Reactions of Peroxovanadates with Amino Acids and Related Compounds in Aqueous Solution

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*Received March 19, 1993**

^{1}H and ^{51}V NMR spectroscopy has been used to study the reactions occurring between mono- and diperoxovanadate and a number of amino acids in aqueous solution. Concentration and pH studies were undertaken in order to establish product stoichiometry and proton requirements. The results revealed two distinct modes of interactions, dependent on whether the vanadate precursor contained one or two peroxide ligands. Monoperoxovanadate reacted with amino acids such as glycine or proline to give two types of bis(amino acid) products. One product had both of the amino acids attached in a bidentate manner while the second type of product had the first amino acid complexed as a bidentate ligand and the second attached only through the amino group. No reaction of monoperoxovanadate with imidazole or the imidazole ring of histidine was observed. In contrast to the results with monoperoxovanadate, no bidentate complexation of amino acids with diperoxovanadate was observed. Complexation occurred through either the carboxyl or the amino groups, with attachment at the amino position being favored. Imidazole, as the free ligand or as the side chain in histidine, was found to complex strongly to diperoxovanadate, as did *N*-methylimidazole and also pyridine, the latter to a lesser extent. Only a weak reaction with tryptophan was observed. The relevance of some aspects of this work to the function of vanadium haloperoxidases is discussed.

Introduction

Over the course of the past decade, interest in the aqueous chemistry of vanadium(V) has expanded dramatically. This interest has been generated, to a large extent, because of the findings that vanadium occurs in a molybdenum-independent nitrogen-fixing system of some bacteria[1] and also that vanadium is a component of the prosthetic group of some bromo- and iodoperoxidases of a lichen and a number of marine algae.[2,3] Furthermore, vanadium activates the function of a number of enzymes and inhibits the function of others.[4,5] It also is concentrated to very high levels by some marine organisms.[2,6,7]

Vanadium gives rise to an insulin mimetic response in live animals[8-10] and generates a large number of other biological responses.[5,11] The insulin-mimetic response is elicited whether the V(IV) or V(V) oxoanions are utilized in the animal experiments. Since, an oxidation/reduction equilibrium will be established within the organs of each animal, it is not known which vanadium oxidation states generate the response.[4]

Recent work showed that there is a large synergistic relationship between vanadate (a V(V) vanadium oxoanion) and hydrogen peroxide, leading to a strongly enhanced insulin-mimetic effect over that of vanadate itself.[12-14] This enhanced effect, in combination with the involvement of vanadium peroxo materials in the haloperoxidases, has raised questions concerning the reactions of peroxovanadates with metabolites and other materials.

Vanadate in the presence of hydrogen peroxide forms several peroxovanadate derivatives, including the mono-, di-, and triperoxomonovanadates and the tetraperoxodivanadate.[15-17] Some, or all, of the above compounds can undergo reactions with additional ligands to give peroxovanadate heteroligand products, some of which have been characterized by X-ray diffraction studies.[18]

Our initial study of the aqueous hydrogen peroxide/vanadate system had concentrated on the various equilibria established in this system and on the dependence of those equilibria on the hydrogen ion concentration. During the course of those initial studies[17] and subsequent studies of peroxovanadates in the presence of peptides,[19] it was found that a number of peptidoperoxovanadate derivatives were formed. An interesting sidelight of this work was the discovery that the well-known vanadate-catalyzed disproportionation of H_2O_2 to O_2 and water[20] was almost completely inhibited by simple dipeptides such as glycylglycine.[17,19]

In this study, the reactions which occur between the peroxovanadates and a selection of amino acids have been investigated. The dependencies of product formation on the pH of the solutions and on the concentrations of the amino acids, vanadate, and hydrogen peroxide have been obtained and the results used to derive product stoichiometries and the relevant formation constants.

* Abstract published in *Advance ACS Abstracts*, September 1, 1993.

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0020-1669/93/1332-4235$04.00/0

Experimental Section

Materials. The amino acids used in this study were obtained from Sigma Chemical Co. and were of pfs origen. The purities of the amino acids were checked by ^{1}H NMR spectroscopy, as thought to be necessary. All were used as supplied. Vanadium pentoxide (99.99%) was purchased from Aldrich Chemical Co. Hydrogen peroxide (3%) was purchased from Fisher Scientific Co., while imidazole and HEPES (*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid) buffer were purchased from Boerhringer Mannheim Gmbh. HEPES buffer was used in these studies because, over the course of our work with vanadate systems, we have found that this buffer tends to be nonreactive toward vanadate and its complexes.

Solutions. All final solutions were prepared at 1.0 M ionic strength using KCl unless indicated otherwise. The pH measurements were made with a pH meter calibrated immediately prior to use with freshly opened pH 4 and pH 10 standards.

Stock solutions of sodium vanadate were prepared by adding 0.5 molar equiv of vanadium pentoxide to a 1.0 M solution of NaOH in distilled water and then stirring until the solution became colorless (generally overnight). This solution was then adjusted to a concentration of 0.1 M with distilled water.

The hydrogen peroxide solution (3% as purchased) was standardized against $KMnO_4$ and then diluted to 0.1 M with distilled water. The 1.0 M HEPES buffer and 2.0 M KCl stock solutions were both prepared at pH 7 in distilled water. Except for phenylalanine, tryptophan, and leucine, which were weighed directly into the peroxovanadate solutions, amino acid stock solutions were prepared and they varied in their concentration from 0.4 to 1.5 M, dependent on the solubility of the amino acid. The amino acid stock solutions were prepared by dissolving the amino acid in distilled water, adjusting the pH to about 7, and then diluting to the desired concentration.

Just before final mixing, an intermediate stock solution, H_2O_2 (75 mM)/HEPES (25 mM), was prepared and the pH adjusted to 7.0. Appropriate proportions of the stock vanadate, buffer, and KCl solutions, to yield the desired final concentrations after the amino acid and peroxide solutions were added, were combined. The H_2O_2/buffer solution was added to the above solution only as these intermediate solutions were required. This procedure minimized the effects of the vanadate-catalyzed decomposition of H_2O_2. The appropriate proportion of the desired amino acid was added last and the volume adjusted to the final volume with distilled water. The pH of the final solution was checked and adjusted as necessary. For the pH studies, the pHs of the KCl and amino acid solutions were adjusted to yield a final pH near the target value after combining all solutions. A further minor pH adjustment was done to yield the desired pH.

Except for those of the vanadium atom or hydrogen peroxide concentration studies, all solutions had the following composition: 3.0 mM total vanadate; 9.0 mM total hydrogen peroxide; 20 mM HEPES buffer, μ = 1.0 M with KCl; pH 7, or variable for the pH studies. The amino acid concentration was varied as required.

Spectroscopy. ^{51}V NMR spectra were obtained at 105.2 MHz at ambient room temperature, 22 ± 1 °C. Vanadium chemical shifts are reported relative to an external reference of $VOCl_3$ assigned to 0.0 ppm. Baseline corrections were applied to all spectra before integrals were obtained. NMR acquisition parameters: pulse width, 60°; spectral width, 80 kHz; acquisition time, 0.05 s; frequency domain size, 16K data points. A 40-Hz line-broadening factor was applied to all spectra before transforming to the time domain using a 32K data point transformation. ^{1}H NMR spectra were obtained at 400.13 MHz under conditions similar to those described above using standard ^{1}H acquisition and processing procedures.

