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# Enzyme Interactions with Labile Oxovanadates and Other Polyoxometalates

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# Enzyme Interactions with Labile Oxovanadates and Other Polyoxometalates

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Aqueous solutions of vanadate contain several labile oxovanadates; each interacts differently with enzymes and other proteins. Vanadate monomer, $V_1$, can act as a ground state or transition state analog of phosphate. Vanadate dimer, $V_2$, inhibits a series of enzymes including dehydrogenases, one aldolase and a phosphatase. $V_2$ can also activate enzymes, and this activation has been observed with a mutase and dehydrogenase. Vanadate tetramer, $V_4$ (often referred to as "metavanadate"), inhibits a large number of enzymes including dehydrogenases, isomerases, and one aldolase. $V_4$ also binds to a series of enzymes including superoxide dismutase, myosin and possibly adenylate kinase. $V_4$ has been identified as the species responsible for the specific UV-light induced photocleavage of the active site of myosin and possibly adenylate kinase. Redox chemistry between $V_1$ and $V_4$ and proteins has been reported. Vanadate pentamer, $V_5$, has not yet shown high affinity for proteins, and studies with related oxomolybdates show that a tetramer interacts more potently with the proteins than the pentamer. Vanadate decamer, $V_{10}$, is a less labile oxometalate that also inhibits and binds to proteins. The potent antiviral and anti-HIV activity of several oxometalates have increased the interest in protein interactions with large polyoxoanions. It is reasonable to expect that proteins catalyzing reactions of large anionic substrates (polymeric nucleic acids) tend to show high affinity for large polyoxometalates. The fact that other proteins, including cytochrome *c*, phosphorylase, phosphatases, dehydrogenases and an aldolase, also show affinity for these large polyoxometalates is perhaps more intriguing: this affinity suggests that some sequences and three dimensional arrays might favor binding of oxometalates.

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**Key Words:** *vanadate, labile oxovanadates, polyoxometalates, enzyme binding sites, enzyme inhibition, protein interaction, enzyme inhibitors*

## VANADIUM IN BIOLOGICAL SYSTEMS: A PERSPECTIVE

The discovery in 1980 that vanadate acts as an insulin mimic and the demonstration in 1985 that it could normalize diabetic rats has significantly increased the interest in interactions of vanadate with proteins from a biological perspective.[1,2] The insulin mimetic properties have now evolved into studies probing the effects of vanadium on protein phosphorylation and signal transduction (reviewed in Refs. 3 and 4). Despite major progress, the mechanism by which vanadate and vanadyl cations act as an insulin mimetic is not understood. Recently, vanadium-based derivatives that maintain the insulin mimetic activity and that could be used as oral substitutes for insulin have been reported.[5–7] It is likely that the complicated aqueous chemistry of vanadate as well as its toxicity have been responsible for the significant time between the discovery of the insulin mimetic activity and the development of compounds with insulin mimetic activity.

Several mechanisms by which vanadium interacts with proteins have been characterized, and some of these have been reviewed elsewhere.[3,8–10] For two decades vanadium has been known to inhibit ribonuclease and phosphatases.[11,12] The potent inhibitory effects of vanadate were attributed to the transition state analogy between a pentacoordinate vanadate complex and the transition state of the enzymatic reaction.[11,12] The potent affinity of vanadate for proteins was further substantiated in 1978 when vanadate was identified as the component in ATP preparations from muscle that inhibits ATPases.[13,14] Recently, studies have shown that organic vanadates can act as enzyme substrates and even as enzyme cofactors.[15–18] Characterization of the aqueous chemistry and the active vanadium compounds is seriously lagging behind the biological discoveries, and this lack of understanding still provides fuel to contradictory results.

The discoveries of the vanadium-dependent bromoperoxidase in algae and seaweed[19–21] and of the vanadium-dependent nitro-

genase in *Azotobacter chroococcum* and *Azotobacter vinelandii*[22] have increased the interest in vanadium from a bioinorganic perspective. Bromoperoxidases are now referred to as haloperoxidases since several of the bromoperoxidases also have activity with other halide ions.[23] Model studies aimed at mimicking the catalytic activity and the binding in the active site have recently been reported.[24-27] The accumulation of vanadium in tunicates and mushrooms and the vanadium-containing natural product, amavadine, have mystified and continue to intrigue scientists. The role of vanadium in these organisms is still not understood,[20,28-30] but the coordination and redox chemistry of related vanadium systems have been studied.[31,32] Early studies of vanadate interactions with proteins focused on the development of vanadyl cation as a probe to study metal binding sites.[33,34] Furthermore, the recent developments of photolysis of protein–vanadate mixtures to probe specific anion binding sites[35,36] make vanadium a potent probe for characterization of proteins.



The very rich vanadium(V) chemistry in aqueous solutions complicates studies aimed at exploring the various mechanisms by which vanadate can act. Prompted by a 1985 report[37] we have explored the biochemistry of vanadate derivatives[16-18,38-43] as well as aqueous vanadium chemistry of relevance to biological systems.[17,37,44-46] The aqueous chemistry is described in the accompanying *Comments* article. Prior to 1990 most studies describing interactions of vanadate with proteins were not designed specifically to examine which oxovanadate was the active species (studies with the more kinetically inert vanadate decamer are exceptions).[9] Interactions of vanadate with several proteins, including selected phosphatases, dehydrogenases, isomerases, one aldolase, a superoxide dismutase and a kinase have been determined by our group.[18,38,40-42,47-50] Although some of the early studies have been reviewed,[9] related work is also described here.[51-55] Ten years ago vanadium was believed to have two roles in biology: as a phosphate analog and as a redox agent.[28] Vanadium is showing a much greater versatility than anticipated since six different types of mechanisms by which vanadium can act have been described.[8] We have studied all six types of mechanisms. Perhaps the interaction of oxovanadates with enzymes is most unexpected, and this type of interaction will be reviewed here.

The specific oxovanadate–protein interactions led us to explore the activity of other oxometalates with similar and related structures.[43] The recent observation that oxometalates have potent activity against AIDS suggests such complexes have some affinity and selectivity for proteins.[56–59] Little is known on the interaction of oxometalates with specific proteins even though work has been carried out evaluating the biological activity of a series of polyoxometalates.[56–63] From the combination of enzyme studies with oxovanadates and enzyme studies with oxometalates, interesting patterns are emerging. It appears that anions with some geometries bind significantly better to proteins than other anions with different geometries and that many proteins share an affinity for these anionic compounds.

## OXOVANADATE SPECIES IN AQUEOUS SOLUTIONS

Vanadate monomer ($H_2VO_4^-$, $HVO_4^{2-}$ and $VO_4^{3-}$ abbreviated $V_1$) is presumed to be a structural and electronic analog of phosphate. In aqueous vanadate solutions $V_1$ oligomerizes to dimer

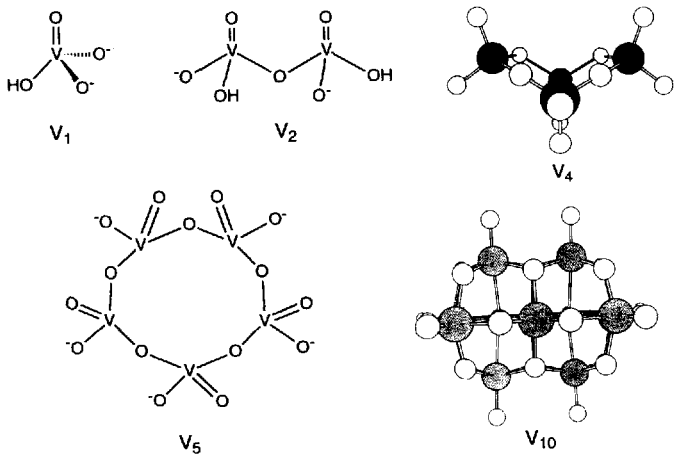



FIGURE 1 The presumed structures are shown for the labile oxovanadates: $V_1$ ($HVO_4^{2-}$), $V_2$ ($H_2V_2O_7^{2-}$), $V_4$ ($V_4O_{12}^{4-}$), $V_5$ ($V_5O_{15}^{5-}$) and the less labile $V_{10}$($V_{10}O_{28}^{6-}$). $V_4$ and $V_{10}$ were drawn from the reported coordinates of compounds characterized in the solid state.

$(H_3V_2O_7^-, H_2V_2O_7^{2-}, HV_2O_7^{3-}, V_2O_7^{4-}$ abbreviated as $V_2$), tetramer ($V_4O_{12}^{4-}$ and $HV_4O_{12}^{3-}$ abbreviated $V_4$) and pentamer ($V_5O_{15}^{3-}$ abbreviated as $V_5$) on a millisecond time scale.[64,65] $V_2$ is presumed to be a pyrophosphate analog. The tetramer is the major species in concentrated solutions and is sometimes referred to as "metavanadate." An X-ray structure has been reported for $V_4$ and it is commonly believed that this cyclic structure represents the $V_4$ observed in aqueous solution.[66,67] In addition to $V_1$, the major oxovanadates present in aqueous solution are $V_2$, $V_4$ and $V_5$ (Fig. 1); other species are present only in low concentrations at neutral pH.[68] In contrast to the labile oxovanadates, vanadate decamer ($V_{10}$) is well characterized (Fig. 1). $V_{10}$ forms between pH 2 and 6,[68-71] but at neutral and basic pH, the $V_{10}$ remains intact for studies of limited duration. Figure 1 shows a drawing of $V_4$ and $V_{10}$ characterized by X-ray, and a schematic representation of $V_1$, $V_2$ and $V_5$.

