Histidine-Containing Bisperoxovanadium(V) Compounds: Insight Into the Solution Structure by an ESI-MS and ^{51}V-NMR Comparative Study

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ESI mass spectrometry and ^{51}V-NMR spectroscopy have been used to study the reactions occurring between bisperoxo vanadates and a number of histidine and histidine-like ligands, in aqueous alcoholic solutions. Coordination of one and two molecules of ligand is observed with all the compounds investigated affording $[VO_5L]^-$ and $[VO_52L]^-$, respectively. Characterization of these species has been achieved by MS^n experiments, which have allowed specific fragmentations of the peroxidic moiety to be distinguished. In particular, with $[VO_52L]^-$, two distinct modes of decomposition were observed, depending on the presence in the ligand of a free carboxylic function. – Possible biochemical implications related to vanadium haloperoxidase enzymes are discussed.

Introduction

Haloperoxidase enzymes catalyze the oxidation of halides by hydrogen peroxide.[1][2] The presence of vanadium in the oxidation state 5+ in vanadium-dependent chloro- and bromoperoxidases has been firmly established by using a variety of techniques. The binding of hydrogen peroxide to the metal, to yield a peroxo species as active intermediate, has been established as the initial step in the catalytic cycle. The oxidized halide is likely formed upon nucleophilic attack of X^- on the protonated peroxide group. In the absence of an organic halogen acceptor, the halogenating intermediate reacts with a second molecule of hydrogen peroxide thus producing halide, water and dioxygen, in its singlet state.

On the basis of several mechanistic studies, the catalytic cycle of the haloperoxidase enzymes can be outlined as it follows in Figure 1.[1][2]

Recently the X-ray structures of the native and peroxide form of V^V containing chloroperoxidase (V-ClPO) from the fungus *Curvularia inaequalis*[3] have been solved and possible implications for the catalytic mechanism have also been offered.[4][5] These recent crystallographic studies confirmed that the apical nitrogen donor bound to the metal center in the active site of the enzyme is an histidine imidazole. The other interactions of the prosthetic group with the proteic residues are hydrogen bonds to surrounding, positively charged or hydrophilic protein functions. A second molecule of histidine (His 404) is close to the active site. A very similar environment around the vanadium atom has been detected in the peroxide form of the enzyme.[4][5]

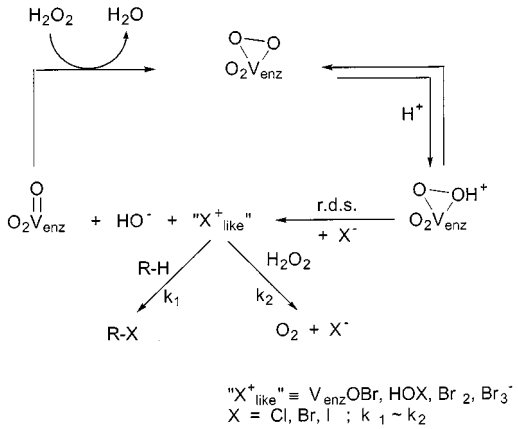

Figure 1. Mechanism for halides oxidation with hydrogen peroxide catalyzed by V-HalPO enzymes

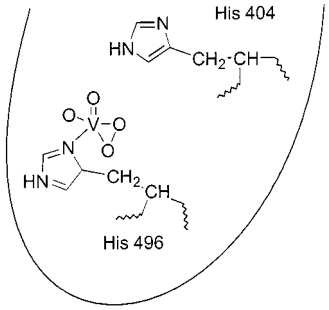

Figure 2. Schematic representation of the vanadium containing active site in the peroxide form of V-ClPO from *Curvularia inaequalis* taken from ref.[5]; hydrogen bonds network with water molecules has been removed for clarity

The occurrence of a protonation step in the catalytic cycle, see Figure 1, is strongly supported from mechanistic

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studies both on the enzyme and on model systems.[2][6] In the enzyme such a role appears to be played by protonated histidine 404, which is in the appropriate position to activate, by protonation, the peroxidic complex through hydrogen bonding interactions.

