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Application of Time-Resolved ^{51}V 2D NMR for Quantitation of Kinetic Exchange Pathways between Vanadate Monomer, Dimer, Tetramer, and Pentamer[†]

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Abstract: A two-dimensional ^{51}V homonuclear NMR exchange experiment (2D-EXSY) has been used to study the oligomerization reactions vanadate undergoes in aqueous solutions. This manuscript describes the *first* quantitative measurement of complex *intermolecular* chemical exchange rates by using ^{51}V ($I = 7/2$) in a 2D-EXSY experiment. Microscopic (pseudo-first-order) rate constants for intermolecular exchange were obtained by using a numerical procedure to solve the 2D exchange matrix. The 2D exchange matrix was converted to a rate matrix that could be used in a kinetic analysis of the four exchanging vanadium species. The major pathway for monomer formation is unimolecular decomposition of the dimer. The major pathway for dimer formation is dimerization of the monomer. The tetramer forms mainly from two monomers and one dimer. At low vanadate concentrations, the pentamer forms from tetramer and either monomer or dimer with similar rate. At higher vanadate concentration, the pentamer exchanges more rapidly with the tetramer. The vanadate monomer is involved in more significant reaction pathways than any other species. The vanadate dimer is inherently more labile than the tetramer and pentamer as illustrated by its rapid hydrolysis rate. Our analysis demonstrates an approach that is applicable to solving other multiexchange systems. The 2D-EXSY method is versatile and may become central to determining the major reaction pathways by which vanadium acts in both chemical and biological systems.

Vanadium is an important trace element that has potent biological effects in mammals.[1] Although vanadium is beneficial at low levels, it can be toxic at higher levels. Despite this, very little characterization of the toxic and beneficial vanadium species have been carried out.[1] Vanadate is a potent inhibitor for ribonuclease, phosphatases, ATPases, and myosin. The inhibiting species for these enzymes is usually monomeric vanadate, and its activity is presumably due to the stable trigonal-bipyramidal structure that mimics the transition-state structure in the phosphate ester hydrolysis reactions.[1] Vanadate polyanions also act as inhibitors. The dimer inhibits phosphoglycerate mutase,[2a] acid phosphatase from seminal fluid,[2b] and glucose-6-phosphase dehydrogenase,[2c] the tetramer inhibits 6-phosphogluconate dehydrogenase[2d] and glucose-6-phosphate dehydrogenase,[2c] and the decamer inhibits Ca^{2+} ATPase,[2e] muscle phosphorylase,[2f] adenylate kinase,[2g] hexokinase,[2g] and phosphofructokinase.[2g]

Aqueous solutions of vanadate anions contain several oligomers including significant concentrations of monomer, dimer, tetramer, and pentamer. The ^{51}V NMR spectra can be used to determine the speciation of vanadate oligomers. The eqs 1–3 describe the

$$2\mathrm{V}_1 \overset{K_{12}}{\rightleftharpoons} \mathrm{V}_2 \tag{1}$$

$$4\mathrm{V}_1 \overset{K_{14}}{\rightleftharpoons} \mathrm{V}_4 \tag{2}$$

$$2\mathrm{V}_2 \overset{K_{24}}{\rightleftharpoons} \mathrm{V}_4 \tag{3}$$

relationships observed in aqueous solutions.[2b,c,3-5] The structures of the vanadate derivatives in solution are somewhat controversial.[3,4] The tetramer and pentamer are generally believed to be cyclic[3,4] although the cyclic structure for the tetramer has recently been questioned.[6] Numerous additional minor species have been observed in ^{51}V NMR spectra and were assigned according to their ^{51}V NMR chemical shifts.[4] The equilibrium position is affected by pH, ionic strength, temperature, and vanadate concentrations. The rates and mechanisms of conversion between various anions are not well understood. Since we believe that the reactions between vanadate species are important in understanding the chemical and biochemical properties of vanadate, we have used a quantitative ^{51}V NMR homonuclear exchange experiment (2D-EXSY) to directly determine exchange rates and rate constants between the vanadate species.

Experimental Section

General Methods. Chemicals were reagent grade (Fisher, Aldrich) and used without further purification. Water was distilled and deionized. A vanadate stock solution was prepared by dissolving vanadium pentoxide with 2 equiv of sodium hydroxide to generate a vanadate solution of 0.25 M; this solution was stored at 4 °C. The concentrations of the vanadate standard solutions were monitored by UV spectroscopy (at wavelengths from 260 to 270 nm), and no changes in concentrations were observed over the course of 6 months.

1D ^{51}V NMR Spectroscopy.[3-5] ^{51}V NMR spectra were recorded at 52.6 MHz on a Bruker WPSY spectrometer (4.7 T) (Figure 1), 79 MHz on a Bruker ACP-300 NMR spectrometer (7.0 T), and 132 MHz on a Bruker AM-500 spectrometer (11.7 T). For the 1D spectra we typically use spectral windows of 150 ppm, a 90° pulse angle, and an aquisition time of about 0.1 s with no relaxation delay between pulses.[5b] An exponential line broadening of 15 Hz was applied to the FID prior to Fourier transformation. The chemical shifts are reported relative to the external reference standard, VOCl$_3$ (0 ppm), although we in practice use an external reference solution (pH 7.5) containing the complex of vanadate and diethanolamine (−490 ppm).[5a] The DEA complex is a convenient reference because the ^{51}V NMR resonance varies only slightly with pH and ionic strength, and the signal appears in the chemical shift range of interest. The 1D spectral integration, using the mole fraction for each resonance combined with the known total vanadium concentration, allowed calculation of the concentrations of various vanadate oligomers.

The T_1's were obtained by using a Freeman–Hill modified inversion recovery experiment. The T_1's were obtained from the slope of semilog

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[†] Dedicated to the late Professor John K. Stille.

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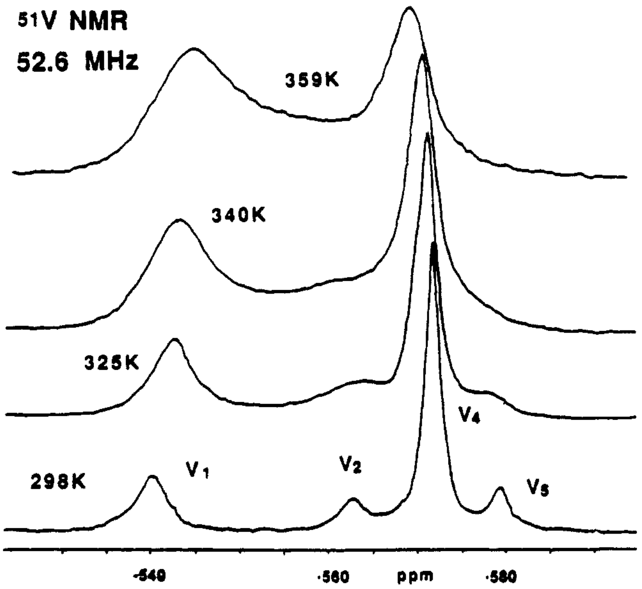

