ISOLATION AND PURIFICATION OF VANADIUM HALOPEROXIDASE

MUTANTS FOR CHARACTERIZATION BY

^{51}V SOLID-STATE NMR SPECTROSCOPY

by

Kristin M. Nuzzio

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Chemistry with Distinction.

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Approved: _____
Tatyana Polenova, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Cecil Dybowski, Ph.D.
Committee member from the Department of Chemistry & Biochemistry

Approved: _____
Calvin Keeler, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____
Alan Fox, Ph.D.
Director, University Honors Program

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ABSTRACT

Vanadium containing compounds have shown excellent potential in the treatment of diabetes, particularly as insulin enhancing compounds, as well as in the treatment of some forms of cancer. However, in order for these compounds to be useful in biomedical applications, the structures of their vanadium active sites and the mechanisms of their biochemical activity need to be determined. Vanadium haloperoxidases (VHPO) are a specific class of vanadium containing enzymes commonly found in marine algae, lichens and terrestrial fungi. These enzymes are the most efficient halide oxidants known to date. The focus of this project is to understand the catalytic mechanism of VHPO and their active site mutants by utilizing ^{51}V solid-state NMR spectroscopy as a site-specific probe of the diamagnetic "spectroscopically silent" vanadium active sites. This knowledge is expected to be important in designing artificial vanadium enzymes with tuned halogenating activities. In this thesis, two VCPO active site mutants as well as the wild type protein have been successfully isolated and purified, and ^{51}V solid-state NMR spectra have been acquired.

Chapter 1

INTRODUCTION

1.1   Vanadium in Biological Systems

Approximately one-third of all proteins require metals as cofactors for biological processes.[1] The transition metal vanadium can exist in a variety of oxidation states, but is found in the +3, +4 and +5 oxidation states in biological systems.[2] Vanadium compounds have several biomedical and therapeutic applications, as they have been used as insulin mimetics for diabetes treatment and tested as possible anti-cancer leads.[3] Several vanadium salts, such as vanadyl sulfate, were shown to improve glucose uptake and improve insulin sensitivity when tested in rat adipocytes.[4] However, in order for these vanadium containing compounds to be useful in biomedical applications, the structure of the vanadium active sites and the mechanism of their biochemical activity needs to be determined.

1.2.1 Vanadium Haloperoxidases

Vanadium is found in several marine biological systems in the range of 30-50 nM in seawater[5], specifically in the form of vanadium haloperoxidases (VHPO). VHPO are a specific class of vanadium containing enzymes also found in terrestrial fungi and several lichens.[6,7] These enzymes catalyze the oxidation of halides in the presence of hydrogen peroxide as outlined in Equation 1.

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX \tag{1}$$
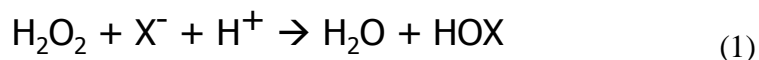
Additionally, VHPO are the most efficient halide oxidants known to date.

VHPO are named for the most electronegative halide that they are able to oxidize. Vanadium chloroperoxidases (VCPO) can oxidize Cl⁻, Br⁻ and I⁻, whereas vanadium bromoperoxidases (VBPO) are only able to oxidize Br⁻ and I⁻. As expected, vanadium iodoperoxsidases (VIPO) can only oxidize I⁻. VCPO are commonly isolated from fungus *Curvularia inaequalis*, while VBPO are found in the seaweed *Ascophyllum nodosum*. Vanadium remains in its +5 oxidation state throughout the entire catalytic cycle and is thus "spectroscopically silent."

1.2.2 Vanadium Chloroperoxidase and Mutants

VCPO is a 67.5 kDa protein with one vanadium active site per molecule. The vanadium site itself has been found to have a trigonal bipyrimidal coordination geometry in the resting state from X-ray crystallography data.[8] The active site consists of the anionic vanadate cofactor, three equatorial ligands and two axial ligands. In the intermediate state, VCPO appears to have a tetragonal pyramidal geometry as shown in Figure 1.[9] In wild type VCPO, the vanadate ion is bonded covalently to the nitrogen atom of a histidine residue (H496). The other residues (R360, R490, K353, S402, and G403) are hydrogen bonded to the surrounding oxygen atoms.[10] The structures of the different types of VHPO are very similar, with only a single residue difference between VCPO and VBPO as shown in Figure 2.[11] The F397 residue is replaced by H411 in VBPO; however, all of the other residues remain conserved in the resting state.[11] R360 has been shown to be bound to one of the oxygen atoms in the vanadate active site from the X-ray crystallographic structure of VCPO.[10] This residue stabilizes the F397 residue in the active site, as it also hydrogen bonds to the carbonyl group of F397. The R360A mutant has a lower chlorinating activity than the wild type by 14%.[10] It has also been found to have a higher optimum pH than the wild type

