Speciation in Vanadium Bioinorganic Systems. 4. Interactions between Vanadate, Adenosine, and Imidazole—An Aqueous Potentiometric and ^{51}V NMR Study

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Abstract: The potential biological activity of vanadium analogs of AMP, ADP, ATP, 2′,3′-cAMP, and 3′,5′-cAMP stimulated the full speciation study of the vanadate−adenosine(AdH) and vanadate−adenosine−imidazole(ImH) systems in aqueous solution, using a combination of potentiometry (glass electrode) and ^{51}V NMR spectroscopy. The study of the $H^+-H_2VO_4^-$−AdH−ImH system was performed in 0.600 M Na(Cl) medium at 25 °C in the pH range 2−11. In the vanadate−adenosine system $V_2Ad_2^{2-}$ and a new complex, $V_2Ad_2^-$, with log $\beta = 7.68 \pm 0.01$ and 11.89 ± 0.08, respectively ($pK_a = 4.21$), explained all experimental observations. Although the $V_2Ad_2^-$-type complex has previously been reported in the vanadate−AMP system, the existence of such a complex in a vanadate−nucleoside system was not previously appreciated. In the vanadate−adenosine−imidazole system a ternary mixed ligand complex, $VAdIm^-$, forms in addition to the $V_2Ad_2^{2-}$ and $V_2Ad_2^-$ species. It exists between pH 5.5 and 11 and has a formation constant log $\beta = 3.04 \pm 0.02$. This is the first ternary complex of this type that has been characterized in a qualitative (stoichiometry) and quantitative (formation constant) manner. Although the complex is fairly weak and requires a large excess of imidazole to form, it is significantly more stable than the 1:1 complexes that previously have been reported to form between vanadate and adenosine. Above all, it is much more stable than the complexes that eventually form between vanadate and imidazole. The possibilities that intramolecular imidazole stacking and/or intermolecular hydrogen bonding explain the enhanced stability in the ternary complex are discussed. Furthermore, the action of various vanadium−adenosine derivatives and the potential role of vanadate−adenosine−imidazole complexes in biological systems is evaluated.

Introduction

The nature and properties of complexes formed between vanadate and adenosine (Figure 1a) have intrigued chemists and biologists for the last decade because of the possible wide range of analogs that can form, including enzyme cofactors, substrates, and secondary messengers (AMP, ADP, ATP, and cAMP).[1] Interest in the formation of the vanadate−adenosine complexes originates in the recognition that vanadate is structurally and electronically analogous to phosphate.[2] The vanadate−phosphate analogy has furthermore been demonstrated in enzyme systems since several vanadate esters act as their corresponding phosphate ester substrates for various enzymes.[3,4] For example, a solution of adenosine and vanadate can act as a substrate analog of AMP for adenylate kinase.[3b] The reaction between vanadate and adenosine has previously been studied with ^{51}V, ^{1}H, and ^{13}C NMR spectroscopy in the neutral to slightly alkaline pH range.[5−12] As in the cases of other vanadate esters, an equilibrium mixture of complexes has been observed depending on the specific conditions employed in the studies. However, to the dismay of those interested in the biological properties of these species, the major complex in solution is a 2:2 complex.[5,8−12]

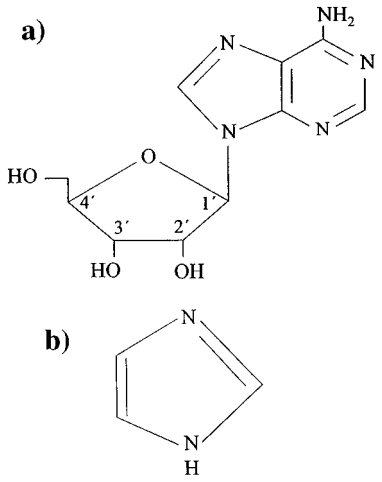

Figure 1. Schematic structure of (a) adenosine and (b) imidazole.

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(1) AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate.

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Such a complex has also been found in a crystalline compound obtained from aqueous solution.[13] The 1:1 complexes with the presumed interesting biological properties are only very weak,[5,8,9,11] if present at all.

Vanadate complexes of other vicinal diols, including ethylene glycol[12,14] and β-methyl riboside,[12] and additional nucleosides and diols, have also been studied. With the exception of two isolated reports[6,7] the concensus is, as above, that the major complexes are 2:2 complexes.[5,8−13] A variety of techniques, including multinuclear[9,11,12] and dynamic NMR spectroscopy,[9,12] circular dichroism,[10] magnetic circular dichroism,[10] IR spectroscopy,[12] and Raman spectroscopy,[12] have been used to characterize the structure of the major 2:2 complex that forms between a vicinal diol and vanadate in aqueous solution. Characterization of most of these materials in the solid state has been limited by the inability to crystallize and to solve diffraction patterns for many of these compounds.[15] However, the X-ray structure of a 2:2 vanadium(V)−adenosine complex has recently been reported,[13] and it shows similar structural characteristics to several model systems that were developed to characterize this system.[16−19] Despite the interest in and the many techniques employed to probe this system,[5−12] there has been no prior examination of the solution speciation by potentiometry or a more accurate combined potentiometry−NMR analysis. In order to probe the vanadate−adenosine system in the presence of imidazole we have carried out the analysis of the vanadate−adenosine system over a wide pH range using a combined potentiometric−^{51}V NMR study.