Figure 1. (a) 1D ^{51}V NMR spectra (at 52 MHz) recorded of a solution containing 10 mM vanadate, 0.4 M KCl, 20% D_2O at pH 8.6 at various temperatures. The vanadate species are marked in the spectrum obtained at 298 K. The chemical shifts are given with respect to $VOCl_3$ at 0 ppm. A minimum of 4000 scans was accumulated per spectrum with a recycle time of 0.1 s.

plots of the intensity difference between equilibrium magnetization and magnetization at the time τ plotted as a function of the recovery time, τ.[7]

EXSY[8] ^{51}V NMR Spectroscopy. The phase-sensitive ^{51}V homonuclear 2D-EXSY experiments were done at 79 and 132 MHz on Bruker spectrometers. The NMR samples contained from 5 to 30 mM vanadate in both 5- and 10-mm NMR tubes. The spectra were run by using temperature control at either 23 or 25 °C. The standard three pulse NOESYPH pulse sequence (90° - t_1 - 90° - τ_m - 90°) supplied with the Bruker software was used to define the experiment. This pulse sequence utilizes time-proportional-phase-incrementation (TPPI) to obtain pure phase line shapes. Variable mixing times, depending on the sample, were used. The best results were obtained when $T_1/2 < \tau_m < 3T_1/2$.[9] The spectra used for calculation of exchange matrices in this work used 8–10 ms as the mixing time. For the 2D spectra we narrow the spectral window down to 100 ppm or less to include only the signal regions of interest. This reduces the size of the data matrix required in the F_1 domain. The acquisition time was about 40 ms, and a relaxation delay of about 40 ms was used to extend the time between transients to at least 5 T_1. Thus the recycle time for each scan was approximately 80 ms. A total of 1000 accumulations was made for each of 256 t_1 increments. The total experimental time therefore varied from 3 to 6 h at 132 MHz. A $\pi/2$ shifted sine-bell weighting function was applied to both F_1 and F_2 domains. We find that this window function introduces very little line-shape distortion and has the advantage of being parameterless compared with exponential and gaussian functions. The F_1 and F_2 were zero-filled to 512 and 1024 points, respectively, prior to Fourier transform. The final resolution was about 60 and 30 Hz along F_1 and F_2, respectively.

The 2D ^{51}V NMR spectrum showed exchange cross-peaks between all the diagonal signals (Figure 2). Integration of the peak volumes was carried out by using two procedures included in the standard Bruker software, DISRNMR. The first method is a feature of the 2D display subroutine incorporated in the DISRNMR software and involves total volume integration of the 2D spectral matrix. However, in practice, we found that integration of the individual row and column slices of the 2D spectral matrix provided greater precision. The volume elements obtained in this manner were normalized by using the relative peak integrals from a 1D ^{51}V NMR spectrum. The normalized intensity matrix was diagonalized by an adapted routine from EISPAC for solving real and general matrices. The exchange matrix calculated in this manner contains specific rate constants for exchange of magnetization between sites. The rate matrix can be obtained from the above rate constants by multiplying each row by the concentration of the specific oligomers.

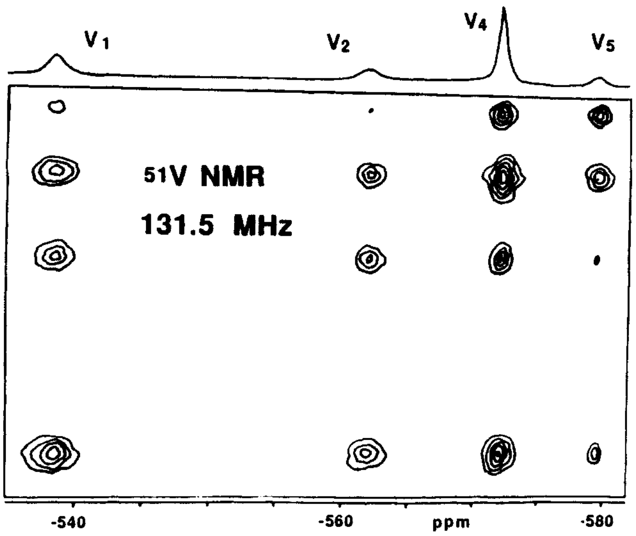

Figure 2. A 2D-EXSY NMR spectrum (131.5 MHz) of a solution containing 10 mM vanadate, 1.0 M KCl, 20% D_2O at pH 8.6 recorded with a mixing time (τ_m) of 10 ms. A standard three pulse sequence with time-proportional-phase-incrementation (TPPI) phase-cycling was used to obtain pure phase spectra. One thousand scans were acquired for each of 256 t_1 increments. The recycle time for each scan was about 80 ms. A $\pi/2$ shifted sine bell and zero-filling were applied in both time domains prior to 2D Fourier transformation. The final resolutions were 30 and 60 Hz/pt in F2 and F1, respectively.

NMR Sample Preparation. The vanadate solutions for ^{51}V NMR studies were prepared by mixing various amounts of vanadate, potassium chloride, and 20% (volume) deuterium oxide. The pH and volume were adjusted to give the final pH (8.6) and volume (3 mL). The ionic strength was maintained essentially constant by using 1.0 M potassium chloride. The vanadate concentration varied from 5 to 20 mM.

Uncertainties. Detailed description of error analysis for the 2D EXSY experiments has been described previously.[10] The errors arise from the limited S/N ratios of the computed spectra, instrumental drift during the experiment or errors in the 2D signal integration. The accumulation time of the ^{51}V NMR experiment is short because vanadium relaxes rapidly, and the experimental drift is therefore small compared to other EXSY experiments. The exchange rate constant matrix is calculated by using the relative intensities of the signals in the 1D spectrum and the intensities of the cross-peaks in the 2D spectrum. We therefore estimate that the S/N ratios and the 2D signal integration are the main sources for errors in our work. We were able to reproduce the rate matrix with less than 2% deviation on the most intense peaks and less than 20% deviation on the smallest peaks. We estimate that the largest rate constants are determined with a 10% accuracy and the smallest rate constants with a 40% accuracy.