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 the results analyzed using standard least-squares techniques. The reported error limits represent three standard deviations.

Results

Simple amino acids such as glycine or alanine were found to undergo rather weak, but clearly observable, reactions with diperoxovanadate to give a mixture of products. The formation of such materials is demonstrated in Figure 1, which also shows NMR signals from several vanadoperoxide complexes. In this particular study, the proportions of vanadate to hydrogen peroxide were 9 mM to 21 mM, respectively, at pH 6.5. Under such conditions, no free vanadate or monoperoxovanadate was observable. Both of these materials were observed if the proportion of hydrogen peroxide was lowered sufficiently. The formation of the predominant peroxo complexes can be represented as in eqs 1–4 where ℓ refers to hydrogen peroxide and V_1 to the vanadate monomer. The formation constants are from ref 17 for pH 6.7. Water and proton stoichiometry is not considered in these equations.

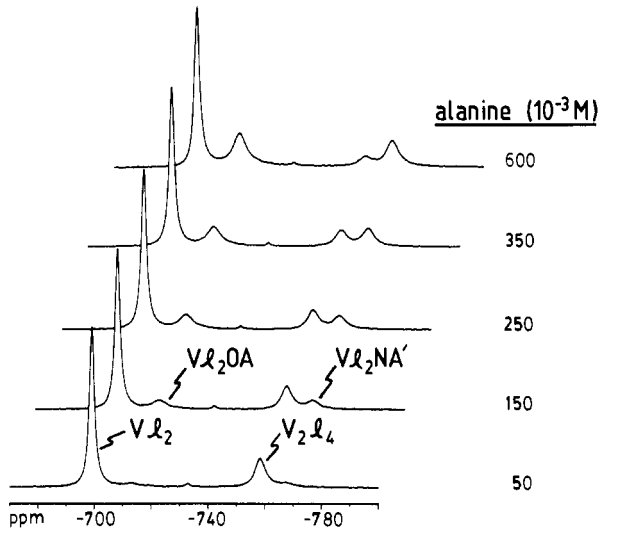


Figure 1. ^{51}V NMR spectra showing the concentration dependence of the formation of the products of the reaction of alanine with diperoxovanadate. All spectra are scaled to a constant amplitude for the Vl_2 signal. Vl_2OA and Vl_2NA' refer to product formation at the carboxylate and amino groups, respectively. Conditions of the experiments: 3.0 mM total vanadate; 9.0 mM total hydrogen peroxide; 20 mM HEPES buffer; pH 6.5; 1.0 M ionic strength with KCl.

$$V_1 + \ell \overset{K_1}{\rightleftharpoons} V\ell \qquad K_1 = (3 \pm 1) \times 10^3\ M^{-1} \tag{1}$$

$$V_1 + 2\ell \overset{K_2}{\rightleftharpoons} V\ell_2 \qquad K_2 = (5 \pm 2) \times 10^8\ M^{-2} \tag{2}$$

$$V\ell_2 + \ell \overset{K_3}{\rightleftharpoons} V\ell_3 \qquad K_3 = 9.4 \pm 0.8\ M^{-1} \tag{3}$$

$$2V\ell_2 \overset{K_4}{\rightleftharpoons} V_2\ell_4 \qquad K_4 = 49 \pm 5\ M^{-1} \tag{4}$$

Figure 1 depicts the results of adding incremental amounts of alanine to an equilibrium solution of peroxovanadates. On the basis of the results shown, it seems that each of the amino acid products have a 1 to 1 stoichiometry with diperoxovanadate, a supposition supported by a hydrogen peroxide concentration study. If this conclusion is sound, then formation of either amino acid product occurs according to eq 5, where the individual product

$$V\ell_2 + A \overset{K_5}{\rightleftharpoons} \text{ppm} \qquad [V\ell_2][A]K_5 = [\text{ppm}] \tag{5}$$

is identified by its ^{51}V chemical shift and A refers to the amino acid. When the results of the concentration studies were plotted according to eq 5 for the −714 ppm product, a reasonable linear correlation was obtained. However, when a pH variation study of this system was made, a discrepancy in the values of K_5 obtained from the two different studies was observed. This suggested that an additional product was formed and that it gave rise to NMR signals under that of diperoxovanadate. This coincidence of chemical shift could arise by means of accidental degeneracy or by a rapid exchange process. Since no extra signal was observed when the position of the Vℓ_2 signal was shifted by varia-

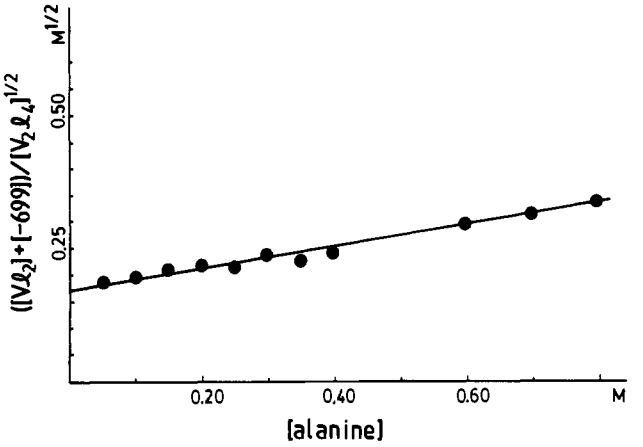


Figure 2. Results of the alanine concentration study plotted according to eq 6. The positive slope of the linear plot illustrates the occurrence of a minor product with its NMR signal superimposed on the signal from Vl_2. V_2l_4 provides the necessary reference concentration. The intercept ($K_4^{-1/2} = 0.17 \pm 0.1$ M^{-1} ($K_4 = 33 \pm 4$)) and slope ($K_4^{-1/2}K_5 = 0.2 \pm 0.03$ M^{-1} ($K_5 = 1.1 \pm 0.2$ M^{-1})) provided the formation constants for the two complexes. Conditions for the experiments were as for Figure 1.

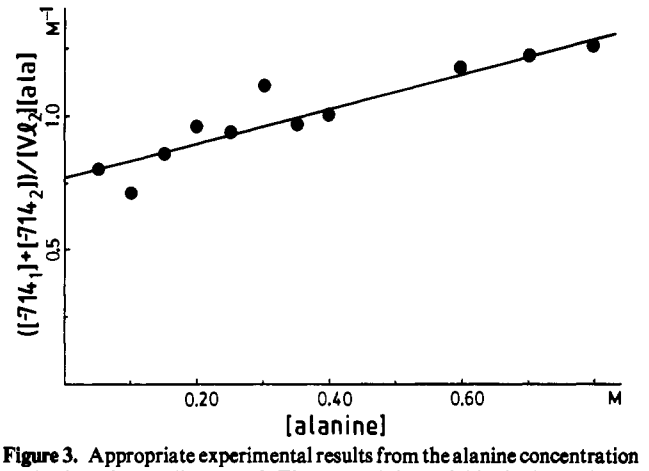


Figure 3. Appropriate experimental results from the alanine concentration study plotted according to eq 8. The upward slope of this plot is consistent with the formation of a secondary bis(ligand) product with its NMR signal superimposed on that of the primary product, Vl_2OA. The intercept ($K_5' = 0.8 \pm 0.2$ M^{-1}) and slope ($K_5'K_6 = 0.7 \pm 0.3$ M^{-2}, ($K_6 = 0.9 \pm 0.5$)) provided the formation constants for the two products. Conditions of the experiments were as for Figure 1.

tion of the pH, it seemed most likely that the product and starting material were in rapid exchange.