Imidazole (Figure 1b), in the form of histidine, plays an essential role in many enzyme mechanisms, including enzymes which are known to interact potently with vanadate.[20,21] For the past few years, evidence has been accumulated which suggests that imidazole, in the form of a histidine derivative, forms vanadium(V) complexes not only in organic solvents[22,23] but also in aqueous solution.[7,24−26] X-ray crystallographic model studies have shown that vanadium(V) forms both six-coordinate[22] and five-coordinate[23] complexes with multidentate ligands containing an imidazole residue. Interestingly, the five-coordinate complex contains an imidazole moiety that is not coordinated to the vanadium and attests to the versatility of the imidazole residues to support binding and catalyze reactions in enzyme active sites. Indeed, a recent X-ray structure of the rat acid phosphatase−vanadate complex shows the histidine residue chelated in the axial position of the trigonal bipyramidal vanadium complex.[27] Indirect evidence has been reported, suggesting that imidazole forms a complex with vanadate.[26,28] This conclusion is based on two lines of evidence. Vanadate is found to be significantly more redox stable in the presence of NADP in imidazole buffers than other buffers, including Tris.[28] Furthermore, aqueous studies of the vanadate monoester formation in the presence and absence of imidazole clearly show differences in both the kinetic and thermodynamic behavior of the equilibrium mixture.[26] Both these observations are consistent with the formation of a vanadate−imidazole complex. The interaction of vanadate with pyridine has been described, and the resulting complex has been found to be exceedingly weak.[29] Although imidazole is a better base than pyridine, a corresponding study with vanadate and imidazole has not been reported. In view of the indirect observations with mixed ligand vanadate systems described above,[26,28] it would perhaps be best to approach the question of vanadium(V)−imidazole interaction in a mixed ligand vanadium(V) complex. Given the fact that both vanadium(IV) and -(V) complexes with nucleosides are potent inhibitors for ribonucleases[7,20] and the fact that the active sites of ribonucleases contain several histidine groups, it seemed possible that complexes containing vanadate and adenosine would be stabilized by imidazole and perhaps even form a complex sufficiently stable for observation and characterization. Indeed, the ^{51}V NMR signal for a complex between nucleosides and vanadate that only formed in the presence of imidazole buffer had previously been reported,[10] and a similar complex can be observed in a vanadate−uridine mixture when histidine is added in great excess.[30] However, none of these complexes has been characterized in a quantitative manner.

The work presented in this paper is a careful speciation study of two systems: the H^+−vanadate−adenosine and H^+−vanadate−adenosine-imidazole systems. A wide pH range has been employed to optimize the analysis and quality of the speciation profiles. The speciation and ^{51}V NMR characteristics of the binary $H^+-H_2VO_4^-$ systems are exactly known,[30,31] which allows pH-independent formation constants to be determined. By using potentiometry and ^{51}V NMR spectroscopy and by simultaneous evaluation of combined experimental data with an advanced computer program, the major species formed in the vanadate−adenosine system was identified as a −2 charged 2:2 complex in agreement with previous studies.[5,8−12] Three isomeric forms of this 2:2 complex were identified by ^{51}V NMR spectroscopy. In addition, a new species, a protonated form of the major 2:2 complex, was found to exist in the slightly acidic pH range. The H^+−vanadate−adenosine−imidazole system generates not only the two species present in the H^+−vanadate−adenosine system but also a new 1:1:1 complex. This complex is interesting not only because of its significantly greater stability than 1:1 complexes in the ternary systems but also because it was of sufficient stability to be characterized in a qualitative and quantitative manner. Thus, this work represents the first characterization of such a ternary complex and demonstrates that significant stabilization can be obtained in such mixed ligand vanadium(V) complexes. Given the central role of imidazole in many enzymes, this work clearly has implications

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for the mechanisms and avenues available to vanadium in interaction and conversions in biological systems.

Experimental Section

Chemicals and Analyses. Adenosine, $C_{10}H_{13}N_5O_4$ (Janssen Chimica 99+%), was dried at 80 °C but found to contain no water. It was thus used as provided. Since the solubility of adenosine in 0.600 M NaCl medium at 25 °C is limited to around 20 mM, this was the highest concentration used in the experiments. Imidazole, $C_3H_4N_2$ (E. Merck p.a.), was recrystallized from water before use. Sodium chloride (E. Merck p.a.) was dried at 180 °C and used without further purification. Boiled distilled water was used for preparation of all solutions. After being boiled, the water was cooled to 25 °C and stored in the absence of CO_2(g). Alkaline and neutral solutions were prepared and stored under argon to protect them from atmospheric CO_2. Vanadate stock solutions were prepared by dissolving sodium metavanadate (E. Merck p.a.) in hot water. The solutions were then cooled to room temperature, filtered through a porous glass G4 filter, and standardized by evaporation to the solid ($NaVO_3$) at 110 °C. Diluted solutions of hydrochloric acid (E. Merck p.a.) were standardized against tris(hydroxymethyl)-aminomethane (TRISMA-base). Diluted sodium hydroxide was prepared from a saturated NaOH solution (50% NaOH and 50% H_2O) and standardized against hydrochloric acid.

Equilibration. In the vanadate−adenosine and vanadate−adenosine−imidazole systems equilibration is fast at neutral and alkaline pH. Measurements showed that the complexes form within a few minutes when mixing the ligands with a vanadate solution. In acid solutions equilibration is slow; at least 24 h are required due to the slow decomposition of initially formed decavanadates.

Notation. The equilibria studied are written with the components H^+, $H_2VO_4^-$, AdH, and ImH. Thus, the complexes are formed according to

$$p\mathrm{H}^+ + q\mathrm{H_2VO_4}^- + r\mathrm{AdH} + s\mathrm{ImH} \rightleftarrows (\mathrm{H}^+)_p\,(\mathrm{H_2VO_4}^-)_q(\mathrm{AdH})_r(\mathrm{ImH})_s^{\,p-q}$$

Formation constants are denoted $\beta_{p,q,r,s}$, and complexes are given the notation (p,q,r,s) or $V_xAd_yIm_z^{n-}$. The total concentrations of vanadium, adenosine, and imidazole are denoted $[V]_{tot}$, $[Ad]_{tot}$, and $[Im]_{tot}$.

Potentiometric Measurements. Emf measurements were carried out as potentiometric titrations or as separate pH measurements. The titrations were carried out in 0.600 M Na(Cl) medium at 25 °C, with an automated potentiometric titrator. The general purpose type glass electrodes, Ingold 201-NS, were used. The free hydrogen ion concentration was determined by measuring the emf of the cell:

$$-\mathrm{Ag,AgCl/0.600\ M\ Na(Cl)//equilibrium\ solution/glass\ electrode} +$$

The measured emf (in mV) is expressed as $E = E_0 + 59.157\ \log[\mathrm{H}^+] + E_j$, where $E_j/\mathrm{mV} = -76[\mathrm{H}^+] + 42.5K_w[\mathrm{H}^+]^{-1}$ for the experimental setup used. E_j is the liquid junction potential at the 0.600 M Na(Cl)//equilibrium solution interface for the experimental setup used. K_w (1.875×10^{-14}) is the ionic product of water in 0.600 M Na(Cl) and at 25 °C.[32] The constant E_0 was determined separately in a solution with known $[H^+]$ in 0.600 M Na(Cl), before and after each titration.

In the pH range 3.5−6.5 equilibration is slow—at least 24 h are required due to formation/decomposition of decavanadates. Therefore, instead of titrating a solution over a period of several days, individual samples were prepared at different pH values, total concentrations, and concentration ratios. After equilibration, pH was measured with an Ingold U402-M6-S7/100 combination electrode that had been calibrated against buffer solutions of known $[H^+]$ in 0.600 M Na(Cl). The same samples were also used for NMR measurements.