protein.[10] R490 is also hydrogen bonded to one of the vanadate oxygen atoms. After site-directed mutagenesis of R360 and R490, the X-ray crystal structures have shown that the mutants remain hydrogen bonded to the vanadate oxygen atoms.[10]

By site-directed mutagenic replacement of H496 with alanine, the protein did not retain its enzymatic activity.[10] Lower activity was also observed for the R360A, R490A and K353A mutants.[9] However, some mutations have been found to enhance the activity of the protein. The VCPO triple mutant of P395D/L241V/T343A showed a 100-fold increase in VCPO activity at pH 8.0.[12] Additionally, the P395T/L241V/T343A mutant and the P395T/L241V mutant, both increased protein activity by 20-fold.[12]

1.2 ^{51}V Solid-State NMR Spectroscopy

The goal of performing ^{51}V NMR on vanadium haloperoxidases is to understand the structure and function of the vanadium active sites in proteins. Solution NMR is a widely developed analytical tool, but its application to large biomolecules, such as proteins, is limited. In solution NMR, the sample must be soluble and relatively small (under 30 kDa for protein applications) in order to achieve full structure determination. There are no such limitations in solid-state NMR, and large proteins can be studied.

Both ^{50}V and ^{51}V can be used in vanadium NMR. However, ^{50}V has a very low natural abundance (0.250%) and a spin 6 nucleus, making it an ineffective NMR probe. ^{51}V is a quadrupolar nucleus with a spin of 7/2.[13] In ^{51}V solid-state NMR, the observables are the quadrupolar and chemical shielding anisotropy tensors.[14] From the spectra, several parameters can be extracted including the quadrupolar coupling constant, C_Q, the asymmetry of the quadrupolar interaction, η_Q, the isotropic chemical shift, δ_{iso}, the chemical shift anisotropy, δ_σ, and the asymmetry of the CSA tensor, η_σ. The quadrupolar coupling constant is given as $C_Q = eQV_{ZZ}/h$, where e is the electronic charge, Q is the quadrupole moment

for vanadium ($-0.052 *10^{-28}$ V/m^2), and h is Planck's constant.[15,16] The asymmetry parameter is represented by $\eta_Q = (V_{XX} - V_{YY})/V_{ZZ}$ with V$_{zz}$, V$_{xx}$, and V$_{yy}$ being the principal components of the electric field gradient tensor.[15] The CSA tensor parameters are expressed as $\delta_\sigma = \delta_{zz} - \delta_{iso}$, $\eta_\sigma = \dfrac{\delta_{yy} - \delta_{xx}}{\delta_{zz} - \delta_{iso}}$, and $\delta_{iso} = \dfrac{1}{3}(\delta_{xx} + \delta_{yy} + \delta_{zz})$, where δ_{zz}, δ_{xx} and δ_{yy} are the principal components of the CSA tensor.[17] These NMR observables provide structural and electronic information about the local environment of the vanadate cofactor.[18,19]

Although there is only 1 vanadium atom per 67.5 kDa-large VCPO molecule,[19] solid-state NMR spectra can still be detected with high sensitivity. ^{51}V solid-state NMR is used experimentally to probe the environment of the vanadium active site in its resting state directly.[19] Even though the X-ray structure is known, important information about the active site is missing, namely the protonation states of the oxygen atoms of the vanadate cofactor. Knowledge of these protonation states is necessary for understanding the enzymatic mechanism of VHPO and for gaining insight into the changes of the mechanism in the active site mutants. Because the amino active site mutants modulate the electronic structure of the vanadium active site, these studies may help to determine the role of the individual amino acid residues on the vanadium center and the active site as a whole.[19]