Several functional models for vanadium dependents HalPO have been reported,[2] the simplest one being *cis*-dioxovanadium(V), VO_2^+, in acidic conditions in the presence of hydrogen peroxide.[7] It is well-known that depending on the acidity of the medium and on the concentration of precursors both mono- and bisperoxovanadium species can be formed.[8][9] We have recently proposed as functional models for V-BrPO a two-phase procedure, the oxidation of bromide is carried out, in the aqueous acidic phase, by either monoperoxo- or bisperoxovanadium derivatives which are formed from NH_4VO_3 and H_2O_2.[10–12] Subsequently, the reactive intermediate can brominate the substrate in the organic phase. The vanadium bisperoxo complex appears to be reactive toward bromide ions only when it is protonated.[6][13]

In almost neutral conditions, bisperoxovanadium complexes are quite stable species,[14] therefore we envisaged the possibility of using such complexes, in the presence of histidine-like ligands,[15] as simple models for the analysis of structural features in solution of peroxovanadium complexes in order to collect information related the behavior of the enzyme. The idea of studying a bisperoxovanadate species as a model of the enzyme is based on the knowledge of the crystal structure of V-ClPO,[5] see Figure 2. In fact, the prosthetic group of the enzyme is a monoperoxo-metavanadate moiety[5] bound to the protein only through histidine 496. Therefore, there is significant correspondence between the structure of a bisperoxovanadate[15] eventually containing an imidazole ring and the prosthetic group of V-ClPO from *C. inaequalis*.

We report here a study on the chemistry in solution of histidine-containing bisperoxovanadium compounds, by using ^{51}V-NMR and ESI-MS analyses. The latter technique has been already successfully used for the analysis of cationic mono-peroxovanadium compounds in acid conditions.[16]

Results and Discussion

Speciation of Bisperoxovanadates

Ammonium vanadate ($4\cdot10^{-3}$ mol L^{-1}), dissolved in water at pH ca. 6.8 shows in ^{51}V-NMR the known distribution pattern of mono- and oligomeric vanadate species.[17] In particular the prevailing species are monomeric, dimeric and tetrameric metavandates, respectively, indicated by the resonances at $\delta = -559$, -572, and -577, in agreement with literature data. When the same solution is diluted with ethanol, signals appear at $\delta = -552$, -556, -561, and -571, thus showing the possible formation also of the pentameric derivative. The addition of alcohol results in downfield shifts of the resonances and a broadening of the signals. This latter behavior can be assigned to fast exchange between water and alcohol molecules in the coordination sphere of the metal.[18]

The analysis of the same solutions by ESI-MS, in negative ion mode, shows the occurrence of the following species: $[VO_3]^-$ (*m*/*z* 99, relative abundance 3%), $[VO_3(H_2O)]^-$ (*m*/*z* 117, 14%), $[VO_3(EtOH)]^-$ (*m*/*z* 145, 44%), $[OV(O)(OEt)_2]^-$ (*m*/*z* 173, 100%), the dimeric $[\{OV(O)\}_2O(OH)]^-$ (*m*/*z* 199, 2%) and $[\{OV(O)\}_2O(OEt)]^-$ (*m*/*z* 227, 28%), as well as the less intense trimeric and tetrameric ions $[\{OV(O)\}_3O(OEt)(OH)]^-$ (*m*/*z* 327, 3%), $[\{OV(O)\}_3O(OEt)_2]^-$ (*m*/*z* 355, 8%), $[\{OV(O)\}_4O(OH)_3]^-$ (*m*/*z* 399, 2%).

When hydrogen peroxide is added to vanadate solutions in water/ethanol, a number of peroxovanadates are formed.[8,9,19] The relative abundance of the different species depends on the ratio H_2O_2/vanadate, pH, and on vanadium concentration. Under acidic conditions, in the presence of equimolar amounts of H_2O_2 with respect to vanadium, the predominant species are cationic monoperoxo complexes with different coordination sphere, whose ESI-MS behavior has been already reported.[16]

Aqueous solutions of peroxovanadium species were initially analyzed by employing mixtures of water and methanol. However, the presence in the coordination sphere of the metal of both the peroxo O_2^{2-} moiety and the methanol molecule having the same molecular weight (32 Da) caused difficulties in assigning some of the fragmentation patterns. Therefore, water and water/ethanol mixtures have been used as solvent. See Experimental Section for details regarding the preparation of the solutions. The preferential formation of bisperoxovanadium monoanion complexes was monitored by increasing the amount of hydrogen peroxide up to a 2.4:1 ratio with respect to vanadium, for pH values of 1.3, 6.9, 8.2. ^{51}V-NMR spectra at pH 1.3 revealed the presence of similar concentration of mono- and bisperoxo complexes, as indicated by signals at $\delta = -521$ ($W_{1/2}$ 501 Hz) and -681 ($W_{1/2}$ 468 Hz), respectively. On the other hand, in ESI-MS experiments, carried out both in positive and negative ion mode, the absence of any detectable species suggests that in these conditions, mono- and protonated bisperoxo compounds do not survive to the electrospray ionization conditions. In agreement with this observation, we have found that the decomposition reaction of bisperoxovanadium solutions at various pHs is faster as the concentration of $HClO_4$ increases, see Figure 3.