Potentiometric Data. The first acidity constant for adenosine ($pK_a = 3.668$) was determined from four automated titrations (51 experimental points). The pH range covered was $2.0 \le \mathrm{pH} \le 5.2$, and two different total concentrations of adenosine were titrated, 5 and 20 mM, respectively. The alkaline pK_a value (12.00) was determined from two manual titrations with a combination electrode specially designed for measurements at high pH values. The electrode was calibrated as described above. The adenosine concentration was 20 mM and the pH range covered was $6.1 \le \mathrm{pH} \le 12.6$. To evaluate the acidity constants for imidazole, four titrations were carried out (66 experimental points). The pH range covered was $2.0 \le \mathrm{pH} \le 10.0$, and the total concentrations of imidazole that were titrated were 10 and 80 mM, respectively.

For the ternary $H^+-H_2VO_4^--$AdH system, data were obtained in six titrations with a total of 68 experimental points. The pH range covered was $2.0 \le \mathrm{pH} \le 9.9$. The $[V]_{tot}$ and $[Ad]_{tot}$ were 5 or 20 mM and 20 mM, respectively. In order to elucidate complex formation in the quaternary $H^+-H_2VO_4^--$AdH−ImH system, two titrations (30 experimental points) were performed. The pH range covered was $5.2 \le \mathrm{pH} \le 11.0$, and the concentrations used were $[V]_{tot} = 5$ mM, $[Ad]_{tot} = 20$ mM, and $[Im]_{tot} = 320$ mM. Moreover, the potentiometric titrations were supplemented by additional measurements where pH values were measured for each solution that was prepared for the NMR measurements (see below).

NMR Measurements. ^{51}V NMR spectra were recorded at 131.5 MHz (11.7 T) and 25 ± 1 °C, using a Bruker AM-500 spectrometer. The chemical shifts are reported relative to the external reference $VOCl_3$ (0 ppm). The field frequency stabilization was locked to deuterium by placing the 8 mm sample tubes into 10 mm tubes containing D_2O. Typically, spectral widths of 200 ppm (26.9 kHz) were used, and data for the FID were accumulated in 4K blocks. A 90° pulse angle was used and, due to short relaxation times, no relaxation delay was used. Unless indicated differently, a 1-Hz line-broadening factor was applied before Fourier transformation.

NMR Data. For the V−AdH system, 41 spectra were recorded in the ranges $3.6 \le \mathrm{pH} \le 8.9$, $1.25 \le [V]_{tot}/\mathrm{mM} \le 40$, and $5.0 \le [Ad]_{tot}/\mathrm{mM} \le 20$. For V−AdH−ImH, a total of 23 spectra were recorded in the ranges $6.7 \le \mathrm{pH} \le 9.6$, $1.25 \le [V]_{tot}/\mathrm{mM} \le 5.0$, and $0 \le [Im]_{tot}/\mathrm{mM} \le 320$ with $[Ad]_{tot} = 20$ mM. Immediately after recording the NMR spectra, the pH of each of the solutions was measured with the carefully calibrated combination electrode mentioned earlier. Spectra were then quantitatively evaluated with the NMRi[33] or the Bruker UXNMR/P program.

Computer Calculations. The emf and quantitative ^{51}V NMR data were evaluated with the least-squares program LAKE.[34] LAKE is able to calculate formation constants with standard deviations from, for instance, emf data obtained in titrations or individual solutions, quantitative integral NMR data, NMR shift data, or combined emf−NMR data. Formation constants for systematically chosen complexes $(H^+)_p(H_2VO_4^-)_q(AdH)_r(ImH)_s^{p-q}$ are varied so that the error squares sum, $U = \sum(W_i\Delta A_i)^2$, is minimized. For NMR data, several complexes with the same or different nuclearities can be included in the same peak integral. Thus, it can be tested if a resonance originates from more than one species. The complex, or set of complexes, giving the lowest U value represents the model that best explains the experimental data. A_i can be either the total concentrations of components, free species concentrations, NMR peak integrals, chemical shifts, or combinations of these. W_i is a weighting factor that must be chosen to give the different types of data their proper weights. In this work we have used a weighting factor that gives NMR peak integrals the largest contribution to the sum of residuals. In addition, a weighting factor has been used that also gives low vanadium concentrations a considerable contribution to the error squares sum. Calculation and plotting of distribution diagrams were performed with the program SOLGASWATER.[35]

Results and Discussion

To determine the speciation in multicomponent systems, the equilibrium conditions in the different subsystems should be precisely known for the same experimental conditions. In order to obtain reliable formation constants, a wide pH range as well as a constant ionic medium (0.600 M Na(Cl)) and constant temperature (25 °C) have been employed in the present study.

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Table 1. Composition, Notation, Formation Constants (β), and Acidity Constants (pK_a) for the H^+-AdH, H^+-ImH, $H^+-H_2VO_4^--AdH$, and $H^+-H_2VO_4^--AdH-ImH$ Systems [0.600 M Na(Cl), 25 °C][a]

(*p*,*q*,*r*,*s*)	notation	log $\beta \pm (3\sigma)$	pK_a
1,0,1,0	AdH_2^+	3.668 (8)	3.668
0,0,1,0	AdH	0	12.00
−1,0,1,0	Ad^-	−12.00 (6)	
1,0,0,1	ImH_2^+	7.124 (2)	7.124
0,0,0,1	ImH	0	
1,2,2,0	$V_2Ad_2^-$	11.89 (8)	4.21
0,2,2,0	$V_2Ad_2^{2-}$	7.68 (1)	
0,1,1,1	$VAdIm^-$	3.04 (2)	

[a] The (*p*,*q*,*r*,*s*) notation is defined in the Experimental Section. Application of the "*p*,*q*,*r*,*s*" notation as given in the Experimental Section means, e.g., that (1,2,2,0) represents the $[(H^+)_1(H_2VO_4^-)_2(AdH^0)_2(ImH^0)_0]^-$ complex, which forms via a condensation reaction.

