Reactions of Mono- and Diperoxovanadates with Peptides Containing Functionalized Side Chains

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Abstract: The equilibria established between the mono- and diperoxovanadates and a number of di- and tripeptides in aqueous solution has been studied by ^{51}V NMR spectroscopy. Both monoperoxovanadate and diperoxovanadate react favorably with a number of these peptido ligands. However, the mode of complexation is quite different for the two types of peroxovanadates. Diperoxovanadate gives rise to unidentate products with condensation occurring through the carboxyl or amino groups and also through the aromatic hydroxyl of tyrosyl residues. Particularly favored complexes are formed with imidazole nitrogens, but complexes that involved tryptophan nitrogens were not observed to form. In contrast with diperoxovanadate, monoperoxovanadate forms multidentate complexes with the peptides studied. These materials form slowly but are highly favored. For the simple dipeptide, glycylglycine, the amino group, the carboxyl group, and the peptide nitrogen comprise the points of attachment. The carboxylate group can be replaced by a hydroxymethyl as in glycylserine. Also, the peptide nitrogen, but apparently not the carboxyl or amino groups, can be substituted by a histidine imidazole nitrogen. Tryptophan nitrogens, as similarly found for diperoxovanadates, do not appear to undergo significant interactions with monoperoxovanadate. Although phenol and tyrosyl hydroxyl groups undergo moderately strong interactions with monoperoxovanadate, no corresponding monodentate reactions were observed with imidazole rings. Some possible biochemical implications of this work are discussed.

Introduction

Over the past several years, the biochemical importance of vanadate oxoanions has become well established.[1] As well, the synergistic influence of hydrogen peroxide on many of the responses generated by vanadate has led to an increased interest in the function of these materials. The influence of peroxovanadate complexes is diverse. They lead to enhanced insulin-like effects in rat adipocyte lipogenesis, protein synthesis, antilipolysis, insulin receptor tyrosine kinase activity,[2-5] and antitumor activity.[6] They are effective oxidants[7] and are important in the function of vanadium-dependent peroxidases.[8]

The molecular basis for many, if not all, of the biological responses of the peroxovanadates is not known. A basic requirement for understanding the biological function of peroxovanadates is the accumulation of information concerning their reactions with metabolites such as amino acids and simple peptides and also other biochemically relevant materials.

Work in this area of research has been initiated and a study of the reactions between peroxovanadate and simple peptides reported.[9] This work revealed that peptides undergo rather simple monodentate reactions with diperoxovanadate through either the carboxylate or the amine functionalities but not both simultaneously. Monoperoxovanadate, however, was found to react in a substantially different manner, apparently forming a tridentate complex as the major product with complexation occurring by means of the terminal amino, the terminal carboxylate, and the peptide nitrogen of a dipeptide. Protection of any of these groups prevented formation of this product. The most notable protecting group is an amino acid, to form a tripeptide, and also in this case, the product was not observed.

A question arises as to whether products similar to those described above will be formed if the individual residues of a polypeptide have functionalized side chains. A study of mono- and diperoxovanadate interactions with di- or tripeptides containing one or two functionalized amino acid residues is reported here.

Experimental Section

Materials. The peptides used in this study were purchased from Sigma Chemical Company and were of PFS quality. Phenol (ACS reagent quality), vanadium(V) oxide (99.99% (Aldrich Chemical Co.)), hydrogen peroxide (3% (Fisher Scientific Co.)), and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Boehringer Mannheim GmbH) were used without further purification.

A stock solution (0.1 M) of sodium vanadate from vanadium(V) oxide was prepared according to previously described procedures.[10] The hydrogen peroxide solution (3% as obtained) was standardized against potassium permanganate and then diluted with distilled water to give a 0.1 M stock solution. The 0.1 M stock solution of hydrogen peroxide was stored in the refrigerator and was stable over a period of 6 months (checked by titrating against $KMnO_4$). Stock solutions of phenol (0.7 M), glycyltyrosine (0.22 M), glycylhistidine (0.08 M), glycylglycine (1.5 M), KCl (2 M), and HEPES (1 M) were prepared in distilled water. Other peptides used in this study were weighed and directly added to peroxovanadate solutions and in some cases warmed in order to dissolve the peptide.

Before the final mixing, a second stock solution of hydrogen peroxide (75 mM)/HEPES (25 mM) was prepared from the 100 mM stock solution of hydrogen peroxide and its pH adjusted to 6.5. The ionic strength of all sample solutions was maintained at 1.0 M with KCl. Appropriate proportions of stock vanadate, buffer, and KCl were taken, and the pH was brought to 6.5. To these solutions was added the required amount of hydrogen peroxide, and the solutions were mixed by shaking. The appropriate amount of peptide or phenol was added to the peroxovanadate solution, and the final concentrations were obtained by adding distilled

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water. In this study, vanadate and hydrogen peroxide concentrations were varied between 3 and 9 mM. To minimize the hydrogen peroxide decomposition catalyzed by vanadate, the required amount of H_2O_2/HEPES solution was added to the vanadate solution only as these solutions were required. The peptide or phenol solution was added last. Mixing in this way allowed equilibrium to be established quickly. The final pH of the solutions was obtained by adjusting with NaOH or HCl.

Monoperoxovanadate–glycylglycine sample solutions were prepared about 7 h before the NMR spectra were obtained because the equilibration of these solutions at pH values over about pH 8.5 requires about 7 h. At lower pH, it takes even longer to establish the equilibrium. NMR spectra of these samples were obtained after 24 h and after 1 week.

Buffers of pH 4.0 and 10.0 were used to calibrate the pH meter. The calibration was verified with a pH 7.0 buffer.

Spectroscopy. ^{51}V NMR spectra were obtained from a Bruker AMX-400 NMR spectrometer operating at 105.2 MHz at ambient room temperature (22 ± 1 °C). Vanadium chemical shifts are relative to the external reference $VOCl_3$ assigned to 0 ppm. NMR spectral parameters: pulse width 60°, spectral width 80 KHz, acquisition time 0.05 sec, line broadening 40 Hz, and frequency domain size 16k data points. Baseline corrections were applied to all spectra before integrals were obtained. ^1H and ^{13}C NMR spectra were obtained using standard techniques.

Methods. The equilibrium equations derived on the basis of the concentration studies were put into the appropriate linear form, as outlined in the text, and results were analyzed using standard least-squares techniques. Errors in the reported parameters are quoted at the 95% confidence level.

Results

When vanadate is combined with hydrogen peroxide in aqueous solution, a number of peroxovanadates are formed.[1,9,11-13] The proportion of products formed is dependent on the total concentration of vanadate and of hydrogen peroxide and also on the pH and ionic strength of the solution. For this work, the ionic strength was maintained at 1.0 M with KCl and pH 6.5 unless a pH variation study was being carried out. The concentrations of both the total vanadate and the total hydrogen peroxide were generally restricted to the range 3.0–9.0 mM. Under these conditions, the peroxovanadate species observable in solution are the monoperoxide ($V\ell$), the diperoxide ($V\ell_2$), and the triperoxide ($V\ell_3$), and also the divanadio tetraperoxide ($V_2\ell_4$). With an increase in pH, the monoperoxovanadate ($V\ell$) becomes increasingly favored relative to the other products.