In order to confirm the formation of this rapidly exchanging product, (–699 ppm), it was necessary to use the tetraperoxodivanadate as a reference and to maintain the pH constant; a value of 6.5 was selected. Assuming that the product is formed in accordance with eq 5, then, from the superposition of starting and product signals, eq 6 is obtained by combination of eqs 4 and 5.

$$\frac{[V\ell_2] + [-699]}{[V_2\ell_4]^{1/2}} = K_4^{-1/2} + K_4^{-1/2}K_5[\mathrm{Ala}] \qquad (6)$$

If the assumption concerning product formation is correct, a plot of the ratio on the left of eq 6 vs the concentration of alanine (Ala) will provide a straight line of intercept $K_4^{-1/2}$ and slope $K_4^{-1/2}K_5$. The results of the plot are shown in Figure 2 . The intercept $K_4^{-1/2} = 0.17 \pm 0.01$ $M^{1/2}$ gave a value of 33 ± 4 M^{-1} for K_4, in good agreement with the value expected for pH 6.5.[17] The slope of the line $K_4^{-1/2}K_5 = 0.20 \pm 0.03$ $M^{-1/2}$ gave 1.1 ± 0.2 M^{-1} ($=K_5$) for the formation of this exchanging product. Figure 2 is fully consistent with the Vl_2A stoichiometry assumed for eq 6 and gives no indication that more than one amino acid is incorporated into this product.

Previous work[19] and other results to be presented here show that the signal at –714 ppm derives from complexation at the carboxylate group. Preliminary analysis of the results of the concentration study according to eq 5 suggested that the –714 ppm signal was a composite signal from a mixture of products, one with one ligand and the second with two. The second product would then be formed as indicated in eq 7. In this equation ppm_1,

$$\mathrm{ppm_1} + \mathrm{Ala} \overset{K_6}{\rightleftharpoons} \mathrm{ppm_2} \qquad [\mathrm{ppm_1}][\mathrm{Ala}]K_6 = [\mathrm{ppm_2}] \quad (7)$$

refers to the mono(ligand) product and ppm_2 to the bis(ligand) derivative. Equation 5 (with K_5' replacing K_5 for this product), in combination with eq 7, leads to eq 8.

$$\frac{[\mathrm{ppm_1}] + [\mathrm{ppm_2}]}{[V\ell_2][\mathrm{Ala}]} = K_5' + K_5'K_6[\mathrm{Ala}] \qquad (8)$$

The concentration of Vl_2 is obtained by calculations using the formation constant, K_5 ($=1.1$ M^{-1}), the known concentration of alanine, and the sum, $[Vl_2] + [-699]$, which is provided by the NMR spectrum. Figure 3 shows the results of plotting the experimental results according to eq 8. The intercept, $K_5' = 0.8 \pm 0.2$ M^{-1}, corresponds to the formation of the monocarboxylate product and is similar in value to that for other carboxylates (this work and ref 19). The formation of a bis(ligand) product from the mono(ligand) precursor ($K_6 = 0.9 + 0.5$ M^{-1}) had not been reported previously, either from acetate or from peptides,[19] and reexamination of the original data for those systems provided no evidence for the formation of such a product.

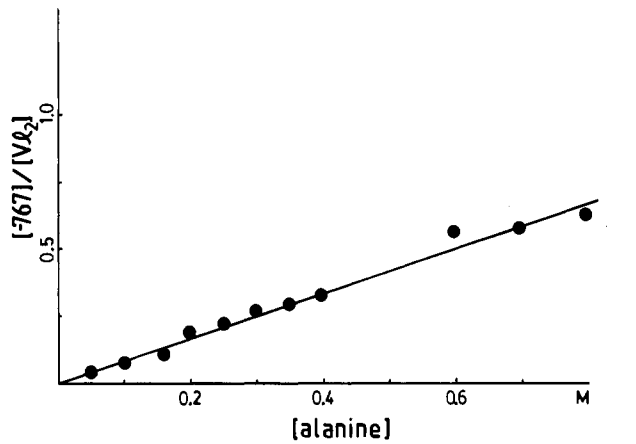


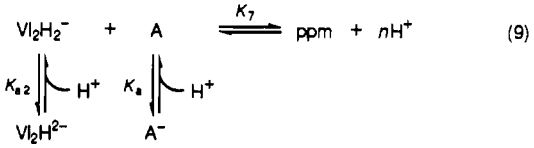
Figure 4. Experimental results relevant to the changes in the –767 ppm NMR signal as a function of alanine concentration plotted appropriately for eq 5. The nonobservation of upward curvature in this graph is consistent with the occurrence of only a mono(ligand) product. The slope ($K_5 = 0.83 \pm 0.12$ M^{-1}) gave the formation constant for the amino-derived product, Vl_2NA'. The experimental conditions were as for Figure 1.

A further product gave an NMR signal at –767 ppm. Signals near this chemical shift derive from reaction at the amino group.[19] When the results were plotted according to eq 5, a good linear relationship was obtained, as shown in Figure 4 . No evidence of a second product was provided by this plot. The formation constant for this product was $K_5'' = 0.83 \pm 0.12$ M^{-1}, as defined by eq 5.

Since two new types of products were observed to form with alanine, a second concentration study was done at pH 7.40. At this pH, the carboxylate-derived products (–714 ppm) were no longer observed. However, a plot of the experimental data according to eq 6 showed that the previously observed rapidly exchanging product was still being formed at this pH, $K_5' = 1.3 \pm 0.2$ M^{-1}. The corresponding formation constant for the amino-derived product (–767 ppm) increased substantially to $K_5'' = 4.6 \pm 0.4$ M^{-1}. Vanadate and peroxide concentration studies carried

out in concert with the alanine concentration studies confirmed the 1:2 vanadium to hydrogen peroxide stoichiometry of all four alanine-derived products.

Details concerning proton requirements for formation of the above products were provided by pH variation studies. For such studies it is necessary to consider the protonation states of both the reactants and the products. Preliminary studies indicated that there was proton release from the −767 ppm product as product formation occurred but not from the other derivatives. If the product complex itself does not have a pK_a within the range of the pH study, its formation can be written as in eq 9, where



(9)

again the chemical shift value is used to specify the product of interest.

The pK_{a2} of $VI_2H_2^-$ was previously reported as 7.36 ± 0.08 for conditions similar to those of this study.[17] The value determined here was 7.38 ± 0.09, using the chemical shift dependence of the VI_2 signal on the pH of the solution as previously described.[17] The two pK_a values of alanine are known to be close to 2.40 and 10.0, dependent slightly on conditions.[21]

For this study the pH was varied between 6.0 and 8.7. Over this range, the −714 ppm product rapidly decreased in relative proportion, being observable only at the lower pH values of the study. This result is consistent with the n of eq 9 being equal to zero for the mono(ligand) carboxylate-derived product. This puts an additional negative charge on the vanadium that is balanced by the positively charged ammonium group. The charge stoichiometry for the reaction at the vanadate center is identical to that previously observed for acetate.[19] From the two points measured at pH 5.97 and 6.51 and the zero intercept, a value of $K_7 = 0.8 \pm 0.3$ M^{-1} was estimated for the formation of the −714 ppm product. This value corresponds to $K_5 = 0.7 \pm 0.2$ M^{-1} when in the form of eq 5 for pH 6.5 and compares favorably to the value of $K_5 = 0.8 \pm 0.2$ M^{-1} measured at that pH.