The vanadate system for these conditions was described several years ago,[31] but has recently been refined by using high-field NMR data (500-MHz spectrometer). No additional species were needed, and the values of the formation constants are in agreement with those obtained in the earlier study. The redetermined formation constants and pK_a values were reported previously.[30] The acidity constants for adenosine and imidazole had to be determined for the same conditions (0.600 M Na(Cl) medium and 25 °C), and the results are summarized in Table 1. The alkaline pK_a value of imidazole is ∼14.5,[36,37] and will thus not affect the present study. The vanadate−adenosine system has been examined previously by other workers at neutral pH.[6,8−11] As detailed knowledge of the speciation in a wide pH range was necessary in order to evaluate the interactions with imidazole, a careful study of this system over a wide pH range was performed.

Self-stacking of adenosine bases near saturating concentrations in aqueous solutions are well-known.[38] Association constants have been determined at significantly different conditions than those employed in the present work and are thus not directly applicable to this system. If such intermolecular stacking would affect complex formation under the conditions used in this study, then these effects would have been apparent when fitting our data. In the absence of such irregularities we have not been able to detect significant contributions by intermolecular stacking with the potentiometric and ^{51}V NMR methods used in the present work.

Speciation in the Vanadate−Adenosine System. Both emf and ^{51}V NMR data were used to determine the speciation in the $H^+-H_2VO_4^--AdH$ system in a wide pH range. ^{51}V NMR spectra show, in addition to the vanadate resonances, a rather broad unsymmetrical resonance at a shift value of −523 ppm. Varying pH, vanadate or ligand concentrations, or concentration ratios affected neither the chemical shift nor the shape of the resonance. To find the complex or set of complexes that best fit the potentiometric and ^{51}V NMR data, different models were tested by using the computer program LAKE.[34] Results from the calculations show that the *U* value for a −2 charged 2:2 species, $V_2Ad_2^{2-}$, of the −523 ppm resonance is almost ten times lower than that for a −1 charged 1:1 species. Deviations for data acquired at acidic pH disappeared, and the *U* value was lowered by another 50%, when an additional protonated complex,$V_2Ad_2^-$, was included in the model. Although one may have expected the pK_a value for the coordinated adenosine

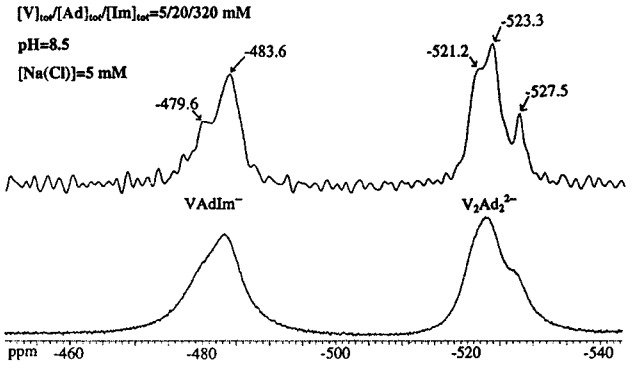

Figure 2. ^{51}V NMR spectra of a solution with extensive V−AdH and V−AdH−ImH complexation. In the upper spectrum, data are processed by using resolution enhancement (LB = −350, GB = 5%). The spectrum was recorded at a low medium concentration ([Na(Cl)] = 5 mM) to decrease the resonance half widths.

to be similar on both adenosine residues, a diprotonated 2:2 complex, $V_2Ad_2^0$, does not fit data as well as $V_2Ad_2^-$. This may reflect that the ability of the coordinated adenosine to protonate is closely linked to the overall charge of the entire 2:2 complex. Thus, the calculations showed that the major resonance is completely explained by two 2:2 vanadate-to-ligand complexes, $V_2Ad_2^{2-}$ and $V_2Ad_2^-$ (Table 1). Resolution enhancement of the resonance reveals three peaks with shifts at −521.2, −523.3, and −527.5 ppm (Figure 2). Since the evaluation of combined emf−NMR data gave V:AdH complexes of 2:2 composition, the three signals must represent isomers. The relative amounts of vanadium in the three subpeaks are difficult to evaluate accurately, but an approximate ratio of 4:1 for the sum of the −521.2- and −523.3-ppm peaks versus the −527.5-ppm peak was found. At alkaline pH (>8) a minor resonance appears at −507 ppm. It represents only a small proportion of the total vanadium concentration (∼2% at pH 8.9 for $[V]_{tot} = 5$ mM and $[Ad]_{tot} = 20$ mM). No attempts were made to determine the composition of this minor species, since it cannot be evaluated with a high degree of accuracy. The resonance might exist at lower pH values as well, if it is superimposed either on the broad −523-ppm resonances or on one of the decavanadate resonances.

The distribution of vanadium-containing species as a function of pH was calculated with the formation constants given in Table 1 and in ref 30 and is illustrated for $[V]_{tot} = 5$ mM and $[Ad]_{tot} = 20$ mM in Figure 3. To simplify the diagram and to make it possible to compare the proposed speciation model with experimental data, the sums for the decavanadate, oligovanadate, monovanadate, and V_2Ad_2 species are shown. As can be seen, the experimental ^{51}V NMR data points, indicated by symbols, show a perfect fit to the calculated model. The $V_2Ad_2^{n-}$ complexes exist from pH around 3.5 to 10. Between pH 5.5 and 8.0 and for the total concentrations given in the figure, almost 75% of the vanadium is bound in these complexes, with the −2 charged species predominating. However, if both $[Ad]_{tot}$ and $[V]_{tot} = 20$ mM, to provide the same 1:1 ratio as in the vanadate−adenosine complexes formed, only 30% of the total vanadium (and adenosine) is bound in the $V_2Ad_2^{n-}$ complexes.

Tracey *et al.*[8] have reported some formation constants in the vanadium−adenosine system. They determined the constant for $2V_1 + 2Ad \leftrightarrows V_2Ad_2$ as $(4.1 \pm 0.2) \times 10^7$ (1 M KCl, pH = 7.00 ± 0.03, 30 mM HEPES buffer, ambient temperature). This value in logarithmic units, $7.61 \pm 0.06(3\sigma)$, is very similar to our value of 7.68 ± 0.01. However, since their constant has been evaluated by using the integral value of the monomeric vanadate signal ($V_1 = H_2VO_4^- + HVO_4^{2-}$), the contribution of the minor HVO_4^{2-} species must be compensated for to obtain