Chapter 2

MATERIALS AND METHODS

2.1 Purification of Wild Type VCPO and Mutants

An adapted version of the procedure developed by Hemrika et al.[10] has been followed to isolate and purify VCPO wild type protein and the mutants R360A and R490A. The procedure outlined below is the most current protocol being followed in the laboratory; however, the purification conditions are still being optimized. *Saccharomyces cerevisiae* cells (Strain BJ1991) with the vector PTNT14 were grown on minimal media plates containing 2% glucose, 10X yeast nitrogen base, leucine and tryptophan and incubated at 30 °C for two days. The cells were replated two times, and then transferred to solutions in four flasks having 2% glucose and minimal media. The cells were grown in the minimal media in the shaker at 250 rpm and 28 °C for approximately 36 hours, until an optical density (O.D.) between 0.6-0.7 was reached. The cells were then transferred into four flasks containing 250 mL of a 1% glucose rich media solution and grown in the shaker at 250 rpm and 28°C for approximately 21 hours until an O.D. of 7.0 was reached. Expression was induced by adding 40% galactose to each rich media flask. The cells were expressed for three days and then were collected via centrifugation for 40 minutes at 3800 rpm and 4 °C.

The cells were resuspended using a minimal amount of 50 mM Tris acetate buffer at pH 8.3. The cells were sonicated in an ice bath for 7 minutes with a 30 second on/off pulse to lyse the cell membranes. 60% isopropanol was added to the supernatant to precipitate the DNA from the cells. The protein was then centrifuged a second time for 40

minutes at 15,000 rpm and 4 °C to remove any other cell debris. DEAE Sephacel resin was prepared for use in column chromatography by washing 75 mL of resin five times with distilled water and then five times with 50 mM Tris Acetate buffer at pH 8.3. The supernatant was then collected and bound to the DEAE Sephacel resin overnight by stirring for use in anion exchange chromatography. A glass column was packed with the resin and protein and 0.1M KCl in 50 mM Tris Acetate buffer at pH 8.3 was used to wash the column. A salt gradient of 0.6M KCl in 50 mM Tris Acetate buffer at pH 8.3 was used to elute the desired protein, and fractions were collected every 5 mL. Since sodium interferes with the vanadium signal in solid-state NMR spectroscopy, the active gravity column fractions were combined and dialyzed against 25 mM piperazine HCl buffer at pH 5.5 for 12 hours to remove any additional salt present.

Additionally, fast protein liquid chromatography (FPLC) with a Mono-Q column was then used to purify the protein by applying a salt gradient with filtered and degassed buffers of 25 mM piperazine/HCl at pH 5.5 and 2 M KCl in 25 mM piperazine/HCl at pH 5.5. The protein was then incubated with 10 mM sodium orthovanadate (Na_3VO_4) to 90% of the protein sample concentration overnight. The protein was dialyzed overnight in 50 mM Tris Acetate buffer at pH 8.3, and then dialyzed two times overnight in 25 mM Tris Acetate buffer at pH 8.3. An Amicon filtration system with a 30 kDa cutoff filter was used to concentrate the protein sample. 200 μL samples of protein were taken throughout the purification process for later use in gel electrophoresis.

2.2 Assessing Protein Purity

The purity of the VCPO samples was tested qualitatively and quantitatively throughout the isolation and purification process.

2.2.1 Qualitative Methods

A phenol red assay was used to detect the presence of VCPO qualitatively. The VCPO assay was prepared using 1M sodium citrate buffer at a pH of 5.0, 1 M KBr, 10 mM Na_3VO_4, 4 mM phenol red and 100 mM H_2O_2. The H_2O_2 must be prepared immediately before the assay is to be run, and the assay solution must be made fresh before use. 20 μL of the protein sample are added to 100 μL of the phenol red assay in a well plate. Upon halognenation, the phenol red assay changes color from yellow to blue, indicating that phenol red has become bromophenol blue. The reaction scheme for this assay is presented in the equation below.[20]
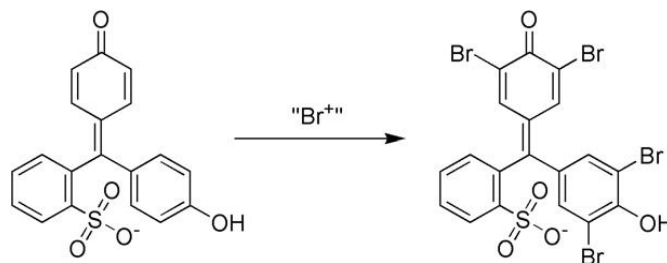
VCPO samples were tested using this assay at different stages during the purification process as a quick, qualitative way to determine if the protein still maintained its halogenating activity.

Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis was performed on the purified samples to assess the amount of pure protein present and assess the level of purity qualitatively. 10 μL of the 200 μL samples collected throughout the purification process were added to 60 μL of the 2x sample buffer. The sample buffer consists of 2% SDS, 20% glycerol, 20 mM Tris-Cl, pH 8, 2 mM ethylene diamine tetraacetic acid (EDTA), 2-mercaptoethanol and 0.1 mg/mL bromophenol blue. The samples were incubated in a boiling water bath for 10 minutes. Each of the lanes was washed with a small portion of cathode buffer and then 10 μL of the BioRad Rainbow

molecular marker were loaded into lane 1 of the acrylamide gel. 10 μL of the incubated samples were loaded into the other lanes in the gel. The inner chamber was completely filled with cathode buffer and the outer chamber was filled with 200 mL of diluted 5X anode buffer. The gel was run for 40 minutes at 140 V with a constant current. The gel was then transferred to fixing solution for one hour, staining solution for one hour, destaining solution for one hour and then placed in distilled water.

2.2.2 Quantitative Methods

The Bradford assay in conjunction with UV-Vis Spectrometry was used to determine the concentration of protein in the sample.[21] Several Bradford standards were made containing different concentrations of bovine serum albumin (BSA) and 25 mM piperazine/HCl buffer having a pH of 5.5. 10 μL of the standard or protein sample was added to cuvettes containing 1 mL of the Coomassie Blue Bradford reagent. The samples were allowed to incubate for 10 minutes, and then the absorbance values were measured at 595 nm. A calibration curve was constructed from the standards and the concentration of the protein sample was determined.

The concentration of the protein sample may also be determined using a UV scan at 280 nm, which is the wavelength of absorbance of the aromatic amino acid side chains in proteins. In this technique, 1 mL of the protein sample is added to a quartz cuvette and the absorbance is measured. Dividing this value by the molar absorptivity of VCPO (91915 M cm^{-1}) and then multiplying by the molecular weight of the protein (67531 g/mol) provides additional concentration information for the sample.

The halogenating activity of the sample was tested using the monochlorodimedone (MCD) assay. The assay reagent for VCPO was prepared with 1M

8

sodium citrate buffer having a pH of 5.0, 1 M KCl, 10 mM Na_3VO_4, 5 mM MCD and 100 mM H_2O_2. The reaction of this assay is presented in the equation below.[22]

$$\text{(monochlorodimedone)} + Cl^\oplus + H_2O_2 \rightarrow \text{(dichlorodimedone)} + H_2O + OH^-$$
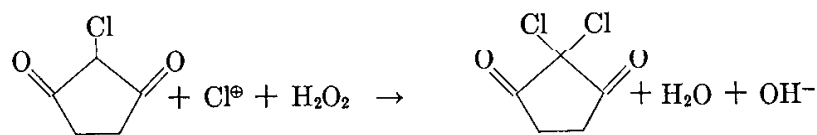
As with the phenol red assay, this reagent must be made and used on the same day. 10 μL of the protein sample was added to 1 mL of the MCD reagent and the change in absorbance was measured for 1 minute at 290 nm. In the presence of H_2O_2, the MCD is halogenated if the haloperoxidase sample is active, and the absorbance decreases from 1 to zero over the 1 minute interval. Using the slope of this graph in units/min, an extinction coefficient of 20.1 $mM^{-1}cm^{-1}$, the volume of the sample used and the concentration data from the Bradford assay in mg/mL, the specific activity of the protein sample in units/mg was calculated.

2.3 Sample Preparation for [51]V Solid-State NMR Spectroscopy

The proteins were lyophilized to produce solid-state NMR samples. Additionally, the hanging drop crystallization method was explored as a potential way to replace the crude lyophilization process. Several hanging drop crystallization screens were prepared in well plates using concentrations of 20% to 45% polyethylene glycol (PEG) and Tris buffer with varying pH values between 5.0 and 8.5. 2 μL of the solution in the well and 2 μL of the protein sample were mixed together and placed on slides above the well plates. Samples were also prepared at different pH values, including pH 6.3 and 8.3 to determine if pH has an effect on the observed NMR signals. The pH values of the samples were adjusted after the final dialysis step in the purification procedure.