Unlike for the product giving rise to the −714 ppm signal, the −767 ppm product was favored with increase in pH, suggesting a nonzero value for n. For the initial analysis, a value of $n = 1$ was assumed. A linear plot should then be obtained on the basis of eq 10 when the ratio on the left is plotted against $1/[H^+]$, if

$$\frac{[-767]}{[V\ell_2H_2^-][A]} = \frac{K_7}{[H^+]} \qquad (10)$$

eq 9 is correct with $n = 1$. The result of the plot is shown in Figure 5. As can be seen, a good linear correlation was obtained. This is consistent with complexation to the amine group of the amino acid, probably by a simple replacement of water by H_2NA'. If n were not equal to 1 or the −767 ppm product had a pK_a within the pH range of this study, the plot would not be linear. A value of $(3.6 \pm 0.4) \times 10^{-7}$ was measured for K_7. The proton stoichiometry of reaction at the ammonium group of the amino acid observed here is the same as that previously established for the reaction of the ethylammonium ion with diperoxovanadate.[19]

Both ligand concentration and pH variation studies were carried out for several ligands other than alanine, including glycinamide, imidazole, and histidine. In addition, ligand concentration studies were carried out for glycine and its ethyl ester.

Interestingly enough, glycinamide gave two products, with chemical shifts of −729 and −749 ppm. The −749 ppm product

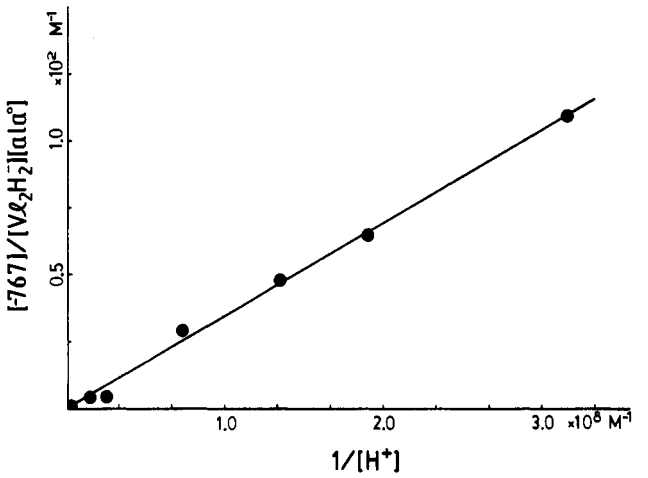


Figure 5. Results of the pH variation study plotted as required by eq 10. The linearity of this graph is consistent with the loss of a single proton as $VI_2NA'^{2-}$ is formed from its precursors. The slope of the line ($K_7 = (3.6 \pm 0.4) \times 10^{-7}$) provided the formation constant for this product. Conditions of the experiments: 3.0 mM total vanadate; 9.0 mM total hydrogen peroxide; 300 mM total alanine; 20 mM HEPES buffer; variable pH; 1.0 M ionic strength with KCl.

arose from interactions with the amino group while the −729 ppm product was found to have a ligand and proton stoichiometry similar to that of the −714 ppm mono(ligand) product of alanine and is tentatively ascribed to product formation with the amido group. Other work has shown that acetamide does not form an observable product with VI_2 at up to 1 M concentrations.[19] However, with glycinamide, the adjacent positively charged ammonium group may be favoring reaction at the amide nitrogen by means of positive interactions with the negatively charged vanadium center. The formation constant for this amide product was 0.64 ± 0.06 M^{-1} ($=K_7$, according to eq 9 with $n = 0$). Condensation with the ammonium functionality of glycinamide ($pK_a = 8.2$[22]) was close to a factor of 10 more highly favored than the corresponding reaction with alanine, the formation constant ($n = 1$ in eq 9) being $(3.5 \pm 0.7) \times 10^{-6}$ compared to $(3.6 \pm 0.4) \times 10^{-7}$ for alanine.

It has previously been shown that complex formation with diperoxovanadate is highly dependent on the electron-donating ability of the ligand, as evidenced by its pK_a. Product formation with the conjugate base of the ligand is favored by a higher pK_a.[19] This trend is followed here. The reaction of vanadate with the amino group of $H_2NCHCH_3CO_2^-$ is favored by a factor of 7 over the corresponding reaction with $NH_2CH_2CONH_2$. Even so, at pH 6.5, more product is observed from reaction with glycinamide than with alanine. This is because there is proportionately much more glycinamide NH_2 than alanine NH_2 in solution at this pH.

On the basis of the pK_a of the imidazole ring, it was not surprising that histidine readily formed a product (−750 ppm) with diperoxovanadate. Because of the possibility of reaction at two imidazole positions as well as at the carboxyl and amino groups, preliminary studies were carried out both with *N*-methylimidazole and with imidazole itself.

Reaction of an equilibrium mixture of peroxovanadates with *N*-methylimidazole provided only one observable product with a ^{51}V NMR signal at −750 ppm. Variation of reactant concentrations showed that the product was formed from VI_2 and one *N*-methylimidazole. A similar conclusion was drawn for the reaction with imidazole. This product provided a chemical shift also at −750 ppm, very close to that of the N-methylated product. Similarly, the corresponding product from histidine gave a signal at −748 pm, while in this case a second signal was found to occur at −737 ppm.

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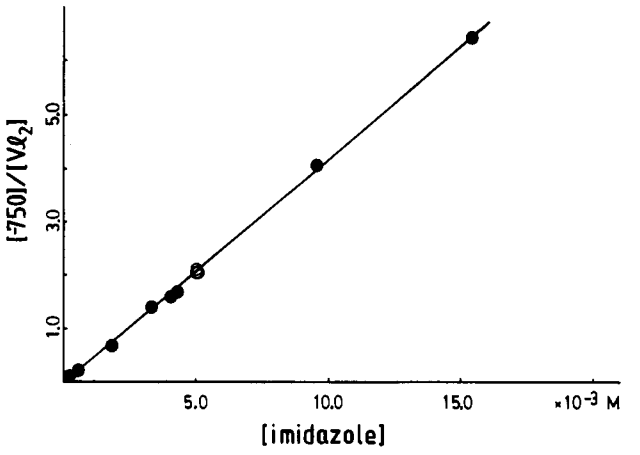
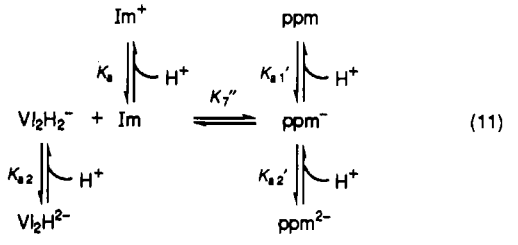


Figure 6. Experimental results for the imidazole/hydrogen peroxide/vanadate study plotted as required by eq 5. The three open circles represent a factor of 3 change in hydrogen peroxide concentration at fixed total vanadate and imidazole concentrations. Conditions of the experiments: variable total vanadate concentration, 3.0–7.0 mM; variable total hydrogen peroxide concentration, 9.0–24.0 mM; variable total imidazole concentration, 0.5–18 mM; 20 mM HEPES buffer; pH 6.5; 1.0 M KCl.