Results and Discussion

1D ^{51}V NMR Spectra Suggest Chemical Exchange. Vanadium-51 is 99.67% abundant and has a favorable gyromagnetic ratio and a relatively small quadrupole coupling constant. Since the frequency for vanadium is near carbon, ^{51}V NMR spectra are easily observed by using a broad band probe. These properties have been successfully exploited for chemical studies with vanadium(V).[3-5] A 10 mM vanadate solution (HVO_4^{2-}) containing 1.0 M KCl and 20% D_2O at pH 8.6 gives the 1D ^{51}V NMR spectrum shown in Figure 1. In accord with the literature, we assign δ −542 to the vanadate monomer, δ −564 to the vanadate dimer, δ −574 to the vanadate tetramer, and δ −582 to the vanadate pentamer.[3-5]

Both longitudinal (T_1) and transverse (T_2) relaxation of vanadium-51 in the polyanions are dominated by quadrupole interactions; motional properties place vanadium in the extreme narrowing limit ($\omega_0\tau_c \ll 1$).[11] Under these conditions $T_1 = T_2$.[11]

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Table I. T_1's and Line Widths for a 10 mM Vanadate Solution at pH 8.6 and 0.4 M in KCl[a,b]

	monomer	dimer	tetramer	pentamer
	T_1			
79 MHz[a] (ms)	14	14	11	12
131.5 MHz (ms)	15	13	10	8
	Predicted Line Width (Hz)[b]			
$\Delta_{1/2} = (\pi T_1)^{-1}$ 79 MHz (Hz)	21	24	32	39
	Measured Line Width (Hz)			
Δ_{12} (Hz) 79 MHz	122	134	85	110
concentration (mM)	2.04	1.06	6.28	0.628

[a] T_1's were obtained from plots of $\ln(M_o-M_A)$ as a function of t measured from modified Freeman-Hill inversion recovery experiments at two field strengths (Freeman, R.; Hill, H. D. W. *J. Chem. Phys.* **1971**, *54*, 3367-77). [b] The expected line widths (full width at half-height) were predicted from the equation $\Delta_{1/2} = (\pi T_2)^{-1}$. Assuming $T_1 = T_2$ and that field inhomogeneity effects contribute very little to the observed peak widths at half-height (that is $\gamma \Delta Bo/2\pi \ll 30$ Hz), $\Delta_{1/2} = (\pi T_1)^{-1}$.

Table II. Microscopic (Pseudo-First-Order) Exchange Rate Constants for a 10 mM Vanadate Solution at pH 8.6 and 1 M in KCl[a-d]

	k			
x	k_{1x}	k_{2x}	k_{4x}	k_{5x}
1		181	6.8	6.0
2	94		6.1	3.3
4	21	36		76
5	1.9	1.9	7.6	

[a] A rate constant labeled k_{4x} is the exchange rate constant that determines the rate by with the vanadate tetramer forms the X oligomer (rate of X formation = $k_{4x}[V_4]$). The units on the rate constants are s^{-1}. [b] Integration of peak intensity was carried out by using both the standard Brucker volume integration software and by integrating individual rows and columns of the 2D matrix. Intensity data were normalized by using peak ratios obtained from a 1D spectrum. The normalized intensity matrix was diagonalized by using an adapted EISPAC routine for real, general matrices. The calculated rate constants are very sensitive to errors in measured intensities. See ref 10 for a formal discussion of sources of errors. [c] Copies of the program used for analysis may be obtained from one of the authors (CDR) upon request. [d] The concentrations (expressed as mM vanadium atoms in a particular vanadate species) of vanadate oligomers were $[V_1] = 2.0$, $[V_2] = 1.1$, $[V_4] = 6.3$, and $[V_5] = 0.63$.

Table III. Exchange Rates for a 10 mM Vanadate Solution at pH 8.6 and 1 M in KCl[a-e]

	k			
x	$v_{1x/yz}$	$v_{2x/yz}$	$v_{4x/yz}$	$v_{5x/yz}$
1		190	43	3.9
2	190		38	2.1
4	43	38		48
5	3.9	2.1	48	

[a-d] See footnotes in Table II. [e] x, y, and z can be 1, 2, 4, and 5.

A Freeman-Hill modified inversion recovery experiment was used to measure T_1's, and only slight variation was observed when changing the field. The expected line widths at half-height were predicted from the equation $\Delta 1/2 = (\pi T_2)^{-1}$. It was assumed that $T_1 = T_2$ and that the field inhomogeneity effects contributed very little to the observed line widths at half-height. That is $\delta \Delta B_0 1/2\pi \ll 30$ Hz.

A comparison of predicted line widths ($\Delta_{1/2} = (\pi T_2)^{-1}$) with the observed line widths shows large discrepancies with the results obtained at 79 and 132 MHz (Table I). It was possible that rapid chemical exchange accounted for the additional NMR line-broadening observed at 298 K.[4] This expectation was supported by ^{51}V NMR spectra that showed increased broadening and the approaching coalescence of the NMR signals at elevated temperatures (Figure 1). The exchange dynamics between the four species observed in this spectrum are complex. It therefore follows that the exchange between various oligomers could not be studied by using one-dimensional dynamic NMR without requiring a large series of experiments at different temperatures and complete bandshape analysis. However, in principle, one two-dimensional, phase-sensitive homonuclear ^{51}V NMR exchange (2D-EXSY) experiment could provide all the pseudo-first-order rate constants of the intermolecular exchange reactions at a given temperature.[8]

The 2D ^{51}V NMR Experiment. Microscopic (pseudo-first-order) rate constants (and relaxation parameters) are derived from the exchange matrix **R**. **R** may be obtained numerically from the normalized EXSY intensity matrix, **I**, as shown in eq 4 for a given mixing time of τ_m. Experimentally most accurate quantifications were obtained when $\tau_m \sim 1/k$.[12]

$$\mathbf{I} = \exp(-\mathbf{R}/\tau_m) \tag{4}$$

Since T_1 is short for vanadium, and $1/k$ is on the order of T_1, a two-dimensional ^{51}V NMR homonuclear exchange experiment (2D-EXSY) will not require long accumulation times. The experiment with vanadium is particularly favorable because homonuclear scalar coupling is not detected, and quadrupole relaxation is the dominant T_1 relaxation mechanism. The magnetization transfer via chemical exchange is the dominant factor creating cross-peak intensity in the 2D spectrum, and kinetic data can conveniently be extracted from the off-diagonal peaks in the 2D exchange matrix.

The phase-sensitive ^{51}V homonuclear 2D-EXSY experiments were done at 79 and 132 MHz on Bruker spectrometers although the higher sensitivity and shorter accumulation times at 132 MHz made this experiment more favorable. Various mixing times, ranging from a minimum of 3 μs to five times T_1, were applied to the 10 mM vanadate sample to determine optimum mixing times (τ_m). The mixing times (τ_m) that provided the best cross-peak sensitivity were larger than $T_1/2$ and smaller than $3T_1/2$.[9] The 2D ^{51}V NMR spectrum showed exchange cross-peaks between all the diagonal signals. Integration of the peak volumes illustrated by the contour plot for the 10 mM vanadate solution shown in Figure 2 were carried out by using two procedures included in the standard Bruker Software, DISRNMR. In practice, the integration of the individual row and column slices of the 2D spectral matrix provided greater precision. The volume elements obtained in this manner were normalized by using the relative peak integrals from a 1D ^{51}V NMR spectrum. The normalized intensity matrix was diagonalized by an adapted routine from EISPAC for solving real and general matrices. The rate constants calculated in this manner for the 10 mM vanadate solution are shown in Table II. The rates calculated for the 10 mM vanadate solutions from such rate constants are shown in Table III, and the rates for addition of vanadate solutions are shown in Table IV.