In the presence of a simple peptide (P) such as glycylglycine and with 3 mM vanadate and 9 mM hydrogen peroxide at pH 6.5, two products are observed. They both are formed according to the equilibrium of eq 1, one product deriving from reaction at

$$V\ell_2 + P \overset{K_1}{\rightleftharpoons} V\ell_2P \qquad [V\ell_2][P]K_1 = [V\ell_2P] \qquad (1)$$

the carboxylate group, the other, at the amino group.[9] The ^{51}V NMR chemical shifts of these products and ones from similar dipeptides are about –717 and –745 ppm for the carboxylate-derived and amino-derived products, respectively.[9] If the peptide has side chains with functional groups, then there is the possibility for formation of additional products as seen, for instance, in the case of glycyltyrosine.

Reaction of glycyltyrosine under conditions of 3.0 mM vanadate, 9.0 mM H_2O_2, and 60 mM glycyltyrosine at pH 6.5 gave three products with ^{51}V chemical shifts of –715, –731, and –744 ppm. The –715 and –744 ppm signals derive from reaction at the carboxylate and at the amino group as discussed above. The –731 ppm product is assigned to reaction at the hydroxyl group of the phenyl ring. The assignment was supported by the finding that phenol also provided a single product with a chemical shift of –731 ppm. Hydroxyl groups tend not to give rise to

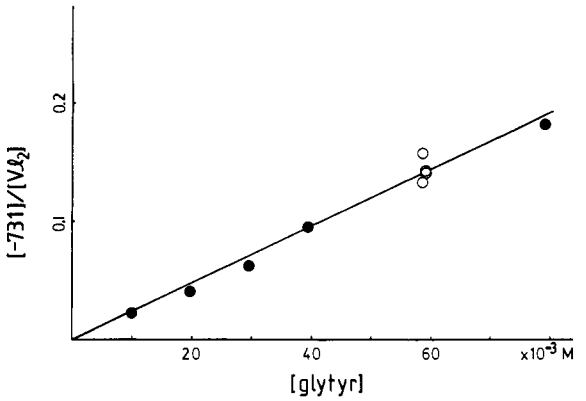

Figure 1. Formation of the –731 ppm product of the reaction of diperoxovanadate with the aromatic hydroxyl group of glycyltyrosine at pH 6.5 plotted according to eq 1. The graph is consistent with a diperoxo mono-ligand product. Conditions of the experiments: total H_2O_2, 9.0 mM; HEPES buffer, 20 mM; pH 6.5; ionic strength, 1.0 M. Solid circles represent total vanadate (3.0 mM) and variable GlyHis. Open circles represent total GlyHis (60 mM) and total vanadate varied from 3 to 7 mM.

diperoxovanadate derivatives; however, the pK_a of phenol (or tyrosine Ar–OH) is low enough to allow the observation of products, as suggested by the rather sensitive linear free energy relationship between the pK_a of the conjugate acid of the ligand and the logarithm of the formation constant.[9]

In order to confirm the $V\ell_2P$ stoichiometry expected for the –731 ppm product, vanadium and phenol concentration studies were carried out at pH 6.5. For this study, the total hydrogen peroxide concentration was maintained at 9.0 mM while the total vanadate concentration was varied from 3 to 7 mM at a constant phenol concentration of 200 mM. The phenol concentration was varied from 50 to 235 mM with 3 mM vanadate and 9 mM H_2O_2. When the results of the concentration study were plotted according to eq 1, an excellent linear relationship was obtained from which a value of 1.5 ± 0.2 M^{-1} was obtained for K_1. A similarly good correlation was obtained for the glycyltyrosine study (product ppm also –731), as can be seen in Figure 1. The formation constant obtained here, K_1 = 2.3 ± 0.1 M^{-1}, is close to that determined for the phenol product. The carboxylate- and amino-derived products, also observed in this study, had formation constants of 1.0 ± 0.2 and 4.1 ± 0.3 M^{-1}, respectively. The excellent linear correlations obtained strongly suggest that equilibrium conditions are obtained in about 5 min. In order to confirm this, a spectrum of one sample was obtained after an additional 6 h. No measurable change in the spectrum was observed.

At the higher vanadate concentrations of the above study, where the monoperoxovanadate is observable (–618 ppm), a product, additional to those discussed above, was formed and gave rise to a ^{51}V chemical shift at –605 ppm. This product was observed both with phenol and with glycyltyrosine and was assigned to an aryl monoperoxovanadate derivative.

In order to characterize all the observed products more completely, pH variation studies were carried out both for the phenol and for the glycyltyrosine systems. Reaction at the terminal carboxyl and the terminal amino groups of dipeptides has previously been described, and it has been shown that no protons are required or released during the reaction of the diperoxovanadate monoanion with either the terminal $-CO_2^-$ or the terminal $-NH_2$ of small peptides.[9] Those conclusions are in accord with all observations for the systems studied here.

Preliminary studies indicated there was proton loss as condensation occurred with the aromatic hydroxyl group so that product formation proceeds as described by eq 2, where it is now necessary to consider the protonation states of all reactants. The pK_a of $V\ell_2^-$ is close to 7.4, dependent to an extent on the reaction

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$$V\ell_2^{1-} + ArOH \underset{}{\overset{K_1'}{\rightleftharpoons}} V\ell_2OAr^{(n+1)-} + nH^+$$
$$K_{a2} \Big\| \; H^+ \quad K_a' \Big\| \; H^+$$
$$V\ell_2^{2-} \qquad ArO^- \qquad (2)$$

conditions.[9,14] The pK_a values obtained here were 7.4 for the 200 mM glycyltyrosine solution and 7.6 for the 200 mM phenol solution. These values are for ~1 M KCl and are about 0.5 of a pK_a unit higher than the value reported for a low ionic strength solution.[13] The pK_a value for the aromatic hydroxyl of phenol is close to 9.9 and that for glycyltyrosine is about 10.35.[15]

Under conditions of 3.0 mM total vanadate and 9.0 mM total hydrogen peroxide, only one product ($V\ell_2OAr$, −731 ppm) of reaction at the hydroxyl group of phenol was observed except at high pH, where a second product ($V\ell OAr$) was observed to form. Figure 2 shows a graph of the results of the pH variation study, for phenol, when plotted appropriately for eq 2, with $n = 1$. This graph is fully consistent with the loss of a single proton as product formation occurred. There was no indication from the graphical results that the product had a pK_a near the range of pH variation, pH 6.5–10.7. Over this pH range, both the $V\ell$ and $V\ell_2$ NMR signals undergo large chemical shift changes,[13,14] while the chemical shift of the phenol-derived product remains constant. Not surprisingly, the product formed with glycyltyrosine also had a pH-independent chemical shift. The formation constants, K_1' of eq 2, determined for phenol and for glycyltyrosine were $(5.1 \pm 0.4) \times 10^{-7}$ and $(8.4 \pm 0.5) \times 10^{-7}$, respectively.

