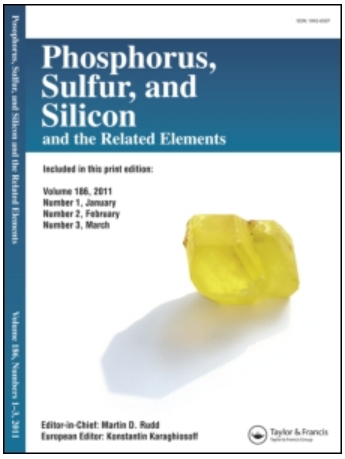

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ORGANIC VANADIUM COMPOUNDS - TRANSITION STATE ANALOGY WITH ORGANIC PHOSPHORUS COMPOUNDS[1]

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Abstract Vanadium compounds, particularly in oxidation state V, are potent inhibitors of phosphoryl group transfer enzymes. In this paper the existance of a correlation between the coordination geometry of a series of vanadium dipicolinate complexes and their potency as inhibitors for chicken intestinal alkaline phosphatase is examined. We find that within a limited series of vanadium compounds the five-coordinate derivatives are the most potent inhibitors.

VANADIUM AND PHOSPHORUS ANALOGY

Introduction Vanadium compounds have been used with great success as tools for studies of phosphoryl group transferases amply illustrated by numerous reports of the inhibition of phosphatases, ATPases, and ribonucleases (for reviews see Refs. 2-4). The early suggestion that a vanadate-uridine complex was a potent, transition state mimic of ribonuclease[5] has been confirmed by an X-ray crystal structure[6] although recent aqueous studies modifyed the initially proposed stoichiometry and structure of the vanadate-uridine-type complexes.[7] In contrast, vanadium derivatives have been reported to act as substrates and a cofactor for enzymes catalyzing reactions other than phosphoryl group transfer.[8-10] Perhaps the most interesting example is the ability of NADV, an analog of NADP, to act as a cofactor for dehydrogenases.[10]

Not all attempts to use vanadium compounds as tools in biological studies have been equally successful. Unfortunately, many of these failures can be attributed to the lack of consideration of the aqueous chemistry of vanadium. Given the rapid interconversion among vanadium compounds, specific measures are typically necessary to ensure the existence of a particular vanadium compound in a biological system.[11] In this paper we will briefly describe the vanadium-phosphorus analogy, potential applications of vanadate esters and other vanadium compounds as transition state probes for phosphoryl group transfer reactions. Finally, new results will be presented in which the potencies of a series of vanadium complexes as inhibitors for chicken intestinal alkaline phosphatase (CIAP) correlate with geometry of these compounds.

Vanadium-Phosphorus Similarities and Dissimilarities. In its highest oxidation state, vanadium exists as vanadate in aqueous solution. As a monomer, vanadate is recognized as a structural and electronic (ground state) analog of phosphate.[4,11] This analogy is also generally believed to exist between the organic phosphates and organic vanadates as suggested by their respective pK_a values and the fact that organic vanadates can substitute for organic phosphates as substrates for many enzymes.[8-10]

As a transition metal, the vanadium atom readily adopts four-, five-, six- and seven-coordinate geometries in oxovanadates and vanadium alkoxides. In contrast to five-coordinate phosphate derivatives, five-coordinate vanadium derivatives are often local or global minima, thus making the latter excellent transition state analogs of the former.

A major difference between vanadium and phosphorus is the wide range of redox reactions vanadium can participate in. Aqueous vanadium(IV), in the form of hydrated VO^{2+}, is stable only at acidic pH. At neutral and alkaline pH it is readily oxidized to vanadium(V) even by trace levels of oxygen or other oxidants. Vanadium(V) on the other hand is readily reduced to vanadium(IV) in the presence of thiols and other reducing agents. Generally it is difficult to conduct biological studies without having both vanadium(IV) and V compounds present.[4,11]

Problems in Applications of Vanadium Compounds as Phosphorus Probes in Biological Systems The complexity of vanadium chemistry under physiological conditions is not widely recognized or considered among life scientists. Often, initial attempts to use vanadium compounds to probe enzyme reactions fail, and the approach is abandoned prematurely although one of the following technical reasons is responsible.

1. Many commonly used buffers, substrates, cofactors and other media additives form stable complexes with both vanadium(IV) and V. Formation of such complexes will prevent vanadate and/or vanadium(IV) from interacting with the biological system. Guidelines for biological studies with vanadium have recently been described.[11]
2. Even well-known vanadium derivatives may not remain intact for the desired/required time periods needed to induce the biological response.
3. Although a structural analog of a particular phosphate derivative may form, its formation constant is often small. Unless the enzyme has sufficiently high affinity for the phosphate analog no response may be observed. More typically, a complex equilibrium mixture of several vanadium derivatives forms, some of which could induce responses other than the expected one.
4. The lack of mechanistic information concerning the biological system under study. The vanadium derivative may be used in an inappropriate capacity.