Formation constants of $K_5 = (4.1 \pm 0.2) \times 10^2$ and $K_5 = (3.7 \pm 0.2) \times 10^2$ M^{-1} were measured for imidazole and *N*-methylimidazole, respectively, from a combination of total vanadate, total hydrogen peroxide, and total ligand concentration variation studies at pH 6.5. Figure 6 shows the results of one such study for imidazole. The excellent correlation strongly supports the proposed Vl_2Im stoichiometry for this product, as expressed by eq 5. Unfortunately, water stoichiometry cannot be established by these studies.

Despite the straightforward analysis of the concentration studies, problems were encountered when an attempt to analyze the results of the pH variation study was made. The initial results suggested that the product itself had a pK_a within the range of the pH study. In this event, eq 9 can be rewritten as eq 11, where Im can be taken to represent either imidazole or *N*-methylimidazole.



Preliminary analysis of the results of the pH study suggested that a proton was being lost from the product at higher pH so that it was necessary to consider both the singly and doubly charged products. In this event, eq 12 follows from eq 11. Plotting the

$$\frac{[\text{ppm}^-] + [\text{ppm}^{2-}]}{[V\ell_2H_2^-][\text{Im}]} = K_7'' + \frac{K_7''K_{a2}'}{[H^+]} \qquad (12)$$

experimental results according to eq 12 provided a graph that apparently consisted of two approximately straight-line segments for which the change in slope occurred at about pH 9. This is not consistent with eq 12. In an attempt to resolve this problem, *N*-methylimidazole concentration studies were done at pH 7.5 and 9.6. The stoichiometry obtained from these two sets of measurements was the same as that determined at pH 6.5, that is, Vl_2Im. Furthermore, it was noted that the ^{51}V chemical shift of the product was constant at -750.2 ± 0.2 ppm over the range of the pH variation study, pH 4.0–10.3. In view of the large protonation shifts for Vl^{2-} and Vl_2^{2-} of +23 and +79 ppm, respectively, the lack of a variation of chemical shift for Vl_2Im seems unlikely if this product has a pK_a.

Imidazole itself has pH-dependent proton chemical shifts for the 2 and 4,5 protons, the shifts being particularly large for the 2 position. From spectra obtained at pH 6.9 and 10.2, the latter signal was found to shift –0.7 ppm while the $H_{4,5}$ signal moved –0.3 ppm. For the same pH values, the product signals remained constant at 8.38, 7.55, and 7.49 for the H_1 and the H_4 and H_5 signals. Again, this is inconsistent with a pK_a for the product. (Interestingly enough, the proton spectra for the imidazole system indicated the formation of a minor product that gave only two signals of intensity ratio 1:2 (8.31 and 7.60 ppm). This product, not observed with *N*-methylimidazole, presumably either is Vl_2ImVl_2 or is derived from V_2l_4 as a symmetrical product. It was not identified in the ^{51}V spectrum. Its formation is favored at higher pH, suggesting the loss of a proton as this product is generated. This material has not been studied in more detail.)

The ^1H chemical shifts of the products derived from *N*-methylimidazole occurred at 8.29, 7.51, and 7.43 ppm for the ring protons and 3.94 ppm for the NCH_3 group. As for imidazole, no variation of these shift positions with change in pH from 5.9 to 10.4 was found and the formation of only one product was observed throughout the pH range of the study.

In a further attempt to resolve the problems encountered in the initial pH studies, a full pD study was undertaken in D_2O solution for the *N*-methylimidazole system. The pD dependence of the ^{51}V chemical shift of the diperoxovanadate signal was used to determine the pK_a of Vl_2, while the ^1H chemical shift variation of the *N*-methyl group was used to give the pK_a of *N*-methylimidazole. The values obtained were 7.73 and 7.70 for the two compounds, respectively. With these values, a much better linear correlation was obtained for the equilibria of eq 9 or, in terms of the ratio on the left of eq 12, a constant value equal to $K_7'' = (6.3 \pm 0.4) \times 10^3$ M^{-1} was obtained. This equals K_7 of eq 9 with $n = 0$. A similar study with imidazole itself was not quite as successful, giving a much larger error, the value of K_7'' being $(4.8 \pm 0.8) \times 10^3$ M^{-1}. Normally it is found that formation constants calculated from pH variation studies agree, to within the experimental error, with the results of the fixed-pH studies. For imidazole, the agreement is well outside the error limits. A large number of alternative reaction schemes were considered, but no better agreement was obtained. The equilibria are established fairly slowly, and it is possible that there are kinetic problems associated with the changes in pH. The results obtained, however, were readily reproduced and time-course studies did not reveal any problems with the experimental work. Despite this, it is felt that the results are being affected by an incorrect procedure, possibly a failure to account for a minor product or products that were not characterized, such as the one suggested above by the ^1H NMR spectra. Not surprisingly, similar problems were encountered in attempts to analyze the results of pH studies with pyridine and histidine.

Histidine provided two diperoxovanadate products with ^{51}V chemical shifts of –737 and –748 ppm. These two products are assigned to condensation with the two different nitrogens of the imidazole ring. Because of the very favorable reaction at these two centers, it did not prove possible to observe product formation with either the carboxylate or the amino group of this amino acid. The values of K_5 determined at pH 6.5 were $(2.3 \pm 0.1) \times 10^2$ and 73 ± 7 M^{-1} for the –748 and –737 ppm products, respectively. In order to confirm that both of these products arose from reaction with the imidazole ring, studies were carried out with derivatives of histidine where either the carboxyl or the amino group were protected. Substitution did not prevent product formation, although it did influence the formation constants as can be seen in Table II. These changes probably have their origins in the conformational changes induced in the amino acid by substitution.

Table I. Formation Constants for Major Complexes of Diperoxovanadate with Selected Amino Acids and Related Compounds[a,b]

ligand	$VI_2H_2^- + AO \rightleftharpoons$ $\delta(^{51}V)$ (ppm)	VI_2OA^- K (M^{-1})	$VI_2H_2^- + A'N^{n-} \rightleftharpoons$ $\delta(^{51}V)$ (ppm)	$VI_2NA^{(n+1)-}$ K (M^{-1})
glycine	−712	0.7 ± 0.1	−758	(4.0 ± 0.5) × 10^3
glycine ethyl ester			−736	(3.3 ± 0.4) × 10^2
glycinamide			−749	(5.5 ± 1.1) × 10^2
alanine	−714	0.8 ± 0.2	−766	(3.6 ± 0.5) × 10^3
histidine			−737	(2.8 ± 0.8) × 10^2
			−748	(8.9 ± 1.0) × 10^2
imidazole			−750	(4.8 ■ 0.8) × 10^3
N-methylimidazole			−750	(6.3 ± 0.6) × 10^3
pyridine			−712	(1.0 ± 0.2) × 10^2

[a] Reactions are for either the carboxylate terminus of the ligand (AO) or the amino terminus (A'N). [b] Conditions of the measurements: variable pH at 3.0 mM vanadate and 9.0 mM H_2O_2; 20 mM HEPES buffer; μ = 1.0 M KCl.