^{51}V-NMR experiments at almost neutral pH indicate the presence of a single species at $\delta = -689$ ($W_{1/2}$ 270 Hz) identified as the bisperoxovanadium compound. When the same solution is analyzed by ESI-MS, most of the observed peaks can be attributed to bisperoxo species containing different solvent molecules in the coordination sphere: $[VO_5]^-$ (*m*/*z* 131, 100%), the solvent bound species $[VO_5(EtOH)]^-$ (*m*/*z* 177, 55%) and $[VO_5(EtOH,H_2O)]^-$ (*m*/*z* 195, 5%), see Figure 4.

Other peroxidic compounds are observed at higher *m*/*z* values such as the dimeric species $[\{VO(OO)_2\}_2(H)]^-$ (*m*/*z* 263, 5%) and the water containing $[\{VO(OO)_2\}_2(H,H_2O)]^-$

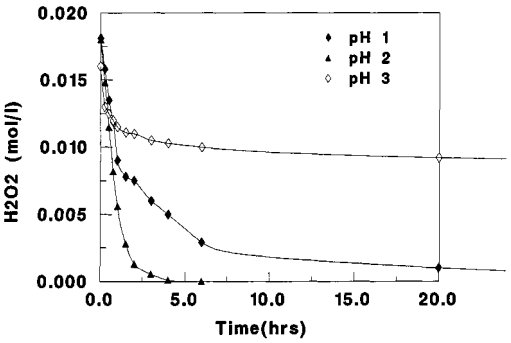


Figure 3. Disappearance of H_2O_2 (0.0.2 mol L^{-1})as a function of the time in the presence of NH_4VO_3 (0.01 mol L^{-1}) in water at 25 °C, at various pH ($HClO_4$); ▲ = pH 1.1 ($[VO(O_2)]^+ \sim [VO(O_2)_2]^-$ as measured by ^{51}V-NMR), ◆ = pH 2.0; (only $[VO(O_2)_2]^-$), ◇ = pH 3.0 (only $[VO(O_2)_2]^-$)

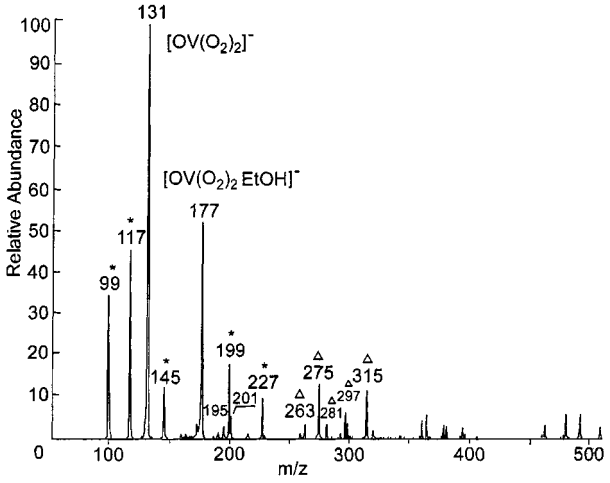


Figure 4. ESI mass spectrum of VO_3^- ($2\cdot10^{-3}$ mol L^{-1}) in the presence of 2.4 equivalents of H_2O_2 in ethanol/water 1:1, pH 6.9; (*) non-peroxidic species, (Δ) peroxospecies with a ratio $O_2^{2-}/V \geq 2$

ion (*m/z* 281, 5%). It is worth of noting that very recently the crystal structure of this latter dimeric compound has been solved.[20] Furthermore, species having a peroxo-to-vanadium ratio higher than two have been detected at *m/z* 315 and 361. As far as the possibility of observing the formation of the previously reported[19] triperoxo compound is concerned, under such experimental conditions even though a small peak is detected at the correct *m/z* value corresponding to $[OV(O_2)_3(H_2O)_2(H)_2]^-$ (*m/z* 201, < 5%), we could not confirm its nature with collision induced-decomposition experiments (MS^n) because of the low intensity of the signal.