The matter is further complicated by the fact the structures of relevant vanadate esters (and, for that matter, other compounds) in aqueous solution are often not known.

DOES THE POTENCY OF VANADIUM COMPOUNDS AS INHIBITORS CORRELATE WITH THEIR COORDINATION GEOMETRY?

Vanadium Compounds; Selection We chose the series of structurally characterized vanadium complexes shown in Fig. 1 which contain five- (V^{V}dipic)[12], six- (V^{IV}dipic)[13] and seven-coordinate (mpVdipic)[14] vanadium atoms complexed to dipicolinic acid (dipic). In addition, the inhibitory potency of phosphate, arsenate, vanadate and peroxovanadate (bpV) were determined for comparison.

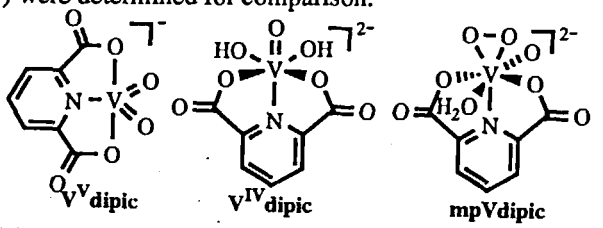

Fig. 1 - The presumed structures of V^{V}dipic,[12] V^{IV}dipic[13] and mpVdipic[14] under alkaline phosphatase assay conditions (pH 8.0).

Stability of Inhibitors During CIAP Assay The stability of phosphate, arsenate, vanadate and peroxovanadate was more than adequate for the duration of the enzyme assay with CIAP. However, the three dipic complexes required careful stability studies. A brief summary will be presented here but the detailed spectroscopic studies of each compound will be reported elsewhere (Crans et al., in preparation).

Dissolving 20.0 mM V^{V}dipic in water generates a stock solution, stable at 4 °C for weeks at pH 5.4 as indicated by ^{51}V NMR spectroscopy. Upon addition of V^{V}dipic to an assay solution, essentially all V^{V}dipic immediately hydrolyzed to form vanadate and free dipic as indicated by the low formation constant under the assay conditions (2.0 ± 0.2 M^{-1}). Two experimental approaches were successful in increasing the V^{V}dipic levels sufficiently at low inhibitor concentrations in the assay solution. First, the pH of the assay

solution was decreased to 7.0 where the formation constant increased to 132 ± 12 M^{-1}. Second, additional free dipic was added significantly increasing the contribution of V^{V}dipic in the assay solution.

V^{IV}dipic (20.0 mM) dissolves readily in aqueous solution at a pH of 3.5, and these solutions are stable at 4 °C for several days as evidenced by both EPR and UV-VIS spectroscopy. Upon addition of V^{IV}dipic to the CIAP assay solution, the complex immediately deprotonates and begins a slower oxidization reaction. The rate of oxidation (disappearance) is monitored by UV spectroscopy at 845 nm and compared to a reference solution. Since the decomposition rate is on the timescale of the enzyme experiment, it is necessary that the enzyme kinetic studies be carried out in a time-specific manner.

The mpVdipic (20.0 mM) dissolves readily in aqueous solution at pH 6.2 and is stable at 4 °C for days as monitored by both ^{51}V NMR and UV-VIS spectroscopy. Upon addition of mpVdipic to the CIAP assay solution, the complex decomposes within a few minutes based on the disappearance of the ^{51}V NMR signal for mpVdipic at -596 ppm and the absorbance peak at 430 nm. The decomposition of mpVdipic to a mixture of mpVdipic, vanadate oligomers, mpV, bpV and $(bpV)_2$ was quantified in a time-dependent manner for the kinetic analysis.

Analysis of Inhibition Data All the inhibitors examined in this study were competitive. For the dipic complexes, more than one inhibitor will be present when measuring the inhibition.[10,11] Assay solutions added V^{V}dipic contains V^{V}dipic, dipic and V_1. The concentrations of these three potential inhibitors in the solution are related through the formation constant, $K_f = [V^{V}dipic]/[V_1][dipic]$, so that the rate expression is simplified (eq. 1). The inhibition experiment is then carried at constant $[V^{V}dipic][/[dipic]$ ratio; the resulting Lineweaver-Burk plot is shown in Fig. 2.

