as averages of two or three determinations.

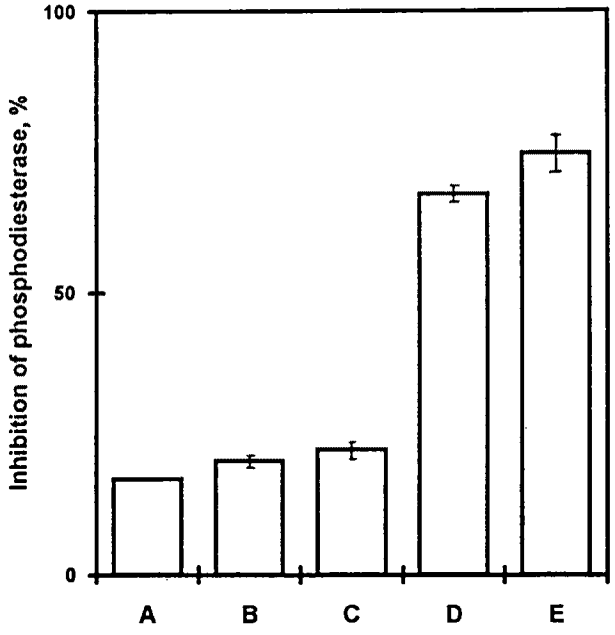

Fig. 3. Effect of quaternary ammonium salts or triethylamine on the inhibitory action of tellurites. The phosphodiesterase (40 milliunits) was inbcubated at 25℃ with tellurite ions (2 μM) in either the presence or absence of the respective organic cation (1.3 mM) in 0.1 M glycine buffer, pH 10 containing 150 μM *p*-NPPC. The value was expressed as the percentage of decreased activity among control (no tellurite). A: with glycerol; B: with triethylamine; C: with tetrabutylammonium salt; D: with tetraethylammonium chloride; E: with tetramethylammonium chloride. The Inhibition by 2 μM tellurite only was 18% of control.

of Zn^{2+}-GPC cholinephosphodiesterase, are consistent with the substrate specificity as observed above. Whereas the glyceryl moiety-binding subsite expresses a broad specificity for non-charged compounds of a limited size, the anionic subsite seems to selectively associate with positively-charged groups.

Inhibition of phosphodiesterase by the hydrolysis products of glycerophosphocholine was examined. While glycerol and phosphocholine had no significant inhibitory action at 20 mM, choline and glycerol phosphate exhibited some inhibition. When inhibition by choline was analyzed according to a Lineweaver Burk plot, choline was found to inhibit phosphodiesterase in a competitive pattern with a K_i value of 1.2 mM. Therefore, it was supposed that inhibition of phosphodiesterase by choline might be due to the association of choline with both a Zn^{2+} subsite and an anionic subsite. Various cationic compounds containing a moiety interacting with a Zn^{2+} site were tested for inhibition of the enzyme. As shown in Table 2, carntine, with both a hydroxyl and a carboxyl group, was a more effective inhibitor than choline. Betaine (K_i, >10 mM) and acetylcholine were weak inhibitors. The K_i value of thiocholine, a thiol derivative of choline, was approximately 500-fold smaller, compared to choline. This might be due to mercapto function binding to the enzyme-bound zinc, since sulfur is a better ligand for zinc atoms than oxygen (Cushman *et al.*, 1977; Lennarz and Strittmatter, 1991). Both dithiothreitol and dimercaptopropanol, a thiol analogue of glycerol, exhibited no significant inhibition, while glycerophosphate showed a K_i value of 1.2 mM. Based on these results, phosphodiesterase may contain a Zn^{2+} subsite between a glyceryl group-binding subsite and an anionic subsite.

The role of Zn^{2+}-associable oxyanions as inhibitors of phosphodiesterase was examined. As shown in Table 3, among the oxyanions tested, the tellurite ion was the most potent inhibitor of the enzyme. Although tellurate ions expressed some inhibition at 1 mM, their inhibitory potenty was much smaller, compared to tellurite ions. These results indicate that tellurites are highly selective for the Zn^{2+}-subsite.