Kinetic Analysis: Derivations. The ^{51}V NMR spectrum of the 10 mM vanadate solution at pH 8.6 contains vanadate monomer,

Table IV. Exchange Rates for Vanadate Solutions at pH 8.6 and 1 M in KCl[a-d]

V_{tot}	$[V_1]^d$	$[V_2]^d$	$[V_4]^d$	$[V_5]^d$	Y_{12}	Y_{14}	Y_{15}	Y_{24}	Y_{25}	Y_{45}
5	1.8	0.70	2.3	0.19	190	20	0.0	8.5	0	15
10	2.0	1.1	6.3	0.63	190	43	3.9	38	2.0	48
12.5	2.2	1.2	8.2	0.93	210	53	3.6	55	7.9	65
15	2.3	1.3	10	1.2	290	79	15	59	7.1	95
20	2.5	1.6	14	1.8	420	110	14	76	8.8	140
estimated uncertainty (%)	5	5	5	5	10	15	30	15	30	15

[a-c] See footnotes in Table II. [d] The concentrations are expressed as mM vanadium atoms present in particular vanadate species.

dimer, tetramer, and pentamer. We will identify the possible statistical rate terms based on mass balance principles for the exchange reactions between monomer, dimer, tetramer, and pentamer. When defining these possible rate terms the exact reaction paths are not implied. Since the system is at equilibrium, the forward and backward rates of the individual exchange reactions, whether they are elementary steps or consist of several reaction steps, are identical. These rate terms are not dictated by specific assumed rate laws. Additional intermediates in the conversion between two or three oligomers may occur, and such possibility will be discussed below. Formation of intermediates presents no problems in this initial analysis since the EXSY ^{51}V NMR measure the overall rate of exchange between two species in equilibrium observed in the ^{51}V NMR spectrum. Determining the rate of exchange between observable species limits the number of possible reactions that must be considered, and the four species exchange problem becomes solvable. By using such analysis the major exchange reaction paths from the monomer, dimer, tetramer, or pentamer to the monomer, dimer, tetramer, or pentamer are identified.

We simplify our analysis by considering reactions at constant pH, temperature, and water concentration. The distribution of protonated vanadate oligomers is therefore constant, and the analysis need only to focus on the number of vanadium atoms in the reacting species. Although the vanadate reactions are likely to depend on $[H^+]$, $[OH^-]$, and/or the particular protonation form of the oligomer, this level of complexity has been ignored at the present time. Furthermore, the mechanisms of exchange are not specified, and only an overall rate term is used to describe a particular pathway. The rate term therefore specifies each pathway, and the question of the reaction paths by which vanadate derivatives exchange can be solved. Progress in understanding the four species exchange system represents a significant contribution in the elucidation of the mechanisms of vanadate oligomer exchange and thus justifies the assumptions above.

Magnetization transfer from vanadate monomer to dimer is represented by a rate constant (k_{12}) by which the vanadate monomer becomes dimer. The measured rate constants represent a composite of rate terms expressing various pathways. These pseudo-first-order rate constants can be converted to the total rate of magnetization transfer by multiplication with the concentration of the species involved. Thus the total rate of magnetization transfer from monomer to dimer is obtained as $k_{12}[V_1]$. We will use the term Y_{12} for this rate. The rate Y_{12} is the sum of all the pathways by which dimer can form from monomer. The possible pathways are obtained by examining the reaction of the monomer with each vanadium species observed in the NMR spectrum. The products of all these pathways must be the dimer and another vanadium species as defined by mass balance. Thus the pathway will be considered if the additional vanadium species is actually observed in the vanadium spectrum. For example, it follows that the monomer can react with either a monomer (V_1), a dimer (V_2), or a pentamer (V_5) and generate the dimer and one other species that is observed in the ^{51}V NMR spectrum (that is either a V_1, V_2, V_4, or V_5). One possibility involves the reaction of two vanadate monomers and results in the formation of the dimer (eq 5). We will call this rate term $v_{11/2}$. Another possibility involves a monomer and a pentamer forming a dimer and a tetramer. This pathway is called $v_{15/24}$. The last possibility represents the direct exchange mechanism that involves the reaction of labeled monomer (V_1) with a dimer and results in the formation of an unlabeled monomer and a dimer (eq 7). The species that was previously

$$V_1 + V_1 \rightarrow V_2 \tag{5}$$

$$V_1 + V_5 \rightarrow V_2 + V_4 \tag{6}$$

$$V_1{}^* + V_1V_1 \rightarrow V_1{}^*V_1 + V_1 \tag{7}$$

a monomer has now become a labeled dimer, and one of the vanadium atoms in the original dimer has become an unlabeled monomer (eq 7). This pathway is called $v_{12/21}$. The above pathways are in contrast to a pathway where monomer reacts with the tetramer (V_4) to generate a dimer and a trimer. Since a trimer is not observed in our ^{51}V NMR spectrum, this reaction does not represent a pathway that is considered in this analysis. Although we describe the reaction pathways as X reacting with Y to form products Z and U, no exact reaction mechanism has been assumed concerning this specific rate term.

The rate of magnetization transfer from monomer to dimer equals the magnetization transfer from dimer to monomer ($Y_{21} = Y_{12}$). The three possible mechanisms for the latter are the reverse reactions for Y_{12}. The first possibility is the spontaneous decomposition of the dimer to generate two monomers (eq 5) ($v_{2/11} = v_{11/2}$). The second possibility involves the reaction of the dimer with tetramer to form monomer and pentamer (eq 6) ($v_{24/51} = v_{15/24}$). The third possibility represents the direct exchange between dimer and monomer (eq 6) ($v_{12/21}$). Since the forward and backward reactions are related, the above relationships yield one equation with three unknown rate terms that express three different pathways for monomer-dimer exchange (eq 8).

$$Y_{12} = v_{12/21} + v_{11/2} + v_{15/42} \tag{8}$$

The magnetization transfer from monomer to tetramer (Y_{14}) has three reasonable mechanisms expressed by three rate terms. One possibility ($v_{14/41}$) is the direct exchange between a labeled monomer ($V_1{}^*$) and a tetramer ($V_1V_1V_1V_1$) to generate a labeled tetramer ($V_1{}^*V_1V_1V_1$) and a monomer (V_1). Another possibility ($v_{112/4}$) is the reaction of two monomers and one dimer to generate the tetramer. This rate term is of particular interest, because it is the only rate term that involves reaction of three vanadium species and is found to be a significant contributor to the exchange reaction. We will discuss this rate term in greater detail below. The third possibility ($v_{15/24}$) involves the reaction of a monomer and pentamer to generate the tetramer and a dimer. The Y_{14} equation therefore contains three unknown rate terms that express three different mechanisms for monomer-tetramer exchange (eq 9).