Each vanadate oligomer gives resolved resonances in the $^{51}V$ NMR spectrum (Fig. 2). Since the vanadate species have similar $T_1$ values,[65] integrating the $^{51}V$ NMR resonances provides mole fractions of each vanadate oligomer that can be used to quantitate concentration. The relationships (1) to (4) define the $H^+$-dependent equilibrium constants and (5) defines the vanadium concentration. The assumptions and simplifications made when applying (1) and (4) were discussed in the accompanying article. The pH value has the greatest effect on the $H^+$-dependent constants, although buffer, ionic strength and temperature also play a role. For specific conditions, $H^+$-dependent formation constants have been reported ranging from 50 to 600 $M^{-1}$ in $K_{12}$, from $3 \times 10^6$ to $1 \times 10^{10}$ $M^{-3}$ in $K_{14}$, from $2 \times 10^8$ to $1 \times 10^{12}$ $M^{-4}$ in $K_{15}$ and from $1 \times 10^4$ to $6 \times 10^4$ $M^{-1}$ in $K_{24}$.

$$2\ V_1 \underset{}{\overset{K_{12}}{\rightleftharpoons}} V_2; \quad K_{12} = [V_2]/[V_1]^2, \tag{1}$$

$$4\ V_1 \underset{}{\overset{K_{14}}{\rightleftharpoons}} V_4; \quad K_{14} = [V_4]/[V_1]^4, \tag{2}$$

$$5\ V_1 \underset{}{\overset{K_{15}}{\rightleftharpoons}} V_5; \quad K_{15} = [V_5]/[V_1]^5, \tag{3}$$

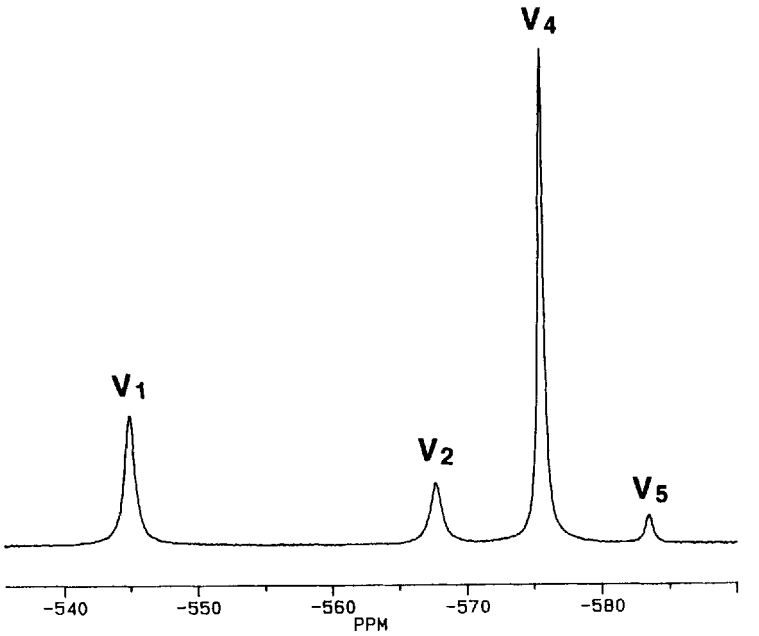



FIGURE 2 $^{51}V$ NMR spectrum (132 MHz) of a 10 mM vanadate solution at pH 8.6 (±0.1) and an ionic strength (KCl) of 0.40 M. The resonances for vanadate monomer ($V_1$), dimer ($V_2$), tetramer ($V_4$) and pentamer ($V_5$) are indicated.

$$2\ V_2 \underset{}{\overset{K_{24}}{\rightleftharpoons}} V_4; \quad (K_{24} = [V_4]/[V_2]^2, \tag{4}$$

$$[V_{tot}] = [V_1] + 2[V_2] + 4[V_4] + 5[V_5]. \tag{5}$$

## ENZYME ASSAYS CONTAINING OXOVANADATES

Problems caused by buffers[44–46] were discussed in the accompanying article. Hepes does not affect the vanadate reactions[46,72–74] or the rates of reactions[75] and is overall the best buffer to use. The major problem is the slow reduction of vanadium(V) in the presence of Hepes and NADH.[76] Tris (tris(hydroxymethyl)amino-

methane), used extensively in early studies of vanadate–ester complexes,[37,77] can be used at low levels, if Hepes is not compatible. Other buffers should be used with caution.[46,77–83]

Studies monitoring the absorption of NAD or NADP (or the corresponding NADH or NADPH oxidation) can be carried out using imidazole since this buffer shows no evidence of the unwanted redox chemistry.[76] Imidazole is thus a preferred buffer when the enzyme reaction involves NADH/NADPH.

Enzyme preparations will often contain additives to increase protein stability.[84] Vanadate forms 1:1 complexes with EDTA even at micromolar concentrations of both EDTA and vanadate.[46] Compounds such as glycerol, ethylene glycol and other protein stabilizers interact weakly with vanadate.[46] Both DTT and 2-mercaptoethanol rapidly reduce vanadate to vanadyl cation. Enzyme substrates such as dihydroxyacetone and fructose will react with vanadate presumably to generate vanadyl cation and oxidized organic ligand.[16,42] The formation of vanadyl cation is cause for concern since vanadyl cation may effect enzyme activity as well as the quantification of oxovanadates.



## REDOX REACTIONS: VANADATE INTERACTIONS WITH ENZYMES

Redox reactions between vanadate and thiol groups are for most practical purposes irreversible and, consequently, are fairly simple to demonstrate experimentally. If the enzyme is inhibited by vanadate through redox reactions, little or no activity is regained by removing the vanadium from the enzyme.[41,42,47] Vanadate and vanadyl cation can conveniently be removed from the enzyme by adding EDTA.[46] The vanadium can also be removed by dialysis or passing the enzyme solution over a Penefsky column.[41,47,85] These types of experiments are critical since the kinetic treatment of the data will depend on whether the inhibitor (or activator) is reversible or irreversible.

An early report described the reduction of vanadate while oxidizing glyceraldehyde-3-phosphate dehydrogenase.[86,87] The reduction was demonstrated by the observation of an EPR signal for vanadium(IV) in the presence of protein. Spectra in the

absence of enzyme or in the presence of deactivated enzyme were not shown or described. It is possible some of the observed reduction is due to conditions (Tris buffer and 9 mM oxalate) even though it is reasonable to expect that the enzyme can reduce vanadate. No evidence was provided to determine which oxovanadate derivative oxidizes glyceraldehyde-3-phosphate dehydrogenase.

The effect of vanadate on glycerol-3-phosphate dehydrogenase (G3PDH)[41] and fructose-1,6-bisphosphate aldolase has been determined.[42] These enzymes contain reactive thiol groups. Both $V_2$ and $V_4$ are inhibitors for G3PDH, but no evidence for redox chemistry was observed with this enzyme, since all activity could be regained upon removal of the vanadium (Fig. 3). This example shows that even with the presence of a thiol group in the active site, redox chemistry may not occur. For fructose-1,6-bisphosphate aldolase it appears that only $V_4$ is oxidizing the enzyme since low concentrations of vanadate have no effect on the enzyme activity. Reactivation buffers containing high levels of DTT will regain the aldolase activity lost by interaction with $V_4$, consistent with the interpretation that $V_4$ is deactivating aldolase through thiol group

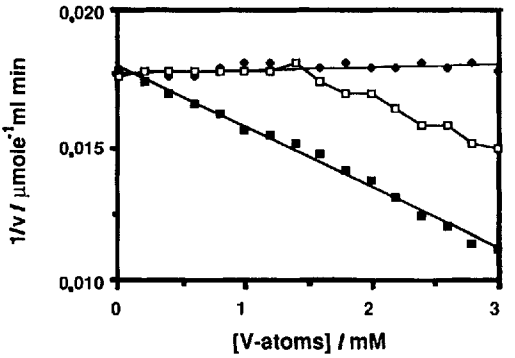



FIGURE 3 Rates of G3PDH-catalyzed oxidation of glycerol-3-phosphate measured as a function of vanadate concentration upon addition of no EDTA (■), 1.5 mM EDTA (□), and 3.0 mM EDTA (▲) in solutions containing 50 mM imidazole, 5 mM semicarbazide, 10.5 mM glycerol-3-phosphate, 3.0 mM NAD, 200 mM KCl, and G3PDH (rabbit muscle) at pH 7.4. Reprinted with permission from Ref. 41 (Copyright 1991 American Chemical Society).

oxidation. The reduction of $V_4$ is of particular interest in view of the fact that $V_2$ also inhibits aldolase (see below).

The reduction of vanadate has been observed on addition of purple acid phosphatase.[47] $V_1$ inhibits this enzyme. Vanadate oxidizes the metal cofactor ($Fe^{2+}-Fe^{3+}$) in this system and probably not the enzyme. Vanadate binds rapidly to the purple acid phosphatase and only after some time oxidizes the cofactor as reflected by the non-linear loss of activity (Fig. 4).[47] This example illustrates that vanadate has the potential to act on proteins following non-linear kinetics, as found for slow binding inhibitors.

In conclusion, vanadate can act both reversibly or irreversibly with enzymes even when thiol groups are present in the active site. It is also clear that specific oxovanadates are involved in redox chemistry with the protein, such as $V_4$ for aldolase and $V_1$ for purple acid phosphatase. Perhaps the observation that not all oxovanadates induce redox chemistry suggests that a specific



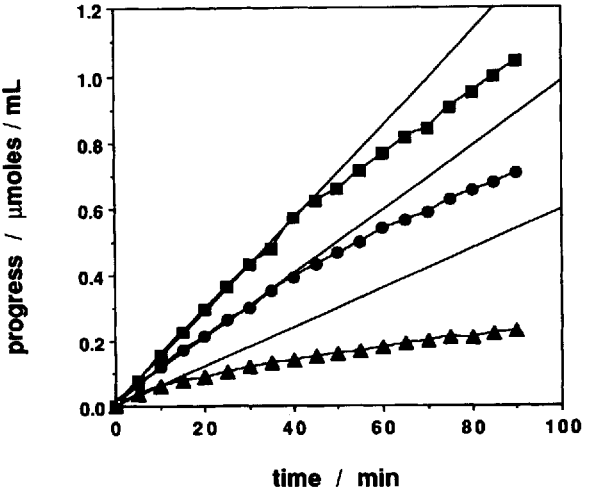



FIGURE 4 Deactivation of uteroferrin (purple acid phosphatase from porcine uterine fluid) as a function of time. Reaction progress measured as hydrolyzed *p*-nitrophenyl phosphate (PNPP) in 200 mM acetate at pH 5.5 and 2.0 mM PNPP. Assays carried out in the presence of vanadate (▲), in the presence of phosphate (●) and in the absence of oxoanion (■). Reprinted with permission from Ref. 47 (Copyright 1992 American Chemical Society).

protein–oxovanadate complex forms prior to the electron transfer step.

