Calculation of Higher Protonation States and of a New Resting State for Vanadium Chloroperoxidase Using QM/MM, with an Atom-in-Molecules Analysis

Gregory A. Anderson*,†, Raghu Nath Behera*,‡, Ravi Gomatam†

† Bhaktivedanta Institute and Institute of Semantic Information Sciences and Technology, Juhu Road, Juhu Mumbai 400049

‡Department of Chemistry, Birla Institute of Technology and Science, Pilani - Goa Campus, Zuarinagar, Goa 403726 India

Corresponding Authors Information:

G.A.A.: E-mail: ganderson@bvinst.edu, Ph: +1 510-520-4777

R.N.B.: E-mail: rbehera@goa.bits-pilani.ac.in, Ph: +91 832 2580331, Fax: +91 832 2557033
ABSTRACT. Earlier QM/MM studies of the resting state of vanadium chloroperoxidase (VCPO) focused on the diprotonated states of the vanadate cofactor. Herein, we report a new extensive QM/MM study that includes the tri- and quadprotonated states of VCPO at neutral pH. We identify certain di- and triprotonated states as being candidates for the resting state based on a comparison of relative energies. The quadprotonated states as well as some of the triprotonated states are ruled out as the resting state. An Atoms-in-Molecules (AIM) analysis of the complex hydrogen bonding around the vanadate cofactor helps to explain the relative energies of the protonation states considered herein, and it also indicates new hydrogen bonding which has not been recognized previously. A Natural Bond Orbital (NBO) study is presented to give a better understanding of the electronic structure of the vanadate co-factor.

Keywords: QM/MM, vanadium chloroperoxidase, atoms-in-molecules, natural bond orbital, resting state

1.0 Introduction:

Vanadium haloperoxidases are found in many algae, lichens, fungi and bacteria, and they also have practical applications in the biotechnology industry.[1,2] In Nature, they have a crucial role in biosynthetic pathways that lead to antibiotics as well as the emission of greenhouse gases (i.e., chloroform and bromoform) into the atmosphere.[3] Within this class of enzymes, we have focused on vanadium chloroperoxidase (VCPO), which catalyzes the two-electron oxidation of halide to hypohalous acid (Scheme 1); the hypohalous acid diffuses out of the active site and reacts in a nonspecific way with electrophilic substrates. In the fungus, Curvularia inequalis, the synthesized hypohalous acid breaks down the cell membrane of plants which the fungus is
attacking.[3] In order to begin the study of the catalytic mechanism [4] of this enzyme, one first has to decide what is its resting state. In many cases, the investigation of the mechanism of catalysis of enzymes requires a determination of the resting state.[5]

\[
\text{X}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{HOX} \\
\text{X} = \text{Cl}, \text{Br}, \text{I}
\]

**Scheme 1.** VCPO catalyzes the two-electron oxidation of halide.

In the case of VCPO, the results from X-ray diffraction studies give only partial information about the resting state. Nevertheless, it is clear that the active site of the enzyme consists of a vanadate in a trigonal bipyramidal configuration with His496 covalently bonded to one of the axial positions of the vanadate, and the vanadate is hydrogen bonded to a sphere of amino acid residues surrounding it, namely, Lys353, Ser402, Gly403, His404, Arg490, and Arg360 (Figure 1).[6] Furthermore, Asp 292 forms a salt bridge to Arg490. The resolution of the X-ray crystal structure (±0.24 Å) is insufficient to reveal the protonation state of the vanadate, and computational studies have been carried out to determine the resting state of VCPO.[7-11]. Spectroscopic and biochemical studies have also established that the oxidation state of vanadium remains +5 throughout the catalytic cycle [12-14]; therefore, it has been thought that the protonation state of the oxo groups coordinating vanadium may be responsible for redox tuning of the cofactor.[15]

Recently, Gupta et al. performed $^{51}$V magic angle spinning (MAS) NMR spectroscopy and determined certain NMR parameters (i.e., the reduced anisotropy, the asymmetry parameter, and the electric field gradient (EFG) tensor parameters) which are influenced by the protonation state of vanadate in the resting state.[15] Then, they constructed models of the active site with the
vanadate in various protonation states, and calculated these NMR parameters by density functional theory using the B3LYP hybrid functional and 6-311G(d,p) basis set while constraining the position of all atoms except for the hydrogens and vanadate. They argued that the protonation state is dependent on the pH in the following way: diprotonated 3 (pH 8.3), triprotonated 10 or 13 (pH 7.3) and quadprotonated 16 (pH 6.3) (Figure 2). At pH 7.3, the observed NMR parameters were consistent with either triprotonated 10 or 13.

From this work of Gupta et al., it is clear that higher protonation states need to be considered for the proper study of the catalytic mechanism of VCPO. Earlier QM/MM studies on VCPO have considered only the mono- and diprotonated states. In this paper, the results from a QM/MM study of the higher protonation states of VCPO are reported.

The QM/MM Hamiltonian, which combines quantum mechanics and molecular mechanics, includes terms for long range electrostatic interactions and can thus model all of the 8,860 atoms in VCPO in comparison to the 126 atom model in Gupta et al.’s study. QM/MM modeling is necessary because it has been found previously that the long range electrostatic interactions substantially influence the protonation state of VCPO, and the effects of the protein environment on the active site model are very important in VCPO.[7] Another advantage of QM/MM modeling of VCPO is that many heavy atoms do not need to be constrained in order to obtain convergence as they do in a QM model.

We describe the results of a QM/MM study starting with input structures, 1-23, in the mono, di-, tri- and quadprotonated states at pH 7. This QM/MM study is conducted with two QM regions consisting of 8 residues and 13 residues. Thus far, no QM/MM study of VCPO with 13 residues in the QM region has been reported. This study provides structural information about
VCPO as well as the stability of the tri and quadprotonated states. It also provides the relative energies of the protonation states. All of this information combined together allows us to indicate what is likely to be the resting state of VCPO.

Next, we apply Bader’s Atoms-in-Molecules (AIM) approach [16] to elucidate the complex hydrogen bonding to the vanadate cofactor. The AIM approach has been increasingly used to determine noncovalent interactions.[17-22] In the literature on VCPO, almost all knowledge of the hydrogen bonding is based on distance considerations, but distance considerations can be misleading. The AIM approach involves a topological analysis of the probability density function. Atoms that are interacting (bonding or secondary interactions) have a single line of locally maximum electron density, and this line is referred to as a bond path. A point on the bond path between two interacting atoms with the lowest value of the electron density is called the bond critical point, and we report herein the values of the electron density, $\rho$, at the bond critical points in units of electrons/bohr$^3$. The bond critical point along a covalent bond, will typically have $\rho= 0.2$ electrons/bohr$^3$, but for a hydrogen bond the $\rho$ value at the bond critical point will be less than 0.1 electrons/bohr$^3$. Knowing the precise hydrogen bonding in VCPO will aid the study of the mechanism, which has so far been limited to the study of the cofactor in isolation or at most by taking into consideration the hydrogen bonding between Lys353 and vanadate.[4,23] In addition to this AIM study, we report a natural bond orbital (NBO) study of the tri- and quadprotonated states of vanadate to gain a better understanding of their electronic structure.
Figure 1. The active site of vanadium chloroperoxidase (VCPO) has a vanadate cofactor which is shown here in an unprotonated state. The vanadate is surrounded by a sphere of residues which hydrogen bond to it. (Three crystallographic water molecules are hidden for clarity.)
Figure 2. The mono-, di-, tri-, quad- protonated states of VCPO, 1-23, which are the input structures for this QM/MM study. The actual QM region used in this QM/MM study is shown in Figures 3-4. Structure 1 shows some of the surrounding amino acid residues so that the stereochemistry is clear in all of the structures. (His496, which is bonded to V, is omitted for simplicity.)
Figure 3. QM Region I with atom numbering. Hydrogen bonding which is present in most of the optimized structures is indicated by dotted lines. The hydrogen bonding between H6 or H7 and the crystallographic water molecules varies between structures and is not indicated in this Figure; this hydrogen bonding is discussed in the text and can be found from the Tables S3-S10 in the Supplementary Material.
Figure 4. QM Region II with atom numbering. Some of the hydrogen bonding is indicated by dotted lines.
2.0 Methods:

QM/MM calculations were carried out with QSite.[24,25] The topological analysis of the electron density, involved in the AIM approach, is built into the QSite code and is invoked with iplotnoncov=1. Natural Bond Orbital calculations were done with NBO 6.0 which is implemented in Jaguar 10.1.[25,26]

The X-Ray crystal structure, 1IDQ, was preprocessed with Schrodinger Protein Preparation Wizard.[27,28] The Schrodinger Protein Preparation Wizard may flip the orientation of Gln, His and Asn residues in the enzyme structure to optimize the hydrogen-bonding network because the orientation of these residues is unclear from the X-ray crystal structure. The titratable residues (i.e., histidines, aspartates, glutamates) outside of the QM region were protonated assuming pH 7.0 because the PROPKA method [29] for calculating the pKa values of amino acid residues failed for this structure.[30] The Protein Preparation Wizard detected that the following residues were incomplete in the X-ray crystal structure: Glu83, Glu119, Gln120, Pro121, Asn122, Pro123, Asn124, Pro125, Asn128, His185. Although they were on the surface of the enzyme and unlikely to affect the calculations, they were repaired. Furthermore, the hydroxyl hydrogen (H28) of Ser402 was rotated to point to the equatorial O3 of the vanadate. (See Figure 3 for atom numbering.) The resulting structure was then relaxed through a restrained minimization to RMSD 0.3 Å on hydrogens to relieve any steric clashes. (Restrained minimization on the heavy atoms was not done because it was found to result in significant and irreproducible changes in the positions of atoms.) This structure was then manually altered to generate input structures, 1-23, which are grouped into four series: mono- (1-2), di- (3-8), tri- (9-14) and quad- (15-23) protonated states. (See Figure 2.)
Two QM regions were used. The first QM region, which is shown in Figure 3, consisted of His496 (side chain), Lys353 (side chain), Arg360 (full), Arg490 (full), Ser402 (full), Gly403 (full), HIE404 (full) [31], Asp292 (side chain), H2O1911, H2O1778 and vanadate (VO₄) in various protonation states. We will refer to this first QM region as “QM Region I” (Figure 3).

The second QM region, “QM Region II” (Figure 4), consisted of these 8 residues and vanadate as well as all of the atoms in the following residues: Ala399-Pro398-Phe397-Pro396-Pro395. It is also included two additional crystallographic water molecules: H2O1832, H2O1628. The series of three water molecules—namely, H2O1911, H2O1778, and H2O1832—form a hydrogen bonded bridge between the axial oxygen (O4) of vanadate and Asp292 (i.e., O13). QM Region I consisted of 149-153 atoms depending on the protonation state of vanadate. QM Region II consisted of 226-230 atoms.

QM optimizations in this QM/MM study were carried out with density functional theory with the B3LYP functional and the lacvp* basis set.[32] In the case of calculations concerning 3-8, the following atoms were constrained for both QM Region I and QM Region II: the backbone atoms of Ser402, Gly403, His404, the methylene atoms of Arg360 and Arg 490, H50 and H51 of the guanidine of Arg360, as well as H52 of HIE404. For the other input structures (1-2, 9-23), all of these atoms as well as the side chain atoms of Asp292 were constrained. These constraints were used to speed up convergence of the geometry optimization and follow the literature.[7] As stated above, the entire Arg360 residue, which is next to Pro361, was included in the QM region, but the only parameterized cut for proline in QSite is along C-Cα; hence, the entire Pro361 would have had to be included. As a result, Pro361 was simplified to alanine following the example of Kravitz et al., and this alanine was constrained during the geometry optimizations.[7] In the case of QM Region II, all of the atoms in the sequence Ala399-Pro395 were constrained except for
the phenyl atoms (i.e., CH2-Ph) of Phe397. The MM region consisted of the remainder of the enzyme; the protein backbone was frozen during the MM optimizations. Furthermore, any MM atoms beyond 20 Å from vanadium were frozen. MM optimization was done with the OPLS_2005 force field.[33] Solvent effects were modeled by neutralizing charged surface residues.[34] The solvent accessible surface area (SASA) of each residue was determined by the method of Lee and Richards[35] which is implemented in Bioluminate, a software of Schrodinger Corporation, and Bioluminate was used to determine the SASA of each residue.[36] Any charged surface residue with a SASA of greater than 1 Å² was neutralized.