pH variation studies were also done for concentrations of 7 mM total vanadate, 3 mM hydrogen peroxide, and 200 mM phenol or 60 mM glycyltyrosine. These concentrations allowed the formation of the monoperoxo products (−605 ppm for phenol, −604 ppm for glycyltyrosine) to be studied over the complete range of pH values. The preliminary indications were that the monoperoxo derivative was formed in a reaction analogous to that of formation of the diperoxo product, that is, as described by eq 3. The pK_a of $V\ell^{1-}$ was determined to be 6.2 from pH dependence of the ^{51}V NMR chemical shift and is in good agreement with previous measurements.[14]

$$V\ell^{1-} + ArOH \underset{}{\overset{K_2'}{\rightleftharpoons}} V\ell OAr^{(n+1)} + nH^+$$
$$K_{a2} \Big\| \; H^+ \quad K_a' \Big\| \; H^+$$
$$V\ell^{2-} \qquad ArO^- \qquad (3)$$

The results of the pH variation study, in a form appropriate to eq 3, are displayed in Figure 3 for glycyltyrosine. The excellent linear correlation is obtained only for a value of $n = 1$, which means that a proton is released as the product forms from $V\ell^{1-}$ and the aromatic hydroxyl group. The formation constant, $(7.1 \pm 0.4) \times 10^{-6} = K_2'$, is about a factor of 10 larger than that for reaction with $V\ell_2^{1-}$ and similar to that determined for phenol, $K_2' = (6.2 \pm 0.3) \times 10^{-6}$. Phenol did not give any observable products other than $V\ell OAr^{2-}$ and $V\ell_2OAr^{2-}$. In addition to products similar to the above, glycyltyrosine also gave, as mentioned previously, products deriving from reaction at the carboxyl and amino groups. An additional minor product with a ^{51}V NMR signal at −624 ppm was also observed, but its formation was not characterized. The pH-independent formation constants measured for these two systems are collected in Table I. There was no indication from these studies that the $V\ell OAr^{2-}$ product either lost or gained a proton throughout the pH range, approximately 6.5–10.6.

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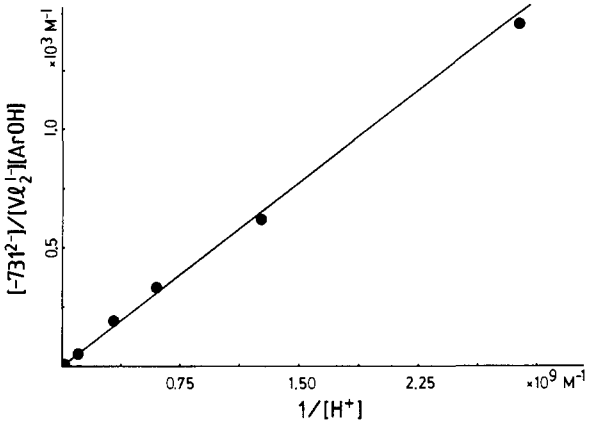


Figure 2. Dependence of the formation of the −731 ppm phenol-derived product on the inverse of the hydrogen ion concentration. This inverse dependence is consistent with proton release as the diperoxovanadium(V) product is formed. Conditions of the experiments: total vanadate, 7.0 mM; total hydrogen peroxide, 3.0 mM; total phenol, 200 mM; KCl, 1.0 M; HEPES buffer, 20 mM; variable pH, 6.51–9.46.

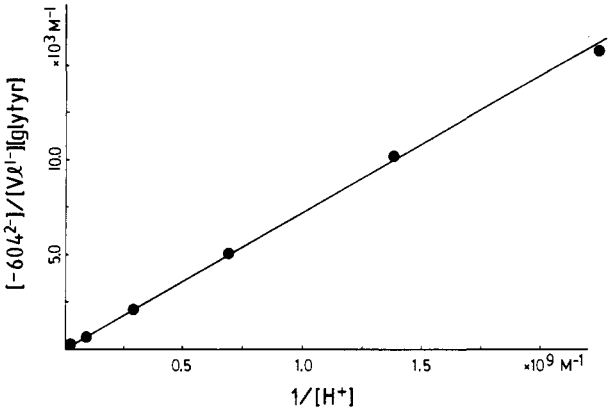


Figure 3. Relationship between the inverse of the hydrogen ion concentration and the formation constant for the −604 ppm product of the equilibrium between the monoperoxovanadate monoanion and neutral glycyltyrosine. The linear plot is consistent with release of a single proton as an aryl monoperoxovanadate product is formed. Conditions of the experiments: total vanadate, 7.0 mM; total hydrogen peroxide, 3.0 mM; total glycyltyrosine, 60 mM; HEPES buffer, 20 mM; variable pH 6.89–9.35.

Table I. Formation Constants for the Reactions of Glycyltyrosine and Phenol with Monoperoxo- and Diperoxovanadate

	Variable pH[a]		
ligand	$V\ell^{1-} + ArOH \rightleftharpoons V\ell OAr^{2-} + H^+$	$V\ell_2^{1-} + ArOH \rightleftharpoons V\ell_2OAr^{2-} + H^+$	$V\ell_2^{1-} + H_3N\text{–}P \rightleftharpoons V\ell_2H_2N\text{–}P^{1-} + H^+$
GlyTyr	$(7.1 \pm 0.4) \times 10^{-6}$	$(8.4 \pm 0.5) \times 10^{-7}$	$(1.5 \pm 0.1) \times 10^{-6}$
phenol	$(6.2 \pm 0.3) \times 10^{-6}$	$(6.1 \pm 0.4) \times 10^{-7}$	

	Fixed pH[b]					
	$V\ell + ArOH \rightleftharpoons V\ell OAr$		$V\ell_2 + ArOH \rightleftharpoons V\ell_2OAr$		$V\ell_2 + N\text{–}P \rightleftharpoons V\ell_2N\text{–}P$	
ligand	pH 6.5	pH 7.0	pH 6.5	pH 7.0	pH 6.5	pH 7.0
GlyTyr	7.5 ± 0.4	9.7 ± 0.6	2.3 ± 0.2	6.7 ± 0.5	4.1 ± 0.3	10.1 ± 0.7
phenol	6.6 ± 0.3	8.5 ± 0.5	1.5 ± 0.2	4.9 ± 0.5		

[a] These pH-independent constants were measured under the following conditions: total vanadate, 7.0 mM; total hydrogen peroxide, 3.0 mM; HEPES, 20 mM; μ = 1.0 M KCl; variable pH; glycyltyrosine (60 mM) or phenol (200 mM). [b] All pH-dependent formation constants (M^{-1}) were calculated using eq 4 and the corresponding pH-independent values except for those measured at pH 6.5 as discussed in the text.