MS^n mass spectra were used to identify some of the above-mentioned species. The "naked" bisperoxovanadium compound, at *m/z* 131, fragments to the non-peroxidic species $[VO_3]^-$ (*m/z* 99). This decomposition is followed by the coordination of water or ethanol to $[VO_3]^-$ affording ions at *m/z* 117 and 145, respectively, Figure 5a. Support for this behavior was obtained by independent experiments on the isolated $[VO_3]^-$ which showed addition of water or ethanol, see Figure 5b. $[VO_5]^-$ with an ethanol molecule in the coordination sphere, is more prone to add neutrals, as found in the MS/MS mass spectrum of $[VO_5(EtOH)]^-$ (*m/z* 177) where coordination of water and ethanol is observed, affording the ions $[VO_5(EtOH,H_2O)]^-$ *m/z* 195 and $[VO_5(EtOH)_2]^-$ *m/z* 223, the latter in much lower relative intensity. An increase of the pH of the medium promotes the formation of oligomeric vanadium peroxo compounds in most cases having a peroxo-to-vanadium ratio higher than two.[19] As expected, also the increase of the concentration of vanadium favors the oligomerization process.

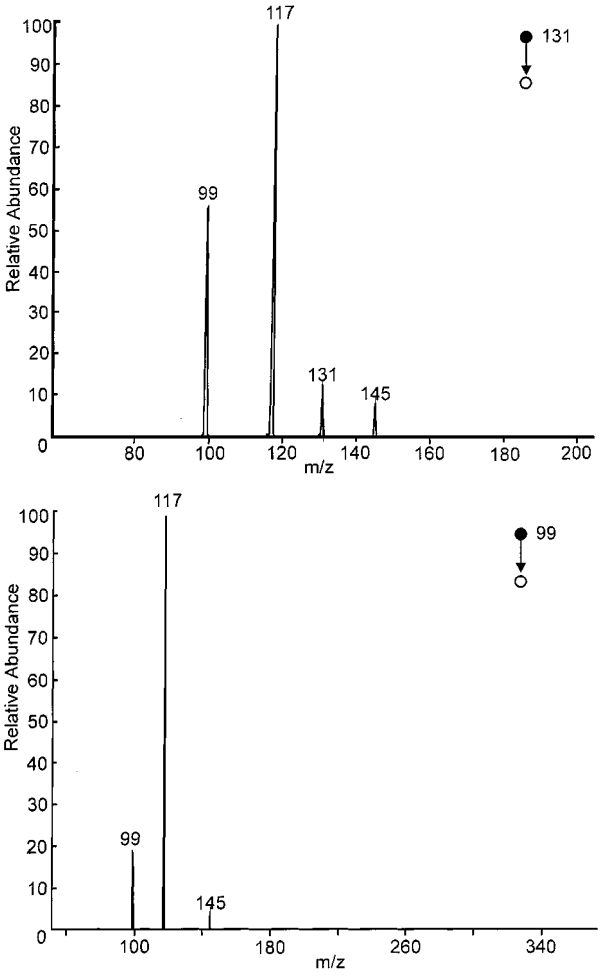
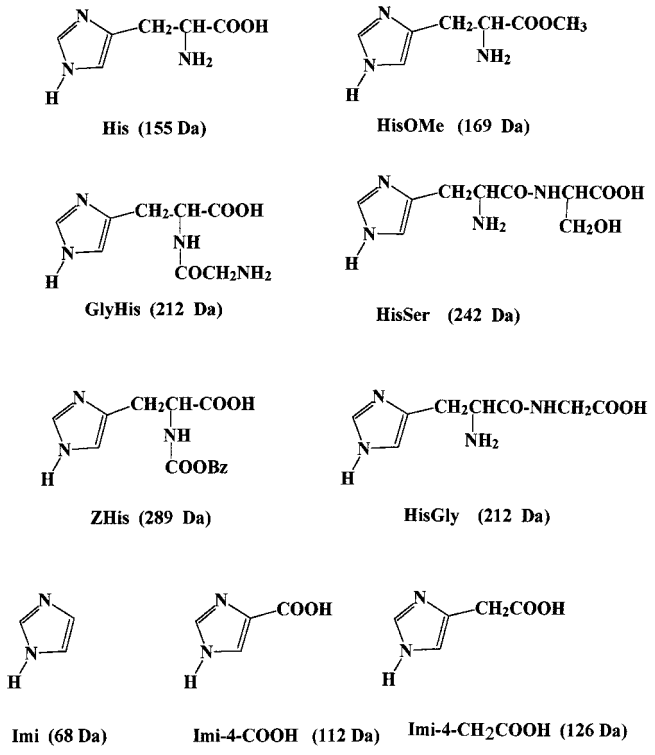


