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Vanadate Monomers and Dimers Both Inhibit the Human Prostatic Acid Phosphatase

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A combination of enzyme kinetics and ^{51}V NMR spectroscopy was used to identify the species of vanadate that inhibits acid phosphatases. Monomeric vanadate was shown to inhibit wheat germ and potato acid phosphatases. At pH 5.5, the vanadate dimer inhibits the human prostatic acid phosphatase whereas at pH 7.0 it is the vanadate monomer that inhibits this enzyme. The pH-dependent shift in the affinity of the prostatic phosphatase for vanadate is presumably due to deprotonation of an amino acid side chain in or near the binding site resulting in a conformational change in the protein. pH may be a subtle effector of the insulin-like vanadate activity in biological systems and may explain some of the differences in selectivity observed with the protein phosphatases.

The insulin-like effects of vanadate on isolated cells, on tissue, on diabetic rats and on the purified insulin receptor may be linked to the possible mechanism of action of vanadate on phosphatases (1). Vanadate and insulin also show some differences in their biological activities which may involve the action of phosphatases (2). Prostatic acid phosphatases from the human prostate gland (3), from seminal fluid (4) and from normal and metaplastic canine prostates (5) have tyrosine phosphatase activity. Vanadate was found to inhibit tyrosine phosphatases much more effectively than threonine and serine phosphatases (1). Vanadate is also a potent inhibitor for most alkaline and acid phosphatases (6,7). A trigonal bipyramidal species was first thought to be the inhibitory species for acid phosphatases and later of the other phosphatases (1,7). The vanadate inhibition of the bovine intestinal mucosa alkaline phosphatase at pH 8.0 has accordingly been correlated directly to monomeric vanadate using enzyme kinetics and ^{51}V NMR Spectroscopy (8). A detailed study of vanadate inhibition of the human prostatic acid phosphatase (9) was undertaken and compared to the vanadate inhibition of acid phosphatases from wheat germ and potato.

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It has been suggested for alkaline phosphatase that vanadate esters are more potent inhibitors than vanadate because alkaline phosphatases recognize both the phosphate moiety and the leaving group in the alkyl or aryl phosphate substrate (1). Acid phosphatases, on the other hand, recognize the phosphate group but not the leaving group (6). Such differences may explain the differences in the vanadate-induced activity of the tyrosine phosphatases compared to threonine- and serine phosphatases (1). We report here an alternative pH modulated mechanism that accounts for decreased vanadate inhibition at low pH (approximately pH 5) and increased inhibition at higher pH (approximately pH 7). It is possible such pH modulation can explain the differences in selectivity of tyrosine phosphatases compared to threonine and serine phosphatases.

We present evidence for monomeric vanadate as the inhibiting species for acid phosphatase from wheat germ and potato. We also show that the acid phosphatase from the human seminal fluid is inhibited by dimeric vanadate at pH 5.5. The vanadate induced inhibition of this enzyme is sensitive to pH, because at pH 7.0 a more potent inhibition is exhibited by monomeric vanadate. Our observaton is particularly interesting since prostatic acid phosphatases have been found to have protein phosphatase activities (3-5). It is possible pH will affect the vanadate inhibition in protein phosphatases and this therefore represents a new variable that should be considered when probing the biological activity of vanadate or using vanadate inhibition to characterize the tyrosine-, threonine or serine protein phosphatases.

Materials and Methods

Reagents and Enzymes: Chemicals were reagent grade (Fisher, Aldrich) and used without further purification. Water was distilled and deionized. Wheat germ acid phosphatase [EC 3.1.3.2] and dicyclohexylammonium *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Human prostate acid phosphatase was prepared as described previously (9). A vanadate stock solution was prepared by dissolving ammonium meta vanadate in distilled and deionized water; this solution was stored at 4°C. At no time was acid added to a solution containing vanadate, since vanadate in the presence of acid generates the orange decamer.

Kinetic Measurements: Spectrophotometric determinations of rates of hydrolysis were obtained at 25°C and 405 nm using a Lambda 4B Perkin-Elmer Doublebeam Spectrophotometer equipped with a constant temperature cell. Inhibition resulting from the production of inorganic phosphate was minimized by maintaining the extent of formation of *p*-nitrophenol to 1-10% hydrolysis of substrate. The rates were normalized at the beginning of each set of kinetic measurements. They were determined in duplicate or triplicates, and at several substrate concentrations. The rates were calculated assuming an $\varepsilon = 18.5\ l \times mmol^{-1} \times cm^{-1}$ for *p*-nitrophenol anion. The protein concentration of enzyme solutions was approximately 0.5 mg/ml and was quantified by the method of Lowry.