$$\text{slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[V^{V}dipic]}{K_{iV^{V}dipic}} + \frac{[V^{V}dipic]}{K_{iV_1}\, K_f\, [dipic]} + \frac{[dipic]}{K_{idipic}}\right) \qquad (1)$$

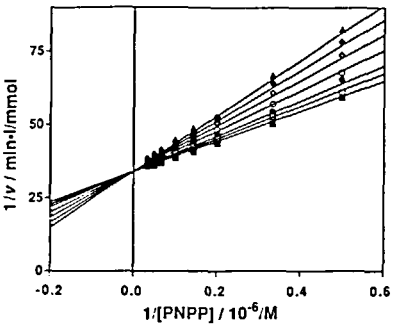

Fig. 2 Lineweaver-Burk plot of inhibition of chicken intestinal alkaline phosphatase by V^{V}dipic (in the presence of V_1 and dipic). The lines shown are calculated with a spreadsheet using the parameters listed in Table I. The experimental points were obtained by adding various amounts of V^{V}dipic and dipic to the assay solution. $[V^{V}dipic]/[dipic]$ added are as follows: 0/0 mM (■), 0.050/0.013 mM (❑); 0.10/0.026 mM (◆); 0.20/0.052 mM (○); 0.30/0.078 mM (◇); 0.40/0.10 mM (◆) and 0.50/0.13 mM (▲).

K_i Values of Vanadium Compounds Acting on CIAP The inhibition constants (K_i) obtained are shown in Table I. The K_i value for phosphate is significantly higher than the K_i value for vanadate as reported previously for yeast and human acid phosphatases[15] but in contrast to studies with *E. coli* alkaline phosphatase.[16,17] This pattern is consistent with the interpretation that vanadate acts as a transition state analog for CIAP.

The K_i value for V^{V}dipic is smaller than the K_i values for V^{IV}dipic, mpVdipic and the other compounds listed in Table I. Also shown is a column in which the K_i values were calculated based on the concentration of complex *added* to the assay solution (in contrast to the concentration of *complex actually present*). K_i values obtained in this manner reveal little information on a possible structure-activity correlation. Inhibition studies with free dipic rule out the possibility that the observed inhibition is caused by dipic alone. In summary, the results in Table I support the hypothesis that the five-coordinate compounds are more potent inhibitors than six- or seven-coordinate compounds for CIAP.

Conclusion and Future Prospects The inhibitor potencies were examined for a series

of five-, six- and seven-coordinate vanadium dipic compounds for chicken intestinal alkaline phosphatase. *A correlation between coordination number around the vanadium atom and inhibitory potency was observed, although it is important to note that all the examined vanadium compounds were strong inhibitors.* The phosphorus-vanadium analogy can be explored successfully when the possible pitfalls in working with labile vanadium compounds are recognized. There is no doubt that when vanadium compounds with the desired structure and stability[18] are prepared, they will have real potential for inhibition of enzymes and generation of catalytic antibodies.

Table I. The K_i values determined for CIAP.[a]

Compound	Coordination number	Charge at pH 8.00	Vanadium oxidation state	K_i value (μM)	Uncorrected[b] K_i value (μM)
P_i	4	-2	-	470±30	
As	4	-3	-	18±1	
V_1	4(5[c])	-1/-2[d]	V	2.8±0.4	
V_1[e]	4(5[c])	-1/-2[d]	V	5.7±1.0[e]	
V^{V}dipic	5	-1	V	1.9±0.6[e]	4.4±0.8[b]
V^{IV} dipic[f]	6	-2[f]	IV	~6[g]	4.9±1.0[b]
mpVdipic	7	-2	V	13±1	4±1[b]
bpV	7	-2	V	23±4	
dipic	-	-2	-	3200±800	

[a] The K_m for CIAP using 5.0 to 50 μM PNPP in 50 mM Hepes, 1.00 M KCl, 5.0 mM $MgCl_2$ at 25 °C and pH 8.00 (±0.05) for CIAP is 9.7 (±1.0) μM. These conditions were used unless otherwise indicated.
[b] These K_i values were calculated assuming 100% of the complex remained after 4 min of incubation in the form they were added to the assay solution.
[c] The coordination number of V_1 may be 4 or 5 (is it in aqueous solution or complexed to an enzyme?).
[d] The two major V_1 species in the assay solution have charges of -1 and -2 (pK_a ranges from 8.0-8.4).
[e] These K_i values were obtained at pH 7.00 (±0.05), under which conditions the K_m for 1.5 to 20 μM PNPP is 1.5 (±0.5) μM for CIAP.
[f] V^{IV}dipic has two pK_a values at 6.7 (±0.2) and 7.0 (±0.2).
[g] Concentrations of inhibitors in this experiment was less than optimal (low accuracy on K_i value).

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