The lyophilized samples were packed into 3.2 mm rotors (Varian) and stored at -20°C. Two sets of spectra of R360A at pH 6.3 were obtained at 14.1 T and 21.1 T on Varian InfinityPlus spectrometers. Spectra of the wild type protein at pH values of 6.3 and 8.3 were obtained. The solid-state spectra acquisition, the numerical simulations of the spectra in SIMPSON[23] and the extraction of the NMR parameters were performed by Kristopher Ooms and Stephanie Bolte.

Chapter 3

RESULTS

3.1 Vanadium Chloroperoxidase Purification Results

3.1.1 Sample Yields

The isolation and purification has been performed successfully on samples of VCPO wild type, R360 and R490. Yields of several expressions as quantified by the Bradford assay together with the specific activities of the samples from the MCD assay are shown in Tables 1 and 2. The Bradford assay was the primary measure of the amount of protein present while the MCD was the measure of protein activity. Yields are reported in units of milligrams and were calculated using a BSA standard calibration curve as well as the final volume of the protein sample. Activities are reported in units/mg and were calculated as described in section 2.2.2.

3.1.2 Gel Electrophoresis Results

SDS-PAGE gel electrophoresis was run on several samples throughout the purification process. In Figure 3, the gel electrophoresis results for a wild type sample expressed on January 23, 2009 and R360A sample expressed on February 27, 2009 are presented. On both gels, the first lane is the molecular marker and the subsequent lanes contain samples of wild type and R360A taken throughout the purification process.

In the wild type gel shown in Figure 3, lane 1 is the molecular marker, lane 2 is the wild type protein after the precipitation of DNA and centrifugation, lane 3 is the active

wild type fractions from the gravity column and lane 4 is the wild type after the third and final dialysis step. In the R360A gel, lane 1 is the molecular marker, lane 2 is empty, lane 3 is the sample after cell lysis, lane 4 is the sample after the second centrifugation step, lane 5 is the sample after DNA precipitation and centrifugation, lane 6 is the sample after the gravity column, lane 7 is the sample after FPLC, and lane 8 is the sample after the final dialysis step. Both gels were run at 140 V for 40 minutes.

3.2 ^{51}V NMR Spectra and Parameters

The ^{51}V solid-state NMR spectra acquired for the R360A mutant (pH 6.3) at 14.1 T and 21.1 T are presented in Figure 4. The experimental spectrum is shown in black and the simulated spectrum is shown in red. Expansions around the central transitions for the experimental and simulated data are presented at each magnetic field as well. Two samples of wild type protein were prepared at different pH values to determine the effects of pH on the observed NMR signals. Figure 5 shows the spectra obtained for wild type VCPO (pH 6.3) at 14.1 T and 21.1 T. Figure 6 displays the spectra obtained for wild type VCPO (pH 8.3) at 14.1 T and 21.1 T. NMR parameters were extracted using SIMPSON and are listed in Table 3 for R360A (pH 6.3), wild type VCPO (pH 6.3) and wild type VCPO (pH 8.3).

Chapter 4

DISCUSSION

4.1 Modifications of Sample Preparation

Protein yields of 80 mg and 100 mg per liter of media have been reported.[10,19] Many modifications to this procedure have been made in an attempt to improve protein purity and yields of the wild type, R360A and R490A sample expressions and to produce protein samples compatible with SSNMR requirements. Sodium commonly interferes with the vanadium signal in SSNMR, making it necessary to eliminate sodium sources from the procedure. Care has been taken to replace sodium with potassium in the buffers used in the purification procedure. Additionally, a bead beater with glass bead media was used to lyse the cell membranes until the summer of 2008, when it was replaced with sonication. Tris sulfate buffers were also used during this time, but they were replaced with Tris acetate buffers because there was concern that the sulfate was competing with vanadate in the active site of the protein. The wide range of protein yields produced in our hands is listed in Tables 1 and 2 (from 7-66 mg). Additionally, the specific activities of the wild type samples from Table 1 are not consistent with the reported literature value of 16 units/mg for the wild type protein.[19] These values indicate that the purification procedure did not work well in our hands. Prior reports from our laboratory and other laboratories,[10,19] including the work of Dr. Neela Pooransingh-Margolis, indicated that pure protein in high yields could be generated consistently and reproducibly.[19]

One way to generate conformationally homogeneous samples is through crystallization. To screen crystallization conditions, the hanging drop approach is commonly followed. We have attempted hanging drop crystallization, but were not successful in producing crystals of the protein samples. We hypothesize that our attempts were not successful because of insufficient protein purity. In the future, this approach will be explored again.