Figure 5. MS/MS mass spectrum of (a) the bisperoxovanadium $[VO_5]^-$ and of (b) the non-peroxidic species $[VO_3]^-$, in ethanol/water, 1:1, pH 6.9

We recently reported[16] that the tandem use of ^{51}V-NMR and ESI-MS analyses allows a better characterization of rapidly exchanging monoperoxovanadium species with different coordination sphere composition. Analogously, in the present study, the evidence that the line width of the NMR signals related to bisperoxocompounds significantly increases in passing from water to water/alcohol mixtures suggests the occurrence of new equilibria, regardless, a detailed snapshot of the equilibrating species, not feasible on NMR time scale, is accessible with the ESI-MS technique.

Histidine-Like Ligands for Bisperoxovanadates

Vanadium imidazole complexes have recently received significant attention in connection with the evidence that haloperoxidases contain histidine residues coordinated to vanadium.[3−5] Taking advantage of the results reported above we analyzed bisperoxo vanadium compounds at neutral pH in the presence of histidine and histidine-like ligands, i.e. methyl histidine (HisOMe), carbobenzoxy histidine (ZHis); dipeptides: GlyHis, HisSer, HisGly; imidazole (Imi), and carboxyimidazole derivatives (Imi−4-COOH) and (Imi−4-CH_2COOH), see Scheme 1. For example, upon addition of 2 to 6 equivalents of histidine to the bisperoxovanadium solution, at pH 6.9, together with the ^{51}V-NMR signal located at ca. $\delta = -690$, two new peaks are observed at $\delta = -729$ ($W_{1/2}$ not measured due to low intensity) and $\delta = -742$ ($W_{1/2} = 291$ Hz). This latter value is very close to the chemical shift ($\delta = -744$) reported by Crans et al.[15] for the bisperoxovanadium imidazole monoanion thus confirming that the coordination of histidine to $[VO_5]^-$ occurs via the imidazole moiety. The former signal has been assigned by Tracey et al.[21], even though with no firm evidence, to a species containing histidine bound to vanadium through the second imidazole nitrogen.



Scheme 1

The coordination properties of all the ligands used, see Scheme 1, were tested via ^{51}V-NMR technique in aqueous solution (pH ca. 6.9) and in water-ethanol mixture, by changing the [ligand]/$[VO_5]^-$ ratio from 1 to 6. In all cases the main peak observed is located between $\delta = -738$ and -750, depending from the ligand and from the solvent, thus indicating that the binding to the bisperoxovanadium complex always occurs through the imidazole sp^2 nitrogen. In agreement with previous data, ^{51}V-NMR analysis of solutions containing Imi and Imi−4-CH_2COOH as ligands shows the occurrence of bisperoxovanadates (at δ ca. -700) and $[VO_5L]^-$ (at δ ca. -740) species. On the other hand, by using Imi−4-COOH, three signals were detected pertaining to bisperoxovanadate ($\delta = -690$), $[VO_5$Imi−4-COOH$]^-$ ($\delta = -740$) and to a new species, located at $\delta = -770$. As far as its nature is concerned, we suggest the formation a dimeric bisperoxovanadate, possibly containing one or two molecules of Imi−4-COOH. Characterization of such species is currently in progress. Solutions of bisperoxovanadates in the presence of two equivalents of histidine were analyzed by ESI-MS, and additional signals are observed at *m/z* 286 and 441, corresponding to $[VO_5$His$]^-$ and $[VO_5$2His$]^-$, respectively, see Figure 6.

