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TETRAVANADATE IS AN INHIBITOR OF PHOSPHATIDYL INOSITOL-SPECIFIC PHOSPHOLIPASE C

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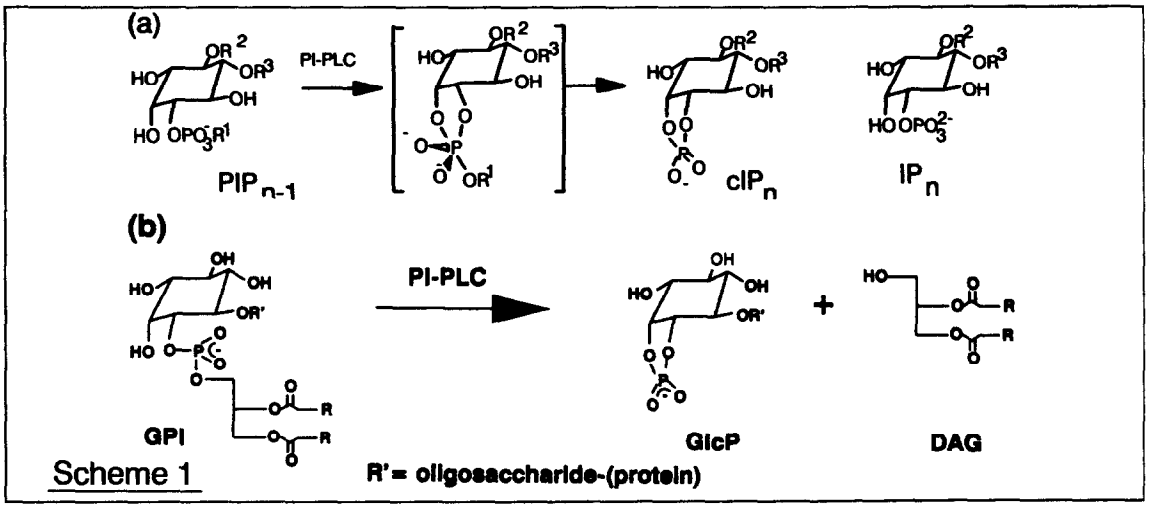
Abstract: Monitoring of oligomer concentrations by ^{51}V nmr demonstrates the tetramer of vanadate ion to be a potent inhibitor of phosphatidyl inositol specific phospholipase C (PI-PLC) from *Bacillus Cereus.*

Within the last few years there has been an explosion of research in the area of inositol phosphates (IP's) and phosphatidylinositols (PI's), stimulated by discoveries of their roles as intracellular messengers[1] and membrane anchors for proteins and oligosaccharides[2]. The key enzyme involved in these biological pathways is PI-PLC, which is responsible for direct generation of the important second messenger inositol-1,4,5,-trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) (Scheme 1a)[1,3]. In addition, it has become apparent that inositol cyclic phosphates (cIP's) may also be released as direct products of PI-PLC action[1b,4]. In some cases these cIP's are the sole inositol-containing product[5]. The action of PI-PLC on PI-linked membrane-bound glycoproteins and oligosaccharides (Scheme 1b) is anticipated to generate cIP's[2]. Indeed we have shown that action of PI-PLC from *B.Cereus* on PI yields inositol cyclic



Scheme 1

-1,2-phosphate (cIP) as product[6]. In this work we define the tetramer of vanadate as an inhibitor of this activity[9]. The potential for inhibition of PI-PLC by tetravanadate must be recognized, in particular in light of recent reports on the effects of decavanadate[10a] and orthovandate[10b] on IP and PI-dependent processes[11].