## INTERACTION OF LABILE OXOVANADATES WITH ENZYMES

Given the rapid exchange rates between the labile oxovanadates, it is not possible to isolate a specific anion for testing.[65] To determine the effect of a specific anion an equilibrium mixture containing this species must be examined. Consequently, the changes in enzyme activity must be associated with changes in concentration of different vanadate oxoanions.[39] The relationships governing the vanadate oligomer populations (1) to (4) are instrumental for this type of analysis. The Lineweaver–Burk slopes or the reciprocal rates, as shown for the inhibition of 6-phosphogluconate dehydrogenase (6PGDH) in Fig. 5, are plotted against each of the oxovanadate populations. As shown in Fig. 5a the inhibition does not correlate with total vanadate concentration since no inhibition is observed at low vanadate concentrations. In Fig. 5, only one species, $V_4$, can account for all the observed inhibition, as evidenced by the linear correlation of reciprocal enzyme activity and $V_4$ concentrations (both at low and high concentrations of $V_4$) (Fig. 5d). $V_1$ (Fig. 5b) and $V_2$ (Fig. 5c) cannot explain inhibition because at low concentrations of these species, no inhibition is observed. $V_5$ is also not a viable candidate because inhibition is observed at concentrations where $V_5$ is not yet present in appropriate concentrations (data not shown). Alternative interpretations involving two molecules of $V_2$ or four molecules of $V_1$ would be consistent with the observed results, but these are not the simplest and most likely explanations. In a few cases, it was not possible to eliminate all viable candidates. However, in most cases, only one reasonable model can explain the observed inhibition (activation or binding) of the enzyme, in part because the other combinations can be discarded. For some enzymes, specific experiments were designed to test various possible models and such approaches have been detailed in the original work in the references cited throughout this review.

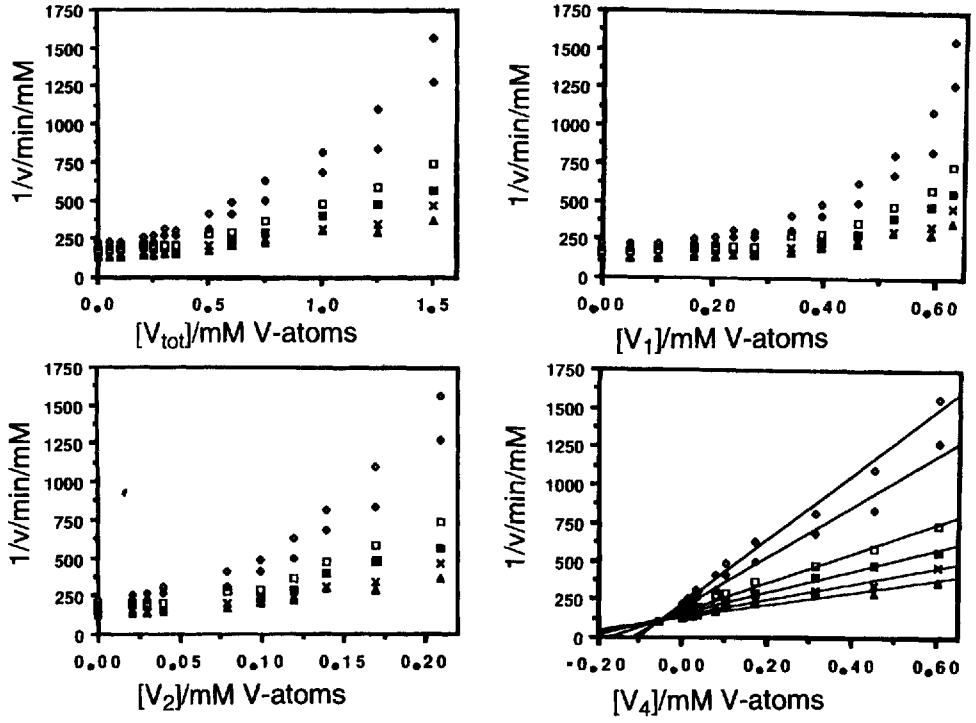



FIGURE 5 Dixon plots showing the reciprocal rates of 6-PGDH from *Torula* yeast catalyzed oxidation of 6-phosphogluconate plotted as a function of (a) total concentration of vanadium atoms, (b) $V_1$, (c) $V_2$, and (d) $V_4$. The oxovanadate populations in the assay solutions were determined by $^{51}V$ NMR spectroscopy. The 6-phosphogluconate concentrations in the figures were: 0.201 (▲), 0.151 (×), 0.101 (■), 0.0774 (□), 0.0504 (♦), and 0.0387 (◇) mM. Only the Dixon plot as a function of $V_4$ is linear through the entire concentration range examined. Adapted with permission from Ref. 40 (Copyright 1990 American Chemical Society).

## Vanadate Monomer, $V_1$

$V_1$ is the phosphate analog and as such is expected to interact with enzymes. In the past, effects induced by "vanadate" were attributed to $V_1$, and indeed in many cases the $V_1$ was inhibiting enzymes.[9] Potent effects observed at micromolar total vanadate concentrations are attributed to $V_1$. Enzymes catalyzing reactions at the phosphorus center, such as phosphatases and ATPases, are likely to be inhibited by $V_1$.[9,10] For example, the $Na^+$, $K^+$ ATPase is inhibited by $V_1$ with a $K_i$ of 4 nM.[14] Since $V_1$ is a much more potent inhibitor than phosphate, $V_1$ has been suggested to form a transition state analog in the active site of the enzyme.[12,88] The effects of $V_1$ on enzymes, which have been recognized for some time and reviewed elsewhere,[10] are beyond the scope of this review.



## Vanadate Dimer, $V_2$

The structural analogy of $V_2$ with pyrophosphate suggests that $V_2$ has the potential to interact with the binding sites of cofactors such as NAD, NADP, FAD, the corresponding reduced species, and CoA. Recently, the interactions of vanadate with a series of enzymes using nicotinamide based cofactors have been examined. The rapid exchange between $V_2$ and the other labile oxovanadates as well as the relationship between $V_2$ and $V_4$ (Eq. (4)) required that careful concentration studies be combined with enzyme kinetics to deduce effects caused by $V_2$ for a series of enzymes (Table I). Several of these enzymes are also inhibited by phosphate and pyrophosphate (Table I). No obvious correlation is observed between enzymes inhibited by pyrophosphate and $V_2$, suggesting $V_2$ is not a good pyrophosphate analog.

The interaction of vanadate with glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* is intriguing because $V_2$ is found to be a fairly good competitive inhibitor against NADP ($K_i$ = 0.090 mM) but only a weak competitive inhibitor against NAD ($K_i$ = 15 mM).[39] This finding suggests the $V_2$ is not binding at the pyrophosphate binding site of the cofactor but perhaps associating with the 3′-phosphate binding site. An earlier study of yeast G6PDH had focused on the interactions of vanadate with the cofactor through the adenine moiety.[89] However, de-

TABLE I

Inhibition constants for inorganic phosphate ($P_i$), pyrophosphate (PP), vanadate monomer ($V_1$) and vanadate dimer ($V_2$) for various enzymes.

| Enzyme | $P_i$/mM | $V_1$/mM | PP/mM | $V_2$/mM | Reference |
|---|---|---|---|---|---|
| GP6DH, torula yeast | >5C | NI | >5C | NI | 40 |
| GP6DH, sheep liver | >5C | NI | >5C | NI | 40 |
| GP6DH, human erythrocytes | >5C | NI | >5C | NI | 40 |
| GP6DH, *L. mesenteroides* | >5C | NI | >5C | NI | 40 |
| G6PDH, *L. mesenteroides* | | | | | |
| G6P (NAD as cofactor) | 70/C | NI | 30/NC | NI to >15 | 39 |
| NAD (NAD as cofactor) | vw | NI | | NI | |
| G6P/NADP as cofactor | ~200/ND | NI | 200/ND | NI | |
| NADP (NADP as cofactor) | | NI | | 0.090 to 0.16/C | |
| G3PDH rabbit muscle | NI (30)[a] | NI | NI (20)[a] | 0.27 ($H_2V_2O^{2}$ )/C 1.5 ($HV_2O_7^{3}$)/C | 41 |
| ADH, *Thermoanaerobium brockii* | NI (3)[a] | 1.1/C | | NI | 18 |
| Fructose 1,6-bisphosphate aldolase, rabbit muscle | 19.5/C | NI | 12.8/C | 0.23/C | 42. |
| Prostatic acid Phosphatase, human semen | 18/C pH 5.5 >0.80 pH 7.1 | 0.013/NC pH 7.1 | >25 | 0.70/NC pH 5.5 | 38 |
| Phosphoglycerate mutase, rabbit muscle | | | | $4 \times 10^{-3}$/C | 93 |

Abbreviations: C—Competitive, NC—non-competitive, ND—not determined, NI—no inhibition, vw—very weak.
[a]Highest concentration at which point no inhibition is observed.

tailed studies of the reactions between vanadate and adenosine and other nucleosides revealed that interactions with the base are minor.[90–92] In the studies with yeast G6PDH no attempt was made to identify which vanadium derivative was causing enzyme inhibition.[89] $K_i$ values were reported encompassing all oxovanadates.[89]

The interactions of vanadate with the cofactor site in yeast and *Leuconostoc mesenteroides* G6PDH enzymes were not corroborated in studies with the Torula yeast 6-phosphogluconate dehydrogenase (6PGDH),[40] lactate dehydrogenase (Crans and Simone, unpublished) and alcohol dehydrogenase (ADH) from *Thermoanaerobium brockii*.[18] $V_2$ was found to inhibit G3PDH with a noncompetitive inhibition pattern with respect to NAD.[41] In these studies $H_2V_2O_7^{2-}$ was found to be ~5 times as effective an inhibitor as $HV_2O_7^{3-}$. Perhaps the high $pK_a$ for $H_2V_2O_7^{2-}$ is in part responsible for the lower affinity of enzymes for $V_2$ than pyrophosphate.