2.1 Justification for the protonation state of amino acids in QM region

The justification for choosing the protonation states of the various amino acid residues in the QM region of the input structures for the QM/MM calculations was the following. In a study of the kinetics of VCPO, van Schijndel et al. observed that at pH<4 H2O2 will not bind to VCPO and chloride inhibits VCPO. From this observation, they argued that a histidine within the active site must be monoprotonated at pH>4.[37,38] Zhang and Gascon argued from this observation that His404 is becoming diprotonated at low pH, and diprotonation of His404 leads to the enzyme being inhibited by halide at low pH.[8] As a result, in their QM/MM study of VCPO, Zhang and Gascon kept His404 in the monoprotonated state, HIE, as shown in Figure 3-4. Secondly, arginine is always considered to be in a protonated state for a variety of protein environments.[39] Asp292 was kept unprotonated because the negatively charged Asp292 can form a stronger salt bridge with positively charged Arg490 than when Asp292 is uncharged. The reasons for keeping Lys353 in a protonated state are given in the Supplementary Material.
3.0 Results and Discussion:

3.1 QM/MM optimization with QM Region I.

QM/MM geometry optimizations were carried out on input structures 1-23 with the 8 residue, QM Region I. With QM Region I, optimization of 3-11 leads to structures in which there is no transfer of hydrogen, and the protonation state of the surrounding amino acid residues in the input structure (as shown in Figure 3) remains the same in the optimized structures. However, geometry optimization of 1-2 results in structures in which a hydrogen (H22) transfers from N19 of Arg360 to equatorial O1 of vanadate. This transfer does not occur for any of the other structures, 3-23. Considering that the pKa of Arg360 is more than 11, one can argue that the monoprotonated 1 or 2 is not stable.

Optimization of triprotonated input structures, 12 and 13, leads to the optimized structures, 12-O and 13-O, which show that the axial hydrogen (H6) transfers from the axial oxygen, O4, to a water molecule (O15) “above” the vanadate as shown in Figure 5 for 12-O; then a proton (H36) from this water molecule transfers to an adjacent water molecule (O16).
Figure 5. QM/MM optimized 12-O (with QM Region I) involves transfer of H6 from O4 to O15 as well as transfer of H36 from O15 to O16. Arg360 has been hidden for clarity. (In Figures 5-8, 12, 14, 15, 16B, bond paths from AIM calculations indicating hydrogen bonding are shown as dotted lines, and bond critical points are shown as small blue cubes on the bond paths.)

In all of the optimized quadprotonated structures (15-O to 23-O), one finds that a proton has transferred to His404 (i.e., to N26). One can argue that these structures may not enter the catalytic cycle since, as discussed earlier, diprotonation of His404 results in inhibition of the enzyme by substrate.[8,37,38] In the other cases (15, 16, 18, 21, and 22), an axial proton (H6 or H7) transfers from axial oxygen, O4, to N26 (e.g., 16-O, Figure 6). Whenever the equatorial oxygen, O3, is protonated (with H10) as in the cases of 17, 19, 20, and 23, the optimized structures show that H10 has transferred to N26 (eg., 17-O, Figure 7).
Figure 6. Optimized structure 16-O involves transfer of H6 from O4 to N26. QM Region I was used. (Arg360 has been hidden for clarity.)
Figure 7. In 17-O, H10 transfers from O3 to N26. QM Region I was used. (Arg360 has been hidden for clarity.)

Even in the case of triprotonated 14, one finds that axial hydrogen, H6, has transferred from O4 to O15 of the water molecule, and then H37 has transferred from O15 to N26. Optimization of 15 results in an unusual transfer of both protons (H6 and H7) on the axial oxygen (O4): H6 transfers from O4 to N26; H7 transfers from O4 to O15, and H36 transfers from O15 to the adjacent water (O16), and then H38 transfers from O16 to O13 of Asp292 (Figure 8). As a result
of these proton transfers, the vanadate is in diprotonated state, and the energy of 15-O cannot be compared with other structures.

Figure 8. Optimized 15-O shows proton transfer of both H6 and H7, resulting in a diprotonated vanadate. (Arg 360 is hidden for clarity. QM Region I was used.)

3.2 QM/MM Optimizations with QM Region II

In order to confirm that the proton transfers are not an artifact of the size of the QM region being used, we carried out a set of QM/MM calculations on input structures 1-23 with the much larger 13 residue, QM Region II (Figure 4). QM Region II included three additional residues (i.e., Pro 395, Phe397, Ala399) modeled in the QM study of Gupta et al.[15] Two more proline residues (Pro396, Pro398) had to be included because QSite allows parameterized cuts of proline only
along the C-Cα bond. The resulting optimized structures have many similarities with the optimized structures found using the 8 residue QM Region I. Table S2 in the Supplementary Material compares the results of these optimizations regarding the transfer of hydrogen(s). There are no transfers of hydrogen in the optimization of diprotonated 3-8 when either QM Region I or QM Region II is used.[40] In the cases of 17-O, 18-O, 19-O, 21-O, 23-O the proton transfer is identical with the two QM regions; the proton transfers in the case of 15-O are very similar with the two QM regions. In some cases, different transfers occur with the triprotonated and quadprotonated input structures when QM Region II is used. Thus, in the case of 10-O and 12-O H6 or H7 transfers to N26 only when QM Region II is used. In all of the quadprotonated cases, one finds with QM Region II that His404 becomes diprotonated except in the case of 16-O. As we have noted previously, diprotonation of His404 leads to the enzyme being inhibited by halide (which is normally the substrate of the enzyme). In the case of 16-O, H6 transfers to O15; therefore, O4 becomes monoprotonated. As a result, 16-O will not be able to enter the first step of the catalytic cycle because the first proposed step involves dissociation of water from vanadate as shown in Scheme 2. In conclusion, the quadprotonated states may not be able to enter the catalytic cycle.

Scheme 2. First two steps of the catalytic cycle of VCPO [4,24]
3.3 Bond lengths and bond angles of optimized structures

The calculated lengths of the V-O bonds and the V-N bonds in the optimized structures with QM Region I are reported in Table 1. (The calculated bond lengths for the optimized structures found with QM Region II are given in Table S1 of the Supplementary Material; these bond lengths do not significantly differ from the results given in Table 1 using QM Region I.) In particular, the trigonal bipyramidal configuration is not rigidly maintained, and the bond angle between axial oxygen (O4), vanadium and the nitrogen (N5) of His496 is bent away from 180° by as much 48° in one case. Furthermore, protonation has the effect of lengthening the V-O bond by about 0.2 Å per hydrogen added. Unprotonated oxygens have a V-O bond length in the range of 1.598-1.691 Å; monoprotonated oxygens have a V-O bond length in the range of 1.732-1.944 Å; diprotonated oxygens have a V-O bond length in the range of 1.971-2.337 Å.

It is not possible to compare the calculated bond lengths (Table 1) with the X-ray crystallographic data found in the PDB because these data have a large error of ±0.24 Å.[6] However, the Extended X-ray Absorption Fine Structure (EXAFS) regions obtained at pH 6.0 at 77 K from vanadium K-edge X-ray Absorption spectroscopy led to more precise bond lengths (±0.02 Å). Namely, two V-O bonds are 1.69 Å, one V-O bond is 1.54 Å, and one V-O bond is 1.93 Å.[13] Based on the calculated bond lengths, one can conclude that this short bond length of 1.54 Å must correspond to an unprotonated O4. Furthermore, the longer bond length of 1.93 Å corresponds to either mono- or diprotonated O4. The bond lengths of 1.69 Å in this spectroscopic data correspond to an unprotonated or monoprotonated oxygen.
Table 1. Calculated bond lengths (in Å) and bond angles (O4-V-N5) for optimized structures (QM Region I) where 1-23 are the input structure as well as bond lengths and bond angle for the X-ray crystal structure data (1IDQ) of VCPO.

| Structure | V-O1  | V-O2  | V-O3  | V-O4  | V-N5  | Angle |
|-----------|-------|-------|-------|-------|-------|-------|
| 1-O       | 1.812 | 1.680 | 1.669 | 1.757 | 2.383 | 168.6°|
| 2-O       | 1.802 | 1.675 | 1.875 | 1.595 | 2.633 | 173.4°|
| 3-O       | 1.939 | 1.660 | 1.647 | 1.731 | 2.299 | 164.2°|
| 4-O       | 1.691 | 1.680 | 1.669 | 2.041 | 2.166 | 174.3°|
| 5-O       | 1.661 | 1.664 | 1.876 | 1.736 | 2.435 | 172.1°|
| 6-O       | 1.648 | 1.953 | 1.640 | 1.789 | 2.264 | 162.9°|
| 7-O       | 1.851 | 1.656 | 1.822 | 1.603 | 2.893 | 173.9°|
| 8-O       | 1.851 | 1.906 | 1.649 | 1.598 | 2.535 | 169.5°|
| 9-O       | 1.898 | 1.625 | 1.622 | 1.972 | 2.150 | 165.7°|
| 10-O      | 1.634 | 1.894 | 1.618 | 1.971 | 2.126 | 164.7°|
| 11-O      | 1.637 | 1.633 | 1.823 | 1.982 | 2.203 | 175.9°|
| 12-O      | 1.875 | 1.640 | 1.820 | 1.623 | 2.594 | 176.0°|
| 13-O      | 1.649 | 1.884 | 1.820 | 1.623 | 2.497 | 170.0°|
| 14-O      | 1.822 | 1.884 | 1.651 | 1.616 | 2.582 | 167.9°|
| 15-O      | 1.857 | 1.850 | 1.638 | 1.628 | 2.602 | 177.5°|
| 16-O      | 1.626 | 2.212 | 1.620 | 1.811 | 2.110 | 149.8°|
| 17-O      | 1.619 | 1.857 | 1.628 | 2.118 | 2.106 | 172.3°|
| 18-O      | 2.144 | 1.616 | 1.626 | 1.813 | 2.211 | 159.8°|
| 19-O      | 1.626 | 1.627 | 1.840 | 2.082 | 2.146 | 166.9°|
| 20-O      | 1.852 | 1.615 | 1.634 | 2.154 | 2.095 | 170.7°|
| 21-O      | 1.803 | 2.337 | 1.625 | 1.608 | 2.116 | 133.0°|
| 22-O      | 1.629 | 2.199 | 1.835 | 1.606 | 2.110 | 140.9°|
| 23-O      | 1.803 | 2.338 | 1.626 | 1.608 | 2.195 | 132.2°|
| 1IDQ      | 1.600 | 1.610 | 1.640 | 1.880 | 2.070 | 174.0°|

3.4 NBO study of Vanadate

To understand the electronic structure of the vanadate protonation states, we undertook a natural bond orbital (NBO) study of an isolated triprotonated 24 and quadprotonated 25 (Figure 9). First, the NBOs of vanadate for 24 and 25 are as follows:

24: V-O1: triple bond; V-O2: double bond; V-N5: single bond

25: V-O1: double bond; V-O2: triple bond; V-O3: double bond; V-N5: single
Although there is no NBO between V and O3 for 24, there is electron density between V and O3 from delocalization as found in the second order perturbative mixing. Namely there is mixing between the lone pair of O3 with the antibonding orbital between V-O1 and with an extremely large E2 stabilization energy of 171 kcal/mol (Table 2). Furthermore, the NBO results for 25 reveal a large amount of perturbative mixing (i.e., delocalization) between the equatorial V-O bonding orbitals with the adjacent equatorial V-O antibonding orbitals which is not present in the triprotonated 24.

![Figure 9. The optimized structures of isolated tri- and quadprotonated vanadate, 24 and 25.](image)

Table 2 lists for 24 the two V-O bond orbitals which mix with the adjacent antibonding V-O bond orbitals. In the case of quadprotonated 25, Table 3 lists sixteen equatorial V-O bond
orbitals which are mixing with adjacent V-O antibonding orbitals. This mixing in 25 is exampified in Figure 10 in which the overlap is illustrated for the case of V-O1 bond orbital as the donor and V-O2 antibonding orbital as the acceptor. It is also interesting that although there is no NBO between axial O4 and V, there is significant delocalization shown in the perturbative mixing. Figure 11 shows this mixing between the donor orbital (i.e., the lone pair on O4) and the acceptor orbital (i.e., the antibonding orbital V-N5) which has significant density in the area of V-O4 bond with stabilization energy (E2) of 60.70 kcal/mol.