Since inactivation of Zn^{2+}-metallohydrolases by EDTA is prevented in the presence of substrates or competitive inhibitors (Cushman *et al.*, 1977), glycerophosphocholine and its hydrolysis products were tested for an ability to prevent inactivation of the phosphodiesterase by EDTA. As shown in Table 4, 1 mM glycerophosphocholine protected the enzyme against inactivation by EDTA. Protection was also observed with phosphocholine and choline. These results support the idea that glycerophosphocholine and its hydrolysis product choline inhibit the enzyme by interacting with a Zn^{2+} site in the active site. Tellurites at a concentration as low as 1.5 μM protected phosphodiesterase against inactivation by EDTA, indicating that oxyanions such as tellurites interact selectively with a Zn^{2+} site in the active site. Thus, the capability of a compound to protect the enzyme from inactivation by EDTA may be determined by its ability to bind to a Zn^{2+} site. In this

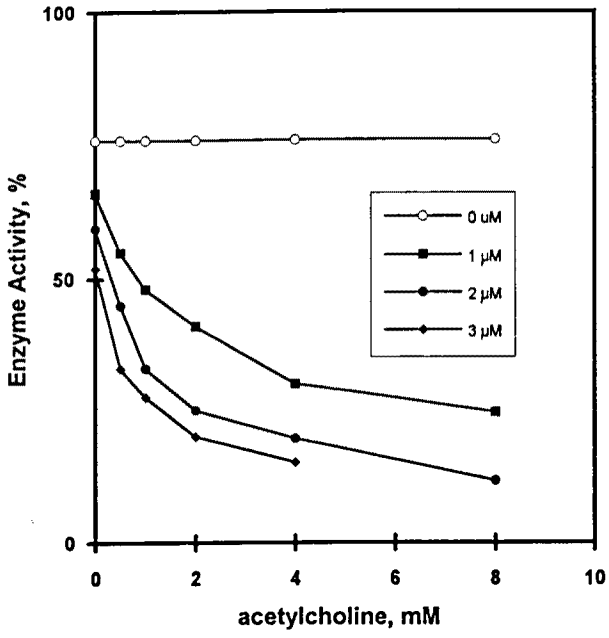

Fig. 4. Combinational effect of tellurites and acetylcholine in the inhibition of the phosphodiesterase. The phosphodiesterase (40 milliunits) was incubated with 150 μM *p*-NPPC in the presence of inhibitors in 0.1 M glycine buffer, pH 10. The concentration of tellurite was fixed at 0, 1, 2, or 3 μM, and that of acetylcholine was varied (0.2~8 mM). The remaining enzyme activity was expressed as the percentage of total activity. Values were shown as the average of duplicates.

Table 5. The protection by Zn^{2+}-GPC cholinephosphodiesterase inhibitors against inactivation of the phosphodiesterase by diethylpyrocarbonate.

Compound	(mM)	Remaining activity, %
none (control)		14.9± 1.1
choline	10	31.2± 0.9
phosphocholine	10	14.5± 1.4
glycerophosphate	10	11.7± 1.5
glycerophosphocholine	1	27.8± 1.3

The phosphodiesterase was preincubated at 30℃ with 3 mM diethylpyrocarbonate for 10 min, and remaining activity was determined as described in Table 4.

respect, it was investigated whether an anionic subsite or a glyceryl moiety-subsite can affect the interaction of tellurites with the Zn^{2+} site. Fig. 3 shows that tetraalkylammonium compounds enhanced the inhibitory action of tellurites in a synergistic manner, whereas glycerol was without effect. Tetraalkylammonium salts such as tetramethylammonium chloride and tetraethylammonium chloride were more effective than triethylamine and tetrabutylammonium salts. The enhancing effect of tetraalkylammonium salts was inversely proportional

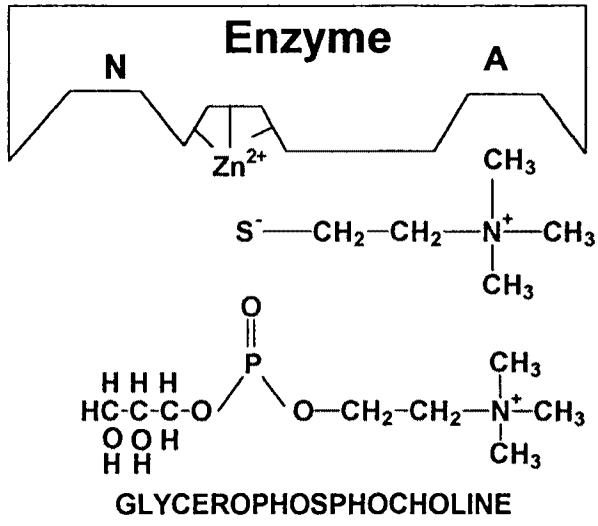

Fig. 5. Proposed model for the active site of Zn^{2+}-GPC cholinephosphodiesterase. A : anionic binding site; N : neutral binding site.