Acid Phosphatase Assay. The rate of hydrolysis was measured as the quantity of 8 mM *p*-nitrophenyl phosphate (or 5 mM MUP, (4-methylumbelliferyl phosphate))

hydrolyzed during a 5 min incubation at 25°C and pH 5.5. The assay solution contained 200 mM acetate, from 0 to 2 mM vanadate and approximately 0.01 mg/ml acid phosphatase. The changes in absorbance were determined against a blank solution and controls were run with each sample. If the absorbance of the control was above 0.004, the assay was repeated with freshly prepared substrate.

^{51}V NMR Spectroscopy. Vanadium-51 is an NMR-active nucleus of 99.75% natural abundance. Although its spin is 7/2 the line widths are relatively narrow and the signals are easily resolved in the vanadium window. Concentrations of various vanadium species are therefore conveniently determined by integration of the spectrum and calculating the concentration from the mole fraction of integrated signals (8). The ^{51}V NMR spectra were recorded at 131.5 MHz on a ^{1}H-500-MHz Bruker (11.7T) Spectrometer. We typically used spectrum widths of 12000 Hz, a 90° pulse angle, and accumulation time of 0.2 s and no relaxation delay. The chemical shifts are reported relative to $VOCl_3$ (0 ppm) although in practice we use a solution containing a complex of diethanolamine and vanadate (- 488 ppm) as the external reference (8).

Results and Discussion

Vanadate was previously found to competitively inhibit the acid phosphatases from potato, wheat germ and human liver (7). In contrast, we found the acid phosphatase from human prostate was noncompetitively inhibited by vanadate at pH 5.5 and a relatively poor inhibitor of the prostatic enzyme. The details of vanadate inhibition of the human prostate acid phosphatase (HPAcP) were therefore compared to that of the wheat germ and potato acid phosphatases.

The inhibition of the acid phosphatases from wheat germ and potato was investigated at pH 5.5 using 0 to 2 mM ammonium vanadate. Vanadate solutions in this concentration range contain a variety of vanadate species (Fig. 1) including monomer (-555 ppm), dimer (-562 ppm), tetramer (-574 ppm) and pentamer (-582 ppm). ^{51}V NMR was therefore used to measure the vanadate oligomers in solution. The reciprocal rates measured at pH 5.5 for the wheat germ acid phosphatase were plotted as a function of the various vanadate oligomer concentrations (Fig. 2). Figure 2 shows that only the monomeric vanadate species correlates linearly with the reciprocal rates suggesting that the monomer is the inhibitory species. This observation is in agreement with the suggestions that the acid phosphatases are inhibited by a trigonal bipyramidal vanadate-enzyme complex (6). The potato acid phosphatase was also found to be inhibited by monomeric vanadate.

The inhibition by vanadate on the prostate acid phosphatase (HPAcP) was measured using 5 mM MUP at pH 5.5 in 0.2 M sodium acetate. Figure 3 shows the reciprocal rates plotted as a function of the concentration of various vanadate oligomers. Since the vanadate dimer gives a linear relationship with the reciprocal rates, the dimer is the inhibiting species of HPAcP (K_i = 0.70 mM).

Vanadate oligomerization reactions vary as a function of pH and the vanadate inhibition was therefore examined at various pH's. The rate measurements were carried out as described above from pH 4.0 to 7.5 using acetate (pH 4.0 to 6.5), and imidazole (pH 7.0 to 7.5). The rate decreases drastically at higher pH (Fig. 4). The

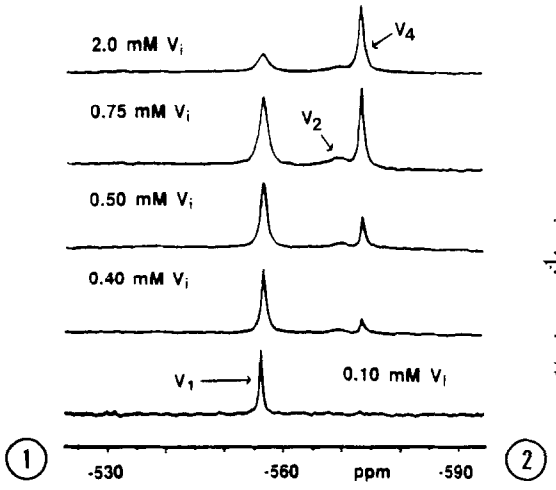

Figure 1. ^{51}V NMR spectra of assay solutions at pH 5.5 containing 0.2 M acetate and various concentrations of vanadate. The integrations of these spectra were used to calculate the concentrations of vanadate oligomers.

Figure 2. The reciprocal rates of wheat germ acid phosphatase catalyzed hydrolysis of 8 mM *p*-nitrophenyl phosphate in the presence of various concentrations of monomeric, dimeric, and tetrameric vanadate (plotted as number of vanadium atoms) at pH 5.5 in 0.2 M acetate.

inhibition by vanadate of the prostatic acid phosphatase was of a mixed type suggesting the binding of vanadate changes significantly with increasing pH. Indeed, at high pH the reciprocal rates were found to correlate with the concentration of monomeric vanadate (K_i = 0.013 mM).