$$Y_{14} = v_{14/41} + v_{112/4} + v_{15/42} \tag{9}$$

The magnetization transfer from monomer to pentamer (Y_{15}) has three possible exchange rate terms. One possibility ($v_{14/5}$) is the reaction of the monomer with the tetramer to generate a pentamer. The second possibility involves direct exchange between the labeled monomer ($V_1{}^*$) and the pentamer ($V_1V_4 = V_5$) to generate the labeled pentamer ($V_1{}^*V_4$) and monomer (V_1). Another possibility ($v_{221/5}$) involves two dimers and one monomer to generate the pentamer. The latter possibility is less likely. The concentration of dimers is small, and thus any term involving two dimers is likely to be significantly less than the rate terms involving either a tetramer ($v_{14/5}$) or a pentamer ($v_{15/51}$). Furthermore, the simultaneous interaction of two vanadate dimers and one vanadate monomer seems less favorable at pH 8.6 than the two other pathways because the dimers have a charge of −3 and the monomer a charge of −2 generating a total charge of −8 for the former path and a lower charge for the two latter pathways. Even if reaction along this pathway ($v_{122/5}$) were to occur it would be very slow. Thus we will assume that the chemical exchange from this reaction is zero. Since we assume the $v_{221/5}$ term is small, the Y_{15} is defined by the sum of the reactions of monomer with either tetramer ($v_{14/5}$) or pentamer ($v_{15/51}$). The reverse reaction, Y_{51}, describes the reaction of a pentamer to generate the monomer and a tetramer or a monomer and a pentamer. These relationships, therefore, give one equation with two unknown rate terms (eq 10).

$$Y_{15} = v_{14/5} + v_{221/5} + v_{15/51} = v_{14/5} + v_{15/51} \tag{10}$$

The magnetization transfer from dimer to tetramer (Y_{24}) has three reasonable possibilities. One possibility ($v_{112/4}$) involves one dimer and two monomers to form the tetramer. This rate term is of particular interest because it is the only rate term that involves reaction of three vanadium species. We will discuss this topic in greater detail below. The other possibility ($v_{22/4}$) involves two dimers generating one tetramer. The third possibility ($v_{24/42}$) is the direct exchange mechanism where a labeled dimer ($V_2{}^*$) reacts with a tetramer (V_2V_2) to generate a labeled tetramer ($V_2{}^*V_2$)

Table V. Specific Exchange Rate Terms for Vanadate Solution at pH 8.6 and 1 M in KCl[a-d]

V_{tot}	$v_{15/42}$ (Y_{25})	$v_{112/4}$ ($Y_{14} - Y_{15}$)	$v_{14/5}$ ($Y_{15} - Y_{25}$)	$v_{22/4}$ ($Y_{24} - Y_{14} - Y_{25} + Y_{15}$)	$v_{11}/2$	$v_{12/21}$ ($Y_{12} - Y_{25} - v_{11/2}$)
5	0	20	0	−10	65	125
10	2.0	39	1.9	7	80	108
12.5	7.9	49	−4.3	14	97	105
15	7.1	64	7.9	2	110	173
20	8.8	96	5.2	−29	130	281
estimated uncertainty (%)	30	25	110	200		

[a-c] See Footnotes in Table II. [d] See Table IV for concentrations of various vanadate oligomers. [e] The best fits for k_p give 1×10^4 M^{-1} < k_p < 4×10^4 M^{-1}. The above calculations are based on an estimated k_p of 2×10^4 M^{-1} ($\pm 2 \times 10^4$ M^{-1}) which yield a k''' of $\sim 13 \times 10^4$ ($\pm 5 \times 10^4$) M^{-1}.

and a dimer (V$_2$). This overall relationship describes the total rate in an equation with three unknown rate terms (eq 9).

$$Y_{24} = v_{24/42} + v_{22/4} + v_{112/4} \tag{11}$$

The magnetization transfer from dimer to pentamer (Y_{25}) has three possibilities. One ($v_{24/51}$) involves the reaction of a dimer and a tetramer to generate a pentamer and a monomer. The other possibility ($v_{221/5}$) is the reaction of two dimers and one monomer to form a pentamer. This reaction rate is likely to be small in comparison with the $v_{24/51}$ term (see above) and as a result is assumed to be zero. The third possibility ($v_{25/52}$) is the direct exchange reaction between a labeled dimer (V_2^*) and a pentamer ($V_2V_2V_1$) to form a labeled pentamer ($V_2^*V_2V_1$) and dimer (V_2). This reaction is likely to be significantly slower and less important than the dimer and tetramer ($v_{24/51}$) reaction because the former involves oligomers of higher concentrations. In addition the larger charge repulsion in the reaction between dimer and pentamer may also decrease the contribution of this term. We therefore assume that the $v_{25/52}$ rate term is insignificant compared to the $v_{24/51}$ rate term and that $v_{25/52} = 0$. The overall rate for relationship Y_{52} therefore yields one equation with one unknown rate term (eq 12).

$$Y_{25} = v_{25/52} + v_{221/5} + v_{24/51} = v_{24/51} \tag{12}$$

The exchange between monomer, dimer, tetramer, and pentamer in solution at ambient temperatures demonstrates that these species have similar stabilities. Equations 9–12 describe four equations with seven unknown rate terms. The seven unknown rate terms can be reduced to four unknown rate terms by using the assumptions expressed in eqs 13 and 14. Two of these rate terms ($v_{24/51}$ and $v_{24/42}$) describe pathways that pass through the same section of the potential energy surface to form identical transition state(s) and possibly identical intermediate(s). The dimer and tetramer will react to form common transition state(s) and possibly identical intermediate(s) which will proceed to generate either pentamer and monomer or tetramer and dimer. The reaction of pentamer with monomer ($v_{51/42}$) is the back reaction for the dimer and tetramer reaction ($v_{24/51}$) and therefore $v_{24/51} = v_{51/24}$. The reaction of the pentamer and monomer is likely to form common transition state(s) and possibly identical intermediate(s) which will proceed to generate either tetramer and dimer or monomer and pentamer. On the basis of the structural and energetic similarities of the appropriate combinations of ground state oligomers, it is reasonable to expect that possible transition state(s) and intermediate(s) also are of similar energy. Therefore, it follows that $v_{51/24} \approx v_{51/15}$ and $v_{24/51} \approx v_{24/42}$ and eq 13 is obtained. It is reasonable to assume similar compositions of oligomers will involve similar structure of the transient state in a one-step reaction. If the reaction involves several steps, the assumption is likely to hold if the two reaction paths have the same rate-determining step. However, even if the assumption is not strictly correct, the rate terms shown in eq 13 are likely to be similar and small, and the error we make therefore small. We could have made the assumptions that some of these rate terms were zero, however, given the structural and energetic similarities of the combination of species involved, we chose to use the assumptions presented. By a similar analysis we will assume the $v_{14/5}$ and $v_{14/41}$ rate terms are equal (eq 14). Also in this case

$$v_{24/51} = v_{15/24} \approx v_{24/42} \approx v_{15/51} \tag{13}$$

$$v_{14/5} \approx v_{14/41} \tag{14}$$

the assumption holds if the reaction mechanism involves only one step or alternatively by a mechanism with several steps but maintaining the same rate-determining step. Also in this case, the assumption may not be strictly correct; however, the rate terms in eq 14 are likely to be similar and small, and thus the error we make is small. By using the assumptions shown in eqs 13 and 14 and substituting eq 12 into eqs 8–11, eqs 15–17 are obtained.

$$Y_{14} - Y_{15} = v_{112/4} \tag{15}$$

$$Y_{15} - Y_{25} = v_{14/5} \tag{16}$$

$$Y_{24} - Y_{14} - Y_{25} + Y_{15} = v_{22/4} \tag{17}$$

By using eqs 12 and 15–17 various exchange rate terms $v_{24/51}$, $v_{24/42}$, $v_{15/51}$, $v_{14/5}$, $v_{14/41}$, $v_{112/4}$, and $v_{22/4}$ can be calculated from our experimental data. These rates are shown in Table V (see below). Consequently we can identify which of these terms are important and which rate terms represent major reaction paths by which the exchange processes occur.