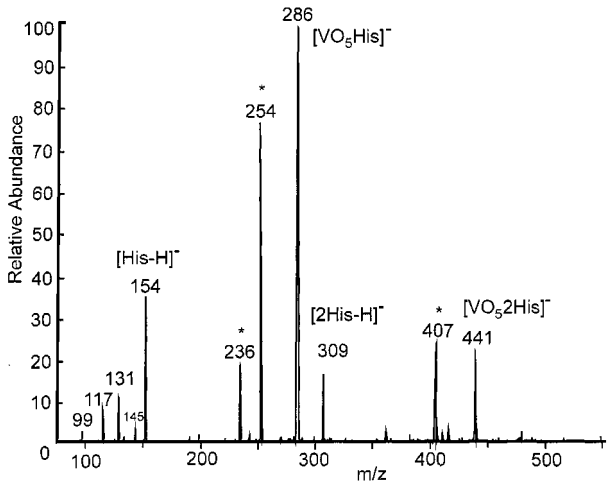


Figure 6. ESI mass spectrum of VO_3^- ($2\cdot10^{-3}$ mol L^{-1})/H_2O_2 (2.4 equivalents) in ethanol/water, 1:1, pH 6.9. in the presence of histidine (2 equivalents); (*) signals due to fragmentation of $[VO_5$His$]^-$ and $[VO_5$2His$]^-$, respectively, see text

It is important to emphasize that both *m/z* values correspond to the insertion of intact histidine molecule(s), although at this point we shall not specifically address where the protons are located. As will be discussed later on in the paper, this is a crucial point in the reactivity of the species. MS^n mass spectra of $[VO_5$His$]^-$ and $[VO_5$2His$]^-$ were also collected and the relevant data are inserted in Table 1. In particular, the different behavior of $[VO_5$His$]^-$ with respect to $[VO_5$2His$]^-$ deserves comments. The collision-induced decomposition (MS^2) of the $[VO_5$His$]^-$ complex shows a new peak at *m/z* 254 corresponding to the loss of 32 Da. On the other hand, $[VO_5$2His$]^-$ under the same CID conditions, shows formation of peaks at *m/z* 407 and 286 related to the loss of 34 and 155 Da. The $[VO_5$2His -155 Da$]^-$ ion is easily attributed to the release of a neutral histidine molecule. In order to better understand the nature of the 32 and 34 Da fragments observed in the decomposition of $[VO_5$His$]^-$ and $[VO_5$2His$]^-$ respectively, we have extended the analysis to the other ligands listed in Scheme 1, see Table 1.

Table 1. Relative abundances of $[VO_5L]^-$ and $[VO_52L]^-$ ionic species in ESI mass spectra of bisperoxovanadium compounds in the presence of histidine and histidine-like ligands and related MS/MS fragmentations[a]

Species	*m/z*	rel. abund.%	MS/MS[a]
[M]$^-$ ≡ $[VO_5His]^-$	286	100	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(His)_2]^-$	441	25	**[M′ - 34]$^-$** **[M′ -His]$^-$**
[M]$^-$ ≡ $[VO_5ZHis]^-$	420	60	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(ZHis)_2]^-$	709	55	**[M′ - 34]$^-$** **[M′ − ZHis]$^-$**
[M]$^-$ ≡ $[VO_5GlyHis]^-$	343	100	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(GlyHis)_2]^-$	555	30	**[M′ - 34]$^-$** **[M′ − GlyHis]$^-$**
[M]$^-$ ≡ $[VO_5HisOMe]^-$	300	35	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(HisOMe)_2]^-$	469	15	**[M′ − HisOMe]$^-$**
[M]$^-$ ≡ $[VO_5HisOMe(HCl)]^-$ [b]	336	45	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(HisOMe)_2(HCl)]^-$ [b]	505	16	**[M′ − HisOMe]$^-$**
[M]$^-$ ≡ $[VO_5HisSer]^-$	373	100	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(HisSer)_2]^-$	614	15	**[M′ - 34]$^-$** **[M′ − HisSer]$^-$**
[M]$^-$ ≡ $[VO_5HisGly]^-$	343	100	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(HisGly)_2]^-$	555	40	**[M′ - 34]$^-$** **[M′ − HisGly]$^-$**
[M]$^-$ ≡ $[VO_5Imi]^-$	199	80	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(Imi)_2]^-$	267	< 1	signal too low
[M]$^-$ ≡ $[VO_5Imi\text{-}4\text{-}COOH]^-$	243	80	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(Imi\text{-}4\text{-}COOH)_2]^-$	355	4	**[M′ - 34]$^-$** **[M′ − Imi-4-COOH]$^-$**
[M]$^-$ ≡ $[VO_5Imi\text{-}4\text{-}CH_2COOH]^-$	257	80	**[M - 32]$^-$**
[M′]$^-$ ≡ $[VO_5(Imi\text{-}4\text{-}CH_2COOH)_2]^-$	383	2	Signal too low