Complexation of the imidazole ring of histidine at pH 7.0 by vanadate results in ^1H NMR complexation shifts opposite to those observed for imidazole itself. This is attributed to the fact that the pK_a values of the two systems are different, so that the imidazole ring of histidine is more fully deprotonated at pH 7 than imidazole at the same pH. The major and minor products, respectively, had chemical shifts of 8.35 and 7.45 ppm and 8.12 and 7.40 ppm to be compared with 8.02 and 7.20 ppm for the uncomplexed histidine, also at pH 7.0.

It was not possible to unambiguously assign the product chemical shifts to specific derivatives. However, since the amino acid residue is attached at the 5 position of imidazole it might be expected that the major product will be formed at N-3 rather than N-1 in order to reduce steric interactions that would exist between adjacent groups.

The results of these ligand and pH variation studies are summarized in Table I. In addition to this, Table II gives the results of studies of product formation results for a number of other amino acids. Complete studies were not done for these remaining systems, but rather each was characterized by only two or three spectra and the formation constants were obtained by assuming there are no changes in the proton and ligand stoichiometry from the fully characterized systems. There is no reason to suspect changes in coordination for any of the amino acids studied.

During the course of these studies, it was observed that amino acids inhibit the vanadate-catalyzed disproportionation of H_2O_2 to oxygen and water. This behavior is similar to that of dipeptides,[19] except that kinetics experiments indicated that the inhibition was much less efficient than for the peptides. Furthermore, formation of the product thought to be responsible for the inhibition occurred much more rapidly than in the case of the dipeptides but still was very slow compared to the reactions with peroxovanadate studied above, for instance, requiring approximately 1 h for equilibrium conditions to be established.

Preliminary vanadate and glycine concentration studies suggested that the "inhibitor" complex contained two glycine residues and one vanadium. Its ^{51}V chemical shift (−662 ppm) and the fact that it can be observed only if the vanadate to hydrogen peroxide ratio is less than 1:2 suggest that this product is a monoperoxovanadate. On this basis, the formation of this inhibitor can be written according to eq 13 where A specifies glycine for the case discussed here.

$$V\ell + 2A \overset{K_8}{\rightleftharpoons} V\ell A_2 \qquad [V\ell][A]^2K_8 = [V\ell A_2] \qquad (13)$$

When the experimental data were plotted according to eq 13, an excellent linear correlation was obtained. The value obtained for K_8 was 8.8 ± 0.3 M^{-2} for pH 8.5 solutions. Neither N-protected nor C-protected glycine gave a product corresponding to the above. The unprotected glycine, however, did give rise to a second product, formed at lower concentrations than the above material. This product gave a ^{51}V NMR signal at −674 ppm and apparently is a bis(ligand) product of mixed coordination. Addition of C-protected glycine to the reaction solution caused the −674 ppm signal to grow in intensity relative to that of the −662 ppm product, while addition of N-protected glycine had no effect. This suggests that the −674 ppm product contains one bidentate glycine with an additional glycine complexed only through the amino group.

It was possible to establish the proton requirements for the major monoperoxo bis(ligand) product (−662 ppm) from a pH variation study. Initial results suggested that this product was formed from the zwitterionic glycine and monoanionic peroxovanadate to form a product that could undergo a subsequent deprotonation reaction at a higher pH as described by eq 14.

$$VI^- + 2A \overset{K_8'}{\rightleftharpoons} VIA_2^- \qquad (14)$$
$$VIA_2^- \overset{K_{a2}'}{\rightleftharpoons} VIA_2^{2-} + H^+$$

Rewriting this equation in a form analogous to eq 12 and plotting the appropriate experimental results gave the graph displayed in Figure 7 . This excellent linear correlation provided the values K_8' = (5.5 ± 0.8) × 10^2 M^{-2} and K_{a2}'' = (6.7 ± 1.3) × 10^{-9} or pK_{a2}' = 8.2 ± 0.1 for the product, VIA_2^-.

Interestingly enough, the mixed-coordination minor product (−674 ppm) maintained its concentration with increase in pH, suggesting that it has a significantly higher pK_a than does the −662 ppm product.

Proline was also found to react favorably with V*I* to similarly give a bis(ligand) product (−632 ppm) and three minor products, −646, −663, and −665 ppm; all of which had the same ligand stoichiometry, as judged from the ligand concentration study. The major product had the formation constant K_8 = 9.4 ± 0.5 M^{-2}, while the minor products, individually, were formed about 10% as efficiently, but in roughly equal proportions to each other. The pK_a determined for the major prolyl product was 9.5 ± 0.2. This pK_a is significantly higher than that of the product formed with glycine (pK_a = 8.2 ± 0.1). This, however, may result from the fact that the pK_a of proline is also higher than that of glycine, 10.6 compared to 9.7. That this type of behavior can occur in vanadate complexes has been shown by studies of vanadate ester formation.[23]

The formation constant of the prolyl product, K_8' = (1.8 ± 0.1) × 10^3 M^{-2}, is larger by a factor of 10 than that of the glycyl derivative. This probably also is a result of the difference in the pK_a values of the ligands. For instance, product formation with the diperoxides is favored by a higher pK_a of the ligand.[19]

Addition of imidazole to a solution containing 7 mM total vanadate and 9 mM total H_2O_2 at pH 6.5 gave rise to product signals deriving only from an (imidazole)diperoxovanadate derivative There was no indication, from the ^{51}V NMR spectra, of the formation of an imidazole derivative of monoperoxovanadate. Similar results were obtained for *N*-methylimidazole and

(23) Tracey, A. S.; Galeffi, B.; Mahjour, S. *Can. J. Chem.* 1988, *66*, 2294–2298.

Table II. Formation Constants for the Major Products of the Reactions of Diperoxovanadate with a Number of Amino Acids at pH 6.5[a]

ligand	VI_2 + AO $\delta(^{51}V)$ (ppm)	$\rightleftharpoons$	VI_2OA K (M^{-1})	VI_2 + A'N $\delta(^{51}V)$ (ppm)	$\rightleftharpoons$	VI_2NA' K (M^{-1})
glycine	−712		0.6 ± 0.1	−758		0.9 ± 0.1
glycine–OEt				−736		5.9 ± 0.5
CBZ–glycine	−714		1.4 ± 0.4			
glycinamide				−749		23 ± 3
alanine	−714		0.8 ± 0.1	−766		0.8 ± 0.1
valine	−712		1.0 ± 0.3	−765		0.8 ± 0.3
leucine	−712		0.6 ± 0.2	−765		0.5 ± 0.2
serine[b]				−763		1.2 ± 0.3
threonine[b]				−765		2.0 ± 0.4
phenylalanine				−757		1.8 ± 0.5
aspartic acid[c]	−715		3.0 ± 0.5	−768		0.5 ± 0.2
lysine	−714		0.6 ± 0.2	−765		0.8 ± 0.3
				−742		0.2 ± 0.2
CBZ–lysine	−716		1.1 ± 0.4			
lysine–OMe[d]				−739		3.6 ± 0.6
arginine	−711		0.6 ± 0.2	−765		1.4 ± 0.3
proline	−712		0.6 ± 0.2	−761		0.9 ± 0.3
histidine				−737		(7.3 ± 0.5) × 10
				−748		(2.3 ± 0.1) × 10^2
histidine–OMe				−740		(1.0 + 0.2) × 10^2
				−749		(2.0 ● 0.2) × 10^2
imidazole				−750		(4.1 + 0.3) × 10^2
N-methylimidazole				−750		(3.7 + 0.2) × 10^2
pyridine				−712		(6.1 + 0.4) × 10
tryptophan	weak reaction observed at 50 mM					
guanidine	no reaction observed to 500 mM					
cysteine	reduced V(V) to V(IV)					

[a] Conditions of the experiments: vanadate, 3.0 mM; H_2O_2, 9.0 mM; pH 6.5; HEPES buffer, 20 mM; μ = 1.0 M KCl; ligands, variable. [b] Both serine and threonine undergo very weak interactions with diperoxovanadate. [c] Aspartic acid gives carboxylate-derived products which have superimposed ^{51}V NMR signals. [d] The signal at −739 ppm was asymmetric, indicating at least two products, presumably from the alkyl nitrogen of the side chain and from the main chain nitrogen; both products should give signals near −739 ppm.