It has been proposed that: (1) vanadates may form stable pentacoordinate trigonal bipyramidal species in solution[12] and at the active site of phosphoryl transfer enzymes such as ribonuclease[13], and; (2) spontaneous esterification is undergone by vanadate in aqueous solution in the presence of a variety of alcohols, including glycols and sugars[14]. Thus, the potential for an inositol:vanadate complex to bind at the active site and inhibit PI-PLC was examined. Initial observations of PI-PLC inhibition by vanadate did not define the inhibitor species[7]. Vanadates and their esters are known to spontaneously oligomerise in solution, the relative amounts being pH and concentration dependent[15]. Previously, oligomeric vanadate species, such as decavanadate[10a] and tetravanadate[16], have been shown to be potentially potent inhibitors of biological processes. This work represents the first report of inhibition of a phosphodiesterase by tetravanadate[17].

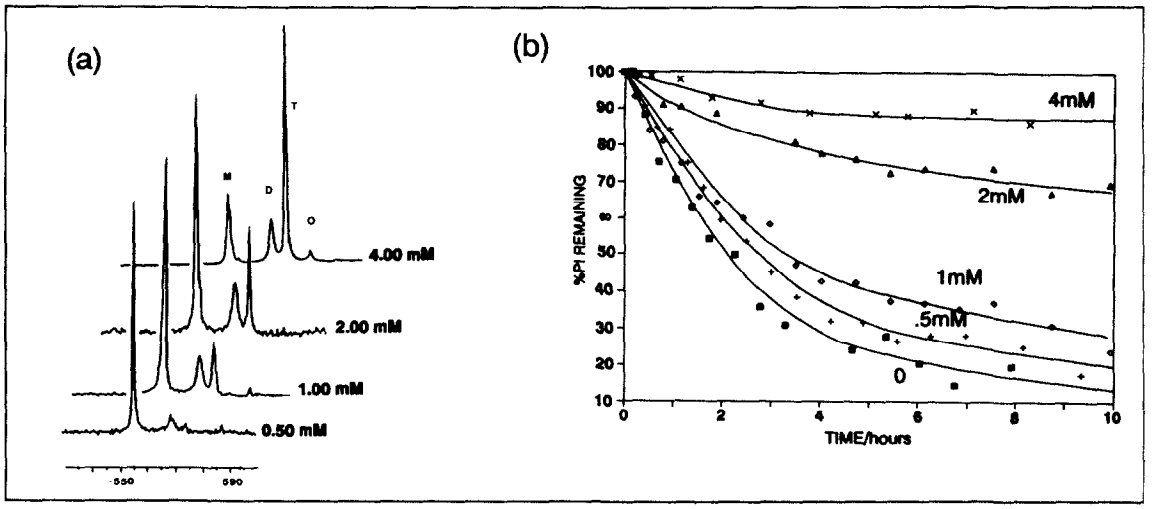


Figure 1a. ^{51}V nmr spectra of PI-PLC reaction solutions at indicated total vanadium concentration: SF=105.25MHz; SI=8k; SW=25KHz; PW=12us; LB=20Hz; RD=.34s; referenced to pH7.5 solution containing vanadate-ethanolamine complex (δ_V =-488ppm relative to $VOCl_3$ external reference at 0ppm).Key: M=monomer; D=dimer; T=tetramer; O=higher oligomer. **Figure 1b.** PI-PLC enzyme assay data. Reactions monitored by ^{31}P nmr from ratio of PI and cIP integrated signals. Conditions: 50mM HEPES (pH 7.31 with NaOH); .19% Triton X-100. Pre-incubation for 1hr prior to addition of PI (8.2mg, Avanti) solution, PI-PLC (1.3mU/ml, Boehringer) used as supplied.