Table 2. NBO result for 24: V-O bond orbitals involved in perturbative mixing between donor and acceptor bond orbital with stabilization energy, E2. There is also significant mixing of the lone pair on O3 with an antibonding orbital between V1-O1.

| Donor     | Acceptor          | E2 (kcal/mol) |
|-----------|-------------------|---------------|
| BD (2) V1-O1 | BD*(1) V1-O2 | 55.86         |
| BD (2) V1-O1 | BD*(1) V1-O1 | 9.74          |
| LP (3) O3   | BD*(2) V1-O1 | 171.46        |
| LP (1) O3   | BD*(2) V1-O1 | 34.03         |
| LP (2) O2   | BD*(1) V1-O1 | 26.82         |
| LP (2) O4   | BD*(1) V1-N5 | 47.75         |
Table 3. NBO results for 25: V-O bond orbitals involved in perturbative mixing between donor and acceptor bond orbital with stabilization energy E2

| Donor          | Acceptor          | E2 (kcal/mol) |
|----------------|-------------------|---------------|
| BD (1) V1- O1  | BD*(2) V1- O2     | 51.96         |
| BD (1) V1- O1  | BD*(2) V1- O3     | 14.61         |
| BD (1) V1- O1  | BD*(1) V1- O3     | 12.27         |
| BD (2) V1- O1  | BD*(2) V1- O2     | 11.07         |
| BD (2) V1- O1  | BD*(3) V1- O2     | 17.9          |
| BD (2) V1- O1  | BD*(2) V1- O3     | 18.65         |
| BD (2) V1- O2  | BD*(1) V1- O1     | 18.32         |
| BD (2) V1- O2  | BD*(1) V1- O3     | 22.76         |
| BD (3) V1- O2  | BD*(2) V1- O1     | 38.6          |
| BD (3) V1- O2  | BD*(3) V1- O2     | 8.62          |
| BD (3) V1- O2  | BD*(2) V1- O3     | 42.87         |
| BD (1) V1- O3  | BD*(1) V1- O1     | 14.12         |
| BD (1) V1- O3  | BD*(2) V1- O2     | 59.03         |
| BD (2) V1- O3  | BD*(2) V1- O1     | 21.78         |
| BD (2) V1- O3  | BD*(3) V1- O2     | 22.74         |
| BD (2) V1- O3  | BD*(2) V1- O3     | 10.01         |
| LP (1) O3      | BD*(2) V1- O2     | 33.90         |
| LP (1) O1      | BD*(2) V1- O2     | 31.20         |
| LP (2) O4      | BD*(1) V1- N5     | 60.70         |

Figure 10. (a) NBO plot of V-O1 bonding orbital of 25; (b) NBO plot of V-O2 antibonding orbital of 25; (c) superimposed image of (a) and (b) showing orbital overlap.
3.5 Atoms-in-Molecules Study

The Atoms-in-Molecules (AIM) approach was applied to clearly determine the complex hydrogen bonding pattern around the vanadate. The electron density, $\rho$, at the bond critical points (BCPs) for each hydrogen bond in the various optimized structures, 1-O to 23-O, was calculated. The results with QM Region I and QM Region II are reported in Tables S3-S10 of the Supplementary Material. The sign of the gradient of $\rho$ ($\nabla^2 \rho$) at all BCPs reported herein is negative, thus indicating covalent or hydrogen bonding. The above Figures 5-8, 12, 14, 15, 16B have included the BCPs and bond paths, and hence the hydrogen bonding can be visualized in these Figures.

The hydrogen bonding around the axial oxygen (O4) is different depending on whether QM Region I or II is used. With QM Region I, in the cases of 1-O to 13-O no hydrogen bonding is found between His404 and H6 or H7. (H6 and H7 protonate axial O4.) However, in the cases of 14-O, 15-O, 16-O, 18-O, 21-O, and 22-O a proton (H6 or H7) transfers from axial O4 to N26. In the cases of 1-O to 7-O and 9-O to 11-O, these axial hydrogens (H6 and/or H7) are connected by strong hydrogen bonds to the crystallographic water molecules. For example, in Figures 12 and 14, the optimized structures of 4-O and 5-O can be seen with the calculated bond paths.
showing the hydrogen bonding, and the strong hydrogen bond between H6 or H7 and water molecules above vanadate can be noted (e.g., 4: \( \rho(O16-H7)=0.03891, \rho(O15-H6)=0.06006; 5: \rho(O15-H6)=0.06714 \)). There is a hydrogen bond between His404 and one of these water molecules (i.e., N26-H37) except in the cases of 7 and 12-13; the value of \( \rho \) for this hydrogen bond (N26-H37) varies from 0.012 to 0.061.

When QM Region II is used, one finds that one of the hydrogens (i.e., H6 or H7) protonating axial O4 now are hydrogen bonded to His404 in most of the diprotonated cases. With the triprotonated and quadprotonated input structures, one of these hydrogens transfers to His404 (N26) in many cases. The key difference between QM Region I and II is that QM Region II has an additional crystallographic water molecule “above” vanadate. Thus, above the vanadate there is a row of three water molecules which form a series of hydrogen bonds between Asp292 and vanadate. Although this row is also formed with only two water molecules in the case of QM Region I, the distances from O4 to O15 and O16 are increased in the case of QM Region II because of this row of water molecules; hence H6 or H7 becomes hydrogen bonded to N26 of His404 (with an average \( \rho=0.046 \)). In some cases, such as 15-O (Figure 8) or 16-O (Figures 6), this hydrogen (H6 or H7) transfers to O15 and in turn the proton transfers from O15 to O16 to O53 to Asp292. This series of proton transfers should have the effect of deactivating the vanadate since the first step of the mechanism is thought to involve dissociation of diprotonated O4 from vanadate and subsequent bonding of vanadate with H\(_2\)O\(_2\) (Scheme 2).

In the cases where the rear equatorial O3 is protonated with H10 (i.e., 5, 7, 11, 12, 13, 17, 19, 20, 22, 23), one finds either that there is a strong hydrogen bond between H10 and N26 of His404 or that H10 has transferred to N26 (as in the quadprotonated states). The average value of
ρ for this hydrogen bond (H10-N26) is 0.044. As mentioned above, diprotonation of His404 leads to the enzyme being inhibited by substrate.[8,37,38]

One finds in the literature [7] the statement that His496 is covalently bonded to vanadium; however, we find that the electron density, ρ, at the bond critical point between V and N5 is actually much less than what one would expect for a covalent bond; for a covalent bond, one would expect ρ to exceed 0.2 electrons/bohr³. The ρ value is actually in the range of 0.0221-0.0801 electrons/bohr³, and this range is more characteristic of hydrogen bonding. The weakness of this bond agrees with the result that the vanadate cofactor can be removed by dialysis against phosphate and then reincorporated.[41]

Some other structurally significant hydrogen bonds become evident from the AIM study. Namely, the carbonyl of Pro401 is strongly hydrogen bonded (ρ=0.04178) to one of the amino hydrogens of Lys353 (i.e., O18-H32). We expect that this hydrogen bond will stabilize the conformation of Lys353 so that Lys353 is strongly hydrogen bonded to the equatorial oxygen (O2) of vanadate; a QM study of the mechanism has shown that this hydrogen bond between Lys353 and vanadate plays a significant role.[4,23] Using QM Region II, we find that the other amino hydrogen of Lys353 is strong hydrogen bonded to the carbonyl of Pro398 (i.e., ρ(O58-H33)=0.033 on average). There are also two significant dihydrogen bonds involving Arg490, namely between H43 and H44 and between H35 and H45; these dihydrogen bonds should restrict the conformation of Arg490. Furthermore, with QM Region II, Pro396 has two hydrogen bonds to Arg360 (ρ(O60-H50)=0.013 and ρ(O60-H66)=0.026 on average); thus, the conformation of Arg360 is also constrained.
3.6 Relative Energies of the Optimized Structures with QM Region I

The relative energies of the QM/MM optimized structures (with QM Region I) in each of the series of mono-, di-, tri- and quadprotonated states of VCPO are shown in Table 4. In the diprotonated series (3-8), optimized structure 5-O (Figure 14) with protonation (H6) of axial O4 and protonation (H10) of equatorial O3 is the lowest in energy. An earlier QM/MM study [7] obtained different results for these relative energies, but it should be noted that this earlier study did not include two crystallographic water molecules in the QM region. Another earlier study used a much smaller QM region and obtained different relative energies.[8] The present results show that these water molecules are involved in strong hydrogen bonds to hydrogens protonating axial O4. In 5-O, H10 is strongly hydrogen bonded to His404 (i.e., $\rho(H10-N26)= 0.03733$) and H6 is hydrogen bonded to a water molecule with $\rho=0.06714$ electrons/bohr$^3$. In comparing diprotonated 3-O to 8-O, one can first notice that the two structures, 6-O and 8-O, in which equatorial O2 is protonated with H11 are probably higher in energy because of steric interactions between Lys353 and H11. Steric interactions are also likely to be responsible for the higher energies in the cases of 3-O, 7-O, 8-O when O1 is protonated with H8. In the space filling model of 3-O (Figure 13) one can note how H8 is forced by steric interaction with Arg360 and Arg490 into a position in which it has repulsive interactions with O4. Similar steric interactions occur in 7-O and 8-O which have even higher relative energies. Structure 4-O (Figure 12) with axial O4 diprotonated is higher energy than 5-O because the bond between V-O4 is significantly weakened and has $\rho=0.07481$. It is no longer a covalent bond as in the case of 5-O.
Table 4. Relative energies in kcal/mol of optimized structures, 1-O to 23-O, when QM Region I is used. In the diprotonated series, the energy is relative to 5-O. For the series of 9-O to 11-O, the energy is relative to 11-O. For the series of 12-O to 14-O, the energy is relative to 13-O. For the series of 16-O to 23-O, the energy is relative to 19-O. (“N/C” means that 15 is “not comparable” with other structures.)

| Monoprotonated states: |   |   |
|-----------------------|---|---|
| 1-O                   | 2-O | 1.7 |

| Diprotonated states:  | 3-O | 4-O | 5-O | 6-O | 7-O | 8-O |
|-----------------------|-----|-----|-----|-----|-----|-----|
|                       | 17.9| 10.5| 0   | 7.8 | 18.8| 33.1|

| Triprotonated states: | 9-O | 10-O | 11-O |
|-----------------------|-----|------|------|
|                       | 24.4| 22.5 | 0    |

| Quadprotonated states: | 15-O | 16-O | 17-O | 18-O | 19-O | 20-O | 21-O | 22-O | 23-O |
|------------------------|------|------|------|------|------|------|------|------|------|
|                        | N/C  | 20.0 | 6.4  | 23.9 | 0    | 1.5  | 41.2 | 38.3 | 35.1 |
Figure 12. Optimized 4-O found using QM Region I.
Figure 13. This space filling model of optimized 3-O shows that H8 is forced by steric interaction with Arg360 and Arg490 into a position in which it has repulsive interactions with O4. QM Region I was used.
In comparing 9-O to 14-O which result from the optimization of triprotonated input structures (9-14), one should first note that the protonation state of the vanadate has become diprotonated in the cases of optimized structures, 12-O, 13-O, and 14-O, because of proton transfers having occurred as described above. Therefore, one can only compare the relative energies of 9-O, 10-O, and 11-O. Separately one can compare the relative energies of 12-O, 13-O and 14-O. In comparing 9-O and 10-O, one finds there are significant steric interactions. The structure of 9-O is similar to 3-O in that Arg360 and Arg490 are bulky and force the hydrogen
(H8) protonating O1 into a position where it forms an angle of 106 degrees with V-O1. H8 is then seen to sterically interact with O4 in a space filling model. In the case of 11-O, the additional hydrogen (H10) is in the back (protonating O3) and forms a strong hydrogen bond with N26 ($\rho=0.04815$), yet it does not sterically clash with other atoms. Thus, 11-O (as shown in Figure 15) is lower in energy than 9-O or 10-O. Regarding 12-O to 14-O, one should recall that the axial hydrogen (H6) transfers to a water molecule “above” vanadate. Structures 12-O and 13-O are stabilized by strong hydrogen bonding of H10 to N26 (with $\rho=0.05330$ and 0.05511, respectively) in comparison to 14-O and are lower in energy than 14-O. Between 12-O and 13-O, it appears that the steric interactions of H8 with Arg360, Arg490 and O4 are less than the steric interactions of H11 with Lys353; therefore, 13-O is lower energy than 12-O. In 14-O, one finds steric interactions for both of the equatorial hydrogens (H8, H11); therefore 14-O is much higher in energy than 12-O or 13-O.
Figure 15. When QM Region I was used in the QM/MM optimization, the resulting optimized 11-O has a lower energy than 9-O or 10-O.

In the quadprotonated series, the energy of optimized 15-O cannot be compared with the other structures for reasons described above. In comparing optimized 16-O to 23-O, one finds that states where O1 or O2 is doubly protonated (16-O, 21-O, 22-O, 23-O) are high in energy because of steric interaction with Lys353. In the case of 16-O, the hydrogens of O2 (H11 and H12) are pushed away from Lys353. Structure 18-O also has a high relative energy +23.9 kcal/mol although O2 is unprotonated and O1 is diprotonated. In the case of 18-O, there are also steric clashes between H8 and H23 and a hydrogen bond forms between H8 and N40 (ρ(H8-N40)=0.03398) of Arg490. This steric clash becomes evident with space filling models (Figure
16A). With any model, one can notice how H23 has been pushed out of the guanidine plane (Figure 16B).

![Image](image_url)

**Figure 16.** A: Space filling model of **18-O** (with QM Region I) shows steric interactions between H8, H9 and Arg360, 490; B: In this representation of optimized **18-O**, H23 is pushed out of the guanidine plane. (Arg360 is hidden for clarity.)

In the case of **19-O** which has the lowest relative energy, one finds that the equatorial hydrogen in the back (H10) transfers from O3 to N26, and this transferal alleviates any steric clashes. Similarly, in the case of **20-O**, H10 transfers from O3 to N26, but the front hydrogen (H8) of **20-O** is sterically clashing with other atoms, and this clash increases the energy **20-O** to 1.5 kcal/mol above **19-O**.

### 3.7 Relative Energies with QM Region II.

When the much larger QM Region II was used in the geometry optimization of **1-23**, we found that the lowest and highest energy structures remain the same in the di- and quadprotonated
series. Table 5 compares the relative energies found with QM Region I and II with input structures, 1-8 and 15-23. One significant difference is that with the larger QM Region II we find that 4-O (with diprotonation of axial O4) is only slightly higher in energy than 5-O (with protonation of the O3). This difference is probably the result of an additional water molecule “above” the vanadate which results in H7 hydrogen bonding to N26 of His404 instead of H7 hydrogen bonding to O16 of a water molecule.