$V_2$ does not only act as an inhibitor. Studies with G6PDH from Torula and bakers yeast show that $V_2$ activates both these enzymes (Crans and Mahroof-Tahir, in preparation). This activation is of interest, not only because it is observed in a purified enzyme system, but also because it illustrates an example of enzyme activation by an oxovanadate anion. In addition, it represents a very different type of enzyme activation than that observed with vanadate substrate and cofactor derivatives.[15–18,93] It is possible that the mechanism of activation of G6PDH by $V_2$ may reflect a mechanism in which binding of $V_2$ will alter the enzyme conformation slightly in the active site such that catalysis becomes slightly faster. The observed vanadate induced activation of G6PDH may represent a general mechanism by which vanadate can activate enzymes. It is conceivable that such a mechanism can account for vanadate-induced activation of protein kinases.[3]

Given the lack of similarity between $V_2$ and most substrates, interaction with substrate binding sites is not anticipated. Striking exceptions to this observation include the competitive inhibition pattern observed for the rabbit muscle fructose-1,6-bisphosphate aldolase.[42] This observation can be rationalized by the highly charged enzyme substrate and corresponding high charge densities at the substrate binding site. An even more dramatic effect is observed with phosphoglycerate mutase (PGM).[93] PGM binds one $V_2$ to each of its two subunits with an intrinsic dissociation constant of

$4 \times 10^{-6}$ M. These studies were carried out using $^{51}V$ NMR spectroscopy. Given the quadrupolar nature of the $^{51}V$ nucleus, the vanadium–protein resonance is broad and only the $+1/2$ to $-1/2$ transition is observable. Quantitation of the binding is thus based on the vanadate oligomer population since the quantitative information of the vanadium–protein signal is lost. Perhaps some of the effects of the vanadium–protein complex are transferred to the oligomer signals, making their quantification no longer exact (Mendz, unpublished). To date several applications of $^{51}V$ NMR spectroscopy to study vanadium–protein complexes have been reported.[49,52,93-101]

Binding constants determined by $^{51}V$ NMR spectroscopy can be compared to $K_i$ values obtained by enzyme kinetic studies even though the latter give only a measure for the affinity of these complexes for the enzyme and are not true intrinsic dissociation constants.[93] Vanadate is known to stimulate 2,3-diphosphoglycerate (2,3-DPG) phosphatase activity of PGM.[53] The binding studies suggest $V_2$ activates 2,3-DPG phosphatase activity consistent with a similar effect of pyrophosphate.[93] It is of interest to note that 0.025 mM vanadate in the presence of 20 mM phosphate also stimulates 2,3-DPG phosphatase activity. This observation is rationalized in terms of activation of the phosphatase activity through the formation of a mixed phosphate–vanadate anhydride, which then activates the PGM.[93] A model for the interaction of $V_2$ with the binding site was proposed (Fig. 6). Studies in hemolysates suggest the activation of human erythrocyte 2,3-diphosphoglycerate phosphatase by vanadate and vanadyl cation is a result of direct

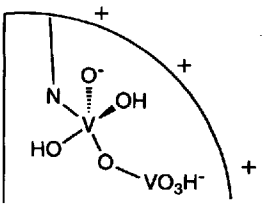



FIGURE 6 Schematic representation of $V_2$ bound at the catalytic site of PGM. Three positive charges indicated at the catalytic site of the enzyme illustrate favorable interaction of three negatively charged groups of the substrate 2,3-diphosphoglycerate. Adapted with permission from Ref. 93 (Copyright 1987 American Chemical Society).

action and not a consequence of changes in intracellular oxidants and reductants.[102] Activation of enzyme activity by vanadium derivatives has been reported for other enzymes and is likely to become more important in the future as the versatility of vanadate becomes more recognized.

An unexpected affinity for $V_2$ was observed by the human prostatic acid phosphatase.[38] Most phosphatases have affinity for $V_1$, but the human prostatic acid phosphatase appears to be inhibited by $V_2$ at low pH. Since this enzyme is inhibited by $V_1$ at neutral and alkaline pH, the observed effects could be associated with protonation of an amino acid residue affecting the binding of $V_2$ at low pH. In any event, the human prostatic and leishmanial acid phosphatases both show significant differences in interaction with a series of oxometalates compared to the interaction of human spleen acid phosphatase with the same compounds.[103]



## Vanadate Tetramer, $V_4$

The vanadate tetramer ($V_4$) has no structural analogy with enzyme substrates or cofactors and, as a consequence, was commonly believed to be an inactive form of vanadate.[9] In a 1990 study of 6PGDH from four different sources, $V_4$ was first shown to be a competitive inhibitor with respect to substrate and a non-competitive inhibitor with respect to the cofactor (Fig. 5).[40] Thus, the observed inhibition did not correlate with $V_{tot}$ concentration; and upon further analysis, no relationship was found with $V_1$ or $V_2$ concentrations. In contrast, $V_4$ showed a linear relationship in the entire concentration range. Other enzymes now known to be inhibited by $V_4$ include dehydrogenase,[18,39–41] isomerase,[43] aldolase[42] and phosphatidyl inositol-specific phospholipase[104] (Table II). $V_4$ was found to be a competitive inhibitor for G6PDH with respect to NAD[40]; however, for other dehydrogenases [lactate dehydrogenase (Crans and Simone unpublished), G3PDH[41] and ADH[18]] this pattern was not confirmed. In most cases (Table II) $V_4$ binding results in a competitive inhibition pattern with respect to the substrate. No obvious structural similarities exist between these substrates and $V_4$ to rationalize this affinity of proteins for $V_4$.

The binding site of $V_4$ on SOD was examined using various forms of spectroscopy and chemically modified SOD.[49] $V_4$ binds very

TABLE II

Inhibition constants for $V_4^a$ and other oxometalates. The $K_i$ values against substrates are given first, and the $K_i$ values against cofactors are given in parentheses.

| Enzyme | $K_i$ for $V_4$/mM | $K_i$ for $Mo_5^c$/mM | $K_i$ for $Mo_5^d$/mM | Reference |
|---|---|---|---|---|
| 6PGDH, torula yeast | 0.013/C<br>(0.15/NC) | 0.019/C | 1.4/C<br>(6.4/NC) | 40 |
| 6PGD, sheep liver | 0.063/C | | | 40 |
| 6PGDH, human erythrocytes | 0.078/C | | | 40 |
| 6PGDH, *L. mesenteroides* | 0.24/C | | | 40 |
| G6PDH, baker's yeast[b] | 0.5/NC[b]<br>(0.5/C)[b] | | | 89 |
| G6PDH, *L. Mesenteroides* | | | | 39 |
| G6P (NAD as cofactor) | 0.45/NC | 0.14/NC | 7.6/C | |
| NAD (NAD as cofactor) | (0.25/C) | (0.0015/C) | (1.3/C) | |
| G6P (NADP as cofactor) | 0.53/NC | 0.26/NC | 2.5/C | |
| NADP (NADP as cofactor) | (0.2/C) | (0.0036/C) | (0.075/C) | |
| G3PDH, rabbit muscle | 0.12/C<br>(0.67/NC) | 1.0/C<br>(2.3/NC) | 1.1/NC<br>(3.7/NC) | 41 |
| ADH, *Thermoanaerobium brockii* | 0.092/C | | | 18 |
| Fructose 1,6-bisphosphate aldolase, rabbit muscle | 0.52/C (irreversible) | 0.56/C | 0.73/C | 42 |
| Phosphoglucose isomerase, baker's yeast | 0.092/C | | | 43 |
| Superoxide dismutase, bovine liver | 0.00005 ($V_4$-binding)<br>0.0002 ($2V_4$-binding) | | | 50 |
| Myosin subfragment, rabbit leg and back muscles | (V1 site) 0.86<br>(V2 site) 0.40 | | | 107 |
| Phosphatidyl inositol, specific phospholipase C *Bacillus cereus* | 0.096/C | | | 104 |

[a]All $K_i$ values are given as mM $V_4$ molecules. This would correspond to four times the concentration as mM vanadium atoms.
[b]This $K_i$ value is estimated from the reported data in which no distinction of oxovanadates were made.
[c]$Mo_4$ abbreviation for $[(CH_3)_2AsMo_4O_{14}OH]^{2-}$.
[d]$Mo_5$ abbreviation for $[(NH_3C_2H_4P)_2Mo_5O_{21}]^{2-}$.

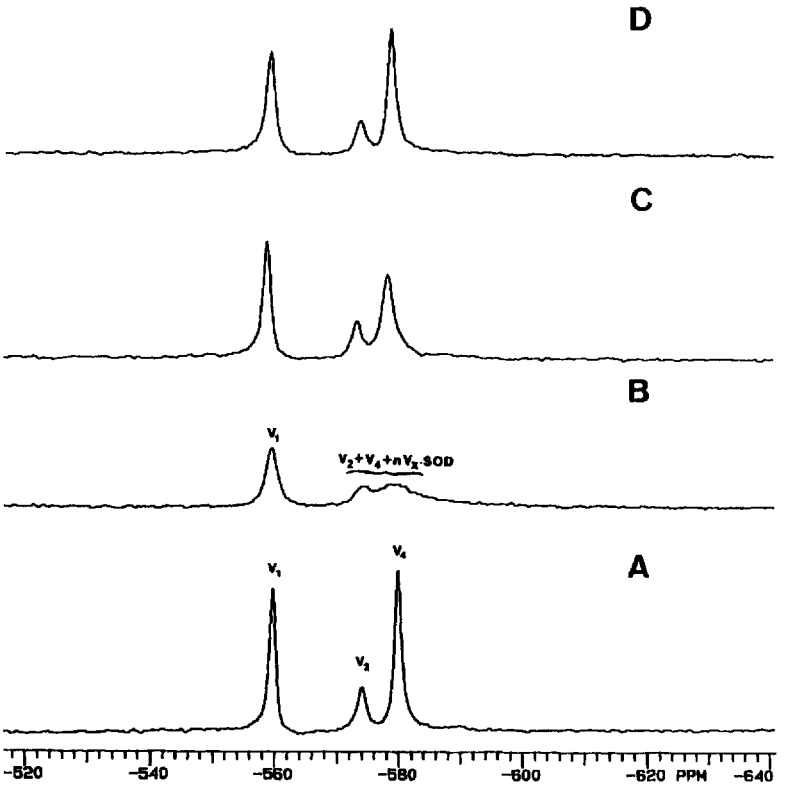



FIGURE 7 $^{51}V$ NMR spectra (79.0 MHz) of 2.0 mM vanadate in 0.10 M Hepes at pH 7.4 and 23°C in the absence (A) and presence of 0.15 mM native SOD (bovine liver Cu,Zn-SOD) (B), 0.15 mM arginine-modified SOD (C) and 0.15 mM lysine-modified SOD (D). Reprinted with permission from Ref. 49 (Copyright 1991 American Chemical Society).

tightly as seen from the disappearance of V-resonance in Fig. 7b compared to Fig. 7a. $V_4$ does not interact directly with the $Cu^{2+}$ center since no change was observed in the absorbance maxima of the enzyme in the presence of vanadate. When SOD was modified at Arg-141, the $^{51}V$ NMR spectra showed the modified protein still had a similar affinity for vanadate (Fig. 7c). SOD modified at Lys-120 and Lys-134, on the other hand, showed no observable binding of vanadate by $^{51}V$ NMR spectroscopy (Fig. 7d). Assuming that the chemical modification reaction did not alter the binding