The relationship between the pH-independent and the observed pH-dependent formation constants, that is between K_1 and K_1' and K_2 and K_2', is given by eq 4, where K_a is the appropriate value

$$K = \frac{K'}{[H^+](1 + K_a/[H^+])(1 + K_{aL}/[H^+])} \quad (4)$$

for $V\ell^{1-}$ or $V\ell_2^{1-}$ and K_{aL} is the corresponding value for the ligand. K is a generic formation constant that can refer to product formation from an aromatic hydroxyl, the terminal $-NH_3^+$, or the terminal $-CO_2H$, since in all cases a single proton is lost as product formation occurs.

Application of eq 4 to the results of the pH study gave the calculated values, $K_1 = 1.7 \pm 0.2$ M^{-1} and $K_2 = 6.6 \pm 0.3$ M^{-1} for the phenol ligand at pH 6.5. The value of K_1 measured at pH 6.5 was 1.5 ± 0.2 M^{-1}, in excellent agreement with the calculated value. For the glycyltyrosine, the calculated values were $K_1 = 2.4 \pm 0.2$ M^{-1} and $K_2 = 7.5 \pm 0.4$ M^{-1}. The measured value of K_1 was 2.3 ± 0.2 M^{-1}, again in excellent agreement. K_2 was not readily measurable at pH 6.5 since lower pH disfavors the formation of $V\ell$ relative to free vanadate and $V\ell_2$; however, the good agreement between the two sets of K_1 values indicates there are no problems with the establishment of proton stoichiometry.

Previous studies[11] have shown that imidazole and histidine undergo highly favorable condensation reactions with $V\ell_2$, giving rise, in the case of histidine, to two observable products. Similarly, in the presence of glycylhistidine, two products were observed to form. The ^{51}V chemical shifts were -742 and -751 ppm, and the products are assigned to reaction at the two different nitrogens of the imidazole ring. A third product resulting from reaction at the terminal amino group was also observed (-746 ppm), but its formation was less favored than that of the other products.

The results of concentration variation studies showed that all of the glycylhistidine products were formed as described by eq 1 and in accordance with the results of studies of imidazole and *N*-methylimidazole.[11] Figure 4 shows the results, obtained at pH 6.5, for the formation of the minor (-742 ppm) glycylhistidine imidazole ring derived product. The excellent linear correlation gave a value of 39 ± 2 M^{-1} for the formation constant. The results obtained for the major (-751 ppm) product were similarly good, and a value of $(2.8 \pm 0.2) \times 10^2$ M^{-1} was obtained for the formation constant. The corresponding value for *N*-methylimidazole is $(3.7 \pm 0.4) \times 10^2$ M^{-1}.[11]

The formation of the imidazole-derived products of glycylhistidine is pH dependent. However, when the results were plotted graphically according to an appropriately modified eq 2 (pK_a of glycylhistidine equals 6.79[15]), a small but systematic deviation from a linear relationship was observed. This deviation from linearity is not caused by neglecting the protonation/deprotonation reaction at the terminal amino group of the peptide because similar behavior was observed for both the imidazole and *N*-methylimidazole ligands.[11] In this latter work, time course studies were also undertaken to ensure that equilibrium conditions had been obtained. Attempts to resolve this problem by considering a number of alternative reaction equilibria have proven unsuccessful. It appears that minor products with pH-dependent formation constants and undergoing rapid exchange kinetics are being formed. The experimental results for both the concentration study at pH 6.5 and the pH variation study are given in Table II.

Because of the interference from the large water signal of the H_2O solutions, attempts were made to observe the proposed minor components by obtaining both vanadium and proton NMR spectra of the products in D_2O solutions. The vanadium spectra, essentially, were indistinguishable from those of the H_2O solutions. The proton spectra revealed two sets of "sharp" product signals (8.28 and 7.33 ppm for the major product and 8.07 and 7.30 ppm for the minor product at pH 7.0) with intensity ratios of about 7:1. The histidine imidazole ring signals of the free ligand were much broader than the signals from the product complexes and also broader than the corresponding signals observed in the absence of vanadium. Cooling the sample to 275 K yielded much better resolved signals. Unfortunately, it appeared that much of the line sharpening was more the result of chemical shifts moving together than the result of slowed exchange. However, the presence of an additional minor product, present in lower relative concentrations, was revealed.

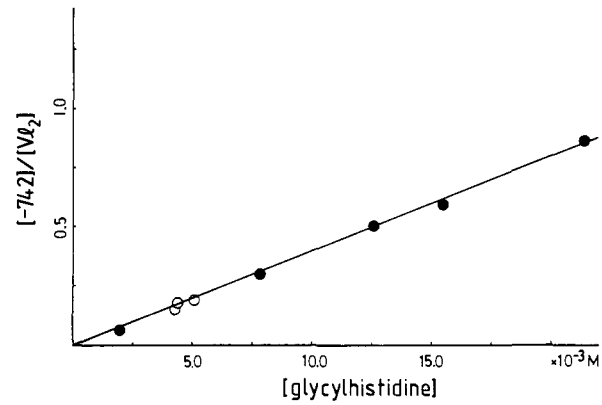


Figure 4. Formation of the -742 ppm product of the reaction of glycylhistidine with diperoxovanadate plotted according to eq 1. The linear relationship observed indicates that the product is a mono-ligand diperoxovanadate. Conditions of the experiments: total H_2O_2, 9.0 mM; HEPES buffer, 20 mM; pH 6.5; KCl, 1.0 M. Solid circles represent total vanadate (3.0 mM) and variable GlyHis (3.0–24.0 mM). Open circles represent total GlyHis (7.0 mM) and variable vanadate (3.0–7.0 mM).

Table II. ^{51}V NMR Data for the Formation of Products from the Interactions of Peroxovanadate with Glycylhistidine[a]

pH	V(t)[b,c]	Gly-His(t)[b]	[Vℓ_2]	[Vℓ_3]	[−742]	[−751]	[−746]	[V$_2\ell_4$]
				pH 6.5				
	3.0	3.0	1.79	0.02	0.11	0.90		0.18
	3.0	7.0	1.09	0.04	0.21	1.66		
	5.0	7.0	1.97		0.30	2.39		0.35
	7.0	7.0	2.03		0.36	2.12		0.33
	3.0	10.0	0.84	0.01	0.25	1.90		
	3.0	15.0	0.58		0.29	2.12		
	3.0	18.0	0.51		0.30	2.20		
	3.0	24.0	0.38		0.32	2.30		
				Variable pH				
6.05	3.0	7.0	1.57		0.16	1.28		
6.49	3.0	7.0	1.09	0.04	0.221	1.66		
6.89	3.0	7.0	0.76	0.04	0.20	2.01		
7.44	3.0	7.0	0.60	0.14	0.25	1.92	0.07	
8.58	3.0	7.0	1.45	0.27	0.16	0.98	0.13	
8.86	3.0	7.0	1.76	0.35	0.10	0.70	0.11	
9.14	3.0	7.0	2.00	0.45	0.04	0.44	0.07	
9.58	3.0	7.0	2.33	0.46		0.21		

[a] All concentrations are given in units of 10^{-3} M. In addition to the indicated concentrations of added vanadate and glycylhistidine, each sample contained 9 mM hydrogen peroxide, 20 mM HEPES buffer, and 1.0 M KCl. [b] Total concentration of the added components. [c] In some cases, reported concentrations of vanadium reactants and products do not add to V(t). This is because free vanadate and peptidovanadate complexes are also present in solution.