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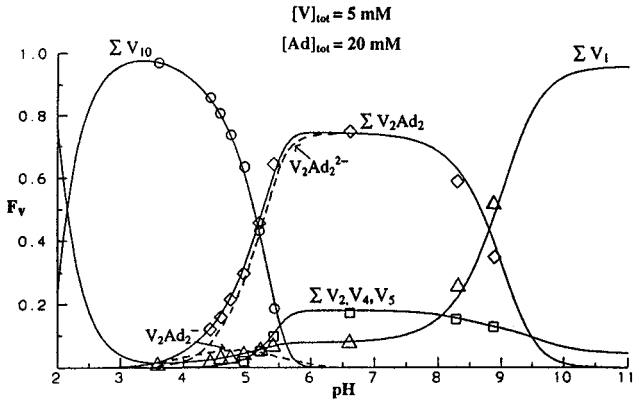

Figure 3. Diagram showing the distribution of vanadium, F_V, vs pH at $[Ad]_{tot}/[V]_{tot} = 4$. F_V is defined as the ratio between [V] in a given species and $[V]_{tot}$ in the solution. The sums for the decavanadate, oligovanadate, monovanadate, and V_2Ad_2 species are shown. The symbols represent experimental NMR data points. The individual distributions of the $V_2Ad_2^{2-}$ and the $V_2Ad_2^{-}$ complexes are shown by dashed curves.

the pH-independent formation constant $\beta_{2,2}$ (the contributions of the $V_2Ad_2^-$ and AdH^+ species are negligible at pH = 7). In 1 M KCl a pK_a value of 8.16 has been reported,[39] which means that 6.5% of the monomeric vanadate is present as HVO_4^{2-} at pH 7.00. The recalculated value, 7.67, is in perfect agreement with ours despite the differences in conditions.

Structure of the $V_2Ad_2^{n-}$ Complexes. The V_2O_8 unit found in the crystal structure of $[N(C_2H_5)_4]_2[\{VO_2Ad\}_2]\cdot 4.74H_2O$[13] probably persists in aqueous solution not only for adenosine but also for other vicinal diol vanadate complexes.[12] In this dimeric structure vanadium is pentacoordinate with one of the two coordinated ribose vicinal oxygens from each adenosine moiety bridging the two vanadium atoms.

The protonated 2:2 complex ($V_2Ad_2^-$) probably has the same structure as the unprotonated $V_2Ad_2^{2-}$ species. Lack of change of the chemical shift for the ^{51}V NMR resonance with pH suggests that the additional proton on this species is likely to be at some distance from the vanadium atom. Since the pK_a value (4.21) differs by only 0.54 unit from adenosine itself, the protonation site is presumably N1 in adenosine. In fact, the pK_a of N1 in adenosine is increased by 0.2−0.4 pH unit upon phosphorylation generating the 3′-phosphate and the 5′-phosphate.

The dimeric structure reported in ref 13 (the cis-2 structure in ref 12) would be expected to give only one ^{51}V NMR resonance. In contrast to most previous studies, three signals are clearly observed under the conditions used in the present work. We offer two explanations for this. First, we used a higher field spectrometer (500 MHz), and when these three specific signals were to be evaluated, signal enhancement was used while processing the FID of the accumulated data. Second, all solutions were prepared, stored, and measured in the absence of atmospheric CO_2, in contrast to most previous studies.[5−12] Although the effect of CO_2 on vanadate equilibria is not well understood, there is no doubt that the presence of HCO_3^- and/or CO_3^{2-} may affect the vanadate exchange reactions and/or equilibria.[40] We make this point because one of the signals observed in the ^{1}H NMR spectrum for the vanadate−adenosine system prepared in the presence of CO_2 is absent when solutions are prepared and stored under nitrogen in the absence of light and carbonate (Ray, W. J., Jr., unpublished results, and ref 12).

Table 2. Composition, Notation, Formation Constants, and Goodness of Fit (expressed in terms of *U*) for Different Species Models[a]

(*p*,*q*,*r*,*s*)	notation	log $\beta \pm (3\sigma)$	10^6U
0,1,1,1	$VAdIm^-$	3.04 (2)	963
0,1,2,1	VAd_2Im^-	4.84 (2)	1248
0,1,1,2	$VAdIm_2^-$	3.60 (4)	4424
0,1,2,2	$VAd_2Im_2^-$	5.43 (4)	3801
0,2,2,2	$V_2Ad_2Im_2^{2-}$	8.68 (4)	5003
0,2,2,1	$V_2Ad_2Im^{2-}$	8.08 (4)	4848
0,2,1,2	$V_2AdIm_2^{2-}$	6.91 (5)	5549
0,2,1,1	V_2AdIm^{2-}	6.30 (4)	5649

[a] The (*p*,*q*,*r*,*s*) notation is explained in Table 1.

The three ^{51}V NMR signals from the $V_2Ad_2^{n-}$ species are likely to be correlated to three of the eight[12] or nine[9] isomers observed by ^{1}H NMR spectroscopy. The major resonance at −523.3 ppm presumably represents the two major isomers observed by ^{1}H NMR spectroscopy, referred to as the cis-1 and cis-2 isomers in ref 12, and as the A and C isomers in ref 9. The two vanadium atoms in each of these isomers have similar environments and differ very little from one isomer to the other. The two minor resonances at −521.2 and −527.5 ppm can be assigned to a trans isomer (see ref 12 for structure). Here, the two vanadium atoms are different and should result in two vanadium signals of equal intensity. The approximate ratio of 4:1 evaluated for the sum of the −521.2- and −523.3-ppm resonances versus the −527.5-ppm resonance points to a cis/trans ratio of 3/2 and is in general agreement with the ^{1}H NMR spectrum reported previously.[12]

Speciation in the Vanadate−Adenosine−Imidazole System. Imidazole is a commonly used buffer and a residue commonly found in enzymes, many of which interact strongly with vanadate. The expectation that the combination of O donors and heteroaromatic N donors, if coordinated to a 3d metal ion, is favorable[41−43] stimulated the study on vanadate−adenosine−imidazole complexation. After determination of the speciation and ^{51}V NMR characteristics of the $H^+-H_2VO_4^-$−AdH system, imidazole was added to examine its effects on vanadate−adenosine complexation. At the moderate concentrations used, no interaction of imidazole with vanadate or adenosine was observed in the ternary subsystems $H^+-H_2VO_4^-$−ImH and H^+−AdH−ImH. In the quaternary $H^+-H_2VO_4^-$−AdH−ImH system, however, mixed ligand vanadate species are formed when imidazole is present in excess.