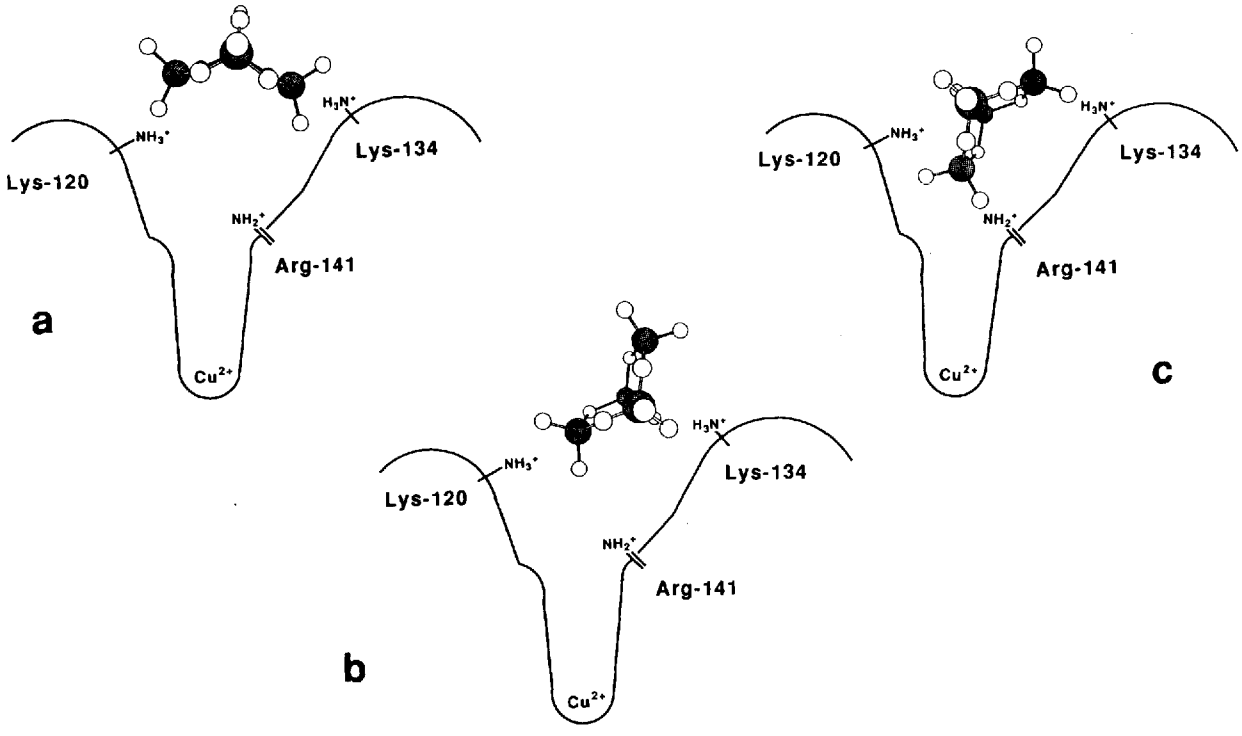



FIGURE 8 Three possible binding modes of the vanadate tetramer in the solvent channel of SOD: (a) $V_4$ spanning the Lys-120 and Lys-134 in inward cup shape mode, (b) $V_4$ Lys-120 and Lys-134 in outward cup shape mode and (c) $V_4$ spanning Lys-134 and Arg-141. Reprinted with permission from Ref. 49 (Copyright 1991 American Chemical Society).

site substantially, these results were interpreted as $V_4$ interacts strongly with the lysine residues in the active site.[49] Interaction of $V_4$ with the two lysine residues would block the entrance to the active site, and it was suggested that $V_4$ span the channel inward cup shape (Fig. 8a) or outward cup shape (Fig. 8b) since the cyclic tetramer has dimensions appropriate for such interaction. A third possible mode of interaction is shown in Fig. 8c.

Studies with the bovine erythrocyte SOD in Hepes and phosphate buffers, respectively, involving enzyme activity studies as well as equilibrium dialysis methods, challenge the high affinity observed by $V_4$ for the bovine liver SOD observed by the $^{51}V$ NMR binding studies.[105] Two reasonable explanations can be offered for this discrepancy. The experiments with the bovine erythrocyte SOD in a non-innocent buffer (phosphate) could mask some of the expected inhibition by $V_4$ in the activity studies. The lack of vanadium content in enzyme samples dialyzed to remove external vanadate suggests that $V_4 \cdot$SOD (and $(V_4)_2 \cdot$SOD) complexes form and dissociate rapidly. The reaction shown in (6) is reversible, and if the rates of dissociation of the protein complex are fast (faster than hours), protein bound $V_4$ will re-equilibrate as illustrated in (7). Due to Le Chatelier's principle, dialysis would eventually remove all the protein bound $V_4$ from the sample [(1) to (4) describe the equilibria summarized in (7)]. Thus, during dialysis, the $V_4$ will effectively be removed before the $(V_4)_2 \cdot$SOD complex can be isolated. This possibility was addressed by dialyzing 0.1 mM SOD against 2 mM vanadate, and a small but significant binding of vanadate was observed after gel filtration and neutron activation analysis.[105] Further dialysis removed this vanadium from the vanadate·SOD complex; however, this experiment shows that SOD is able to bind vanadate but that the complex is labile. It furthermore illustrates that even the brief gel filtration manipulations may have been sufficient to remove the SOD-bound vanadium. Another possible explanation is that the bovine liver and erythrocyte SOD enzymes respond differently to vanadate.[105]

$$2\ V_4 + \text{SOD} \rightleftharpoons V_4 + V_4 \cdot \text{SOD} \rightleftharpoons (V_4)_2 \cdot \text{SOD} \qquad (6)$$

$$V_4 \rightleftharpoons \text{equilibrium oligomer mixture } (V_1, V_2, V_4, V_5) \qquad (7)$$

A $^{51}V$ NMR binding study has also been carried out with the sarcoplasmic reticulum.[95] Solutions containing vanadate oligomers as well as decamer were added to isolations of sarcoplasmic reticulum containing $Ca^{2+}$-ATPase. The disappearance of the resonances of $V_2$, $V_4$ and $V_5$ were interpreted to indicate that vanadate oligomers bind to the protein.[95] However, since these oxovanadates are rapidly exchanging, the disappearance of oxovanadate signals implies that one of the labile oxovanadate species is binding to the protein, but does not indicate which one. A study exploring the interaction of vanadate with the sarcoplasmic reticulum $Ca^{++}$-ATPase using equilibrium binding techniques and radioactive $^{48}V$ shows that one V-atom binds per enzyme molecule.[106] This study confirms that one of the labile oxovanadates binds to the enzyme. Furthermore, it also provides an example of a vanadium–protein complex stable enough to be isolated by equilibrium binding techniques.

It has been suggested that $V_4$ is the species responsible for the vanadate-induced photolytic cleavage of myosin subfragment 1 (S1).[36,107,108] The vanadate induced photolysis results in cleavage of the protein backbone at a specific active site serine residue (Ser-180).[36,109] A similar cleavage site has been described for the dynein heavy chain.[110] Two cleavage sites termed V1 and V2 in myosin S1[111] describe the active site (V1) and a separate site (V2) that can be cleaved even when the active site is blocked.

Binding of $V_4$ to the protein is consistent with the possibility that this species is the photolytic active species; however, interaction of $V_4$ with the protein could occur in the absence of irradiation or photoinduced cleavage. Broadening and disappearance of the $V_4$ $^{51}V$ NMR signal upon addition of increasing amounts of myosin S1 (in the absence of radiation) was interpreted as conclusive evidence that $V_4$ is the active species.[107] The rapid exchange between vanadate oligomers and the changes in $^{51}V$ NMR signal linewidths at different conditions makes $^{51}V$ NMR spectroscopy a sensitive tool to examine interactions between proteins and $V_4$. However, the disappearance of the $V_4$ resonance upon binding to myosin S1 is dictated by the total population of free exchanging oxovanadates and would result whether the $V_4$ or other labile oxovanadate were bound to myosin S1. It is likely that $V_4$ is the active species responsible for the photoinduced cleavage[107,108];

however, the quantitative analysis supporting this suggestion has not yet been reported for any enzyme system.

An alternative explanation for the mechanism of cleavage has been put forward based on the observation that other cations such as Fe(III) and Rh(III) also induce similar chemistry.[35] It was therefore suggested that vanadium may mimic a cation substituting for $Mg^{2+}$.[35] Recent studies show vanadium(IV) solutions were not competent to cleave S1 at either the V1 or V2 site.[107] The suggestion by Cremo is finding support in studies associating $V_4$ populations with cleavage of the dynein heavy chain,[110] actin (Dabroviak, unpublished) and 6PGDH (Crans, Francova and Zamborelli, unpublished).

Adenylate kinase is inhibited by a vanadate oligomer which presumably is vanadate decamer (see below). The suggestion that the vanadate tetramer is the species responsible for interacting with adenylate kinase from the chicken muscle is thus very intriguing.[112,113] Vanadate-induced photocleavage of the chicken muscle enzyme was reported to occur at low vanadate concentrations, where only little $V_4$ is present.[112] However, no quantitative analysis or $^{51}V$ NMR data were reported. The photocleavage of adenylate kinase occurs at a proline residue and not at a serine residue and Cremo *et al.* point out that the proline is located in the sequence GGPGSGKGT (sequence no. 15–23) related to the sequence of the cleavage site in myosin (GESGAGKTV (sequence no. 178–186)).[112] The reasons for the differences in the cleavage reaction, as well as the nature of the oxovanadate interaction with the enzyme, is not yet understood. The photolytic studies were carried out on chicken muscle enzyme,[112] the inhibition studies on the rabbit skeletal muscle enzyme[114] and X-ray studies on pig muscle enzyme.[51] Given the similarity of the sequences in this region of the peptide chain, the different enzyme sources are not likely to account for the differences in the observations. In any event, the successful application of vanadate-induced photocleavage with enzymes such as adenylate kinase and myosin suggests that other enzymes, which would be expected to favor interactions with $V_1$, may very well show specific interactions with more complex oxoanions.

The possibility that $V_4$ interacts specifically with myosin S1 to cleave the enzyme at Ser-180 could be important. How would this

interaction occur? Formation of covalent $V_4$-protein derivatives is an intriguing possibility and chemical precedence exists for such complexes with $V_1$,[37,115-118] $V_2$ [83,119,120] and $V_{10}$ (Tracey, unpublished, and Ref. 121). No such complexes have yet been reported for $V_4$.[119,120,122] Alternatives to the $V_4$-protein complex include vanadium–protein complexes that formed as a result of the rapid decomposition of $V_4$ in the active site. One could envision that an active species is generated which would react with the protein to form a covalent vanadium–protein derivative that then would undergo the observed peptide cleavage. More information on the hydrolytic and redox chemistry of the reactions of $V_4$ and the other vanadate oligomers with and without ligands is desirable, since vanadium-based redox and/or radical chemistry is probably involved in the vanadate-induced photolytic cleavage reaction of proteins.