With QM Region II, a proton transfer occurs in each of the following optimized triprotonated structures: 10-O, 12-O, 13-O and 14-O. The relative energies are as follows: 10-O (0 kcal/mol), 12-O (6.8 kcal/mol), 13-O (11.2 kcal/mol), 14-O (22.4 kcal/mol). Gupta et al. [15] could not distinguish between 10-O and 13-O, but we find that 10-O is lower in energy than 13-O with the larger QM Region II. With QM Region II, 9-O is now 6.4 kcal/mol lower in energy than 11-O.

3.8 What is the resting state?

In the above discussion, it has been argued that the quadprotonated forms of VCPO are unlikely to enter the catalytic cycle since geometry optimizations of quadprotonated 15-23 show a proton transferring in each case to His404, thus forming diprotonated His404. Since there is evidence that diprotonation of His404 leads to the enzyme becoming inhibited by substrate, we rule out the quadprotonated states as the resting state. [8,37,38]

With the larger QM Region II, the relative energy of 4-O and 5-O is 0.66 kcal/mol, favoring of 5-O. One would expect significant concentrations of both 4-O and 5-O to be present in the
resting state. Thus both 4-O and 5-O are likely to be the resting state of VCPO.[42] Since the first step of the mechanism involves dissociation of water from the vanadate (Scheme 2), only 4-O can in principle enter the catalytic cycle.

In the triprotonated series, we can rule out 10-O, 12-O, 14-O as the resting state since the optimized structures show proton transfers to His404. Regarding the remaining structures, one can compare the relative energies of 9-O and 11-O; however, 13-O cannot be compared with 9-O and 11-O because the vanadate in 13-O is only diprotonated. In comparing 9-O and 11-O, one

|   | QM Region I | QM Region II |
|---|-------------|-------------|
| 1-O | 0           | 2.1         |
| 2-O | 1.7         | 0           |
| 3-O | 17.9        | 25.8        |
| 4-O | 10.5        | 0.66        |
| 5-O | 0           | 0           |
| 6-O | 7.8         | 6.9         |
| 7-O | 18.8        | 15.0        |
| 8-O | 33.1        | 28.4        |
| 15-O | N/C        | NC          |
| 16-O | 20.0        | 27.2        |
| 17-O | 6.4         | 3.1         |
| 18-O | 23.9        | 28.5        |
| 19-O | 0           | 0           |
| 20-O | 1.5         | 15.8        |
| 21-O | 41.2        | 46.0        |
| 22-O | 38.3        | 11.0        |
| 23-O | 35.1        | 18.9        |

*Diprotonated structures 3-O to 8-O are relative to 5-O. Structures 15-O to 23-O are relative to 19-O. “N/C” means the energy of this structure cannot be compared to other structures.*
notes two different results depending on whether QM Region I or QM Region II is used. IN the
case of QM Region I, \textbf{11-O} is lower in energy, but in the case of QM Region II, \textbf{9-O} is lower in
energy. Since QM Region II is a larger model and more likely to model the actual enzyme, it is
proposed that \textbf{9-O} may also be resting state. Based on the present results, it is not possible to
determine whether \textbf{9-O} or an equilibrium of \textbf{4-O} and \textbf{5-O} is the resting state.

\textbf{4.0 Conclusions:}

We have studied the protonation states of VCPO using two QM/MM models. By a process of
elimination, we have argued that \textbf{4-O/5-O} or \textbf{9-O} is likely to be the resting state of VCPO. The
quadprotonated states and many of the triprotonated states can be eliminated since the optimized
structures show transfer of a proton from vanadate to His404. (As discussed above, diprotonated
His404 results in the enzyme becoming inhibited by what is supposed to be the substrate, that is,
halide.)

Across the various series, we find that the state in which the rear O3 is protonated is often the
lowest energy structure because of strong hydrogen bonding to His404 and because of minimal
steric interactions which are present in forms where O1 or O2 is protonated. When O1 or O2 is
protonated, steric interactions with Lys353 and Arg360/490 destabilize these protonation states
based on an examination of space filling models.

We have also reported the results of an AIM study to ascertain the complex hydrogen
bonding in VCPO. Many hydrogen bonds which are not apparent by consideration of distances
became apparent. It is particularly notable that the conformations of Lys353, Arg360 and Arg490
are restricted because of various hydrogen bonds, and this restriction of conformations may enhance the rate of catalysis.

**Supplementary Material:**

**Justification for the protonation state of Lys353 in the QM region:**
Lys353 was included in the QM region in a fully protonated state. The justification for including Lys353 in the fully protonated state is as follows. First, we found that geometry optimization of 10-NH2 (Figure S1) with Lys353 in an unprotonated state led to 4 in which H11 from equatorial O2 has transferred to N12 of Lys353. Similarly, we deleted a hydrogen from Lys353 of optimized 16-O to give 16-NH2 (Figure S2). When 16-NH2 was geometry optimized, the result was 10, in which a hydrogen (H11) has again transferred from O2 to N46. We also studied cases where O2 is unprotonated. Thus, we prepared input structures by removing H33, from Lys353 in the cases of diprotonated 4, triprotonated 9 and quadprotonated 18. Geometry optimization of these structures resulted in structures which were 300-400 kcal/mol higher in energy than the corresponding structures with Lys353 in the protonated state. It is clear that VCPO with protonated Lys353 is much lower in energy than in the case of VCPO with unprotonated Lys353. Because of these results, we used protonated Lys353 for the calculations described in this study, and indeed other computational studies have kept Lys353 in the protonated state.[7]
Figure S1. Geometry optimization of **10-NH2** results in **4** by a transfer of H11 from O2 to N46. Distance between N46-H11 goes from 2.025 Å in **10-NH2** to 1.049 Å in **4**. (Arg360, Asp292, Ser402, Gly403, His404 and other atoms of the QM region have been hidden for clarity.)

Figure S2. Geometry optimization of **16-NH2** results in **10** by a transfer of H11 from O2 to N46. The distance between N46-H11 goes from 3.034 Å in **16-NH2** to 1.032 Å in **10**. (Arg360, Asp292, Ser402, Gly403, His404 and other atoms of QM region have been hidden for clarity.)
Table S1. With QM Region II, the following bond lengths (in Å) and bond angle (O4-V-N5) were calculated for optimized structures where 1-23 are the input structures.

| Structure | V-O1  | V-O2  | V-O3  | V-O4  | V-N5  | Angle  |
|-----------|-------|-------|-------|-------|-------|--------|
| 1-O       | 1.720 | 1.705 | 1.700 | 1.799 | 2.486 | 174.5  |
| 2-O       | 1.725 | 1.710 | 1.914 | 1.616 | 2.645 | 176.6  |
| 3-O       | 1.932 | 1.656 | 1.651 | 1.762 | 2.290 | 165.6  |
| 4-O       | 1.681 | 1.683 | 1.682 | 2.062 | 2.161 | 172.1  |
| 5-O       | 1.657 | 1.653 | 1.886 | 1.770 | 2.362 | 170.4  |
| 6-O       | 1.668 | 1.897 | 1.655 | 1.738 | 2.525 | 175.3  |
| 7-O       | 1.824 | 1.653 | 1.865 | 1.614 | 2.524 | 172.5  |
| 8-O       | 1.847 | 1.907 | 1.646 | 1.610 | 2.383 | 171.1  |
| 9-O       | 1.888 | 1.627 | 1.635 | 1.952 | 2.168 | 164.8  |
| 10-O      | 1.643 | 1.923 | 1.649 | 1.809 | 2.236 | 165.7  |
| 11-O      | 1.636 | 1.633 | 1.819 | 2.048 | 2.147 | 176.9  |
| 12-O      | 1.875 | 1.643 | 1.898 | 1.605 | 2.380 | 168.8  |
| 13-O      | 1.636 | 1.861 | 1.874 | 1.632 | 2.362 | 160.7  |
| 14-O      | 1.881 | 1.876 | 1.649 | 1.606 | 2.445 | 170.9  |
| 15-O      | 1.862 | 1.865 | 1.637 | 1.636 | 2.392 | 174.7  |
| 16-O      | 1.635 | 2.185 | 1.614 | 1.816 | 2.126 | 152.8  |
| 17-O      | 1.622 | 1.847 | 1.654 | 2.121 | 2.059 | 170.2  |
| 18-O      | 2.077 | 1.612 | 1.630 | 1.842 | 2.209 | 163.0  |
| 19-O      | 1.622 | 1.618 | 1.888 | 2.098 | 2.083 | 163.2  |
| 20-O      | 1.768 | 1.605 | 1.901 | 1.797 | 2.128 | 155.8  |
| 21-O      | 1.837 | 2.317 | 1.622 | 1.694 | 2.069 | 132.2  |
| 22-O      | 1.629 | 2.228 | 1.621 | 1.813 | 2.052 | 138.4  |
| 23-O      | 1.797 | 2.309 | 1.623 | 1.627 | 2.084 | 132.3  |
| Table S2. Comparison of proton transfers in the QM/MM optimized structures 9-O to 23-O when either QM Region I or QM Region II is used. |
|---------------------------------------------------------------|
| **QM Region I** | **QM Region II** |
| 9-O | No transfer |
| 10-O | No transfer |
| 11-O | No transfer |
| 12-O | H6 transfers to O15; H36 transfers to O16 |
| 13-O | H6 transfer to O15; H36 transfers to O16 |
| 14-O | H6 transfers to O15; H37 transfers to N26 |
| 15-O | H7 transfers to N26; H6 transfers to O15; H36 transfers from O15 to O16; H38 transfers from O16 to O13 |
| 16-O | H7 transfers to N26 |
| 17-O | H10 migrates to N26 |
| 18-O | H6 migrates to N26 |
| 19-O | H10 migrates to N26 |
| 20-O | H10 transfers to N26 |
| 21-O | H6 transfers to N26 |
| 22-O | H6 transfers to N26 |
| 23-O | H10 transfers to N26 |

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Table S3. ρ values in units of e/bohr³ for monoprotonated (1-2) and diprotonated (3-8) forms [43] calculated using QM Region I. Atom numbering is given in Figure 3.a

|     | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     |
|-----|-------|-------|-------|-------|-------|-------|-------|-------|
| O1-H23 | 0.03902 | 0.03179 | 0.02229 | 0.02857 | 0.02032 | 0.03054 | 0.01214 | 0.02472 |
| O1-H22 | NCᵇ   | NCᶜ   | 0.05413 | 0.06046 | 0.06953 | 0.04634 | 0.01327 | 0.02015 |
| O1-H21 | 0     | 0.00995 | 0.01411 | 0.00912 | 0.00996 | 0.00000 | 0.02510 | 0.01105 |
| O2-H29 | 0.03757 | 0.03673 | 0.04027 | 0.03058 | 0.02601 | 0.01288 | 0.04363 | 0.03699 |
| O2-H30 | 0.087  | 0.00739 | 0.01252 | 0.00810 | 0.00620 | 0.00430 | 0.00872 | 0.00774 |
| O2-H31 | 0.05623 | 0.05898 | 0.05337 | 0.05134 | 0.04738 | 0.04659 | 0.05171 | 0.05209 |
| O3-H27 | 0.01946 | 0.01898 | 0.01506 | 0.01101 | 0.01513 | 0.00860 | 0.01446 | 0.01082 |
| O3-H28 | 0.04449 | 0.02698 | 0.04071 | 0.03353 | 0.02677 | 0.02595 | 0.01770 | 0.03576 |
| O3-H24 | 0.01489 | 0.02247 | 0.01400 | 0.02396 | 0.02505 | 0.02561 | 0.02267 | 0.02026 |
| O4-H36 | 0     | 0.01626 | 0.00000 | 0.00000 | 0.00000 | 0.03288 | 0.00000 | 0.00000 |
| O4-H37 | 0     | 0     | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.01901 | 0.01214 |
| O16-H36 | 0.03335 | 0.00896 | 0.03689 | 0.01086 | 0.04243 | 0.00000 | 0.04155 | 0.03357 |
| O16-H7 | n/a   | n/a   | 0.00000 | 0.03891 | n/a   | n/a   | n/a   | n/a   |
| O16-H8 | n/a   | n/a   | 0.00000 | 0.03891 | n/a   | n/a   | n/a   | n/a   |
| O15-H6 | 0.05746 | n/a   | n/a   | 0.06006 | 0.06714 | n/a   | n/a   | n/a   |
| O18-H32 | 0.03655 | 0.03524 | 0.04099 | 0.03969 | 0.04017 | 0.04506 | 0.04506 | 0.04580 |
| H43-H44 | 0.01089 | 0.0109 | 0.01085 | 0.01086 | 0.01088 | 0.01089 | 0.04089 | 0.01089 |
| O13-H41 | 3761   | 0.03725 | 0.03808 | 0.03477 | 0.03612 | 0.04184 | 0.04176 | 0.04136 |
| O13-H38 | 0.02694 | 0.02589 | 0.04282 | 0.04020 | 0.03101 | 0.04210 | 0.04531 | 0.03630 |
| O14-H42 | 0.3887 | 0.04048 | 0.03427 | 0.03541 | 0.03856 | 0.03150 | 0.03511 | 0.03384 |
| V-N5 | 0.03823 | 0.0221 | 0.04727 | 0.06402 | 0.03369 | 0.05143 | 0.00000 | 0.02776 |
| V-O4 | NC   | NC   | NC   | 0.07481 | NC   | NC   | NC   | NC   |
| N26-H37 | 0.03078 | 0.01204 | 0.02836 | 0.03892 | 0.01837 | 0.01944 | 0.00000 | 0.01225 |
| N26-H6 | 0   | n/a   | n/a   | 0.00000 | 0.00000 | n/a   | n/a   | n/a   |
| N26-H10 | n/a   | 0.04017 | n/a   | n/a   | 0.03733 | n/a   | 0.05815 | n/a   |
| H45-H35 | 0.01255 | 0.01417 | 0.01545 | 0.01524 | 0.01434 | 0.01249 | 0.01578 | 0.01276 |
| O17-H34 | 0.0217 | 0.02023 | 0.02077 | 0.01934 | 0.02010 | 0.01849 | 0.01670 | 0.01988 |
| N5-H22 | 0   | 0     | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.01274 | 0.01042 |
| O15-H7 | n/a   | n/a   | 0.08131 | 0.00000 | n/a   | 0.00000 | n/a   | n/a   |
| H21-O2 | 0   | 0     | 0.00000 | 0.00000 | 0.00000 | 0.02063 | 0.00000 | 0.00000 |

ᵃn/a means “not applicable”: one or both of the atoms were not present. “NC” means ρ value was not calculated, and it is expected to be greater than 0.1. A value of ρ=0 indicates that there is no density between these atoms, and hence there is no bond between these atoms.