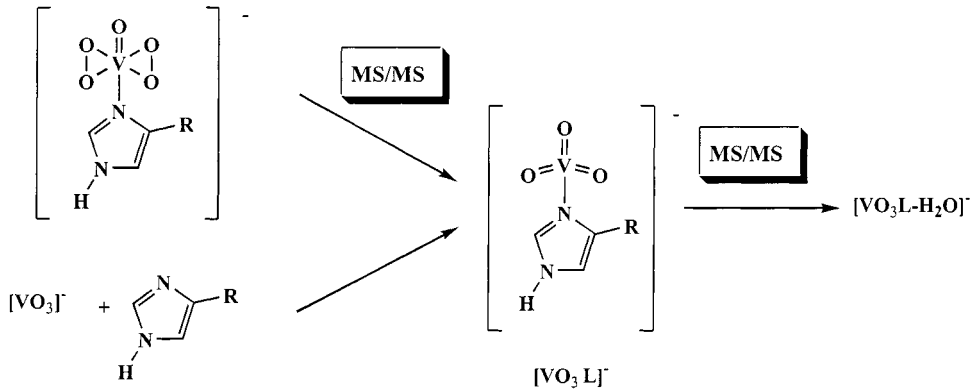
[a] MS/MS fragmentations of the selected $[M]^-$ and $[M']^-$ ions, respectively. − [b] These signals are due to the use as ligand of the commercially available HisOMe(HCl).

The overall picture obtained with these ligands by ESI-MS is very close to that observed for histidine. The data reported in the Table show that all the vanadium bisperoxo species containing one heteroligand in the coordination sphere are prone to release the 32 Da fragment, whereas the presence of two heteroligands on $[VO_5]^-$ appears to favor the expulsion of 34 Da only when a carboxy group is present in the framework of the ligand. The loss of 32 Da fragment from $[VO_5L]^- \equiv [M]^-$ has been attributed to the decomposition of both peroxidic moieties, which gives rise to the formation of the non-peroxidic species $[VO_3L]^-$ on the basis of the identical decomposition pattern of $[M - 32\ Da]^-$ ions and the isobaric ions obtained from ligands containing vanadates solutions, see Scheme 2. On the other hand, the loss of 34 Da fragment from $[VO_52L]^- \equiv [M']^-$, observed when L has a free carboxylic function, has been attributed to the release of H_2O_2. In fact upon isolation of the $[M' - 34]^-$ ion, loss of an intact L molecule is observed, thus proving that fragmentation of the ligands is not taking place, Scheme 3. Furthermore, the $[M' - 34]^-$ ions have to have an intact "side on" peroxidic group otherwise they would not be monoanions and therefore observed at a different *m/z* value.

A plausible explanation for the above-reported data takes into account the protonation a peroxidic oxygen by the carboxylic function of the ligands. When two ligands are involved in the coordination, the loss of hydrogen peroxide is made possible. Protonation of a peroxidic moiety likely results in an opening of the three-membered ring.[14] Such a process may allow a direct coordination to the metal of a second molecule of ligand. The resulting species has the peculiar ESI-MS fragmentation pattern discussed above, i.e. 34 Da loss. In the absence of a free carboxy group, as it is the case of HisOMe, HisOMe(HCl), and IMI, the second molecule of ligand in $[VO_5L_2]^-$, can be seen as external to the first coordination sphere of the metal, as it is in the crystal structure of $[VO_5Imi]^-(ImiH)^+$, where the imidazolium cation acts as counterion. Ab initio calculations are currently in progress in order to obtain further support to this hypothesis.