Reactions were followed by ^{31}P nmr and vanadium species were identified by ^{51}V nmr. At 6.2mM total vanadium concentration ($[V]_t$) in HEPES buffer at pH 7.3 with and without inositol, the inhibition plot and ^{51}V spectra are virtually indistinguishable. Causes for the absence of a signal due to an inositol/vanadate complex must be considered. Firstly, signal overlap has been observed in cases where there is rapid ligand exchange with vanadate and the signals therefore coalesce[18]. This is doubtful for a 1:1 complex involving a

cyclic diester, since the vanadium in this case should be pentacoordinate[19], showing the typical ^{51}V nmr signal downfield from vanadate (-520 ppm). No signal was observed in this region. The presence of a 1:1 monoester cannot be ruled out on these grounds, because signal coalescence is possible, and has indeed been quantified in other cases[18]. In general, a high concentration of alcohol is found to be required for observation of complex formation[12,13c]. In the case of ribonuclease, the inhibitor, a 1:1 uridine:vanadate complex, was undetectable by ^{51}V nmr, although a 2:2 dimer complex was observed[20]. In our case at $[V]_t$ = [inositol] = 6.2mM, too little complex is present to be detectable by ^{51}V nmr: at 100mM inositol no complex is observed; at 0.6M inositol ($[V]_t$=3mM; pH 7.5) appropriate ^{51}V signals are detected, including major signals at δ=-501, -514 ppm. The evidence does not rule out the existence of inositol:vanadate complexes at low $[V]_t$, but clearly such a complex is not required for inhibition of PI-PLC by vanadate.

The ^{51}V nmr spectrum illustrates that, at 6.2mM in the enzyme assay medium, vanadate is present almost exclusively in tetrameric form, in agreement with previously reported spectra[14,16a]. This led to a series of studies using total vanadium concentrations ranging from 0.50 - 4.00 mM. The degree of inhibition was analysed in parallel to the ^{51}V spectrum of each PI-PLC solution. At lower $[V]_t$, the dominant species in solution is the monomer[15b,16a], and almost no tetramer exists (Fig.1a). There is little inhibition of PI-PLC activty by vanadate at these levels (Fig 1b). However, the amount of tetramer begins to rise sharply near 2.00 mM $[V]_t$ (Fig.1b). A corresponding increase in enzyme inhibition indicates that tetravanadate may be the inhibitory species.

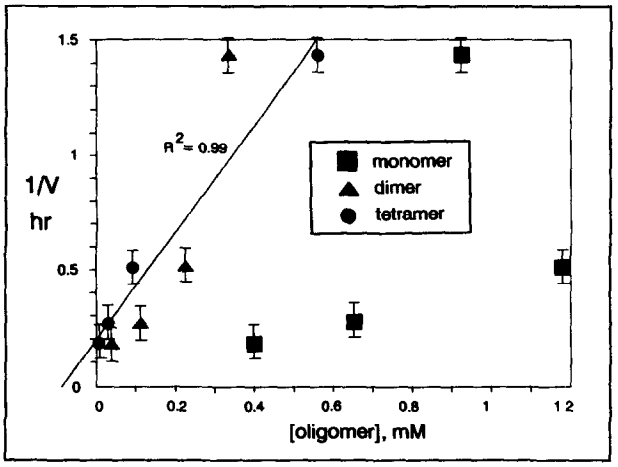


Figure 2. Dixon-type plot showing correlation of inhibition with concentration of each vanadate species. Inverse rate data obtained at 10% reaction. Linear regression for dimer and monomer yield R^2 = 0.86 and 0.25 respectively. Correlations at 5% and 15% reaction yield identical results.

Due to potential error in integration of ^{51}V nmr spectra[15] and the kinetic data (followed by ^{31}P nmr), it would be dangerous to extract detailed rate and inhibition constants from these data[21]. The observed scatter (Fig. 2) clearly demonstrates that inhibition does not correlate with concentration of monomer. Only inhibition with respect to tetravanadate yields a linear relationship. Molecular mechanics calculations and comparison with crystal structure data[22] suggest tetravanadate to be approximately isosteric and isoelectronic with cIP_3[23]. Specific vanadate-protein interactions are as yet unmapped, although interaction with lysine and arginine residues is to be expected[16d,24]. Detailed inhibition studies are in progress to define the mechanism of inhibition. The present study demonstrates that tetravanadate does inhibit the PI pathway

at the PI-PLC level, with the corrollary that significant total vanadate concentrations (>2mM) are required to observe potent inhibition[25].

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