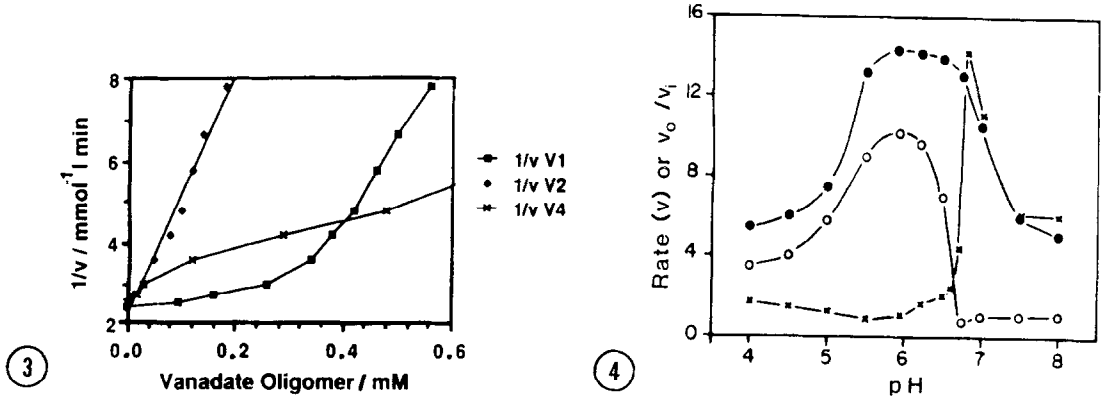

Figure 3. The reciprocal rates of human prostate acid phosphatase catalyzed hydrolysis of 5 mM 4-methylumbelliferyl phosphate (MUP) in the presence of various concentrations of monomeric, dimeric, and tetrameric vanadate (plotted as number of vanadium atoms) at pH 5.5 in 0.2 M acetate.

Figure 4. The uninhibited rate (•), inhibited rate (o), and rate ratio (V_o/V_i) (x) were plotted as a function of pH. The rates were measured using 5 mM 4-methylumbelliferyl phosphate (MUP) in the presence of 0.2 M acetate from pH 4.0 to 6.5 and in 0.1 M imidazole from pH 6.5 to 8.0). The vanadate concentration in the inhibited rate measurements was 0.5 mM.

The changes in the preference of HPAcP from the vanadate dimer to the monomer as the pH increases could be explained by the deprotonation of a residue in or near the binding site of the enzyme resulting in a conformational change in the protein. Protonation of such residue may be required for binding of the highly anionic vanadate dimer. This interpretation was supported by the observation that the binding of pyrophosphate is also stronger at the low pH.

The human prostatic acid phosphatase represents the first phosphatase for which changes in pH have been shown to alter the affinity for particular vanadate oligomers. At low pH the vanadate dimer is a weak inhibitor for HPAcP, whereas at higher pH the monomer is the more potent inhibitor. Since threonine and serine and a few tyrosine phosphatases are inhibited to a lesser extent than most tyrosine phosphatases by vanadate, it is possible similar changes in the active sites are responsible for the lower affinity of the threonine and serine and selected tyrosine phosphatases for vanadate. It is possible pH may be a key factor one should consider when examining the effects of vanadate in biological systems, and one must be aware that subtle pH changes alter the strength of the vanadate-protein interaction.

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References

1. Gresser, M.J.; Tracey, A.S.; Stankiewicz, P.J. (1987), *Adv. in Prot. Phosphatases*, **4**, 35-57, and references therein.
2. Bernier, M.; Laird, D. M.; Lane, M. D. *J. Biol. Chem.* (1988), **263**, 13626-34.
3. Li, H.-C.; Chernoff, J.; Chen, L. B.; Kirschonbaum, A. *Eur. J. Biochem.* (1984), **138**, 45-51.
4. Lin, M.-F.; Clinton, G. M. *Biochem J.* (1986), **235**, 351-7.
5. Tessier, S.; Chapdelaine, A.; Chevalier, S. *Mol. Cell. Endocrin.* (1989), **64**, 87-94.
6. Lopez, V.; Stevens, T.; Lindquist, R.N. (1976), *Arch. Biochem. Biophys.*, **175**, 31-8.
7. VanEtten, R.L.; Waymack, P.P.; Rehkop, D.M. (1974), *J. Am. Chem. Soc.*, **96**, 6782-5.
8. Crans, D.C.; Bunch, R.L.; Theisen, L.A. (1989), *J. Am. Chem. Soc.*, **111**, in press.
9. Mukhopadhyay, N. K.; Saha, A. K. ; Smith, W.; Dowling, J. N.; Hiserodt, J. and Glew, R. H. *Clin. Chem. Acta*, (1989), **182**, 31-40.
10. This reference serves as an example: Crans, D.C.; Shin, P.K. (1988) *Inorg. Chem.* 27, 1797-806. See further references reported therein.