The ^{13}C spectrum of the D_2O solution was also measured, and the carbon chemical shifts of the major product were obtained. The chemical shifts were assigned as follows: 169.8 ppm, glycyl CO; 43.7 ppm, glycyl CH_2; 179.7 ppm, histidine CO_2; 57.6 ppm, histidine CH; 30.4 ppm, histidine CH_2; 139.9 ppm, histidine imidazole 2′-carbon; 134.0 ppm, histidine imidazole 4′-carbon; 120.1 ppm, histidine imidazole 5′-carbon. The change in chemical shifts of the imidazole carbons as complexation occurred provided direct evidence for the position of complexation. The complexation shifts are as follows: +2.5 ppm, 2′-carbon; +7.6 ppm, 5′-carbon; −2.6 ppm, 4′-carbon. The two carbons that underwent a positive shift are those on either side of N_1', while the carbon that underwent the negative complexation shift is the next-removed carbon. No complexation shifts were observed for either the carboxylate group of the histidine residue or the amido carbonyl

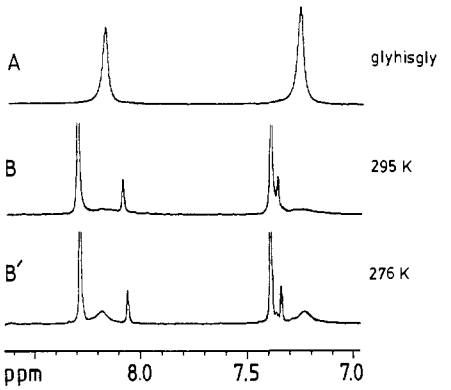


Figure 5. Aromatic region of the proton NMR spectra of glycylhistidylglycine in the absence (A) or presence (B, B') of diperoxovanadate. The spectra were obtained at temperatures of 295 (A, B) and 276 (B'). The result is consistent with chemical exchange broadening of the free ligand signal in spectrum B. Conditions of the experiments for spectrum A: total GlyHis, 20 mM; HEPES buffer, 20 mM; KCl, 1.0 M; pH, 7.0; temperature, 295 K. For spectrum B: as for A but with vanadate, 20 mM; H_2O_2, 40 mM; temperature, 295 K. For spectrum B': as for B but obtained at 276 K.

Table III. Formation Constants for the Products of Reaction of Various Imidazole Ligands with Diperoxovanadate at pH 6.5[a,b,c]

	$V\ell_2 + L' \rightleftharpoons V\ell_2L'$		$V\ell_2 + L'' \rightleftharpoons V\ell_2L''$		
ligand	K (M^{-1})	ppm	K (M^{-1})	ppm	reference
imidazole			$(4.1 \pm 0.3) \times 10^2$	−750	11
N-methyl-imidazole			$(3.7 \pm 0.3) \times 10^2$	−751	11
GlyHis	39 ± 4	−742	$(2.8 \pm 0.3) \times 10^2$	−751	
HisGly	97 ± 10	−739	$(2.7 \pm 0.5) \times 10^2$	−749	
HisSer	80 ± 7	−741	$(2.5 \pm 0.5) \times 10^2$	−749	
GlyHisGly	64 ± 7	−740	$(2.7 \pm 0.3) \times 10^2$	−751	
CBz–HisGly	21 ± 3	−738	$(9.4 \pm 0.9) \times 10^1$	−748	

[a] Conditions for the experiments: HEPES buffer, 20 mM; pH, 6.5; μ = 1.0 M KCl; variable concentrations of reactants. [b] $V\ell_2$ is diperoxovanadate; L' and L'' symbolize the two different nitrogens of the imidazole ring in the histidine moiety of a peptide. [c] Quoted errors represent three standard deviations in the measured parameter.

of the glycyl group. The pattern of these complexation shifts strongly suggests that the major product derives from complexation at N_1' and does not involve the other functional groups of the glycylhistidine. From structural considerations, this should be the least sterically hindered nitrogen and thus more accessible to complexation. On this basis, the minor product will derive from complexation at the N_3' position.

^1H NMR studies of GlyHisGly did not provide any additional information. In this case, the room temperature ligand imidazole signals were even broader than for GlyHis. They sharpened up considerably when the samples were cooled to 275 K. Representative spectra are shown in Figure 5. It does seem clear, from this evidence, that at least one rapidly exchanging product occurs at pH 7.0. Further characterization of this product, or products, has not yet proven successful. The neglect of these products introduces an error into the equilibrium calculations. This error is estimated to be comparable to that of the error introduced by the inaccuracy of knowing the pK_a of the ligand but could be more severe if the formation of minor products is strongly pH dependent. Table III gives the formation constants measured for a selection of histidine-containing dipeptides. For this table, formation of minor products, if they are formed, has been neglected.

A number of other peptides with functionalized side chains were also studied, including dipeptides such as glycyltryptophan, tryptyltryptophan, glytyllysine, and others. The results were consistent with reaction at the terminal carboxylate and the terminal amino groups and with the side chain carboxylate and side chain amino groups if they were present. No products, distinct from those already characterized, were observed to form with tryptophan ring systems. A summary of the results obtained is provided in Table IV.

Table IV. Formation Constants for the Products of Reactions between the Carboxylate and Ammonium Groups of Various Di- and Tripeptides with Diperoxovanadate[a,9]

	$V\ell_2^- + RCO_2^- \rightleftharpoons V\ell_2^{2-}$		$V\ell_2^- + RNH_3 \rightleftharpoons V\ell_2P^{2-} + H^+$	
ligand	K (M^{-1})	ppm	K (M^{-1})	ppm
GlyTyr	1.1	−715	1.5×10^{-6}	−744
GlySer	1.3	−716	2.1×10^{-6}	−748
GlyThr	1.5	−716	1.3×10^{-6}	−746
GlyGlu[b]	2.2	−720	5.5×10^{-7}	−747
	0.8	−716		
GluGlu[b]	9.3	−720	5.1×10^{-7}	−744
GlyLys[b]	4.8	−718	6.2×10^{-7}	−745
GlyTrp	1.5	−717	1.2×10^{-6}	−745
TrpGly	1.4	−713	4.8×10^{-7}	−742
TrpTrp[c]			9.9×10^{-7}	−743
TrpTyr	0.9	−713	6.1×10^{-7}	−740
GlyGlySer	1.1	−712	1.3×10^{-6}	−742
TyrGlyGly	1.3	−713		−736
TrpGlyGly	1.2	−712	9.6×10^{-7}	−735

[a] Formation constants, unless discussed in detail in the text, were obtained from two or more NMR spectra from solutions of different reactant concentrations and put into the pH-independent form of the equations using the known pK_a values of diperoxovanadate and the peptide. The errors in formation constants are estimated to be less than 30% of the reported values. [b] NMR signals of the products from the two carboxylate groups of GlyGlu were resolved in the ^{51}V spectra. GluGlu and GluLys did not give resolved spectra so that the formation constant is a composite value. [c] TrpTrp was not soluble enough to obtain a formation constant for the reaction with the carboxylate group.