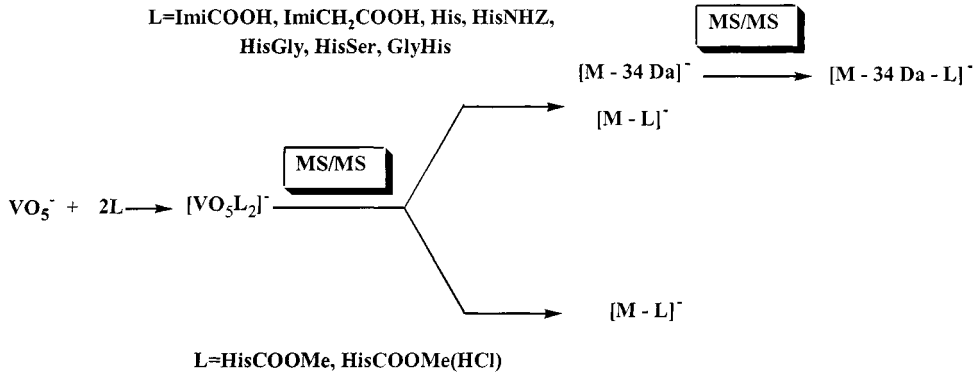
Conclusion

In summary, this study shows that the correct choice of the model for V-HalPO enzymes and its analysis with appropriate techniques allows the collection of significant structural and mechanistic data. In this respect, we have obtained direct[6] and independent evidence that the protonation of the peroxidic moiety is a crucial step in the activation of vanadium peroxocompounds. Such a mechanism of activation is also involved in the reactivity of V-HalPO enzymes.[5] The role of two different molecules of histidine and histidine-like ligands as proton donors has been also directly observed.



R = CO_2H, CH_2CO_2H, $CH_2CH(NH_2)CO_2H$, $CH_2CH(NH_2)CO_2CH_3$, $CH_2CH(NHCOOBz)CO_2H$, $CH_2CH(NH_2)CONHCH_2CO_2H$, $CH_2CH(NH_2)CONHCH(CH_2OH)CO_2H$, $CH_2CH(NHCOCH_2NH_2)CO_2H$

Scheme 2. Decomposition pattern of $[VO_3L]^-$ ions obtained from $[VO_5L]^-$ (MS^3 experiment) or of ligand containing vanadate solutions (MS^2 experiment), respectively



Scheme 3. Decomposition patterns of $[VO_5L_2]^-$ ions (MS^2 and MS^3 experiments) as a function of the ligand L

Experimental Section

General Remarks: The ESI-MS measurements were obtained using a Finnigan MAT LCQ instrument (San Jose, CA) by using the following parameters: solution flow rate 8 μL min^{-1}, capillary temperature 120−140 °C, spray voltage 3.4 kV, capillary voltage comprised between −5 and −20 V, nebulizing gas N_2 (40 units flow rate). Parameters related to octapoles and detector were those achieved by the automatic set-up procedure. Collision induced decompositions of selected ions (MS^n, n = 2, 3) were obtained by applying a supplementary r.f. voltage (tickle voltage) to the end cap electrodes of the ion trap (resonance activation). Typical tickle voltage values for fragmentation were 3−5 V. The vanadate and peroxovanadate solutions were introduced into the instrument by direct injection. The vanadate solutions at pH 6.9 are prepared by dissolving NH_4VO_3 ($2\cdot10^{-3}$ mol L^{-1}), in CH_3CH_2OH/H_2O, 1:1. The peroxovanadium solutions are prepared by dissolving NH_4VO_3 ($4\cdot10^{-3}$ mol L^{-1}) in water. 2.4 equivalents of H_2O_2 are subsequently added and pH is finally adjusted to the desired value [1.3 ($HClO_4$), 6.9 (natural value), and 8.2 (NH_3)]. The solutions are then diluted 1:1 with ethanol. Ligands are added in due time (1−8 equivalents with respect to V^V) to the aqueous solution of peroxovanadates before pH adjustment.

No significant variation on the qualitative distribution of the species was observed by repeating the measurements. However, the intrinsic nature of the ESI-MS technique does not allow to estimate the relative concentration of the ions detected. This event, nevertheless, does not interfere with the conclusion drawn in the present work.

Decomposition Reactions: The appropriate amount (2 equivalents) of H_2O_2, in water, is added to a aqueous solution of NH_4VO_3 ($1\cdot10^{-3}$ mol L^{-1}), at pH = 1 and 25 °C. When needed, pH adjustment (at values of 2 and 3) is settled with NH_3. Consumption of peroxide content is analyzed at chosen time interval with standard iodometric titration. Reproducibility of the kinetic behavior was checked for each pH with triplicate runs.

NMR experiments were carried out as already described.[9][12]

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