The affinity for $V_4$ by most enzymes is modest, since $K_i$ ranges between micro and millimolar concentrations (Table II). In contrast, the affinity of $V_4$ for bovine liver SOD[49] is so strong that it binds significantly to SOD even at low $V_4$ concentrations. Any affinity of these enzymes for $V_4$ was surprising considering its structure and the fact that vanadate pentamer ($V_5$) does not interact similarly with these enzymes.

The affinity of this series of enzymes for $V_4$ and $V_5$ was further examined by inhibition studies using compounds with similar structures.[43] Cyclic four- and five-membered oxomolybdates have similar shapes as $V_4$ and $V_5$, respectively. Although the conformation of the ring varies somewhat, the X-ray structures for $V_4$ and a cyclic oxomolybdate ($[(CH_3)_2AsMO_4O_{14}OH]^{2-}$, abbreviated $Mo_4$) show very similar bond distances and bond angles.[123] Accordingly, $V_5$ is expected to have similar structure as the five-membered cyclic oxomolybdate ($[NH_3C_2H_4P)_2Mo_5O_{21}]^{2-}$, abbreviated $Mo_5$).[124] When tested as inhibitors, $Mo_5$ is significantly poorer than $Mo_4$, reflecting the observed lower affinity in the vanadium derivatives (Table II). 6PGDH from Torula yeast was found to interact with $V_4$ with similar affinity as $Mo_4$. However, for G6PDH from *Leuconostoc mesenteroides* $Mo_4$ is a significantly better inhibitor than $V_4$.[43]

The specific vanadate-induced photocleavage of myosin S1 suggests that $V_4$ may be used as a specific probe of anion binding to enzymes such as those listed in Table II.

## INTERACTION OF LESS LABILE OXOVANADATES WITH ENZYMES

$V_{10}$ has been recognized to interact with enzymes since 1973 when it first was reported as a inhibitor for rabbit skeletal muscle adenylate kinase.[114] More recently, the decamer has been found to inhibit phosphorylase *a* and phosphorylase *b*,[52] hexokinase,[125] adenylate kinase from other sources,[51,125] phosphofructokinase from several sources,[125,126] fructose-1,6-bisphosphate aldolase,[42] and cAMP dependent protein kinase[50] (Table III).

TABLE III

Observed inhibition by vanadate decamer ($V_{10}$) of various enzymes.[a,b,c]

| | $K_i$ /M | $[V_{10}]$ for 50% inhibition /M | Reference |
|---|---|---|---|
| Adenylate kinase (rabbit muscle) | $1 \times 10^{-6}$ | | 114 |
| Fructose 1,6-bisphosphate aldolase (rabbit muscle) | $0.92 \times 10^{-3}$ | | 42 |
| Adenylate kinase (rabbit muscle) | | $11 \times 10^{-6}$ | 125 |
| Hexokinase | | | |
| *S. cerevisiae* | | $62 \times 10^{-6}$ | 125 |
| Rabbit muscle | | $62 \times 10^{-6}$ | 125 |
| Phosphofructokinase | | | |
| Sheep heart | $0.45/(5.5) \times 10^{-7}$ | | 126 |
| Rabbit muscle | | $10 \times 10^{-6}$ | 125 |
| Tunicate (*S. clava*) blood cells | | $12 \times 10^{-6}$ | 125 |
| *B. subtilis* (CE) | | $23 \times 10^{-6}$ | 125 |
| *E. coli* (WT; CE) | | $22 \times 10^{-6}$ | 125 |
| *E. coli* (mutant; CE) | | $26 \times 10^{-6}$ | 125 |
| *S. cerevisiae* (CE) | | $75-170 \times 10^{-6}$ | 125 |
| Phosphorylase b (Rabbit skeletal muscle) | $4 \times 10^{-6}$ (glucose 1-phosphate) | | 53 |
| | $6 \times 10^{-6}$ (activator, phosphite) | | 53 |

[a]Abbreviation CE—crude extract.

[b]Binding of $V_{10}$ was also observed with sarcoplasmic reticulum $Ca^{2+}$-ATPase (rabbit skeletal muscle) (Ref. 95), adenylate kinase (pig muscle) (Ref. 51), and cAMP-dependent serine-threonine protein kinase (Ref. 51).

[c]No inhibition was observed for galactokinase (*K. fragilis* (CE)), glycerolkinase (*C. mycoderma*), creatine kinase (rabbit muscle), pyruvate kinase (rabbit muscle), inorganic pyrophosphatase (*S. cerevisiae*) (Ref. 125).

The potent inhibition of adenylate kinase from rabbit skeletal muscle by $V_{10}$ was followed up by crystallographic studies of $V_{10}$ and pig liver adenylate kinase. These studies assigned the "$V_{10}$" binding as encompassing the phosphate binding sites. However, given the electron density observed, the authors point out that a species smaller than $V_{10}$ could possibly be bound.[51] In view of the proposed binding of $V_4$ to chicken muscle adenylate kinase, it is tempting to speculate that perhaps the smaller vanadate oligomer was indeed bound to the pig muscle adenylate kinase crystals. However, the reported structure was only refined to 6 Å, and some inconsistencies were observed upon further refinement of the structure of the protein only.[127] The high affinity of adenylate kinase for $V_{10}$ could explain the differences that are observed between the adenylate kinase and myosin photolytic reaction since a different oxovanadate would be responsible for the reaction. However, the latter interpretation would be inconsistent with the deliberate efforts to prepare the vanadate solutions free of decamer.[112]

In binding studies of the $Ca^{2+}$-ATPase in sarcoplasmic reticulum preparations using $^{51}V$ NMR spectroscopy, the decamer signals disappeared upon addition of protein.[95] The decamer, in contrast to the labile oxovanadates ($V_1$, $V_2$, $V_4$, and $V_5$), is sufficiently long-lived that the disappearance of NMR signals can be attributed to protein binding. Interestingly, the resonances disappeared upon addition of less $Ca^{2+}$-ATPase than the resonances did for the labile oxovanadates. This observation shows that $V_{10}$ has a greater affinity for the protein than $V_1$ or the other labile oxovanadates.[95]

The inhibition of phosphorylase by oxoanions is particularly intriguing because the studies clearly reveal a preferential interaction with larger oxoanions such as the decavanadate ($V_{10}O_{28}^{6-}$) and the corresponding decatungstate ($W_{10}O_{32}^{4-}$).[52] The pH and concentration profile of the inhibition pattern suggests that $V_4$ also may be interacting with this enzyme. A time-dependent inactivation by both the decavanadate and the decatungstate was attributed to deformation of the coenzyme site on the basis of UV studies with decatungstate and phosphorylase *b*. From the inhibition patterns and the spectroscopic studies the authors suggest that the bound oxoanions encompass the glucose-1-phosphate and pyridoxal 5′-phosphate binding sites.[52] The authors question whether the clustering of positively charged residues in the regions of the substrate

and cofactor binding sites is sufficient for binding of the oxoanions or whether other structural and conformational features are important.

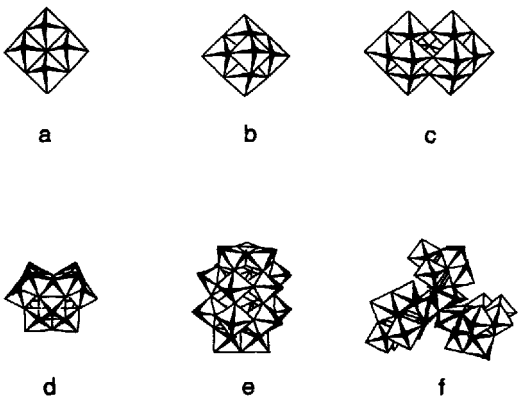
A recent study with the cAMP-dependent protein kinase suggested yet another mode of action for oxovanadates.[50] $V_{10}$ was found to be a non-competitive inhibitor against substrate, kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly). When exploring this interaction further it appeared that $V_{10}$ interacts with the kemptide and prevented the kemptide from binding to the protein kinase. Not only does this represent a novel mechanism by which oxovanadates can act, but it also shows that these types of oxoanions can have affinity for specific amino acid sequences.

## INTERACTION OF OTHER OXOMETALATES WITH ENZYMES



For the past two decades most of the biological studies with other oxometalates (including polyoxometalates) were concerned with the *in vivo* and *in vitro* antiviral, anti-leukemic and anti-human immunodeficiency viral properties of these compounds.[56–63,128–136] One compound, HPA-23 ($(NH_4)_{17}Na[Sb_9W_{21}O_{86}]$) has been extensively examined as a potential anti-HIV-1 agent[60] and with respect to its affinity for various proteins.[137,138] Characterization of the interactions of various polyoxoanions from the point of view of correlating structure with biological activity is difficult, and attempts to do so with the anti-HIV activity have met some success and difficulties.[56,57] The binding of the polyoxometalates to the cell surface may be responsible for the anti-HIV activity.[128] Oxometalates (oxovanadates, $[W_3O_2(O_2CCH_3)_6]^{2+}$) and polyoxometalates ($[NaP_5W_{30}O_{110}]^{4-}$) have also found use in the crystallization of proteins,[51,139–141] as well as cytochemical stain for localizations of proteins (representative reference 142). When combined, these studies demonstrate the high affinity of some oxometalates for proteins, and the need for understanding details of the protein interactions. Identifying the molecular sizes, shapes, surface charge and species of polyanions that are particularly favorable for protein interactions should be useful for the more complex task[143] of successful drug design.