Kinetic Analysis: Experimental. In order to examine the exchange reactions between vanadate oligomers we measured the exchange matrix at various vanadate concentrations (5, 10, 12.5, 15, 20 mM) at constant pH 8.6. The ionic strength was kept essentially constant at 1.0 by using 1.0 M KCl during these measurements. The resulting rate constant matrices (see Table II for the 10 mM experiment) were converted to the rate matrices (see Table III for the 10 mM experiment), and the resulting rate terms Y_{12}, Y_{14}, Y_{15}, Y_{24}, Y_{25}, and Y_{45} are listed in Table IV. The reverse rates Y_{21}, Y_{41}, Y_{51}, Y_{42}, Y_{52}, and Y_{54} are identical with the reactions listed in Table IV. By using eqs 12 and 15–17 we have calculated the terms $v_{15/24}$, $v_{112/4}$, $v_{14/5}$, and $v_{22/4}$ at various vanadate concentrations, and the results are listed in Table V.

The experimental data analyzed by using the approach described above yields results which are consistent with chemical expectations and is examined in two cases. We have subjected our data to such tests, in order to increase the reader's confidence in our data and data analysis. First, $v_{24/51}$ describes the reaction between dimer and tetramer. This reaction is likely to occur by a second-order reaction mechanism between dimer and tetramer. We therefore examined the expected concentration dependence of this term $v_{24/15} = Y_{25} = k^{I}[V_2][V_4]$. We do indeed find that Y_{25} (eq 12) (within experimental uncertainly) is proportional to the dimer concentration multiplied by the tetramer concentration (Figure 3). We also consider the rate term $v_{22/4} = v_{4/22}$ which describes the reaction of two dimers to form a tetramer (eq 17). Because the forward reaction involves two dimers of low concentration with the charge of −3 each we expect this rate term will be small. This particular rate term is calculated from four measured magnetization transfer rates ($Y_{24} - Y_{14} - Y_{25} + Y_{15}$). Consequently the rate term is determined with very little accuracy over the studied concentration range (±200%). However, despite the large experimental uncertainty we indeed found the rate terms were very small as anticipated. These two correlations are in accord with expectations and thus support the validity of our analysis.

Tetramer–Pentamer Exchange. The exchange paths between the tetramer and pentamer have four statistical possibilities that satisfy the mass balance principle. The reaction between tetramer and dimer will form pentamer and monomer ($v_{42/51}$), reaction of tetramer with monomer will generate pentamer ($v_{41/5}$), reaction

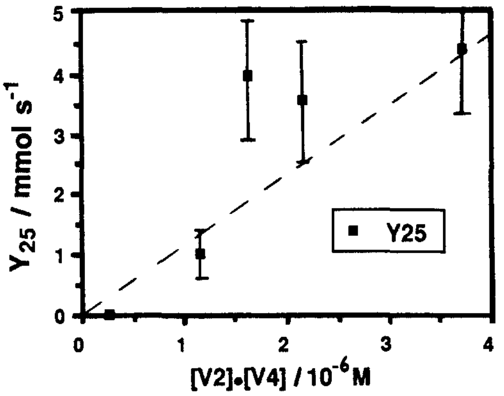

Figure 3. The magnetization transfer from a dimer to a pentamer ($Y_{25} = v_{24/51}$) is plotted as a function of the concentration of tetramer multiplied with the concentration of dimer. Estimated error bars are indicated.

of two tetramers will generate pentamer, monomer, and dimer ($v_{44/521}$), and the reaction of labeled tetramer with unlabeled pentamer will form a labeled pentamer and an unlabeled tetramer ($v_{45/54}$). We therefore anticipate the magnetization transfer from tetramer to pentamer occurs by using the four major rate terms shown in eq 18. By using eqs 12 and 15–17, eq 19 follows.

$$Y_{45} = v_{42/51} + v_{41/5} + v_{44/521} + v_{45/54} \tag{18}$$

$$Y_{45} - Y_{52} - Y_{15} + Y_{25} = Y_{45} - Y_{15} = v_{44/521} + v_{45/54} \tag{19}$$

Further exploration of quantification of $v_{44/521}$ and $v_{45/54}$ requires detailed studies over a larger concentration range than we have currently carried out. Therefore, we do not attempt to distinguish between the two paths $v_{44/521}$ and $v_{45/54}$ at the present time.

Involvement of Labile Reaction Intermediates. In conducting our analysis we have focused on the equilibrium reaction between vanadium oligomers present in significant concentrations and observable in the ^{51}V NMR spectrum at millimolar oligomer concentrations. This approach led us to examine reaction paths in which unspecified labile intermediates may be important to the mechanism of the exchange reaction. In addition to the monomer, dimer, tetramer, and pentamer discussed in this work, several additional minor oligomeric forms have been identified by ^{51}V NMR.[4] These species and possibly others could be intermediates in the reaction mechanisms of conversion between vanadate oligomers. Consider, for example, the rate term $v_{112/4}$. This rate term expresses a pathway that converts two monomers and one dimer to a tetramer. Mechanistically it is unreasonable to expect this pathway proceeds via a termolecular complex. It is, however, likely that the species react stepwise to generate a labile intermediate. Specifically, one can propose a reasonable mechanism where one monomer reacts with one dimer to generate a trimer. This trimer is too unstable to be observed in significant quantity and thus invisible by NMR.[4] However this trimer could react with the other monomer to ultimately form the tetramer. Since the solution is in equilibrium, the concentration of trimer is constant at a given total vanadate concentration. Consequently the rate term ($v_{112/4}$) represents the overall exchange. The formation of a labile trimer could be the explanation for why the $v_{112/4}$ pathway involves the reaction of three vanadium species and still represents a major reaction path.

If indeed a trimer were to form as an intermediate, would this intermediate also react with higher vanadate oligomers and possibly present alternative acceptable mechanisms for the rate terms $v_{221/5}$ and $v_{25/52}$? Such a presumed trimer intermediate is likely to be noncyclic and thus have a negative charge of −4.[4] If indeed a pentamer ($v_{221/5}$) were formed from such a noncyclic trimer reacting with a dimer, it would require two species present in low concentrations, and since the trimer is not observable under our conditions, its concentration is significantly lower than the dimer. In addition these compounds have −4 and −3 charges distributed over five vanadium atoms suggesting that reaction with each other will be slow. This reaction is therefore as unfavorable as the intermolecular mechanism requiring two dimers and a monomer. In the case of the direct dimer–pentamer exchange, a plausible mechanism could involve unimolecular decomposition of pentamer to generate the noncyclic trimer and a dimer. The reverse reaction involves the reaction of a trimer with a dimer to form a pentamer. The latter reaction step would require the reaction between two oligomers in low concentrations and with high negative charges. Since the rate term for the reaction between two dimers ($v_{22/4}$) is small, the reaction between a dimer and a trimer is likely to be even smaller. The assumption described in eq 12 ($v_{25/52} = 0$) is therefore in accord with such considerations. This suggests that even if the formation of low concentrations of additional vanadate oligomers were required to explain the detailed exchange reaction mechanisms, the considerations we have made concerning the rate terms in the overall exchange between oligomers are still likely to apply.