ᵇH22 transfers from N19 to O1; H22-O2 distance is 1.000 Å and ρ(N19-H22)=0.04895
ᶜH22 transfers from N19 to O1; H22-O2 distance is 1.002 Å and ρ(N19-H22)=0.04922
Table S4. ρ values in units of e/bohr³ for triprotonated forms, 9-14 calculated using QM Region I

|          | 9     | 10    | 11    | 12    | 13    | 14    |
|----------|-------|-------|-------|-------|-------|-------|
| O1-H23   | 0.02448 | 0.02933 | 0.02078 | 0.01637 | 0.02555 | 0.02787 |
| O1-H22   | 0.04854 | 0.04170 | 0.04506 | 0.02838 | 0.04084 | 0.01172 |
| O1-H21   | 0.01164 | 0.00000 | 0.01231 | 0.02322 | 0.01002 | 0.00896 |
| O2-H29   | 0.03445 | 0.02534 | 0.02243 | 0.04082 | 0.03136 | 0.03717 |
| O2-H30   | 0.00764 | 0.00620 | 0.00579 | 0.00832 | 0.00000 | 0.00755 |
| O2-H31   | 0.03684 | 0.04082 | 0.03752 | 0.04239 | 0.04289 | 0.04280 |
| O3-H27   | 0.01458 | 0.00835 | 0.01510 | 0.01347 | 0.01042 | 0.01145 |
| O3-H28   | 0.03047 | 0.02111 | 0.02122 | 0.02174 | 0.01999 | 0.02894 |
| O3-H24   | 0.01422 | 0.02730 | 0.02289 | 0.02490 | 0.03345 | 0.02408 |
| O4-H39   | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.01400 |
| O16-H36  | 0.00000 | 0.00000 | 0.00000 | NCb    | NCc    | 0.04329 |
| O16-H7   | 0.06463 | 0.05616 | 0.05569 | n/a    | n/a    | n/a    |
| O16-H8   | 0.00000 | n/a    | n/a    | 0.00000 | n/a    | 0.02571 |
| O15-H6   | 0.08555 | 0.09942 | 0.06561 | NCd    | NCe    | NCf    |
| O18-H32  | 0.04347 | 0.04421 | 0.04202 | 0.04272 | 0.04443 | 0.04374 |
| H43-H44  | 0.01083 | 0.01086 | 0.01084 | 0.01086 | 0.01087 | 0.01083 |
| O13-H41  | 0.04503 | 0.04680 | 0.04244 | 0.03544 | 0.03490 | 0.04536 |
| O13-H38  | 0.05085 | 0.04479 | 0.04475 | 0.00000 | 0.00000 | 0.04554 |
| O14-H42  | 0.03916 | 0.03700 | 0.04172 | 0.03774 | 0.03719 | 0.04003 |
| O14-H38  | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| V-N5     | 0.06940 | 0.07354 | 0.06064 | 0.02468 | 0.03043 | 0.02530 |
| V-O4     | 0.09263 | 0.09394 | 0.08844 | NC     | NC     | NC     |
| N26-H37  | 0.05086 | 0.06052 | 0.02285 | 0.00000 | 0.00000 | NC (d) |
| N26-H10  | n/a    | n/a    | 0.04815 | 0.05330 | 0.05511 | n/a    |
| H45-H35  | 0.01518 | 0.01476 | 0.01754 | 0.01641 | 0.01367 | 0.01368 |
| O17-H34  | 0.02000 | 0.01880 | 0.01948 | 0.01774 | 0.01849 | 0.04374 |
| N5-H22   | 0.00000 | 0.00000 | 0.00000 | 0.00095 | 0.00000 | 0.01157 |

a n/a means “not applicable”: one or both of the atoms were not present. NC means ρ value was “not calculated” and is expected to be greater than 0.1. A value of ρ=0 indicates that there is no density between these atoms, and hence there is no bond between these atoms.
b H6 transfers from O4 to O15; H36 transfers from O15 to O16; O16-H36 distance is 1.056 Å; O15-H36 distance is 1.471 Å
c H6 transfers from O4 to O15; H36 transfers from O15 to O16; distance between O16-H36 is 1.047 Å; distance between O15-H36 is 1.494 Å
d H6 transferred from O4 to O15; H36 transferred from O15 to O16; H6-O15 distance is 0.993 Å; O4-H6 distance is 1.704 Å
e H6 has transferred from O4 to O15; distance between O15-H6 is 0.992 Å; distance between O4-H6 is 1.693 Å
H6 has transferred from O4 to O15; H37 has transferred from O15 to N26; O15-H6 distance is 0.969; O4-H6 distance is 2.017; N26-H37 distance is 1.019; O15-H37 distance is 2.484
Table S5. ρ values in units of e/bohr\(^3\) for quadprotonated states 15-19\(^a\) calculated using QM Region I

|      | 15  | 16  | 17  | 18  | 19  |
|------|-----|-----|-----|-----|-----|
| O1-H23 | 0.02796 | 0.03164 | 0.02879 | 0\(^c\) | 0.01856 |
| O1-H22 | 0.02421 | 0.04012 | 0.02823 | 0.02088 | 0.03306 |
| O1-H21 | 0.01149 | 0.00000 | 0.00515 | 0.00853 | 0.01324 |
| O2-H29 | 0.04212 | 0.00536 | 0.03357 | 0.03439 | 0.02888 |
| O2-H30 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00602 |
| O2-H31 | 0.03425 | 0.03367 | 0.03524 | 0.02529 | 0.03334 |
| O3-H27 | 0.01309 | 0.00635 | 0.01056 | 0.01395 | 0.01321 |
| O3-H28 | 0.02422 | 0.01356 | 0.02199 | 0.04390 | 0.00000 |
| O3-H24 | 0.02232 | 0.02823 | 0.02547 | 0.01458 | 0.02670 |
| O4-H11 | 0.00000 | 0.04260 | 0.00000 | n/a | n/a |
| O16-H36 | NC\(^c\) | 0.03341 | 0.00000 | 0.00000 | 0.04649 |
| O16-H7 | NC\(^c\) | n/a | 0.05506 | 0.01767 | 0.06948 |
| O16-H8 | 0.00000 | n/a | 0.00000 | 0.03263 | n/a |
| O15-H6 | NC\(^c\) | 0.00000 | 0.04106 | 0.00000 | 0.03850 |
| O18-H32 | 0.04657 | 0.04985 | 0.04636 | 0.04497 | 0.04039 |
| H43-H44 | 0.01079 | 0.01089 | 0.01080 | 0.01082 | 0.01079 |
| O13-H41 | 0.03322 | 0.05178 | 0.05154 | 0.05551 | 0.04615 |
| O13-H38 | NC\(^c\) | 0.04217 | 0.04429 | 0.03367 | 0.04649 |
| O14-H42 | 0.03463 | 0.03722 | 0.04039 | 0.04996 | 0.04306 |
| V-N5 | 0.02476 | 0.76490 | 0.07834 | 0.06026 | 0.07073 |
| N26-H6 | NC\(^c\) | NC\(^b\) | 0.00000 | NC\(^f\) | 0.00000 |
| N26-H10 | n/a | n/a | NC\(^d\) | n/a | NC\(^e\) |
| H45-H35 | 0.01406 | 0.01422 | 0.01433 | 0.01550 | 0.01614 |
| O17-H34 | 0.01774 | 0.01670 | 0.02001 | 0.02351 | 0.01725 |
| N5-H22 | 0.01019 | 0.00000 | 0.00000 | 0.01214 | 0.00000 |
| N20-H12 | n/a | 0.03742 | 0.00000 | n/a | n/a |
| O3-H10 | n/a | n/a | 0.01344 | n/a | 0.01278 |
| O15-H7 | 0 | 0.00000 | 0.00000 | 0.02433 | 0.00000 |
| O15-H10 | n/a | n/a | 0.00000 | n/a | 0.00000 |
| O4-H10 | n/a | n/a | 0.01124 | n/a | 0.02718 |

\(^a\)n/a means “not applicable”: one or both of the atoms were not present. NC means ρ value was not calculated and is expected to be greater than 0.1. A value of ρ=0 indicates that there is no density between these atoms, and hence there is no bond between these atoms.

\(^b\)H6 has transferred from O4 to N26, and the distances are as follows: d(N26-H6)=1.034 Å; d(O4-H6)=3.552 Å.

\(^c\)H7 has transferred from O4 to O15; H36 has transferred from O15 to O16; H38 has transferred from O16 to O13. H6 has transferred from O4 to N26. The distances and additional ρ values are as follows: d(O4-
H7 = 1.990 Å; d(O15-H36) = 1.714 Å; d(H36-O16) = 0.995 Å; d(O16-H38) = 1.429 Å; d(H38-O13) = 0.9264 Å; d(O4-H6) = 2.178 Å; d(H6-N26) = 1.019 Å; ρ(O4-H7) = 0.02272; ρ(O4-H6) = 0.01617; ρ(O15-H36) = 0.04621; ρ(H38-O13) = 0.0924 Å

\[^d\]H10 has transferred from O3 to N26; d(O3-H10) = 2.261 Å; d(H10-N26) = 1.013 Å

\[^e\]H23 is normally hydrogen bonded to O1, but in this case O1 is doubly protonated and H23 is forced up and hydrogen bonds to O4 with ρ(H23-O4) = 0.02477 Å; there is an additional unusual hydrogen bonds in 18: ρ(H9-N40) = 0.03398.

\[^f\]H6 has transferred from O4 to N26; d(O4-H6) = 1.756 Å; d(H6-N26) = 1.029 Å; ρ(O4-H6) = 0.04115;

\[^g\]H10 has transferred from O3 to N26; d(O3-H10) = 2.292 Å; d(H10-N26) = 1.022 Å; in 19 O3 is doubly protonated (with H10 and H47), and there is hydrogen bond between H47 and O17 of Ser401 with ρ = 0.03726.
Table S6. ρ values in units of e/bohr$^3$ for quadprotonated states 20-23$^+$ calculated using QM Region I

|        | 20-O | 21-O | 22-O | 23-O |
|--------|------|------|------|------|
| O1-H23 | 0.02363 | 0.02503 | 0.02130 | 0.02378 |
| O1-H22 | 0.03027 | 0.01349 | 0.02905 | 0.01328 |
| O1-H21 | 0.01192 | 0.00636 | 0.00689 | 0.00721 |
| O2-H29 | 0.03836 | 0.00000 | 0.00000 | 0.00000 |
| O2-H30 | 0.00677 | 0.00000 | 0.00000 | 0.00000 |
| O2-H31 | 0.02781 | 0.03554 | 0.03259 | 0.03211 |
| O3-H27 | 0.01439 | 0.00607 | 0.00000 | 0.000593 |
| O3-H28 | 0.03109 | 0.01994 | 0.02444 | 0.01847 |
| O3-H24 | 0.01659 | 0.03657 | 0.03690 | 0.03691 |
| O16-H36 | 0.00000 | 0.04070 | 0.04228 | 0.03899 |
| O16-H7 | 0.06464 | 0.00000 | 0.00000 | n/a |
| O16-H8 | 0.01520 | 0.03619 | n/a | 0.03725 |
| O15-H6 | 0.04135 | 0.03150 | n/a | n/a |
| O18-H32 | 0.04448 | 0.04873 | 0.04748 | 0.04601 |
| H43-H44 | 0.01079 | 0.01084 | 0.01085 | 0.01084 |
| O13-H41 | 0.04668 | 0.04710 | 0.05176 | 0.04639 |
| O13-H38 | 0.04662 | 0.04422 | 0.03970 | 0.04343 |
| O14-H42 | 0.04045 | 0.03967 | 0.04504 | 0.04055 |
| V-N5 | 0.08010 | 0.07472 | 0.07664 | 0.07461 |
| V-O4 | 0.05792 | NC | NC | NC |
| N26-H6 | 0.00000 | NC$^c$ | NC$^d$ | n/a |
| N26-H10 | NC$^b$ | n/a | 0.01348 | NC$^e$ |
| H45-H35 | 0.01541 | 0.01518 | 0.01334 | 0.01502 |
| O17-H34 | 0.02096 | 0.01862 | 0.01999 | 0.01857 |
| N5-H22 | 0.00797 | 0.00560 | 0.00000 | 0.00000 |
| N20-H12 | n/a | 0.05009 | 0.05125 | 0.05007 |
| O2-V | NC | 0.03744 | 0.05190 | 0.03763 |
| O4-H29 | 0.00000 | 0.02906 | 0.01680 | 0.00000 |
| O3-H10 | 0.01081 | n/a | NC | 0.00000 |
| O15-H7 | 0.00000 | n/a | 0.04624 | n/a |
| O15-H10 | 0.00000 | n/a | 0.00000 | 0.03278 |
| O4-H27 | 0.00000 | 0.00000 | 0.00520 | 0.03091 |
| O4-H10 | 0.02554 | n/a | 0.00000 | 0.00000 |