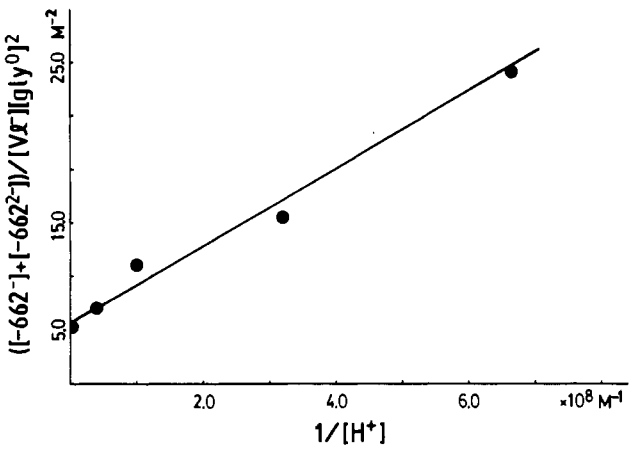


Figure 7. pH dependence of the formation of the bis(glycine) complex with monoperoxovanadate. The upward slope of the graph is consistent with a product pK_a. Conditions of the experiments: total vanadate, 7.0 mM; total hydrogen peroxide, 3.0 mM; glycine, 200 mM; HEPES buffer, 20 mM; ionic strength, 1.0 M with KCl; variable pH.

for histidine. Variation of the pH up to pH 9.5 and changes in the relative concentrations of reactants gave no indication of a monoperoxo product. The formation of an (imidazole)monoperoxovanadate cannot be ruled out; however, compared to the corresponding diperoxide, it very clearly is not readily formed. It is possible that it is more easily formed under more strongly acidic conditions than those utilized in this study.

Discussion

Amino acids without functionalized side chains condense, in aqueous solution, with diperoxovanadate to give four products. At least two of these materials arise from monodentate complexation. One set of two products gives ^{51}V NMR signals near −715 ppm. The main component of this set derives from complexation with the carboxylate group. This conclusion is supported by previous studies of C-terminal-blocked peptides,[19] of acetic acid,[19] and of C-protected glycine and lysine (this work). Furthermore, a crystalline diperoxovanadate derivative of glycine has been reported to be obtained from the reaction of glycine with vanadium pentoxide and hydrogen peroxide. This material is complexed to vanadium by means of the carboxylate oxygen.[24]

The NMR signal from the second component of the above pair of carboxylate-derived products is superimposed on that of the major carboxylate product. This superposition most probably is a result of rapid exchange. This is proposed because a third product gives a signal that is superimposed on that of free diperoxovanadate even when the signal position of VI_2 is moved by varying the pH. Products displaying this rapid kinetics behavior have not previously been observed and apparently are unique to amino acids. They require both the carboxylate and ammonium groups for their formation and were not observed to form if either group was protected. Similar products also were not found when the ligand was a dipeptide. The fourth product was obtained when complexation of the mono(ligand) precursor occurred via the amino group rather than through the carboxylate group.

It is not at all clear what the rapidly exchanging products are. The fact that these products are not favored with increase in pH suggests that complexation requires the acid form of the carboxylate group. The requirement for the vicinal ammonium group strongly suggests that there is bidentate complexation, but it is not clear why exchange would be rapid for such case, particularly in view of the fact that exchange is slow for the monodentate complexes. There could, however, be a change in coordination geometry. Of course, it is not certain that the −714 ppm mono(ligand) product is complexed in a monodentate fashion to a single oxygen of the carboxyl group. However, crystalline diperoxovanadate derivative of glycine has been obtained and the IR spectrum showed that complexation occurs through only one oxygen of the carboxylate group.[24]

(24) Bhattacharjee, M.; Chaudhuri, M. K.; Islam, N. S.; Paul, P. C. *Inorg. Chim. Acta.* 1990, *169*, 97–100.

In Table I, formation constants have been written for condensation with either the carboxylate or the amine form of the ligand. They could as well have been written for the acid or ammonium forms. If K_7 represents product formation from the carboxylic acid (or ammonium) group, then $K_7' = K_7/K_a$ where K_a is the pK_a of the conjugate acid and K_7' is the formation constant written for reaction with the deprotonated form of the ligand, i.e. A = RCO_2^- or $R'NH_2$ from eq 9 with $n = 0$. With these pH-independent formation constants, K_5 of eq 5, which is a pH-dependent equilibrium constant, can be calculated for any pH by utilizing eq 15, where K_{a2}' represents the second pK_a of the product

$$K_5 = \frac{K_7'(1 + K_{a2}'/[H^+])}{(1 + K_{a2}/[H^+])(1 + [H^+]/K_a)} \qquad (15)$$

if it has one within the pH range of the study. For the amino acids studied here, $K_{a2}'/[H^+]$ can be ignored. An analogous equation is readily obtainable when the formation constant is written for reaction with RCO_2H or RNH_3^+ where the n of eq 9 is now equal to 1. For the sake of comparison, the K_5 values calculated for alanine are 0.7, 0.6, and 0.3 M^{-1} for reaction at the carboxylate group; correspondingly, the values are 1.0, 10, and 48 M^{-1} for complexation at the amino functionality (pK_{a2} = 9.9[21]) for pH values of 6.5, 7.5, and 8.5, respectively. It is evident from this that reaction at the carboxylate group drops off significantly over this pH range, while product formation at the ammonium functionality increases substantially. At higher pH, where both $VI_2H_2^-$ and RNH_3^+ become deprotonated, formation of the amino-derived product will decrease.

It has previously been shown that there is a rather strong free energy relationship between formation of peptido-derived diperoxo products and the pK_a of the conjugate acid of the reactant group.[19] The same pattern is followed for the amino acids studied here. This is not surprising, since chemically the same types of products are formed; i.e., monodentate products formed either with the carboxylate group or with the amino group. In fact the imidazole-derived products from histidine or imidazole itself are more favored by about a factor of 10 than would have been predicted solely on the basis of the previously mentioned free energy relationship. However, the nitrogens of the imidazole ring are part of an aromatic system, and this could well account for the discrepancy.

A major difference in reactivity between the amino acids studied here and the peptides previously studied[19,25] is the reactivity with monoperoxovanadate. The previous work had shown that if either the carboxylate group, the terminal amino group, or the peptide nitrogen of a dipeptide was protected, then product formation with the dipeptide was not observed. The results were consistent with the peptide acting as a tridentate ligand in a monoperoxo monopeptido complex.[19] For the case of the amino acids studied here, a slowly formed monoperoxovanadate derivative was also observed, but it formed considerably more rapidly than the peptido complex. It was formed only if the carboxyl or amino groups were not protected, in accord with the amino acid acting as a bidentate ligand. Studies of the formation of the product showed that it contained two amino acid units.