to the size of the alkyl group, supporting the idea that the anionic site of the phosphodiesterase may accomodate tetraalkylammonium salts with an alkyl group of a limited size, in contrast to the anionic site of acetylcholinesterase (Sok *et al.*, 1994). Tellurite action was efficiently enhanced in the presence of endogeneous organic cations such as choline and acetylcholine. The binding affinities of choline and acetylcholine were also enhanced in the presence of tellurites (Fig. 4). Although the mechanism for the the enhancement of tellurite action by organic cations was not investigated, it might be due to occupation of the anionic site by cationic compounds, followed by a charge neutralization which facilitates the binding of tellurite anions to a positively-charged Zn^{2+} site. Also, it is possible that the neutralization of a Zn^{2+} site by oxyanions favors the association of cationic compounds with the anionic site. Based on these results, it is proposed that the Zn^{2+} subsite cooperates in a positive manner with the anionic subsite.

Phosphodiesterase was preincubated with various modifying reagents to characterize the amino acid residues of the catalytic substrate (Bell and Bell, 1988). Among the compounds tested, diethylpyrocarbonate (3 mM) was found to inactivate phosphodiesterase, while neither N-acetylimidazole (5 mM) nor N-ethylmaleimide (5 mM) had any effect. Thus, the presence of a cysteine or a tyrosine residue in the catalytic site is excluded. Also, the involvement of a histidine residue is excluded because phosphodiesterase, which was inactivated by diethylpyrocarbonate, was not reactivated by hydroxylamine (100 mM). As shown in Table 5, inactivation by diethylpyrophosphate (3 mM) was partially prevented by glycerophosphocholine (1 mM) and choline (10

mM), but not by either phosphocholine or glycerophosphate. Glycerophosphate slightly enhanced the inactivating action of diethylpyrocarbonate. These results suggest that a diethylpyrocarbonate-sensitive amino acid residue is present between the Zn^{2+} site and the anionic site.

Taken together, it is concluded (Fig. 5) that the anionic subsite and the Zn^{2+}-subsite express a stringent specificity, whereas a glyceryl moiety-binding subsite shows a broad specificity among neutrally-charged compounds of a limited size. Moreover, the Zn^{2+}-subsite is proposed to cooperate with the anionic subsite on the same surface of the active site.

References

Bell, J. E. and Bell, E. T. (1988) *In Proteins and Enzymes*, pp. 148-164, Prentice-Hall, INC. New Jersey.

Cushman, D. W., Cheung, H. S., Sabo, E. T. and Ondetti, M. A. (1977) *Biochemistry*, **16**, 5484.

Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) *Biochem. Phamacol.* **7**, 88.

Janzen, L., Tourtellotte, W. W. and Kanfer, J. N.(1990) *Exp. Neurol.* **109**, 243.

Kanfer, J. N. and McCartney, D. G. (1989) *J. Neuro. Res.* **24**, 231.

Kitz, R. and Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245.

Lennarz, W. J. and Strittmatter, W. J. (1991) *Biochim. Biophys. Acta*, **1071**, 149.

Martinez, A., Olafsdottir, S., Haavik, J. and Flatmark, T. (1992) *Biochem. Biophys. Res. Comm.* **182**, 92.

Orning, L., Krivi, G., Bild, G., Gierse, J., Aykent, S. and Fitzpatrick, F. A. (1991) *J. Biol. Chem.* **266**, 16507.

Sok, D.-E. and Kim, M. R. (1992a) *Biochem. J.* **286**, 435.

Sok, D.-E. and Kim, M. R. (1992b) *Biochem. J.* **284**, 641.

Sok, D.-E. Kim Y.-B., Jung C.-H., Choi, S.C. and Cha, S.-H. (1994) *Biochem. J.* **301**, 713.