Product formation at the amino and carboxylate groups is insensitive to adjacent substituents. This can be judged from the formation constants given in Table IV. At pH 6.5, they are close to about 1.4 M^{-1} per carboxylate ligand and about 0.9×10^{-7} for each ammonium group. The formation of diperoxopeptidovanadates, however, has been shown to be sensitive to the pK_a of the ligating group,[9] and this parameter should be considered when detailed comparisons are being made.

Products have also been observed that arise from the reaction of monoperoxovanadate with the above peptides. Such products form quite slowly, requiring many hours for equilibrium conditions to be established. These materials strongly inhibit the vanadate-catalyzed disproportionation of hydrogen peroxide and seemingly are tridentate complexes.[9] Under conditions of 3.0 mM vanadate and 9 mM H_2O_2 and with no peptide present at pH 7, the vanadium-catalyzed hydrogen peroxide decomposition goes to completion in about 12 h. Under similar conditions, in the presence of many dipeptides, decomposition requires several months.

There is an ambiguity here in that, although the time for equilibrium to be established and the time for complete disproportionation are comparable, the amount of hydrogen peroxide decomposition is minimal.[9] This suggests that there is an energetically unfavorable structural modification preceding the disproportionation. It follows that, as this product is generated, it is rapidly complexed by the free ligand, thus effectively blocking the next steps leading to decomposition and providing the observed product.

At elevated pH, the complexation reaction was found to occur more rapidly than at lower pH. In the case of glycylglycine, time course studies showed that equilibrium was established in about 6 h at pH 9. Actually, from an experimental point of view, of even more importance is the fact that higher pH increasingly favors formation of $V\ell$ over $V\ell_2$, thus allowing the formation of the monoperoxopeptido product to be more easily studied. However, constraints placed on the equilibria by proton stoichiometry can not be ignored.

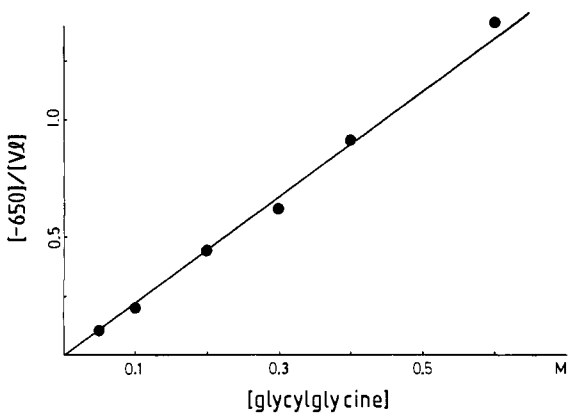
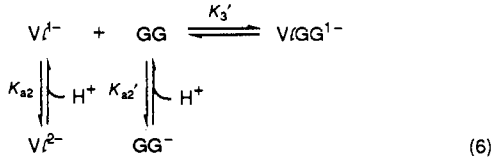


Figure 6. Results from studies of the formation of the –650 ppm product plotted in a format corresponding to eq 7. The linearity of this plot is consistent with the proposal that this product is a mono-glycylglycine derivative of monoperoxovanadate. Conditions of the experiments: total vanadate, 9.0 mM; total hydrogen peroxide, 9.0 mM; HEPES buffer, 20 mM; pH, 9.0; KCl, 1.0 M. Spectra were obtained 8 and 26 h after sample preparation; no significant changes were observed.

With glycylglycine, a single product giving rise to a ^{51}V NMR signal at –650 ppm was observed. Formation of this material was studied by maintaining the total vanadate and total hydrogen peroxide constant at 9.0 mM, each at pH 9.0, and varying the glycylglycine concentration from 50 to 600 mM. The results of the study were consistent with a 1:1 stoichiometry, so that the product is formed as described by eq 5, where GG represents

$$V\ell + GG \overset{K_3}{\rightleftharpoons} V\ell GG \qquad [V\ell][GG]K_3 = [V\ell GG] \quad (5)$$

glycylglycine. Figure 6 shows the results when plotted appropriately for eq 5. This pH 9 formation constant, 2.2 ± 0.2 M^{-1}, is much smaller than that required to account for the inhibition of decomposition of H_2O_2 at pH 6.5. However, both Vℓ and glycylglycine have a pK_a whereas the constancy of the ^{51}V chemical shift of the product suggests that the product does not have a pK_a. This can be tested by a pH variation study where now the equilibria of eq 6 must be considered. For this study, the pH was varied from pH 8.0–9.5.



(6)

The pK_a of Vℓ^{1-} and of glycylglycine have previously been determined for conditions similar to those of this study and are 6.2[14] and 8.2,[9,13] respectively. With these pK_a values, the concentrations of the Vℓ monoanion and neutral glycylglycine can be calculated from the appropriately determined total concentrations so that the results can be displayed according to eq 6 to give K_3'. The steep slope of this line is unusual and gives a value of $(8.3 \pm 0.8) \times 10^3$ M^{-1} for K_3'. It is evident from this large value that formation of the product complex will be highly favored under the pH conditions (pH 6.5) of the previous study of the inhibition of the disproportionation of H_2O_2.[9] In fact, K_3' is related to the overall formation constant, K_3, by eq 7, which

$$K_3 = K_3'/[1 + K_{a2}/[H^+])(1 + K_{a2}'/[H^+]) \quad (7)$$

derives from eq 6. Equation 7 differs from eq 4 by a term for proton generation. From this equation, K_3' gives a value of 1.9 ± 0.2 for K_3 at pH 9, a value in excellent agreement with the value of 2.2 ± 0.2 M^{-1} actually measured at that pH (Figure 6).

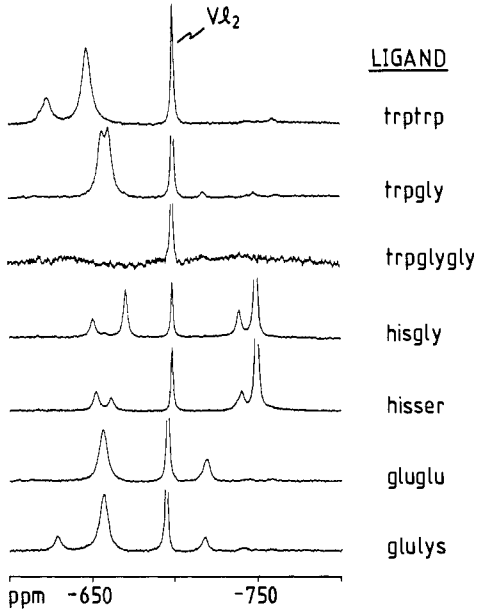


Figure 7. ^{51}V NMR spectra showing the formation of products assigned to monoperoxovanadate derivatives (–620 to –675 ppm) and those arising from diperoxovanadate interactions at the carboxyl group (~–720 ppm) and at the individual nitrogens of the histidyl imidazole group (~–745 ppm). The relatively low signal intensity of Vℓ_2 in the tryptylglycylglycine solution is a result of the uninhibited decomposition of H_2O_2. Conditions of the experiments: total vanadate, 7.0 mM; total H_2O_2, 9.0 mM; KCl, 1.0 M; HEPES buffer, 20 mM; pH, 6.5; time after sample preparation, 14–20 h.