$n/a$ means “not applicable”: one or both of the atoms were not present. NC means ρ value was not calculated and is expected to be greater than 0.1. A value of ρ=0 indicates that there is no density between these atoms, and hence there is no bond between these atoms.
bH10 has transferred from O3 to N26, and the distances are as follows
\( d(O3-H10)=2.368 \, \text{Å} \); \( d(H10-N26)=1.021 \, \text{Å} \);
\( \rho(O15-H5991)=0.01159 \)

cH6 has transferred from O4 to N26; 
\( d(H6-O4)=3.046 \, \text{Å} \); 
\( d(H6-N26)=1.029 \, \text{Å} \)

dH6 has transferred from O4 to N26; 
\( d(H6-O4)=3.068 \, \text{Å} \); 
\( d(H6-N26)=1.052 \, \text{Å} \),

eH10 has transferred from O3 to N26: 
\( d(H10-N26)=1.030 \, \text{Å} \); 
\( d(O3-H10)=2.807 \, \text{Å} \)

Table S7. QM Region II was used to calculate \( \rho \) values in units of e/bohr\(^3\) for mono- and diprotonated states 1-O to 8-O

|       | 1-O | 2-O | 3-O | 4-O | 5-O | 6-O | 7-O | 8-O |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| O1-H23| 0.03210 | 0.02697 | 0.02675 | 0.03060 | 0.01939 | 0.02992 | 0.01233 | 0.02922 |
| O1-H22| 0.06526 | 0.07297 | 0.04572 | 0.04819 | 0.04328 | 0.04847 | 0.02575 | 0.03421 |
| O1-H21| 0.01053 | 0.01529 | 0.01026 | 0.00815 | 0.01623 | 0.00000 | 0.02690 | 0.00885 |
| O2-H29| 0.03657 | 0.03409 | 0.04197 | 0.03680 | 0.02886 | 0.03408 | 0.04124 | 0.03957 |
| O2-H30| 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.01474 | 0.00000 |
| O2-H31| 0.06157 | 0.07264 | 0.04349 | 0.04666 | 0.05508 | 0.03109 | 0.05981 | 0.05315 |
| O3-H27| 0.00988 | 0.01484 | 0.01282 | 0.00961 | 0.01334 | 0.00000 | 0.01410 | 0.00967 |
| O3-H28| 0.04423 | 0.02316 | 0.04766 | 0.04254 | 0.02505 | 0.03374 | 0.02509 | 0.03659 |
| O3-H24| 0.02653 | 0.02759 | 0.01509 | 0.02524 | 0.02581 | 0.02964 | 0.02192 | 0.02141 |
| O16-H36| 0.01683 | 0.01313 | 0.01686 | 0.04283 | 0.00000 | 0.03544 | 0.00000 | 0.00000 |
| O16-H8| 0.00000 | n/a | n/a | 0.04845 | 0.00000 | n/a | n/a | n/a |
| O15-H6| 0 | n/a | n/a | 0.04845 | 0 | n/a | n/a | n/a |
| H43-H44| 0.01088 | 0.01087 | 0.01085 | 0.01086 | 0.01086 | 0.01086 | 0.01086 | 0.01087 |
| O13-H41| 0.03313 | 0.03312 | 0.04018 | 0.03757 | 0.03869 | 0.03889 | 0.03932 | 0.04169 |
| O13-H38| 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| O13-H55| 0.04544 | 0.04639 | 0.04973 | 0.04791 | 0.04151 | 0.04501 | 0.04567 | 0.04965 |
| O14-H42| 0.03878 | 4058.00000 | 0.03898 | 0.03943 | 0.04159 | 0.03814 | 0.04233 | 0.03757 |
| O14-H38| 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| V-N5| 0.02906 | 0.02098 | 0.04870 | 0.06508 | 0.04120 | 0.02750 | 0.02872 | 0.03934 |
| V-O4| NC | NC | NC | 0.07357 | NC | NC | NC | NC |
| N26-H37| 0.00551 | 0.01205 | 0.00000 | 0.00000 | 0.01201 | 0.00000 | 0.01436 | 0.01678 |
| N26-H6| 0.03228 | n/a | n/a | 0.00000 | 0.00000 | n/a | n/a | n/a |
| N26-H7| n/a | n/a | n/a | 0.04203 | 0.04982 | n/a | 0.06149 | n/a |
| N26-H10| n/a | 0.03610 | n/a | n/a | 0.03298 | n/a | 0.03499 | n/a |
| H45-H35| 0.01145 | 0.01493 | 0.01327 | 0.01263 | 0.01568 | 0.00975 | 0.01504 | 0.01090 |
| O17-H34| 0.01649 | 0.01613 | 0.01945 | 0.01837 | 0.01744 | 0.01673 | 0.01695 | 0.01758 |
| O60-H66| 0.02560 | 0.02559 | 0.02560 | 0.02560 | 0.02559 | 0.02560 | 0.02560 | 0.02563 |
| O60-H50| 0.01236 | 0.01236 | 0.01244 | 0.01242 | 0.01243 | 0.01242 | 0.01244 | 0.01244 |
| O61-H67| 0.03145 | 0.03147 | 0.03125 | 0.03125 | 0.03124 | 0.03126 | 0.03127 | 0.03155 |
| O58-H33| 0.02710 | 0.02811 | 0.02771 | 0.02893 | 0.03128 | 0.02675 | 0.02960 | 0.03684 |
| O59-H68| 0.02174 | 0.01596 | 0.01627 | 0.02005 | 0.010483 | 0.02533 | 0.01474 | 0.01958 |
| Bond         | C69-H30 | N20-H70 | H8-H71 | H62-O17 | O63-H72 | O3-H74 | O4-H57 | O2-H57 | O4-H36 | O15-H39 | O16-H6 | H75-O16 | O15-H11 | O4-O16 | O4-O15 | O16-H73 | O4-H39 | O16-H57 |
|--------------|---------|---------|--------|---------|---------|--------|--------|--------|--------|---------|--------|---------|---------|--------|--------|---------|--------|---------|
| Value        | 0.00000 | 0.01246 | n/a    | 0.02881 | 0.01407 | 0.00000| 0       | 0.0141 | 0.01401 | 0       | 0.00000| 0       | n/a     | 0      | 0      | 0       | 0      | 0       |
| Angle        | 0.01028 | 0.01281 | 0.01228| 0.02625 | 0.01392 | 0.00000| 0.00991| 0.01391| 0.01639 | 0       | n/a    | n/a    | 0.00000| n/a    | 0      | 0      | 0       | 0      | 0       |
| Orientation  | 0.00948 | 0.01253 | n/a    | 0.02847 | 0.01590 | 0      | 0      | 0.01035| 0.01423 | 0.02825 | n/a    | n/a    | 0.00000| n/a    | 0      | 0      | 0       | 0      | 0       |
| Temperature  | 0.00000 | 0.01252 | n/a    | 0.02731 | 0.01530 | 0      | 0      | 0.01182| 0.00000 | 0.0285  | n/a    | n/a    | 0.06497| N/A    | n/a    | 0      | 0       | 0      | 0       |
| Deviation    | 0.01002 | 0.01158 | n/a    | 0.02506 | 0.01474 | 0      | 0      | 0.00954| 0.03223 | 0.04103 | n/a    | n/a    | 0.02525| n/a    | n/a    | 0      | 0       | 0      | 0       |
| Variance     | 0.00000 | 0.01240 | n/a    | 0.02600 | 0.01460 | 0      | 0      | 0.01585| 0.00000 | 0.04103 | n/a    | n/a    | 0.02525| n/a    | n/a    | 0      | 0       | 0      | 0       |
| Standard     | 0.00000 | 0.01201 | n/a    | 0.02539 | 0.01500 | 0      | 0      | 0.01585| 0.00000 | 0.04103 | n/a    | n/a    | 0.02525| n/a    | n/a    | 0      | 0       | 0      | 0       |
Table S8. QM Region II was used to calculate $\rho$ values in units of e/bohr$^3$ for triprotonated states 9-O to 14-O

|          | 9-O     | 10-O    | 11-O    | 12-O    | 13-O    | 14-O    |
|----------|---------|---------|---------|---------|---------|---------|
| O1-H23   | 0.02671 | 0.03215 | 0.02292 | 0.01843 | 0.02578 | 0.02920 |
| O1-H22   | 0.03651 | 0.02930 | 0.03948 | 0.03961 | 0.03509 | 0.04475 |
| O1-H21   | 0.00845 | 0.00000 | 0.01070 | 0.01911 | 0.00934 | 0.00000 |
| O2-H29   | 0.04213 | 0.03710 | 0.02435 | 0.03792 | 0.01075 | 0.04003 |
| O2-H31   | 0.02937 | 0.04213 | 0.03786 | 0.04300 | 0.05210 | 0.03633 |
| O3-H27   | 0.01341 | 0.00000 | 0.01558 | 0.00000 | 0.01099 | 0.01232 |
| O3-H28   | 0.04213 | 0.03424 | 0.01972 | 0.03300 | 0.02465 | 0.03045 |
| O3-H24   | 0.01396 | 0.03230 | 0.02260 | 0.02305 | 0.02662 | 0.02209 |
| O16-H36  | 0.05147 | 0.00000 | 0.05019 | 0.03694 | 0.08644 | 0.02483 |
| O16-H7   | 0.00000 | 0.05011 | 0.02614 | n/a     | n/a     | n/a     |
| O15-H6   | 0.06887 | 0.06270 | 0.06384 | 0.04404 | NC$^c$  | 0.01083 |
| H43-H44  | 0.01083 | 0.01085 | 0.01084 | 0.01082 | 0.01085 | 0.01083 |
| O13-H41  | 0.04373 | 0.04322 | 0.04249 | 0.04316 | 0.04257 | 0.04496 |
| O13-H55  | 0.04917 | 0.04895 | 0.04335 | 0.04773 | 0.05169 | 0.04648 |
| O14-H42  | 0.03827 | 0.03929 | 0.04166 | 0.04264 | 0.04909 | 0.03828 |
| V-N5     | 0.06726 | 0.05560 | 0.06995 | 0.04074 | 0.04227 | 0.03480 |
| V-O4     | NC      | NC      | 0.07538 | NC      | NC      | NC      |
| N26-H37  | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.05420 | 0.00000 |
| N26-H6   | 0.00000 | 0.00000 | 0.00000 | NC$^b$  | 0.00000 | NC$^d$  |
| N26-H7   | 0.09704 | NC$^a$  | 0.00000 | n/a     | n/a     | n/a     |
| N26-H10  | n/a     | n/a     | 0.04980 | 0.01193 | 0.02526 | n/a     |
| H45-H35  | 0.01375 | 0.01200 | 0.01578 | 0.01419 | 0.01423 | 0.01203 |
| O17-H34  | 0.02059 | 0.01870 | 0.01818 | 0.01848 | 0.01787 | 0.01763 |
| N20-H11  | n/a     | 0       | n/a     | n/a     | 0.01034 | 0.00000 |
| H57-H11  | n/a     | 0.01269 | n/a     | n/a     | 0.00000 | 0.01357 |
| O60-H66  | 0.02563 | 0.02561 | 0.02562 | 0.02560 | 0.02562 | 0.02561 |
| O60-H50  | 0.01251 | 0.01240 | 0.01253 | 0.01254 | 0.01256 | 0.01249 |
| O61-H67  | 0.03125 | 0.03127 | 0.03126 | 0.03120 | 0.03126 | 0.03125 |
| O58-H33  | 0.03157 | 0.03599 | 0.03255 | 0.03279 | 0.02743 | 0.03461 |
| O59-H68  | 0.01535 | 0.02078 | 0.01452 | 0.01454 | 0.02107 | 0.01934 |
| C69-H30  | 0.00000 | 0.00000 | 0.01057 | 0.00000 | 0.01088 | 0.01001 |
| N20-H70  | 0.01223 | 0.01122 | 0.01231 | 0.01256 | 0.01160 | 0.01207 |
| H8-H71   | 0.01214 | n/a     | n/a     | 0.01573 | n/a     | 0.01715 |
| H62-O17  | 0.02600 | 0.02436 | 0.02330 | 0.02497 | 0.02324 | 0.02483 |
| O63-H72  | 0.01658 | 0.01560 | 0.01608 | 0.01637 | 0.01537 | 0.01602 |
| Bond     | d1   | d2   | d3   | d4   | d5   | d6   |
|----------|------|------|------|------|------|------|
| H7 O7    | 0.00000 | 0.01439 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| O4 H57   | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.01071 | 0.00000 |
| O2 H57   | 0.01012 | 0.00000 | 0.01142 | 0.01160 | 0.01126 | 0.00000 |
| H75 O16  | 0.00000 | 0.00000 | 0.00000 | 0.01531 | 0.00000 | 0.01364 |
| O4 O15   | 0.00000 | 0.00000 | 0.00000 | 0.01354 | 0.00000 | 0.00000 |
| H27 H10  | n/a    | n/a   | 0.00000 | 0.01195 | 0.00000 | n/a   |