For the purpose of discussing the effects of various compounds, we first illustrate the structural categories of the oxometalates and the polyoxometalates. Oxometalates and polyoxometalates are polyanionic, condensed oligomeric aggregates of transition-metal ions in the $d_0$ electronic configuration and oxide ions (for a recent review see Ref. 144). Five types of ions form these types of complexes, $V^V$, $Nb^V$, $Ta^V$, $Mo^{IV}$ and $W^{VI}$ in which the $MO_6$ octahedra is the principal unit. Scheme I illustrates these complexes, the cyclic tetrameric unit exemplified by $R_2AsMo_4O_{14}OH^{2-}$ or $W_4O_{10}(O_2)_6^{4-}$ (a), the hexametalate (Lindquist structure), $W_6O_{19}^{2-}$ (b), the decametalate structure, $W_{10}O_{32}^{4-}$ (c), the Keggin structure, $(X^{n+}W_{12}O_{40})^{(8-n)-}$ (d), the Dawson (or Wells–Dawson) structure, $(P_2W_{18}O_{62})^{6-}$ (e) and the HPA-23 (9-antimonio(III)-21-tungsten(VI)-sodate), $[NaSb_9W_{21}O_{86}]^{18-}$ structure (f). The smaller derivatives tend to carry a high localized charge in a fairly flexible and loose geometry and are likely to respond to protein surfaces with localized charges. The larger, more compact and inflexible Keggin and Dawson structures delocalize the charge. Unless the latter polyoxoanions are modified, they are less likely to interact through simple localized coulumbic charge interactions than the smaller oxoanions. For the purpose of this review we will




SCHEME I Schematic illustrations of various polyoxometalates using octahedrons to represent one metal atom surrounded by six oxygen atoms. (a) simple tetrameric oxometalate, (b) hexametalate (Lindquist structure), (c) decametalate, (d) the "Keggin" structure, (e) the "Dawson" (or "Dawson–Wells") structure and (f) the 9-antimonio(III)-21-tungsten(VI)-sodate structure (present in HPA-23).

focus on the structure of the oxoanion, although reports have documented that the cations can affect the biological activity.[57,103]

The effects of a series of oxometalates were examined with the purpose of identifying compounds that showed selective affinity for acid phosphatases of parasite (*Leishmania* spp) and human prostate gland origin. Early studies of polyoxomolybdates of several types (Scheme I) showed remarkable selectivity in their interaction with acid phosphatases from several sources.[145,146] The activities of simple oxomolybdates, as well as a Dawson structure, led these workers to pursue a systematic study of a series of polyoxomolybdates.[103] The simple tetrameric unit was found to interact specifically with both the Leishmanial and the human prostatic acid phosphatases compared to the human spleen acid phosphatase and other enzymes.

Studies with a series of dehydrogenases (and one aldolase) have also been carried out with the purpose of determining whether the cyclic $Mo_4$ derivatives had greater affinity than cyclic $Mo_5$ derivatives (see above).[43] As shown in Table II, the enzymes examined have a much greater affinity for the former than the latter. The protein affinity for $Mo_4$ over $Mo_5$ mimics the protein affinity of $V_4$, suggesting that the tetrameric unit interacts particularly well with proteins. Whether these observations are only related to the shape of the molecule or are also based on other factors remains to be determined. Some enzymes (6PGDH, aldolase) respond similarly to both the $V_4$ and $Mo_4$ units, suggesting the shape is most important for their affinity, whereas others (G6PDH, G3PDH) show differences affecting the $K_i$ up to a factor of 10, suggesting that other factors are also important.[43] No attempts have yet been made to correlate amino acid sequence with these observations.

The Leishmanial and the human prostatic acid phosphatases had little affinity for the Lindquist structure. However, the addition of one or two $MO_6$ units generated a derivative that showed medium to high affinity and selectivity toward Leishmanial and the human prostatic acid phosphatases.[103] Little anti-HIV-1 activity was observed for compounds with four and eight metal atoms as well as the Lindquist structure in contrast to observations with the decametalates.[57,128] Interactions of decavanadate and decatungstate with rabbit skeletal muscle phosphorylase were strong and resulted in

TABLE IV

Inhibition of HIV-1 reverse transcriptase activity by polyoxometalates. Adapted from Ref. 128.

| Compound | $IC_{50}$[a] Recombinant Reverse Transcriptase /μM | $IC_{50}$[a] Native Reverse Transcriptase /μM |
|---|---|---|
| Hexametalate | | |
| $[Na/K]_6[Nb_4W_2O_{19}]\cdot 12H_2O$ | >12.2 | >12.2 |
| Keggin | | |
| $K_5[BW_{12}O_{40}]$ | 0.58 | 1.85 |
| $K_6[BGa(H_2O)W_{11}O_{39}]\cdot 15H_2O$ | 0.02 | 0.19 |
| $K_5[Si(TiCp)W_{11}O_{39}]\cdot 12H_2O$[b] | 0.28 | 1.74 |
| $[Me_3NH]_8[Si_2Nb_6W_{18}O_{77}]$ | 0.23 | 1.06 |
| Keggin Sandwich | | |
| $Na_7[PrW_{10}O_{35}]\cdot 18H_2O$ | >6.6 | >6.6 |
| $K_{13}[Ce(SiW_{11}O_{39})_2]\cdot 26H_2O$ | 0.04 | 1.25 |
| $K_{10}[P_2Zn_4(H_2O)_2W_{18}O_{68}]\cdot 20H_2O$ | 0.23 | 3.6 |
| Dawson | | |
| $K_{12}H_2[P_2W_{12}O_{48}]\cdot 24H_2O$ | 0.16 | 0.9 |
| $K_8H[P_2V_3W_{15}O_{62}]\cdot 34H_2O$ | 0.01 | 0.08 |
| Others | | |
| $[NH_4]_{17}Na[NaSb_9W_{21}O_{86}]\cdot 14H_2O$[c] | 0.18 | 1.48 |
| [(+)-(*S*)-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazol[4,5,1-jk][1,4]-benzodiazepin-2(1*H*)thione] | 0.14 | 0.24 |

[a]$IC_{50}$ is defined as the concentration required to inhibit reverse transcriptase activity by 50%. The template/primer was poly(C)-oligo$(dG)_{12-18}$ and the enzyme preparations tested were either recombinant reverse transcriptase (p66) or HIV-1(111[b]) virion-derived native reverse transcriptase.

[b]Cp = cyclopentadienyl.

[c]HPA-23.

similar inhibition patterns both quantitatively and with respect to the time-dependent inactivation.[52]

The Leishmanial and the human prostatic acid phosphatases showed high selectivity toward the heteromolybdate compounds with the Keggin structure as long as the heteroatom was fairly small. Both $GeMo_{12}O_{40}^{4-}$ and $PMo_{12}O_{40}^{4-}$ interacted strongly, whereas $SiMo_{12}O_{40}^{4-}$, $ThMo_{12}O_{40}^{8-}$ and $CeMo_{12}O_{40}^{8-}$ interacted more weakly

and/or showed lower specificity.[103,145] Anti-HIV-1 activity of compounds with Keggin or Keggin sandwich type structure is also high, and the toxicity to uninfected cells is low.[56,57,128] The compounds inducing the highest activities are heteropolytungstates, whereas the heteropolymolybdates showed a much lower affinity. The hybrid $PMoW_{11}O_{40}^{3-}$ shows greater activity than the parent polyoxotungstate, $PW_{12}O_{40}^{3-}$, suggesting that in a few cases similar subtle structural changes may affect the whole cell activity.[57] Compounds with the Keggin structure are in general potent inhibitors of HIV-1 reverse transcriptase requiring submicromolar concentrations to reduce the activity to 50% (Table IV).[128] However, there is a poor correlation between the anti-HIV activity of the compounds in cell culture and the inhibitory effects on reverse transcriptase activity. In contrast, the ability of the compounds to inhibit binding of anti-gp120 mAb to pg 120 correlated well with their anti-HIV activity.[128] It was therefore concluded that these compounds occupied sites on the cell surface required for the viral envelope to interact with the cell surface. These conclusions were supported by recent studies with PM-104 ($(NH_4)_{12}H_2(Eu_4(MoO_4)(H_2O)_{16}(Mo_7O_{24})_4) \cdot 13\ H_2O$).[147]

The Leishmanial and human prostatic acid phosphatases showed strong affinity and selectivity for the Dawson structure as exemplified by $As_2Mo_{18}O_{62}^{6-}$ [103]; however, the selectivity was lost if As was substituted with P.[145] The small change in shape and electronics of these clusters suggests the protein affinity is very selective to both factors. Corresponding heterotungstates also have potent anti-HIV activity and exhibit low toxicity.[56,57,128] They are potent inhibitors for HIV-1 reverse transcriptase (Table IV), but also absorb potently to the cell surface.[128]

The interaction of HPA-23-derived polyoxoanions (the central $Na^+$ substituted by another cation) with a series of polymerases including the reverse transcriptase from murine Moloney leukemia virus (M.MLV) and from mouse mammary tumor virus, and the *E. coli* DNA and RNA polymerase, was characterized.[137] The HPA-23 derivatives inhibit the M.MLV reverse transcriptase and DNA polymerases competitively with respect to the template, so these polyoxoanions are presumably binding at the template binding site. The kinetic analysis suggested that the inhibition of the *E. coli* RNA polymerase was irreversible, probably as a result of

the dissociation of the RNA pol-HPA complex. Substituting the central $Na^+$ in HPA-23 with $K^+$, $Sr^{2+}$ or $Ca^{2+}$ changed the inhibition somewhat. It appears that the charge ratio, and minor changes in the structural parameters of the polyoxoanion, induced by the central cation, did affect the interaction with the protein. The $K^+$ derivative was surprisingly poorer than the others. These authors also examined the effects of varying the central cation in a series of tungstoarsenates (TAs, $MAs_4W_{40}O_{140}^{27-}$) with these enzymes; however, in this case they found no evidence for similar dramatic effects of the protein affinity when the central cation varied from $Na^+$, $K^+$ to $Ba^{2+}$.[137] The $K_i$ values for the M-MLV reverse transcriptase are $2 \times 10^{-8}$ M for HPA and $10^{-8}$ M for TAs. The $K_i$ values for the *E. coli* DNA POL I are $3 \times 10^{-7}$ M for HPA and $2 \times 10^{-9}$ M for TAs. Thus, despite the large differences in these polyoxoanions, little difference was observed for the reverse transcriptase, although a large difference was observed for the DNA POL I. The enzymes tested are also very different, although enzymes converting large anionic substrates presumably enjoy similar structural strategies in the substrate binding sites. The similar high affinities observed are perhaps a reflection that enzymes with such binding sites will show high affinity for any polyoxoanion of a certain size. It is possible that a better reflection of peptidic affinity for polyoxoanions will be observed in studies of polyoxometalates with other types of protein. Preliminary results examining the interactions of a series of lanthanide oxotungstates with proteins such as bovine serum albumin do show significant interaction (Francesconi, unpublished).