The value of K_3, calculated for pH 6.5, is $(2.9 \pm 0.3) \times 10^3$ M^{1-}. A formation constant of this magnitude adequately accounts for the almost complete inhibition of hydrogen peroxide decomposition by glycylglycine and other dipeptides at pH 6.5, since relatively only very small amounts of Vℓ will be left free in solution. Presumably it is Vℓ that is responsible for the disproportionation of H_2O_2.[13,16,17]

The results reported here have been obtained for 1 M KCl solutions. Systematic variation of the electrolyte content revealed only a small change in the formation constant of the Vℓ product. The values, given in the pairs (K_3 (M^{-1}), [KCl]), were as follows: 2.5, 0.0; 2.3, 0.2; 2.2, 0.45; 2.2, 0.61; 2.1, 0.95 (all measured at pH 9.5). From the same solutions, the formation of the amino-derived product, Vℓ_2NP, remained constant within the range, K_1 = 11.7 ± 0.8 M^{-1} (eq 1). At lower pH (pH 6.5), the formation of the amino-derived Vℓ_2 product was more sensitive to electrolyte content, rising from 4.4 to 5.3 M^{-1} over a KCl range similar to that given above. Over the same range, the formation of the carboxylate-derived product was relatively much more highly favored, the formation constant doubling from 0.7 to 1.4 M^{-1} in an almost monotonic fashion with increase in electrolyte content.

Formation of the monoperoxo derivative, VℓGG, is not observed if either the carboxylate, the amino, or the peptido nitrogen is substituted. On the other hand, the presence of a side chain has a relatively small influence on overall formation of this product. Side chains, however, often lead to the formation of isomers. ^{51}V NMR spectra from a number of systems are displayed in Figure 7. The ^{51}V chemical shifts of the observed products, along with those from several other ligands, are collected into Table V.

A relevant observation from Figure 7 and also evident from Table V is that always at least one of the products gives a ^{51}V signal near –650 ppm. These are assigned to complexes that are structurally similar to the glycylglycine derivative, and they frequently correspond to the major product.

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Table V. ^{51}V Chemical Shifts of the Products of Complexation of Monoperoxovanadate with Selected Dipeptides at pH 6.5[a]

peptide	chemical shifts		
GlyGly		–649	
ValGly		–643, –657	
GlySer	–625	–652	
GlyThr	–632	–650	
GlyTyr[b]		–651	
GlyGlu	–625	–647, –651	
GluGlu		–657	
GluLys	–629	–657	
GlyTrp	–620	–651	
TrpGly		–652, –656	
TrpTrp	–623	–648	
TrpTyr	–628	–644	
GlyHis		–650	–673[c]
HisGly[c]		–651	–675[c]
HisSer		–652	–663[c]
No products observed: TrpGlyGly, CBz–HisGly, GlyHisGly, TyrGlygly,[b] GlySar, GlyGlySar			

[a] Phenols and tyrosines do form products with V*l*, but they are highly unfavored compared to the above products. [b] The GlyTyr solution becomes black after about 1 h, presumably from oxidation of its phenolic group. [c] These signals had a chemical shift dependence on pH. The value quoted is the high pH limiting value.

A second series of products provides ^{51}V signals close to –625 ppm. The resonance frequency of monoperoxovanadate is also close to –620 ppm at pH 6.5, but it has a pH-dependent chemical shift and the signal itself is considerably sharper that of the above signals. In addition, because of the large product formation constants, it is unlikely that an NMR signal from Vℓ will be observable once the system approaches equilibrium. A very low intensity Vℓ signal, however, is generally found during the early stages of the equilibration. The products giving rise to this series of compounds tend to be minor products compared to the –650 ppm series.

A third set of products arises from histidine-containing compounds. These products have a pH-dependent chemical shift. This pH dependence is consistent with a product pK_a, and the values obtained were pK_a = 6.9 ± 0.2 and pK_a = 6.2 ± 0.2 for the glycylhistidine- and histidylglycine-derived products, respectively. The corresponding low and high pH limiting chemical shift values were –649 and –673 ppm and –664 and –675 ppm, respectively. The pH dependence of the chemical shift of the histidylserine-derived product was not sufficiently large (~3 ppm) to properly determine a product pK_a.

Neither CBz–histidylglycine nor glycylhistidylglycine provided observable amounts of any product. This suggests that the imidazole nitrogen can replace the peptide nitrogen to generate this third series of product complexes. The pH dependence of the product chemical shift would then be caused by the protonation/deprotonation of the uncomplexed nitrogen (presumably N_1) of the imidazole ring.

There is no obvious reason why glycylglycine should give rise to only one geometrically defined Vℓ product, and it may well be that particular side chains favor alternative possibilities. In this event, the signals near –625 ppm can be assigned to such complexes. It is otherwise difficult to understand why the dipeptides in Table V, with such different side chains, give rise to products with similar chemical shifts. Some evidence for this is provided from studies of glycylthreonine.

Glycylthreonine provides the possibility of replacing a carboxylato group in the complex by an alkoxo functionality. In accord with this expectation, four product signals were observed at elevated pH (~9.5). With a systematic decrease in pH, two of the signals increased in magnitude while the others became unobservable. The two products that were favored by decrease in pH (–632 and –650 ppm) are assigned to carboxylate-derived products. The other two complexes (–637 and –656 ppm) are assigned to alkoxo-derived products since their formation would require release of a proton. As a consequence of this requirement, their formation is disfavored at lower pH. These results are, then, consistent with two geometric isomers within each product set. The pH study does not rule out the possibility that one product of each set derives from tridentate binding while the other arises from a bidentate reaction, but if this were so, it would be reasonable to expect similar products from some of the nonreactive ligands listed in Table V. They are not observed. Amino acids, however, do form bidentate complexes with Vℓ. In this case, two ligands are incorporated into the product rather than one.[11] It is possible that a mono-ligand product is formed with amino acids but that it is not readily observed because of low relative concentrations. Picrate, for instance, yields both mono- and bis-picrate products, dependent on conditions.[7]

Discussion

Aqueous vanadate, in the presence of hydrogen peroxide, gives rise to a number of peroxovanadate derivatives.[12,13] It is becoming increasingly clear, from this and other work,[9,11] that, in the context of ligands with potential biochemical interest, the mono- and diperoxovanadates are important substrates for further reactions. Furthermore, the solution chemistry of these two materials is surprisingly different.