In order to elucidate the V−AdH−ImH complexation, a pH range from 5.2 to 11.0 and varied total concentrations and concentration ratios were employed. ^{51}V NMR spectra show, in addition to the vanadate and vanadate−adenosine resonances, the presence of a broad and asymmetric resonance at −483 ppm. The signal persists from pH ∼5.5 to ∼10.5 with maximum area at pH ∼8.5. To obtain the same amount of vanadium bound in this V−AdH−ImH complex as in the V−AdH complexes, a large excess of ImH is needed. The amount of complex formed for different imidazole concentrations is shown in Figure 4. To determine the speciation and formation constant of the complex or complexes giving rise to ^{51}V NMR resonances at this chemical shift, different models were tested by using the LAKE program. Selected results are summarized in Table 2, and as can be seen the 1:1:1 vanadate−adenosine−imidazole stoichiometry has the lowest *U* value. The possibility of an additional protonated complex (1,1,1,1) was tested, but it was rejected. Thus, the −1 charged, mononuclear, mixed ligand $VAdIm^-$

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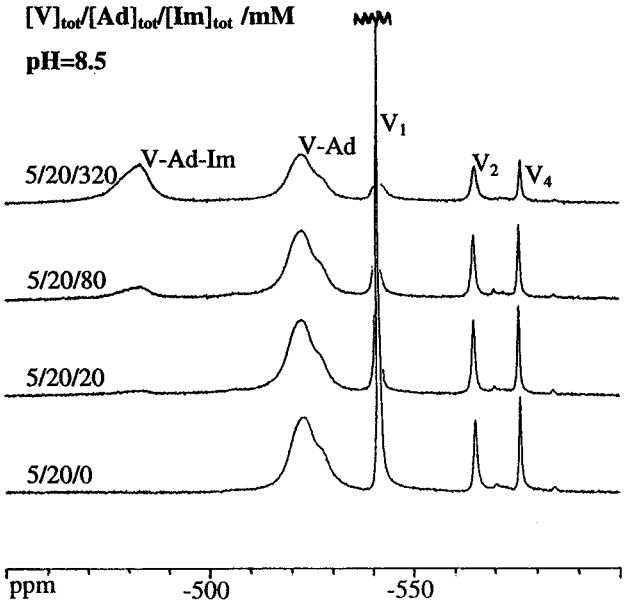

Figure 4. ^{51}V NMR spectra of aqueous solutions containing vanadate, adenosine, and imidazole at different $[V]_{tot}/[Ad]_{tot}/[Im]_{tot}$ ratios at pH 8.5. Spectra are plotted in an absolute intensity mode.

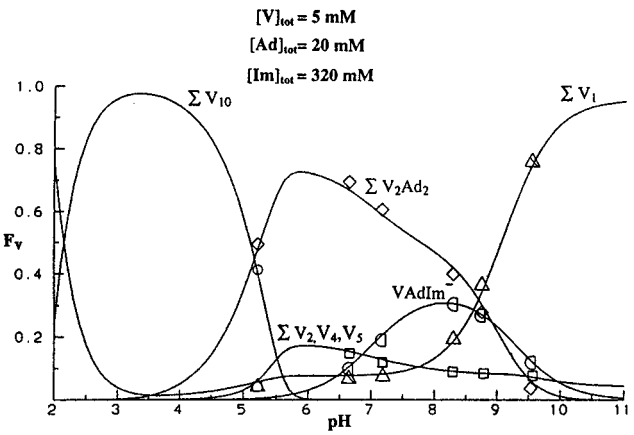

Figure 5. Diagram showing the distribution of vanadium, F_V, vs pH at $[V]_{tot}$ = 5 mM, $[Ad]_{tot}$ = 20 mM, and $[Im]_{tot}$ = 320 mM. F_V is defined as in Figure 3. The sums for the decavanadate, oligovanadate, monovanadate, and V_2Ad_2 species are shown. The symbols represent experimental NMR data points.

species (0,1,1,1) alone represents the model that best explains experimental data. ^{51}V NMR resonances for this and related complexes containing vanadium(V), vicinal diols, and a second ligand, such as imidazole[10] or Tris,[7,8,44] have been observed previously. However, this is the first time that a complex of this type has been characterized in detail in aqueous solution.

The distribution of vanadium-containing species from pH 2 to 11, at the same $[V]_{tot}$ and $[Ad]_{tot}$ concentrations as in Figure 3 but with a large excess of ImH (ImH/AdH = 16/1), is shown in Figure 5. The constants used for the calculations are collected in Table 1 and in ref 30. As can be seen, the calculated model fits experimental ^{51}V NMR data points perfectly. The distribution curve for the $VAdIm^-$ complex is shifted slightly toward the alkaline pH range compared to that for the $V_2Ad_2^{2-}$ complex. The $VAdIm^-$ complex appears at pH 5.5, has a maximum at pH around 8, and persists up to pH 11. Even at high excess of ImH, the $V_2Ad_2^{n-}$ species predominates and it is only in the alkaline pH range (>9) that $VAdIm^-$ is more prevalent than $V_2Ad_2^{2-}$. However, a lower total concentration of vanadium favors the mononuclear mixed ligand complex, as illustrated in

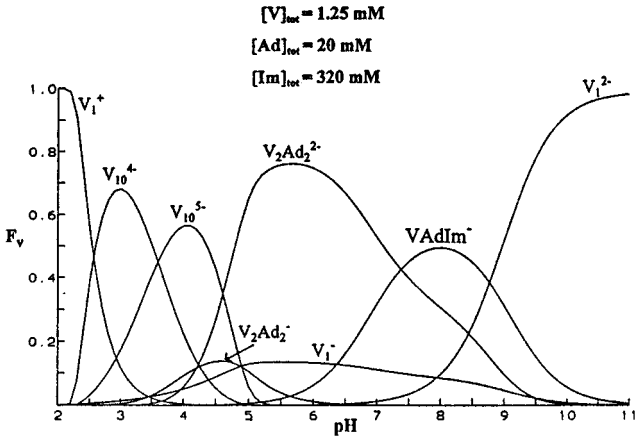

Figure 6. Diagram showing the distribution of vanadium, F_V, vs pH at $[V]_{tot}$ = 1.25 mM, $[Ad]_{tot}$ = 20 mM, and $[Im]_{tot}$ = 320 mM. F_V is defined as in Figure 3. All vanadium-containing species are shown except those containing <5% of $[V]_{tot}$. $V_1^+ = VO_2^+$, $V_1^- = H_2VO_4^-$, $V_1^{2-} = HVO_4^{2-}$, $V_{10}^{4-} = H_2V_{10}O_{28}^{4-}$, $V_{10}^{5-} = HV_{10}O_{28}^{5-}$.