A second type of bis(ligand) product was also observed to form. Mixed-ligand studies showed that this second product contained one amino acid complexed in a bidentate fashion via the amino and carboxylate groups, while the second ligand was attached only through the amino group.

Available X-ray structural data on monoperoxo bis(bidentate ligand) complexes of vanadate suggest that such products are seven-coordinated. This is found, for instance, in the bis(oxalato)[26] and the 2,2'-bipyridyl pyridine-2-carboxylato[27] derivatives. However, pH variation studies showed that both of the bis(ligand) products that are formed here had the ability to lose protons. This implies, at least for the bis(bidentate) product, that it incorporated a water and is eight-coordinated, possibly as $-V(OH)_2$ rather than $-VO(H_2O)$. If no water were taken up, the product would have no labile protons. Eight-coordination is not well-known in vanadium(V) chemistry, although it apparently occurs in the tetraperoxovanadate derivative, $V(O_2)_4^{3-}$.[15,16,28] Some systematic error in the experimental procedures could result in the misinterpretation of the data giving rise to Figure 7, and the case might be similar for the corresponding study with proline. However, the individual sets of equilibrium studies were internally self-consistent so such an error seems unlikely. Deprotonation of the amino functionality as the pH of the solution was raised also seems unlikely.

The observation of two bis(ligand) derivatives, for one of which the carboxylate of the second ligand is left uncoordinated, does suggest the presence of significant amounts of steric crowding as might be expected with eight ligating groups. Somewhat surprisingly, a mono(ligand) product was not found to occur, at least in significant quantities. In fact, there was a low-intensity signal in the appropriate region of the ^{51}V NMR spectra (−638 ppm) that could not be properly studied. Whether this corresponds to a mono(ligand) product is not certain. However, its formation as a function of ligand concentration was consistent with such a product. The low abundance of the mono(ligand) product relative to the bis(ligand) derivative suggests there may be a coordination change, but this certainly is not definitive. It is perhaps of interest to note in this regard that eight-coordination is well established in V(IV) chemistry, being found, for instance, in several sulfur complexes, in the natural product amavadin and in related compounds.[29,30]

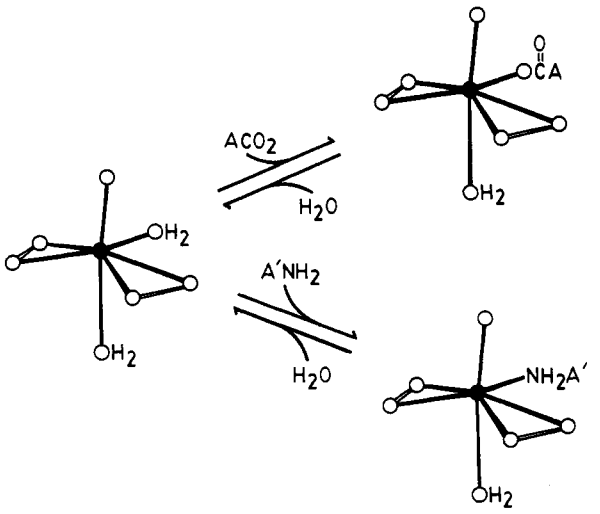
The reaction of imidazole with the peroxovanadates was somewhat puzzling. ^{51}V and ^1H NMR spectroscopy indicated the formation of a single diperoxovanadate derivative. The stoichiometry of this product was determined at pH 6.5, 7.4, and 9.6 and found to be VI_2Im. The chemical shifts of both the ^1H and ^{51}V NMR signals of the product were found to be invariant with changes in pH, indicating it does not have a pK_a. The equilibria established as a result of the variation in pH also were not interpretable in terms of a product pK_a but were not able to unequivocally rule out the possibility.

An unexpected result of this study was the finding that there was no indication of any reaction of monoperoxovanadate with imidazole. This is in marked contrast to the very favorable reaction between imidazole and diperoxovanadate. The reason for this difference is not obvious. It may, however, be a consequence of the pK_a of imidazole; phenol for instance reacts quite favorably with the monoperoxo- but comparatively much less so with the diperoxovanadate.[25]

The structures of the amino acid and imidazole derivatives of diperoxovanadate are, to a degree, conjectures, since it is not known how much water is incorporated into the coordination shell. It has been proposed on the basis of ^{17}O NMR studies that diperoxovanadate is eight-coordinate.[15,28] However, a seven-coordination alternative has been proposed[19] as this coordination is typically observed in single-crystal X-ray analysis of diperoxovanadate derivatives.[18] On the basis of the X-ray work, the reaction of carboxylate or amino groups of amino acids is suggested to occur as depicted in Scheme I. A similar reaction scheme has been proposed for peptides.[19] There does not appear to be any reason to modify this scheme for secondary amines or for aromatic heterocyclic amines such as imidazole.

(25) Jaswal, J. S.; Tracey, A. S. *J. Am. Chem. Soc.* **1993**, *115*, 5600–5607.
(26) Stomberg, R. *Acta Chem. Scand.* 1986, *A40*, 168–176.
(27) Sventivanyi, H.; Stomberg, R. *Acta Chem. Scand.* 1983, *A37*, 709–714.
(28) Howarth, O. W. *Prog. Nucl. Magn. Reson. Spectrosc.* 1990, *22*, 453–485.
(29) Bayer, E.; Koch, E.; Anderegg G. *Angew. Chem., Int. Ed. Engl.* 1987, *26*, 545–546.
(30) de C. T. Corrondo, M. A. A.; Duarte, M. T. L. S.; Pessoa, J. C.; Silva, J. A. L.; da Silva, J. J. R. F.; Vaz, M. C. T. A.; Vilas-Boas, L. F. *J. Chem. Soc., Chem. Commun.* **1988**, 1158–1159.

Scheme I



The results of this study are relevant to the coordination in the active center of vanadium haloperoxidases. Apparently, the haloperoxidase active site requires a single peroxo group for its function.[2,31] The present study has shown that imidazole, at best, is complexed only weakly by monoperoxovanadate. Although the products studied here are poor models for the active site of the haloperoxidases, their chemistry does suggest that imidazole is not found in the reactive site of the active form of the haloperoxidase although it may be a component when the enzyme is in its inactive form. The evidence of weak imidazole binding is in keeping with a ^{51}V NMR study of a vanadium haloperoxidase that suggested there is an oxygen-rich environment about the vanadium.[32] It does argue against X-ray absorption spectroscopy studies of the reduced form of the enzyme (for which V(IV) is contained in the active site) that suggest imidazole is an active-site component.[33] Unfortunately, neither the vanadium NMR nor the X-ray work actually studied the reactive form of the haloperoxidase, so rearrangements within the active site as either the hydrogen peroxide or the halide undergoes complexation cannot be ruled out. On the other hand, the rapid production of oxygen when H_2O_2 is in the presence of the peroxidase[33] may preclude such studies.

Acknowledgment. Thanks are gratefully extended to the NSERC of Canada for its financial support of this work in the form of an operating grant to A.S.T.

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