Diperoxovanadate reacts with potential multidentate dipeptides (or amino acids[11]) in a monodentate fashion to give carboxylate- and amino-derived products. If the amino group is aromatic, as in imidazole, the complexation reaction is particularly favorable. Bidentate complexation, however, does occur, as for instance with dipyridine, the product of which has been characterized by X-ray diffraction studies.[18] Whether such a product is the favored one in aqueous solution is not known.

The reaction occurring between diperoxovanadate and amino or carboxylate groups proceeds with loss of a single proton if product formation is taken to occur from the acid form of either ligand. No evidence for a product pK_a was obtained. Product formation is, however, dependent on the pK_a of the ligand, with a higher pK_a of the conjugate acid of the ligand favoring product formation.[9] This favorability is counterbalanced by the fact that a higher pK_a also generally means that there is less of the reactive form of the ligand present in solution. Thus, Vℓ_2^- has not been observed to form a product with alkyl alcohols. On the other hand, reaction with both phenol and tyrosyl hydroxyls was observed.

The phenols are also oxidized in a slow reaction that has not been characterized in detail, but might well derive only from oxidation by free hydrogen peroxide. Tyrosine oxidation by peroxovanadates could, potentially, be very important since this function might well be responsible for some of the biochemical behavior observed for peroxovanadates. Oxidation of aromatic alcohols by vanadate is a well-characterized reaction,[19,20] but the oxidation by peroxovanadate probably follows a chemically quite different pathway. Sulfhydryl oxidation is another potential source of biochemical action.[21]

The aromatic hydroxyl group of tyrosine also serves as a reactive center for condensation with monoperoxovanadate. The formation constants vary only by about a factor of 3 between the Vℓ_2OAr and VℓOAr products, being 2.4 and 7.5 M^{-1}, respectively for glycyltyrosine. The pH-independent formation constants, however, vary by about a factor of 10. The rather small differences between these two formation constants suggest that very little selectivity would be conferred, by a tyrosyl group, on the binding

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of mono- or diperoxovanadate in the active site of an enzyme. The situation changes substantially, however, if the ligating group is an imidazole moiety.

At near neutral pH, the product formation constant for the reaction of diperoxovanadate with a histidyl imidazole is about 100 times more favorable than that with the tyrosyl phenol group (Tables I and III) and this suggests that histidyl residues are relatively more important that tyrosyl derivatives in determining the biochemical function of diperoxovanadate. Somewhat surprisingly, despite the very favorable reaction of imidazole with $V\ell_2$, no reaction of $V\ell$ with imidazole was observed. It is not clear why this should be, but it does seem unlikely that no reaction at all occurs. This finding does suggest that the relative biochemical importance of the mono- and diperoxovanadates might, to a large extent, be governed by the presence or absence of imidazole in the active site of the enzyme. Of course, dependent on the binding pattern in the active site, the overall difference in the ability of the two different peroxides to accept multiple ligands could well also be an important, if not overriding, factor. Monoperoxovanadate, for instance, will accept imidazole as one component of a multidentate ligand as in its complexation by CBz–glycylhistidine.

In contrast to the favorable reaction of the histidyl imidazole ring nitrogen with diperoxovanadate, no reaction with the nitrogen of the indole ring of tryptophan was observed. This suggests that tryptophan will not be an important active-site component of enzymes that are sensitive to diperoxovanadate.

Monoperoxovanadate reacts quite differently from diperoxovanadate both with amino acids[11] and with dipeptides. Dipeptides complex very strongly, in a tridentate manner, with $V\ell$ so that reaction occurs via the terminal nitrogen, amide nitrogen, and terminal carboxylate to give a highly favored product.[9] It seems that this product is responsible for the peptide-induced inhibition of the vanadate-catalyzed disproportionation of H_2O_2 to oxygen and water. This inhibition extends the lifetime of hydrogen peroxide in these solutions from a few hours to several months.[9] A system that does not utilize either the peptide terminal amino or terminal carboxy group for complex formation has not yet been studied. However, as shown by the work here, a side chain hydroxymethyl can replace a terminal carboxyl group and a histidyl imidazole ring nitrogen can replace a peptide nitrogen in these complexes. There is no apparent reason to expect that a side chain amino or side chain carboxyl cannot replace the equivalent main chain group, so it seems evident that, if amino acid side chains are appropriately substituted, complexation could occur without recourse to the terminal groups of the peptide. Detailed studies of relevant systems have not been reported here because of the difficulties in obtaining and studying equilibrated systems. The slow formation and even slower decomposition of such monoperoxovanadate products does, however, give credance to the proposal[9] that a possible source of action of peroxovanadates in living systems is that peroxovanadate ligand complexes provide a mechanism that allows transport to the appropriate location where the biochemical action is initiated. For this process, the complex itself is not necessarily active, but if not, the free vanadate or peroxovanadate would be. Clearly, the utilization of appropriate organ-specific ligating groups might usefully enhance the biochemical function of vanadate or peroxovanadate.

In aqueous solution, the vanadium-catalyzed diproportionation of hydrogen peroxide is strongly inhibited by unprotected dipeptides.[9] Other work has shown that oxygen-transfer reactions from monoperoxovanadate are very inefficient when the complex contains the tridentate ligand dipicolinate, being slower by a factor of at least 10^4 when compared to the corresponding picolinate complex.[22] As of yet, we have observed no significant amount of inhibition of disproportionation of H_2O_2 by monodentate complexes while bidentate complexes, at least of amino acids, represent an intermediate case.[9,11] This is of some relevance to understanding the function of vanadium-dependent haloperoxidases for, if it can be assumed that oxygen-transfer efficiency is correlated with disproportionation, then this suggests that the ligating groups in the active site of the haloperoxidases are not multidentate in character or that a selective type of multidentate binding somehow enhances the reactivity. Vanadium NMR studies of bromoperoxidase have suggested the presence of η^2 complexation of vanadium by carboxylate groups.[23] Such a reaction mode might well enhance oxygen-transfer capability.

As of yet, only unidentate complexation of additional ligands to diperoxovanadate has been observed with the systems studied here. Although bidentate complexes of oxalatooxodiperoxovanadate and 2,2′-bipyridyloxodiperoxovanadate have been prepared under strongly forcing conditions,[18] it is not known whether any of the ligands of the present study form appreciable proportions of bidentate products under similar conditions. Contrary to what might have been inferred from the results of numerous X-ray investigations of peroxovanadate complexes,[24] it is clear that, under the aqueous conditions of this study, the chemistry of mono- and diperoxovanadates is very different with respect to additional ligating groups and it seems highly likely that there also will be significant differences in the biological chemistry of these two materials.

Acknowledgment. Thanks are gratefully extended to the Natural Sciences and Engineering Research Council of Canada for its financial support of this work.

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