Figure 6. For these conditions, $VAdIm^-$ becomes the prevalent complex at pH values down to approximately 7.4 (i.e. in the physiological pH range).

Stability and Structure of the $VAdIm^-$ Complexes. The high stability of the $V_2Ad_2^{2-}$ complex makes observation of the $VAdIm^-$ complex, albeit at large excesses of ImH, unexpected. This 1:1:1 complex is clearly significantly more stable than the 1:1 complexes reported previously for the vanadate−adenosine system[5,8,9,11] (which are below the detection limit of this study). The $VAdIm^-$ complex is most stable in the neutral to slightly alkaline pH range where imidazole is present in its uncharged form, perhaps suggestive of formation of a covalent bond between vanadium and imidazole at neutral pH, whereas electrostatic interactions between the imidazolium ion and the $V_2Ad_2^{2-}$ complex are more favorable at low pH. This fact supports the interpretation that forces opposing the covalent bond formation in the complex are easily overcome. The question arises why the 1:1:1 complex with imidazole is so much more stable than any of the 1:1 complexes between vanadate and imidazole or vanadate and adenosine.

Base-stacking properties of imidazole in ternary metal complexes have been documented in both the solid state[45] and solution.[46] No precedence for this type of stabilization has been offered for vanadium(V) compounds, including the $V_2Ad_2^{2-}$ complexes and vanadium−imidazole complexes, in part due to the complex solution chemistry involving multiple species. Only a limited amount of solid state structural data is available for vanadium−imidazole complexes, and combined with the weak formation constants of vanadium(V)−imidazole complexes in aqueous solution,[26] information based on structural data supporting imidazole stacking is thus not yet available. It is the case, however, that the adenine groups trans to each other in the X-ray structure for the $V_2Ad_2^{2-}$ complex[13] are too far apart to be involved in base stacking, and that the other $V_2Ad_2^{2-}$ isomers with adenine groups in the cis configuration are far apart since they are separated by a V_2O_2 moiety. This is in contrast to the mononuclear ternary mixed ligand complexes where stacking effects have indeed been demonstrated. The possibility thus exists for gaining the extra stability offered by stacking when a 1:1:1 complex containing Ad, ImH, and V is formed. In view of the fact that such stacking effects have been reported to increase the formation constant by a factor of 1000,[47]

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this type of stabilizing effect may indeed be an important factor stabilizing the 1:1:1 complex sufficiently for observation.

Alternative explanations for the increased stability of the $VAdIm^-$ species exist. Hydrogen bonding may assist in stabilizing a specific complex. Evidence for intermolecular hydrogen bonding in vanadium complexes in the solid state has been reported,[48,49] and both inter- and intramolecular hydrogen bonds have previously been shown to affect metal complex stability and stoichiometry in solution.[50,51] In addition, a recent study of a series of vanadium(V)−diethanolamine based complexes showed that the enthalpic and entropic contributions oppose each other. Ligand modification resulting in larger entropic terms, decreasing the enthalpic contribution, led to an overall decrease in complex stability.[52] In the absence of more quantitative information on these mixed ligand complexes and/or the nature of the inherent stability, the role of stacking in these types of complexes cannot be evaluated.

Evaluation of the possibility for base stacking and/or hydrogen bonding would be assisted by structural information on the $VAdIm^-$ complex. Unfortunately, the large excess of imidazole required to form significant concentrations of $VAdIm^-$, the fact that this complex is only favored over the $V_2Ad_2^{2-}$ complex at low vanadium concentrations, and overlapping signals in the spectra precluded observation of the 1:1:1 complex by ^{13}C and ^1H NMR spectroscopy at this time. However, considering the five-coordinate geometry for the 2:2 vanadate−adenosine complexes[12,13] and the fact that the chemical shift of −483 ppm is similar to the chemical shifts of a series of amino alcohols which form five-coordinate complexes with vanadate,[53] it seems reasonable to speculate that the $VAdIm^-$ complexes contain a five-coordinate vanadium atom. In Figure 7, two possible five-coordinate geometries with the imidazole axial (**1** and **2**) are shown, along with two geometries with the imidazole equatorial (**3** and **4**). The adenosine ligand in these structures is simplified by omitting the C1 and C4 substituents, which makes isomer **1** different from **2** (and **3** different from **4**). Precedence exists for five-coordinate complexes of related ligands with both amine, imine, and pyridine functionalities in both axial and equatorial positions.[54−57] In the X-ray structure of an acid phosphatase−vanadate complex the histidine group is in the axial position.[27] The V−AdH−ImH resonance is asymmetric and resolution enhancement shows two resonances (Figure 2) indicating the presence of two isomers, one at −483.6 ppm (major) and one at −479.6 ppm (minor). The possibility exists that the two isomers form because of imidazole stacking, and one signal represents the complex without (the "open form") and one the complex with (the "closed form") stacking stabilization. Alternatively, the two isomers may represent two of the four structures shown in Figure 7, since the chemical shift difference of approximately 4 ppm between the two 1:1:1 isomers is similar to the observed chemical shift difference between the 2:2 vanadate−adenosine isomers. Additional structural information of these types of complexes must await identification of a ternary mixed ligand complex more amenable to structural analyses.

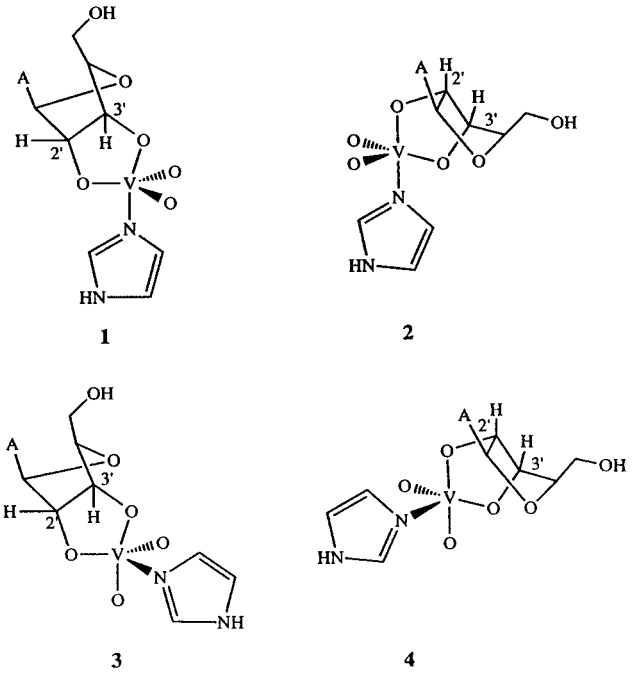

Figure 7. Possible geometries for the $VAdIm^-$ complex. The representation for the adenosine group is simplified (cf. Figure 1a).