Assumptions in the Kinetic Analysis. In the kinetic analysis we assumed $v_{25/52}$ and $v_{221/5}$ were zero in the Y_{25} term eq 12. It is expected that $v_{25/52}$ and $v_{221/5}$ are considerably smaller than $v_{24/51}$, based on the lower concentrations of species involved and less favorable charge distribution of reactants in the former two rate terms. Since the Y_{25} is small even at high vanadate concentrations it follows that $v_{25/52}$, $v_{221/5}$, and $v_{24/51}$ will all be small. These assumptions have therefore been reasonable. The terms $v_{15/51}$ and $v_{15/5}$ are considerably larger than $v_{221/5}$, and since $v_{15/51}$ and $v_{14/5}$ are small, $v_{221/5}$ must be even smaller. The assumption of $v_{221/5} = 0$ in the rate term Y_{15} (eq 12) is therefore also reasonable.

We also made the assumptions described in eq 13 and 14. We assumed eq 13 would apply if the reaction was a one-step reaction or if the rate-limiting step in the reaction of $v_{24/51}$ would pass through a transition state similar in energy with the rate-limiting transition state(s) of $v_{15/51}$ and $v_{24/42}$ pathways. As seen from Table IV the rate term $v_{24/51}$ is small, suggesting that $v_{15/51}$ and $v_{24/42}$ will also be small and thus support the above assumption. We also assumed that eq 14 indeed would hold if this was a one-step reaction or the same rate-limiting step governed both reactions in a multistep mechanism. Since the rate term ($v_{14/5}$) was also found to be small the rate term $v_{15/51}$ is also likely to be small (Table V), suggesting that indeed the assumptions were reasonable.

Mechanistic Considerations. The rate of dimerization of vanadate monomer, k_D, was previously determined to be 3.1 × 10^4 M^{-1} under similar conditions (pH 7.0) with use of temperature jump relaxation times.[13,14] At constant pH the dimerization of vanadate monomer is likely to follow the rate law expressed as $v_{11/2} = k_D[V_1]^2$. The monomer–dimer exchange is likely to follow a rate law expressed as $v_{12/21} = k^{\text{III}}[V_1][V_2]$. Since $Y_{12} - Y_{25}$ equals the sum of the rate term for the dimerization reaction and the rate term for the direct exchange reaction of monomer with dimer, the rate can be expressed as eq 20. Consequently by solving the

$$Y_{12} - Y_{52} = v_{12/21} + V_{11/2} \cong k^{\text{III}}[V_1][V_2] + k_D[V_1]^2 \tag{20}$$

above equation for the various rates and monomer concentrations one can determine the dimerization rate constant. By using the data shown in Table IV, one should be able to calculate k_D. However, the accuracy of the current data is not sufficiently high to yield a single best fit for k_D and k. We observe that better fits are obtained when $k_D < k^{\text{III}}$. On the basis of the fits to eq 20 we estimate $k_D = 2 \times 10^4$ ($\pm 2 \times 10^4$) M^{-1}. Using these values we have calculated the contributions of dimerization and direct exchange in the Y_{12} term (Table V). Our estimate is in agreement with the previous dimerization rate constant determination of 3.1 × 10^4 M^{-1}.[13,14]

Our calculated exchange rates are in accord with the measured distribution of the vanadate species as a function of overall vanadate concentrations. At low vanadate concentrations monomer

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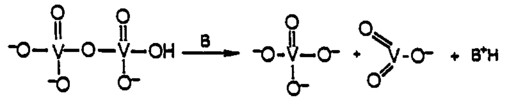
is the major species in solution. The rapid rates of conversion of monomer into dimer suggest that dimeric vanadate will be the next vanadate derivative formed in solution. As total vanadate concentrations increase it is experimentally found that the vanadate dimer appears, accompanied by the vanadate tetramer. The appearance of the tetramer is also reflected in the large rate term ($v_{112/4}$), where two monomers and one dimer are required for tetramer formation. The rate of tetramer formation from reaction of pentamer with monomer approaches the rate of tetramer formation from two monomers and one dimer at high total vanadate concentrations. However, at low vanadate concentrations the latter is the major mechanisms by which the tetramer forms.

When a tetramer reacts with monomer, dimer, tetramer, or pentamer, a new pentamer forms. At low vanadate concentration the two former mechanisms dominate. Previously the pentamer has been proposed to form from a tetramer and a monomer through an insertion mechanism.[4] Such a mechanism is indeed possible, and the rate term for the reaction of a tetramer with a monomer is measured. Our results show that this rate term is similar in size to the rate terms described by a tetramer and a dimer. Pentamer appears only in solutions with high total vanadate concentrations and high tetramer concentrations. This is consistent with the exchange rates mentioned above.

Our results demonstrate that the reactivity of the vanadate monomer is larger than that of the other oligomers. The monomer is involved in the largest number of exchange reactions, although at high vanadate concentrations the rate of tetramer to pentamer exchange grows more rapidly than any of the reactions involving a monomer. It is therefore conceivable that the tetramer–pentamer rate (Y_{45}) would become faster at even higher vanadate concentrations. Vanadate is known to have many biological effects and often these effects are observed at micromolar concentrations. At such concentrations vanadate monomer is essentially the only species present (vanadate dimer emerges approximately at or above 0.2 mM). It is possible that some of the observed biological effects of vanadate result from the high reactivity of the vanadate monomer with proteins or other cellular components.

The high lability of the vanadate dimer was somewhat surprising (Table V). The dimer hydrolyzes very rapidly suggesting that little energy is required to break the anhydride bond ($E_{a,25^\circ C}$ = 6.3 kcal/mol). This reactivity may be due to the reaction mechanism by which the dimer hydrolyzes. A hydrolysis mechanism analogous to the metaphosphate hydrolysis mechanism is one possibility (eq 21). Metavanadate has been observed by X-ray crystallography in some vanadate salts. A more likely hydrolysis mechanism involves nucleophilic attack of hydroxide (or water) on the dimer (eq 22). It is possible the high reactivity of the dimer may be important for interactions of vanadate in biological systems. The vanadate dimer has recently been reported to have biological activity as an inhibitor for phosphoglycerate mutase,[2a] acid phosphatase,[2b] and glucose-6-phosphate dehydrogenase.[2c] The dimer has also been implicated in the inhibition of yeast growth.[15]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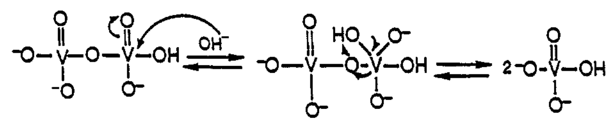
(22)