a: H7 transfers to N26; d(H7-N26)=1.047, d(O4-H7)=1.676; \( \rho \) (O4-H7)=0.05009
b: H6 transfers to N26; d(H6-N26)=1.054, d(O4-H6)=2.548; \( \rho \) (O4-H6)=0
c: H6 transfers to O15; d(H6-O15)=1.016, d(H6-O4)=1.550, \( \rho \) (O4-H6)=0.06271
d: H6 transfers to N26; d(H6-N26)=1.025, d(H6-O4)=2.218, \( \rho \) (O4-H6)=0.01405
Table S9. QM Region II was used to calculate ρ values in units of e/bohr³ for quadprotonated states 15-O to 19-O

|       | 15-O      | 16-O      | 17-O      | 18-O      | 19-O      |
|-------|-----------|-----------|-----------|-----------|-----------|
| O1-H23| 0.03353   | 0.03126   | 0.03128   | 0.00000   | 0.02193   |
| O1-H22| 0.03364   | 0.02942   | 0.02841   | 0.02215   | 0.02981   |
| O1-H21| 0.00664   | 0.00000   | 0.00495   | 0.00716   | 0.00991   |
| O2-H29| 0.03863   | 0.00795   | 0.03308   | 0.04098   | 0.02515   |
| O2-H30| 0.00000   | 0.00000   | 0.00000   | 0.00645   | 0.00000   |
| O2-H31| 0.03589   | 0.03248   | 0.03702   | 0.02580   | 0.03245   |
| O3-H27| 0.01219   | 0.00000   | 0.01045   | 0.01529   | 0.01202   |
| O3-H28| 0.02728   | 0.02464   | 0.02267   | 0.04357   | 0.03155   |
| O3-H24| 0.02120   | 0.03865   | 0.02371   | 0.01288   | 0.02307   |
| O4-H11| 0.00000   | 0.00000   | 0.00000   | n/a       | n/a       |
| O16-H36| NC⁹      | NCb⁹     | 0.03369   | 0.00000   | 0.02674   |
| O16-H7 | 0.00000   | 0.00000   | 0.02976   | 0.00000   | 0.04235   |
| O16-H8 | 0.00000   | 0.00000   | n/a       | 0.05636   | n/a       |
| O15-H6 | 0.979A    | NCb⁹     | 0.05666   | 0.04916   | 0.05694   |
| H43-H44| 0.01081   | 0.01088   | 0.01080   | 0.01081   | 0.01078   |
| O13-H41| 0.03290   | 0.03550   | 0.04893   | 0.05111   | 0.04779   |
| O13-H38| 0.00000   | NCb⁹     | 0.00000   | 0.00000   | 0.00000   |
| O13-H55| NC⁹      | NCb⁹     | 0.04301   | 0.04508   | 0.04461   |
| O14-H42| 0.03374   | 0.03160   | 0.04096   | 0.04977   | 0.04377   |
| O14-H38| 0.00000   | 0.00000   | 0.00000   | 0.00000   | 0.00000   |
| V-N5  | 0.03996   | 0.07331   | 0.08882   | 0.06175   | 0.08320   |
| V-O4  | NC       | NC       | 0.06111   | NC       | 0.06628   |
| N26-H37| 0.00000   | 0.00000   | 0.00000   | 0.00000   | 0.00000   |
| N26-H6 | 0.00000   | 0.00000   | 0.00000   | 0.00000   | 0.00000   |
| N26-H7 | NC⁹      | 0.03586   | 0.00000   | NC⁹      | 0.00000   |
| N26-H10| n/a      | n/a      | NC⁹      | n/a      | NC⁹      |
| H45-H35| 0.01295   | 0.01300   | 0.01390   | 0.01450   | 0.01414   |
| O17-H34| 0.01709   | 0.01881   | 0.01859   | 0.02135   | 0.02309   |
| N5-H22| 0.00854   | 0.00000   | 0.00000   | 0.01115   | 0.00000   |
| N20-H12| n/a      | 0.03154   | n/a      | n/a      | n/a      |
| O2-V  | NC       | 0.05352   | NC       | NC       | NC       |
| O4-H29| 0.00000   | 0.00511   | 0.00000   | 0.00000   | 0.00000   |
| O3-H10| n/a      | n/a      | 0.01345   | n/a      | 0.00000   |
| O15-H7| 0      | 0.00000   | 0.00000   | 0      | 0.00000   |
| O15-H10| n/a     | n/a      | 0.00000   | n/a     | 0.00000   |
| Bond            | Bond Distance 1 | Bond Distance 2 | Bond Distance 3 | Bond Distance 4 | Bond Distance 5 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| O4-H27          | 0.00000         | 0.00000         | 0.00000         | 0.00000         | 0.00000         |
| O4-H10          | n/a             | n/a             | 0.01016         | n/a             | 0.03612         |
| C56-H65         | 0.01007         | 0.01043         | 0.01019         | 0            | 0.00936         |
| H57-H11         | 0.01275         | 0.01589         | 0.01522         | 0             | n/a             |
| O60-H66         | 0.02562         | 0.02568         | 0.02565         | 0.02565        | 0.02564         |
| O60-H50         | 0.01269         | 0.01284         | 0.01273         | 0.0127         | 0.01265         |
| O61-H67         | 0.03119         | 0.03133         | 0.03128         | 0.03125        | 0.03125         |
| O58-H33         | 0.04062*        | 0.03422         | 0.04024         | 0.03418        | 0.03623         |
| O59-H68         | 0.01763         | 0.02274         | 0.01948         | 0.01294        | 0.01766         |
| C69-H30         | 0.0100          | 0.01104         | 0.01013         | 0             | 0.00000         |
| N20-H70         | 0.01204         | 0              | 0.01119         | 0.01132        | 0.01183         |
| C21-H70         | 0.01562         | 0.0192          | 0.00000         | 0             | 0.00000         |
| H8-H71          | 0.02283         | 0.02134         | 0.02271         | 0.02533        | 0.02083         |
| H62-O17         | 0.01696         | 0.01574         | 0.01692         | 0.01783        | 0.01706         |
| H9-N40          | n/a             | n/a             | n/a             | 0.0334         | n/a             |
| O4-H73          | 0.00752         | 0.01517         | 0.00000         | 0             | 0.00000         |
| O1-V            | NC              | 0               | NC              | 0.07174        | NC              |
| O3-H74          | 0.00752         | 0.01517         | 0.00000         | 0             | 0.00000         |
| O4-H57          | 0.00000         | 0               | 0               | 0             | 0               |
| O4-H37          | 0.00000         | 0               | 0               | 0             | 0               |

a: H6 transfers to O15; H36 transfers from O15 to O16 with d(H36-O16)=0.991A; H38 transfers from O16 to O53; H54 transfers from O53 to O13; rho(O15-H36)=0.04064; rho(O16-H38)=0.05307; rho(O53-H55)=0.09737; rho(O4-H6)=0.03806); H7 transfers to N26: d(H7-N26)=1.020 A, d(O4-H7)=2.142, rho(O4-H7)=0.01767

b: H6 has transferred to O15, d(H6-O15)=0.977; H36 has transferred from O15 to O16 with d(H36-O16)=0.991; H38 has transferred from O16 to O53; H54 has transferred from O53 to O13,d(H54-O13)=1.060; rho(O4-H6)=0.03425, rho(O15-H36)=0.04232, rho(O16-H38)=0.05514, rho(O53-H54)=0.08818

c: H10 has transferred to N26, d(H10-N26)=1.014

d: H7 has transferred to N26, d(H7-N26)=1.036; d(O4-H7)=1.723; rho(O4-H7)=0.04581; rho(O4-H23)=0.02018

e: H10 has transferred to N26, d(H10-N26)=1.024; d(O3-H10)=2.444; rho(O3-H11)=0.01443
Table S10. QM Region II was used to calculate \( \rho \) values in units of e/bohr\(^3\) for quadprotonated states 20-O to 23-O

|       | 20-O     | 21-O     | 22-O     | 23-O     |
|-------|----------|----------|----------|----------|
| O1-H23| 0.01814  | 0.02798  | 0.02846  | 0.02577  |
| O1-H22| 0.02137  | 0.02039  | 0.02845  | 0.01689  |
| O1-H21| 0.01276  | 0.00672  | 0.00540  | 0.00665  |
| O2-H29| 0.02943  | 0.00000  | 0.00000  | 0.00000  |
| O2-H30| 0.00675  | 0.00000  | 0.00000  | 0.00000  |
| O2-H31| 0.03325  | 0.04400  | 0.04603  | 0.04338  |
| O3-H27| 0.00000  | 0.00000  | 0.00788  | 0.00637  |
| O3-H28| 0.04035  | 0.01923  | 0.02223  | 0.01836  |
| O3-H24| 0.02421  | 0.03606  | 0.02912  | 0.03239  |
| O4-H11| n/a      | 0.00000  | 0.04711  | 0.00000  |
| O16-H36| 0.00669  | 0.04498  | 0.00000  | 0.03327  |
| O16-H7 | 0.00000  | n/a      | n/a      | n/a      |
| O16-H8 | 0.06188  | 0.00000  | n/a      | 0.05096  |
| O15-H6 | 0.05516  | 0.03985  | 0.06892  | n/a      |
| H43-H44| 0.01082  | 0.01083  | 0.01081  | 0.01083  |
| O13-H41| 0.04152  | 0.05021  | 0.05031  | 0.04316  |
| O13-H58| 0.00000  | 0.00000  | 0.00000  | 0.01717  |
| O13-H55| 0.04185  | 0.05181  | 0.03863  | 0.03492  |
| O14-H42| 0.04176  | 0.04031  | 0.04136  | 0.04023  |
| O14-H38| 0.00000  | 0.00000  | 0.00000  | 0.00000  |
| V-N5  | 0.07587  | 0.08430  | 0.08871  | 0.08143  |
| V-O4  | NC       | NC       | NC       | NC       |
| N26-H37| 0.00000  | 0.00000  | 0.00000  | 0.00000  |
| N26-H6 | 0.00000  | NC\(^e\)  | 0.00000  | n/a      |
| N26-H7 | NC\(^f\)  | n/a      |          |          |
| N26-H10| 0.00000  | n/a      | NC\(^h\)  | NC\(^i\)  |
| H45-H35| 0.01345  | 0.01367  | 0.01367  | 0.01338  |
| O17-H34| 0.01893  | 0.01809  | 0.01861  | 0.01793  |
| N5-H22 | 0.00000  | 0.01809  | 0.00000  | 0.00000  |
| N20-H12| n/a      | 0.05387  | 0.0491   | 0.05320  |
| O2-V  | NC       | 0.03952  | 0.04921  | 0.04074  |
| O4-H29| 0.01234  | 0.02932  | 0.01881  | 0.02947  |
| O3-H10| NC       | n/a      | 0.00000  | 0.00000  |
| O15-H7| 0.00000  | n/a      | n/a      | n/a      |
| O15-H10| 0       | n/a      | 0.00000  | 0.04380  |
|     |     |     |     |     |
|-----|-----|-----|-----|-----|
| O4-H27 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| O4-H10 | 0.00000 | n/a | 0.00000 | 0.00000 |
| C56-H65 | 0.00876 | 0.01005 | 0.01048 | 0.00970 |
| H57-H11 | n/a | 0.01363 | 0.00000 | 0.01221 |
| O60-H66 | 0.02564 | 0.02568 | 0.02567 | 0.02569 |
| O60-H50 | 0.01261 | 0.01280 | 0.01277 | 0.01279 |
| O61-H67 | 0.03125 | 0.03135 | 0.03134 | 0.03137 |
| O58-H33 | 0.03623 | 0.03337 | 0.03492 | 0.03192 |
| O59-H68 | 0.01702 | 0.02203 | 0.02253 | 0.02143 |
| C69-H30 | 0.00000 | 0.01023 | 0.01004 | 0.01030 |
| N20-H70 | 0.01176 | 0.00000 | 0.00000 | 0.00000 |
| H21-H70 | 0.00000 | 0.01867 | 0.01679 | 0.01797 |
| H8-H71 | 0.00000 | 0.00000 | n/a | 0.00000 |
| H62-O17 | 0.02378 | 0.02177 | 0.02171 | 0.02177 |
| O63-H72 | 0.01682 | 0.01641 | 0.01632 | 0.01649 |
| H9-N40 | n/a | n/a | n/a | n/a |
| O4-H73 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |
| O1-V | NC | NC | NC | NC |
| O3-H74 | 0.01472 | 0.01031 | 0 | 0.00882 |
| O4-H57 | 0 | 0 | 0.01271 | 0 |
| O4-H37 | 0.00000 | 0.00000 | 0.00000 | 0.02876 |

f: H7 has transferred to N26, d(H7-N26)=1.032; d(O4-H7)=1.720; rho(O4-H7)=.04567, d(O1-H71)=0.01131, rho(H73-O16)=.01023

g: H6 transfers to N26; d(H6-N26)=1.041, d(H6-O4)=3.133; rho(O1-H71)=.01221

h: H10 has transferred from O3 to N26 with the following distances: d(N26-H10)=1.024, d(O3-H10)=2.759

i: H10 has transferred from O3 to N26 with the following distances: d(N26-H10)=1.041, d(O3-H10)=2.993