At this time the origin of the unusual stability of the $VAdIm^-$ complex has not been determined. Other ternary mixed complexes of this type must be characterized both quantitatively and structurally before the respective roles of stacking, H-bonding, and other effects such as solvent organization in complex stability can be determined.

Reduction. Reduction of vanadium has frequently been reported for solutions with organic ligands.[30,58−60] With adenosine or adenosine and imidazole, no reduction was observed during the time of investigation. However, after approximately 2 months partial reduction was found in some acid and neutral solutions. If the solutions were stored exposed to UV light, reduction was markedly enhanced. Since vanadate is known to reduce to vanadium(IV) when becoming cell associated,[61] imidazole-type ligands may play a role in the rate and type of vanadium(IV) complexes that form under physiological conditions.

Biological Implications. The present work confirms results of previous studies that the major vanadate−adenosine complex in solution has a 2:2 stoichiometry, and that the biologically relevant 1:1 complexes, if present in solution, are exceedingly weak. Perhaps this explains why, despite the many studies describing the reactions between vanadate and adenosine and other nucleosides, few examples have emerged with use of the mixture of vanadate and adenosine as a replacement for AMP, 2′,3′-cAMP, 3′,5′-cAMP, or NAD. Solutions containing NAD and vanadate have been used successfully as cofactor analogs

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for 2′-NADP with enzymes including glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and alcohol dehydrogenase.[3b,4,62,63] 2′-NADP is derived from NAD by phosphorylation of the adenosine portion of the molecule at the oxygen atom at C2′, and the mixture of NAD and vanadate presumably forms such a derivative as suggested by the ^{51}V NMR analysis of this system.[4]

Characterization of the vanadium(V) analogs of AMP, 2′,3′-cAMP, and 3′,5′-cAMP has been limited to ^{51}V NMR spectroscopy. Although other techniques have been used to study the reaction of adenosine with vanadate, the 1:1 complexes are so weak that so far they have eluded direct spectroscopic observation by any experimental method. The structures of some 1:1 complexes that have been observed are presumed to be similar to AMP and the other four possible monoesters.[5,8,9] Some evidence has been presented for the vanadium analog of 2′,3′-cAMP,[11] whereas no information has been reported supporting the formation of 3′,5′-cAMP.

Since vanadate analogs of AMP, 2′,3′-cAMP, and 3′,5′-cAMP, at best, form in extremely low concentrations, could these types of compounds be an active component in biological processes? Enzymes with millimolar and micromolar affinities for substrates and/or cofactors are not likely to be affected by compounds at such low concentrations. However, enzymes with nanomolar and lower affinity constants could respond to such derivatives. Examples of enzymes with extremely high affinity for the vanadium complexes include ribonucleases. The most studied ribonuclease (ribonuclease A) binds the vanadate−uridine or vanadate−cytosine complex,[64] and ribonuclease T binds the vanadate−guanosine complex.[7] In the first study describing the inhibition of the ribonuclease by the vanadate−uridine complex, a tight inhibition by a 1:1 complex was reported. However, considering the current understanding of the reaction, it is clear that the affinity of ribonuclease for the vanadium(V) complex is significantly higher than previously anticipated.[20b]

The work presented here (and elsewhere) raises several questions with respect to the interaction of the vanadate−nucleoside complex with ribonuclease. Can ribonuclease abstract the monomeric unit directly from the dimeric complex? Must the $V_2Ad_2^{2-}$ complex form a VAd^- complex in solution? How does the protonated $V_2Ad_2^-$ species affect the enzyme reaction—is it also an inhibitor? Since the evidence for the monomeric unit in solution is based on indirect measurements, perhaps other approaches can be used to probe this question. Buffers such as imidazole and Tris significantly stabilize a monomeric (in vanadium) complex by forming V−AdH−ImH/Tris complexes. This demonstrates that a moiety such as ImH, free in solution or in an enzyme active site, can form stable monomeric vanadium−nucleoside−imidazole complexes.

Although the characterization of the 1:1:1 vanadate−nucleoside−imidazole complex is the first, the approach of using a stabilizing ligand for preparation and characterization of complexes in the solid state has been used extensively.[22,23,65] It is possible that metabolites can act in a similar manner and thus significantly increase the concentration of 1:1 complexes under physiological conditions. However, imidazole or Tris are not buffers customarily used for studies of ribonuclease, and to our knowledge no information is available concerning whether the vanadate−nucleoside complexes are more or less potent inhibitors in the presence of imidazole or Tris.

The complexation of vanadate by imidazole,[26] histidine,[7,30,66,67] and peptides containing histidine[7,24,62] has been recognized for some time, although only a few quantitative studies have been available. The presence of a low concentration of a V−ImH complex is insignificant, unless such a complex modifies the enzyme reaction under examination. Indeed, imidazole has been a preferred buffer in systems where the redox chemistry of the vanadium(V) is to be kept at a minimum.[28,68] Since the vanadate−imidazole complex formation affects both the kinetics and equilibria of the vanadate derivatives in solution,[26] both aspects may be important for the interaction of vanadate with enzymes containing imidazole residues in the active site. The formation of the vanadate−histidine protein complex could affect the rate of complex formation and/or the potency with which the vanadium(V) complex inhibits the enzyme. For example, the acid phosphatases[21] are much more inhibited by vanadate than both *E. coli*[69] and mammalian alkaline phosphatases (Crans *et al.* in preparation). This would be consistent with initial formation of a vanadate−histidine acid phosphatase complex, whereas the fewer histidine residues in the active site of the alkaline phosphatases preclude formation of similarly favorable complexes.

Several haloperoxidases use vanadium(V) as a cofactor, and the active sites of these enzymes contain several histidine and carboxylate groups. Model and enzyme studies which probe both the structure and the halogenation reaction catalyzed by the haloperoxidases have been performed.[70] A recent X-ray crystallographic characterization shows that his-496 in the chloroperoxidase from the fungus *Culvularia inaequalis* is coordinated axially to the VO_3 fragment trans to the azide group.[71] Accordingly, it has been suggested that the peroxide binds trans to this histidine group since the position is accessible from the solvent channel and azide is an inhibitor for chloroperoxidase. These studies combined with those presented in the present work suggest that the histidine residue is crucial to the stability of the enzyme−vanadium−substrate complex.

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