A study of horse heart cytochrome *c* and its interaction with $SiW_{11}O_{39}^{8-}$, $NaSb_9W_{21}O_{86}^{18-}$ and $KAs_4W_{40}O_{140}^{27-}$ shows high affinity for these large polyoxometalates.[138] The silicotungstate forms two complexes with cytochrome *c*, a 1:1 and a 2:1 complex, and these complexes show little structural perturbation of the protein upon binding of the Keggin polyoxoanion. Interaction of cytochrome *c* with the Keggin structure ($\alpha$-$K_8SiW_{11}O_{39}$) did, however, increase the reversibility of the electrochemistry of cytochrome *c* on gold and glassy carbon electrodes.[148] The interaction with the two larger polyoxoanions resulted in a greater structural perturbation on the protein. Two forms of the tungstoarsenate–cytochrome *c* (1:1) complex are generated. Three forms of the tungstoantimonate–

cytochrome *c* (1:1) complex are generated. It was suggested that these complexes mimic the states the protein occupies during the catalytic reaction.[138] The complexes that form from the two largest polyoxoanions have association constants about the size of the complex that forms between cytochrome *c* and cytochrome $b_5$. The authors propose that polyoxoanions can be used to mimic protein–protein electrostatic interactions.[138]

Correlation of biological activities with redox chemistry has been described for a series of oxomolybdates.[149,150] The oxomolybdate $Mo_7O_{24}^{6-}$ has potent *in vivo* antitumor activity against Meth-A sarcoma, MM-46 adenocarcinoma, and MX-1 human breast, OAT human lung, and CO-4 human colon cancer xenografts.[149] When $Mo_7O_{24}^{6-}$ was photochemically reduced it generated a $Mo^{V}O_5(OH)$ site within the $Mo_7O_{24}^{6-}$ ion (in contrast to the $Mo^{VI}O_6$).[149] Since the solutions of $Mo_7O_{24}^{6-}$ photochemically treated are found to be more toxic, it is possible that the reduced oxometalate is responsible for the toxicity.[149] $Mo_7O_{24}^{6-}$ interacts with a flavin mononucleotide (FMN) to yield a 1:1 complex with a redox potential 0.1 more positive than FMN.[150] The authors propose that the $Mo_7O_{24}^{6-}$ directly or as a $Mo_7O_{24}^{6-}$ FMV complex may interfere with the electron transfer chain in mitochondria.[149,150] Although the latter mechanism may not directly involve oxoanion protein interactions, the viability of such a mechanism will require that this oxometalate does *not* interact significantly with proteins in order to be free to interact with the cellular FMN. Human seminal fluid and *Leishmania donovani* acid phosphatases showed high affinity for $Mo_7O_{24}^{6-}$, whereas neither the human spleen acid phosphatase, α-mannosidase and β-glucuronidase showed affinity for this ion.[103] Further studies characterizing the interactions of $Mo_7O_{24}^{6-}$ with cellular metabolites and proteins are needed to substantiate the proposed mechanism. In any event, the observations are intriguing and raise the possibility that the redox chemistry of oxometalates could be an important factor for inducing biological activities.

The studies described above show that with the exception of the $Mo_5$ and Lindquist structures most oxometalates and polyoxometalates examined bind strongly to some protein. In many cases the oxometalates and polyoxometalates are presumed (on the basis of kinetic studies) to bind at the substrate or cofactor site in the protein. Perhaps these types of interactions take place because of

specific protein–oxometalate interactions, including both electronic and structural fits. Such interactions are therefore very different than the cases in which the protein binds a polynucleotide of polydeoxynucleotide substrate. These enzymes will interact strongly with all the large anions and will show a deceptively high and similar affinity for all polyoxovanadates. In the case of such enzymes, structural relationships of oxoanions with observed enzyme activity require that a wide range of oxometalate geometries be examined. At the present time, only the effects on polymerases of large polyoxometalates with some structural similarities have been examined. It is possible that should a systematic study include smaller oxometalates, as in the study with the Leishmanial phosphatase, some different protein affinities will emerge. The high affinity of Leishmanial and human prostatic acid phosphatase for heteromolybdates is intriguing, particularly since the cell surface appears to have a much stronger affinity for corresponding heterotungstates.

Protein interaction with oxovanadates show many similarities with those of other oxometalates. When the corresponding oxovanadate derivative exists, such a compound presents a unique possibility for monitoring the interactions by $^{51}V$ NMR spectroscopy. Since the number of oxovanadates that are stable under physiological conditions is limited, additional heteropolyoxoanions will be needed for any systematic study. The limited stability of some of these materials can complicate the enzyme studies. Studies with the Leishmanial and human prostatic acid phosphatase showed a subtle effect in enzyme activity upon the increase of the size of the center metal ion in the Keggin structure. Similar changes in reverse transcriptase activity studies with the larger polyoxoanions were not observed. It is possible these differences are a result of geometric parameters being affected more by central metal ion in the smaller polyoxometalates, or alternatively the insensitivity of reverse transcriptase to changes in large polyoxometalates. Other factors could also play a role. One such factor, redox potential, is an intriguing possibility, although the experimental evidence currently supporting redox chemistry as a contributor to the activities and toxicities of polyoxometalates is difficult to evaluate in a structural manner (Yamase, unpublished). In view of the studies with oxovanadates and the similarities of proteins interacting with ox-

ovanadates and other polyoxometalates, it is likely that some amino acid sequences and three-dimensional structural array will have high affinity for specific oxometalates.

## PHYSIOLOGICAL RELEVANCE OF OXOVANADATES

The oxovanadates form at significant concentrations only at millimolar total vanadate concentrations, and thus will not commonly be observed under physiological conditions. It is beyond the scope of this article to review extensively the literature on the presence of vanadium in biological systems (recent reviews include Refs. 10 and 151). A few points apparent from metabolic studies in yeast are relevant to the enzyme interactions of oxovanadates and other oxometalates described here will be discussed briefly (for a review see Ref. 10).[152-154]

*In vivo* studies of vanadium metabolism in yeast show that under conditions where the yeast are growing (1 mM vanadate) there is no evidence for significant concentrations of vanadium(V) inside the cells (that is "cell-associated vanadium").[152] One resonance now assigned to $V_1$ was observed for the cell sample. Given the difficulties in conducting these experiments, some carry-over of the media can be anticipated and the low levels of observed cell-associated vanadium may originate from the media trapped between the cells. At high concentrations of $V_1$ the cells had ceased to grow, and the toxicity was attributed to the presence of $V_1$.[152] The presence of $V_4$ as cell-associated vanadium is evidenced by a shoulder on the $V_1$ resonance. The fact that little (if any) $V_4$ was observed in growth inhibited cells in a media containing excess vanadate (and $V_4$) suggests that the cell will prevent the accumulation of high concentrations of free $V_4$. It is not yet known whether the lack of $V_4$ accumulation is related to a low intracellular vanadium(V) concentration (suggesting $V_1$ is the toxic species) or complexation of $V_4$ (as $V_4$ or $V_2$ being the toxic species) or a combination thereof. The broad $^{51}V$ NMR signal expected for vanadium bound to a macromolecule would prevent the observation of a protein-bound $V_4$ (or $V_2$). The possibility that either $V_2$ or $V_4$ is the toxic species is consistent with the observations and should be explored.

Mutants capable of growing at higher concentrations of vanadate have now been reported; however, the observed cell-associated vanadium in the form of $V_1$ and $V_4$ levels is never high.[152,153] It appears that the ratio of labile oxovanadates to $V_{10}$ is decreased in the mutant. The reduction of vanadate to vanadium(IV) and its subsequent release from the cell have been suggested to be a detoxification mechanism. These observations are also consistent with the interpretation that $V_4$ (or $V_2$) are toxic species since the levels of these species are also decreased in the mutant.

$V_{10}$ is not likely to be present at significant concentrations under normal physiological conditions. It has, however, been observed as cell-associated vanadium in yeast in the presence of high concentrations of vanadate (Willsky, unpublished). It is possible that vanadium is accumulated in cell organelles where the acidic conditions will promote $V_{10}$ formation. $V_{10}$ could thus form as a brilliant detoxification mechanism because once formed in a cellular compartment, $V_{10}$ will remain there since it is not likely to be transported across membranes easily. Eventually $V_{10}$ will be excreted to the cell medium where it no longer will be able to enter the cell. Control experiments have shown that $V_{10}$ does not significantly affect the growth of yeast until very high concentrations (Willsky, unpublished). This observation is of particular interest in view of the recent studies with oxometalates that suggested heteropolytungstates with Keggin and Dawson structures bind potently to the cell surface.[128,147] Although the cell surfaces in the two systems are significantly different, some kind of transport mechanism across membranes is required if the anion is to enter the cell. One possibility, illustrated by these *in vivo* studies, involves formation of $V_{10}$ inside the cell from smaller components. An alternative possibility requires that the polyoxometalate is covered by organic and lipophilic residues such that membrane transport can be feasible.



## CONCLUDING REMARKS

Various types of interactions of oxovanadates ($V_1$, $V_2$, $V_4$, $V_5$, and $V_{10}$) with proteins have been reviewed. The labile oxovanadates ($V_1$, $V_2$, $V_4$ and $V_5$) bind with varying affinities to enzymes, and

although these species exchange on a millisecond time scale in aqueous environments, experiments can be designed to probe which derivative has the greatest affinity for the protein. Both hydrolytic and redox chemistry can be observed with these oxovanadates. The vanadate tetramer ($V_4$) is of interest because even if it shows only a modest affinity for most proteins, this species may be useful as a probe to study anion interactions with enzymes through photolytic cleavage reactions. The $V_{10}$ is less labile and is also found to interact with surprising affinity with several proteins. The decametalate and several other complex polyoxometalates are of particular interest because of their potent antiviral activities. At this time we divide the enzyme interactions with oxometalates and polyoxometalates into two major categories. The first category involves those oxometalates and polyoxometalates which mimic substrates and cofactors and which may be involved in hydrogen bonding, other types of electrostatic interactions, and steric interactions. Enzymes such as phosphatases, dehydrogenases, aldolase, isomerases, and superoxide dismutase have exhibited this type of interaction with oxometalates and/or polyoxometalates. The high affinity of some of the oxometalates described here for proteins would suggest certain affinity for specific peptide sequences. The second category involves oxometalates and polyoxometalates that interact with proteins by mimicking protein–protein interactions or by utilizing other types of interactions in which the charges are no longer as localized on the surface of the protein or the polyoxoanion. In several cases, polymerases and transcriptases exhibit these types of interactions with polyoxometalates. Depending on the enzyme and the type of reaction the enzyme catalyzes, a particular oxometalate may interact differently. Understanding the nature of protein interactions with oxovanadates and other oxometalates is important for further development of drugs based on vanadium complexes and polyoxoanions.



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