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REFERENCES

[1] J.-B. Fournier, C. Leblanc, Halogenation and vanadium haloperoxidases, in: S. L. Barre and J.-M. Komprobst (Eds.) Outstanding Marine Molecules: Chemistry, Biology, Analysis, First Edition, Wiley-VCH Weinham, 2014, 225-242.

[2] V. Vreeland, Recombinant minimal catalytic vanadium haloperoxidases and their uses, US Patent No. 6,998,257, February 14, 2006.

[3] R. Wever, P. Barnett, Vanadium Chloroperoxidases: The Missing Link in the Formation of Chlorinated Compounds and Chloroform in the Terrestrial Environment? Chem. Asian J. 2017, 12, 1997–2007.

[4] G. Zampella, P. Fantucci, V.L. Pecoraro, L. De Gioia, Insight into the catalytic mechanism of vanadium haloperoxidases. DFT investigation of vanadium cofactor reactivity, Inorganic Chemistry 2006, 45, 7133-7143.

[5] Some examples are as follows: M. R. A. Blomberg, P. E. M. Siegbahn, Protonation of the binuclear active site in cytochrome c oxidase decreases the reduction potential of CuB, Biochim. Biophys. Acta, Bioenerg., 2015, 1847, 1173-1180; W. Lee, B. Engels, The Protonation State of Catalytic Residues in the Resting State of KasA Revisited: Detailed Mechanism for the Activation of KasA by Its Own Substrate, Biochemistry 2014, 53, 919-931; B. Benediktsson, R. Bjornsson, QM/MM Study of the Nitrogenase MoFe Protein Resting State: Broken-Symmetry States, Protonation States, and QM Region Convergence in the FeMoco Active Site, Inorg.
Chem. 2017, 56, 13417-13429; C. N. Morrison, T. Spatzal, D.C. Rees, Reversible Protonated Resting State of the Nitrogenase Active Site, J. Am. Chem. Soc. 2017, 139, 10856-10862.

[6] A. Messerschmidt, L. Prade, R. Wever, Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from the fungus Curvularia inaequalis by X-ray structures of the native and peroxide form. Biol. Chem. 1997, 378, 309-315.

[7] J. Y. Kravitz, V. L. Pecoraro, H. A. Carlson, Quantum mechanics/molecular mechanics calculations of the vanadium dependent chloroperoxidase. J. Chem. Theory Comput. 2005, 1, 1265-1274.

[8] Y. Zhang, J. A. Gascon, QM/MM investigation of structure and spectroscopic properties of a vanadium-containing peroxidase, J. Inorg. Biochem. 2008, 102, 1684-1690.

[9] K. R. Geethalakshmi, M. P. Waller, W. Thiel, M. Buhl, $^{51}$V NMR chemical shifts calculated from QM/MM models of peroxo forms of vanadium haloperoxidases, J. Phys. Chem. B 2009, 113, 4456-4465.

[10] M. P. Waller, M. Buhl, K. R. Geethalakshmi, D. Wong, W. Thiel, $^{51}$V NMR chemical shifts calculated from QM/MM models of vanadium chloroperoxidase, Chem. Eur. J. 2007, 13, 4723-4732.
[11] N. Pooransingh-Margolis, R. Renirie, Z. Hasan, R. Wever, A. J. Vega, T. Polenova, $^{51}$V Solid-state magic angle spinning NMR spectroscopy of vanadium chloroperoxidase, *J. Am. Chem. Soc.* 2006, **128**, 5191-5208.

[12] E. de Boer, K. Boon, R. Wever, Electron Paramagnetic Resonance Studies on Conformational States and Metal Ion Exchange Properties of Vanadium Bromoperoxidase, *Biochemistry* 1988, **27**, 1629-1635.

[13] R. Renirie, J. M. Charnock, C. D. Garner, R. Wever, Vanadium K-edge XAS studies on the native and peroxo-forms of vanadium chloroperoxidase from Curvularia inaequalis, *J. Inorg. Biochem.* 2010, **104**, 657-664.

[14] J. W. P. M. Van Schijndel, E. G. M. Vollenbroek, R. Wever, The chloroperoxidase from the fungus Curvularia inaequalis; a novel vanadium enzyme, *Biochim. Biophys. Acta* 1993, **1161**, 249.

[15] R. Gupta, G. Hou, R. Renirie, R. Wever, T. Polenova, $^{51}$V NMR crystallography of vanadium chloroperoxidase and its directed evolution P395D/L241V/T343A mutant: protonation environments of the active site, *J. Am. Chem. Soc.* 2015, **137**, 5618-5628.

[16] R. F. W. Bader, *Atoms in Molecules: A Quantum Theory*, Oxford University Press, New York, 1994.

[17] C. F. Matta.; R. J. Boyd (Eds.) *The Quantum Theory of Atoms in Molecules*, Wiley-VCH., Weinheim, Germany, 2007.
[18] A. Panda, R. N. Behera, Comparative study of E\cdot\cdot\cdotN (E = Se/Te) intramolecular interactions inorganochalcogen compounds using density functional theory, *J. Hazard. Mater.* 2014, **269**, 2-8.

[19] Y. Aray, R. Aguilera-Garcia, D. R. Izquierdo, Exploring the nature of the H-bonds between the human class II MHC protein, HLA-DR1 (DRB*0101) and the influenza virus hemagglutinin peptide, HA306-318, using the quantum theory of atoms in molecules. *J. Biomol. Struct. Dyn.* 2017, **35**, 1-17.

[20] A.T. Ayoub, T. J. A. Craddock, M. Klobukowski, J. Tuszyński, Analysis of the strength of interfacial hydrogen bonds between tubulin dimers using Quantum Theory of Atoms in Molecules. *Biophys. J.* 2014, **107**, 740–750.

[21] J. Held, S. van Smaalen, The active site of hen egg-white lysozyme: flexibility and chemical bonding, *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2014, **D70**, 1136–1146.

[22] J. R. Lane, J. Contreras-Garcia, J.-P. Piquemal, B. J. Miller, H. G. Kjaergaard, Are bond critical points really critical for hydrogen bonding? *J. Chem. Theory Comput.*, 2013, **9**, 3263–3266.

[23] G. Zampella, P. Fantucci, V. L. Pecoraro, L. De Gioia, Reactivity of peroxo forms of the vanadium haloperoxidase cofactor. A DFT investigation. *J. Am. Chem. Soc.* 2005, **127**, 953-960.

[24] Schrodinger Release 2018-3: QSite, Schrodinger, LLC, New York, NY, 2018.
[25] A. D. Bochevarov, E. Harder, T. F. Hughes, J. R. Greenwood, D. A. Braden, D. M. Philipp, D. Rinaldo, M. D. Halls, J. Zhang, R. A. Friesner, Jaguar: A high-performance quantum chemistry software program with strengths in life and materials sciences. *Int. J. Quantum Chem.* 2013, **113**, 2110-2142.

[26] NBO 6.0. E. D. Glendening, J. K. Badenhoop, A. E. Reed, J. E. Carpenter, J. A. Bohmann, C. M. Morales, C. R. Landis, and F. Weinhold (Theoretical Chemistry Institute, University of Wisconsin, Madison, WI, 2013); [http://nbo6.chem.wisc.edu/](http://nbo6.chem.wisc.edu/)

[27] [http://www.rcsb.org/pdb/explore/literature.do?structureId=1IDQ](http://www.rcsb.org/pdb/explore/literature.do?structureId=1IDQ)

[28] There is another structure (1VNI) of VCPO in the Protein Data Bank, but it lacks important crystallographic water molecules.

[29] M. H. M. Olsson, C. R. Sondergaard, M. Rostkowski, J. H. Jensen, PROPKA3: Consistent treatment of internal and surface residues in empirical pKa predictions. *J. Chem. Theory Comput.* 2011, **7**, 525-537.

[30] The PROPKA method fails because of the presence of vanadium. One can do PROPKA calculations if vanadium is deleted or if it is substituted with some other metal such as iron. In the iron is substituted, one finds that the pKa of His404 is 1.63. One can also delete vanadium and run PROPKA, and in this case one can calculate the pKa of His404 is 3.63. If the pKa of His404 is much less than what is found when histidine is in isolation from a protein environment.

[31] HIE is ε²-histidine.
[32] P. J. Hay, W. R. Wadt Ab initio effective core potentials for molecular calculations. Potentials for K to Au including the outermost core orbitals *J. Chem. Phys.* 1985, **82**, 299-310.

[33] J. L. Banks, H. S. Beard, Y. Cao, A. E. Cho, W. Damm, R. Farid, A. K. Felts, T. A. Halgren, D. T. Mainz, J. R. Maple, R. Murphy, D. M. Philipp, M. P. Repasky, L. Y. Zhang, B. J. Berne, R. A. Friesner, E. Gallicchio, R. M. Levy, Integrated Modeling Program, Applied Chemical Theory (IMPACT). *J. Comp. Chem.* 2005, **26**, 1752.

[34] R. A. Friesner, V. Guallar, Ab Initio quantum chemical and mixed quantum mechanics/molecular (QM/MM) methods for studying enzymatic catalysis, *Annu. Rev. Phys. Chem.* 2005, **56**, 389–427.

[35] B. Lee, F. M. Richards, The interpretation of protein structures: estimation of static accessibility, *J. Mol. Biol.* 1971, **55**, 379-490.

[36] BioLuminate, version 2.5, Schrödinger, LLC, New York, NY, 2017.

[37] J. W. P. M. Van Schijndel, P. Barnett, J. Roelse, E. G. M. Vollenbroek, R. Wever, The stability and steady-state kinetics of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*, *Eur. J. Biochem.* 1994, **225**, 151-157.

[38] S. Macedo-Ribeiro, W. Hemrika, R. Renirie, R. Wever, A. Messerschmidt, X-ray crystal structures of active site mutants of the vanadium-containing chloroperoxidase from the fungus *Curvularia inaequalis*, *J. Biol. Inorg. Chem.* 1999, **4**, 209–219.
[39] C. A. Fitch, G. Platzer, M. Okon, B. Garcia-Moreno, L. P. McIntosh, Arginine: Its pKa value revisted. *Protein Science*, 2015, **24**, 752-761.

[40] There is no transfer of H22 (of Arg360) to equatorial O1 for monoprotonated 1-O or 2-O when QM Region II is used, as there was when QM Region I was used.

[41] A. S. Tracey; G. R. Willsky; E. S. Takeuchi, *Vanadium: Chemistry, Biochemistry, Pharmacology and Practical Applications*, CRC Press, New York, 2007, 162.

[42] It is not unprecedented to suggest that two species in equilibrium are the resting state. For example, Kravitz et al. argued that the resting state of VCPO is an equilibrium of 3-O and 4-O.[7] In comparing the present work with Kravitz et al., it should be noted that they used the PDB structure, 1VNI, to build their input structures. But 1VNI does not have the crystallographic water molecules of 1IDQ. These crystallographic water molecules have a significant effect on the calculations. Furthermore, it has been shown that water molecules are likely to be involved in the catalytic mechanism.

(43) Unfortunately, QSite does not determine bond critical points and corresponding ρ values for covalent interactions (i.e., ρ>0.099). If we believed ρ>0.099 along any bond length, we have written “NC” or “not calculated”. In general, any ρ<.01 has been ignored and not reported except in some cases where a particular bond has ρ>.01 for some of the structures.