The identity of the major vanadium species in neutral solutions has been controversial for several decades.[3,4] Recent evidence has favored a tetrameric species over a trimeric species.[4] If the cyclic tetramer is the most favorable species then one may ask why the cyclic trimer does not form in similar quantities. A noncyclic trimer has been observed as a minor oligomer at 0.1 M vanadate concentrations in the pH range 9–11.[4a] The structure of this trimer may be bent or linear depending on whether the favored extended conformation of tri- and tetraphosphates is also valid for the vanadate trimer. It is possible that the charge repulsion in the linear trimer increases as the two ends approach each other to form a cyclic trimer. Consequently, the energy of activation for cyclization should increase as the length of the vanadate chain decreases. It is possible cyclization in aqueous solutions of a linear trimer may not occur over in the observed temperature range. The fact a cyclic trimer is not observed may therefore result from unfavorable kinetics rather than from unfavorable thermodynamics. This is consistent with the cyclic triphosphates that also do not form in aqueous solution from linear triphosphates. The cyclic trimetaphosphate is prepared at 500 °C in nonaqueous media.

The linear tetramer has also been reported as a minor vanadate oligomer (0.5% of total vanadate).[4] The low stability of both the linear trimer and tetramer follows the low stability of the dimer compared to the cyclic tetramer or pentamer. The high rate by which the dimer hydrolyzes may be increased with additional vanadium groups. The cyclic tetrameric derivative represents a more stable form of the tetramer, and its rate of decomposition should be correspondingly less.

Conclusions

Two-dimensional ^{51}V homonuclear NMR exchange has been used for quantitative studies of exchange reactions between four vanadate oligomers. The 2D ^{51}V NMR spectra show that all the major vanadate oligomers observed at pH 8.6 (monomer, dimer, tetramer, and pentamer) exchange with each other. A kinetic analysis combined with exchange rates determined by using the EXSY method allowed quantification of several exchange paths. The major exchange paths responsible for formation of various oligomers could therefore be identified.

The vanadate dimer is mainly formed from two monomers. Dimerization and direct monomer–dimer exchange have the highest exchange rates. A minor route for formation of dimer is the reaction of monomer with pentamer. Tetramer is mainly formed from the overall reaction between two monomers and a dimer. The actual mechanism may involve formation of a noncyclic trimer. At higher vanadate concentrations the exchange reactions between a tetramer and another tetramer or a pentamer increase rapidly. The vanadate pentamer forms by three mechanisms. At low vanadate concentrations, the pentamer mainly forms from the reaction of tetramer with either monomer or dimer. The rate terms of these pathways are similar. However, as the vanadate concentration increases the tetramer–pentamer exchange rate term increases and becomes dominant.

We found that the vanadate monomer is the most reactive vanadate derivative. It is involved in reactions with all vanadate oligomers, and the rates are all significant. The dimer has the highest unimolecular decomposition rate. We speculate that noncyclic monoprotonated trimer is more unstable than the dimer because of high charge density. The trimer's high and unfavorable charge density can be relieved if a V–O–V bond is cleaved and the third vanadium group is lost.

This work presents the first quantitative analysis of exchange reactions between four species. We have been able to identify (by quantification) the major reaction paths for formation of various vanadate species by using the EXSY method. The quantitative rate determination has yielded information about the kinetic stability of the vanadate oligomers. This method clearly has tremendous potential for achieving further insight into complex multipath exchange reactions. It is possible that some of the biological activities exhibited by vanadate are a result of the kinetic properties of vanadate oligomers. The method and analysis we have described in this manuscript will allow studies of both chemical and biological samples, and such studies will yield further insight into how vanadium interacts in both chemical and biological systems.

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Conformational Dynamics of Proline Residues in Antamanide. *J* Coupling Analysis of Strongly Coupled Spin Systems Based on E.COSY Spectra

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Abstract: The conformational dynamics and individual conformations of the four proline residues in the cyclic decapeptide antamanide in solution are determined from vicinal proton coupling constants combined with the measurement of carbon-13 relaxation times. The coupling constants, obtained by a least-squares analysis of a two-dimensional E.COSY spectrum, are interpreted in terms of generalized Karplus equations. It is found that Pro^3 and Pro^8 are conformationally rigid, while Pro^2 and Pro^7 are mobile with a significant population of a second conformation. The time constants that govern the conformational dynamics of the proline residues are estimated to be between 30 and 40 ps.

1. Introduction

It is known that the conformational dynamics of peptides can be relevant for their biological activity. Often, binding to a substrate leads to additional conformational constraints. In many cases, it is the interconversion rate between conformations that determines the rate of complex formation.

The cyclic decapeptide antamanide, cyclo-(-Val^1-Pro^2-Pro^3-Ala^4-Phe^5-Phe^6-Pro^7-Pro^8-Phe^9-Phe^{10}-), is known to exhibit several conformations which can dynamically interconvert.[1] This molecule has been studied in great detail by various research groups. The crystal structure has been determined by Karle et al.[2] The structure in solution was investigated by Tonelli,[3] Dorman and Bovey,[4] Pook et al.,[5] and most recently in an extensive study by Müller[6] and Kessler et al.[7-10] It was discovered in 1973 by Patel[11] that antamanide is involved in a dynamic equilibrium between different backbone conformations. The rate constant of interconversion ($\sim 10^6$ s^{-1}) has later been measured by Burgermeister et al.[12,13] by means of ultrasonic absorption experiments. It has also been found that complexation with ions, such as Na^+ and Ca^{2+}, inhibits the conformational dynamics.[12] Evidently this type of motion is related to its binding properties.

In addition, antamanide shows degrees of motion that are associated with the rotational isomerism of side chains, such as phenylalanine ring flips. Rate processes that can be associated with this type of motion have been observed in ultrasonic absorption measurements.[13] These processes have correlation times on the order of the inverse nuclear Larmor frequency in typical NMR experiments and are therefore relaxation-active.

This paper is concerned with a further type of conformational dynamics in antamanide, the proline ring flips. This process is expected, if it occurs at all, to be considerably faster than the backbone motion and the side-chain mobility mentioned above. The conformation of proline rings has already been determined in different types of peptides.[14-22] It has been found that the free proline ring without external constraints can occur in two almost equal-energetic conformations that rapidly interconvert.[18,19] Upon introduction of external constraints, such as in cyclic peptides or in proteins with a confined structure, the conformational mobility can be restricted or inhibited. Conditions for rigidity versus flexibility have been summarized by Cung et al.[22]

In antamanide it has been found that two of the four proline rings, Pro^3 and Pro^8, are fixed in the $^{\beta}_{\gamma}T$ conformation,[7,8] while the other two proline systems, Pro^2 and Pro^7, could not be analyzed due to strong coupling in the proton resonance spectrum which complicates the spectrum to an extent that inhibits a straightforward analysis.

In this paper, we present a complete analysis of the structure and dynamics of all four proline ring systems in antamanide. A new strategy is introduced that combines the knowledge of proton spin coupling constants and carbon-13 relaxation times. A recently developed least-squares computer-fitting procedure[23] is used to

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