4.2 Gel Electrophoresis

A single, thick band on the gel near the 67 kDa region has to be observed in the pure protein sample. From the data on the wild type gel in Figure 3, it is apparent that as the purification process progresses, the wild type samples become more pure. There are fewer bands present below the 67 kDa region in lane 4 than lane 2. The sample in lane 3 has a high degree of purity as well, but there are still additional bands below the desired protein band. This indicates that further purification steps are necessary past the gravity column step. Additionally, it appears that the sample in lane 4 is pure since the desired protein band is the only band which appears on the gel in that lane.

For the R360A gel in Figure 3, a similar pattern is observed up until lane 6. It is clear that the protein samples do become more pure as the purification process continues because there are fewer bands present in lanes containing theoretically purer protein. Although there is a thick desired protein band in lane 8, there are still other bands present in the lane from the final dialysis step in the purification procedure. The presence of this band indicates that some impurities remain in the R360A sample, and these impurities may have been reintroduced into the protein sample during the FPLC and dialysis steps.

Another trivial and anticipated observation can be made. The data for the wild type and R360A gels also reveal that samples taken at the same point in the procedure for

different protein samples are similar. The sample collected after DNA precipitation and centrifugation (lane 2 on wild type and lane 5 on R360A) looks the same on both gels. Additionally, lane 3 in wild type and lane 6 in R360A both have one thicker band near the desired 67 kDa region and several very light bands further down the gel. The appearance of similar bands for samples taken at points during the procedure indicates that the current methods of DNA precipitation and anion exchange chromatography are consistent among different protein sample expressions.

4.3 ^{51}V SSNMR

The ^{51}V NMR parameters summarized in Table 3 provide insight into the local environment of the active site. The isotropic chemical shift (δ_{iso}) for R360A is -470 ± 10ppm. The values of δ_{iso} for wild type at pH 6.3 and 8.3 are -580 ± 10 ppm and -507 ± 2 ppm. The resonance position for the wild type pH 6.3 sample is more deshielded by about 100 ppm than the resonance of the R360A pH 6.3 sample. We note that we inadvertently prepared the R360A sample with high salt. This might result in formation of a chloride adduct in the active site. In the future, low salt samples of the R360A mutant will be prepared. Additionally, the pH of the sample affects the isotropic chemical shift for vanadium in the two wild type samples, suggesting that the protonation states of oxygen atoms and/or the active site amino acid residues are pH dependent.

The values of the quadrupolar coupling constant (C_Q) for the samples are different, with the value for R360A being 18.0 MHz, the highest C_Q we have detected so far. For the two wild type VCPO samples prepared at different pH values, the C_Q value of the sample prepared at the more basic pH is much smaller. From our previous work, the C_Q value for VCPO at pH 8.3 was 10.5 ± 1.5 MHz.[19] In our current experiments at 21.1 T,

$C_Q = 10.0 \pm 0.2$ MHz for the same sample, in excellent agreement with the original measurements. For the sample prepared at pH 6.3, $C_Q = 15 \pm 1$ MHz. These differences between the three samples suggest distinct protonation environments in the wild type and mutant VCPO, which are also a function of pH.

The chemical shift anisotropy can be used to characterize hydrogen bonding in the protein.[24] Our previous studies have found that for the wild type VCPO, $\delta_\sigma = -530 \pm 10$ ppm at pH 8.3. Our current work indicates that at pH 6.3 in wild type VCPO, $\delta_\sigma = -380 \pm 30$ ppm. For the R360A mutant, $\delta_\sigma = -420 \pm 10$ ppm. These are very large differences, also indicating that protonation environments in VCPO change as a function of pH and as active site residues are mutated.

These NMR parameters have been measured for the first time in VHPO. In the future, parameters for additional VHPO samples will be measured. Understanding the relationships between the experimental NMR parameters and the molecular structure of the vanadium active sites is expected to provide insights into the reaction mechanisms of halide oxidation catalyzed by VHPO.

Chapter 5

CONCLUSIONS

5.1 Conclusions

Samples of vanadium chloroperoxidase and its active site mutants, R360A and R490A, have been prepared following an isolation and purification protocol reported by Hemrika et al.[10] Despite the high level of purity according to the SDS-PAGE gel, there is a concern that vanadate is not being effectively incorporated into the active site of the protein, since the activity of the protein remains low and inconsistent between trials.

[51]V SSNMR spectra have been acquired for the wild type and R360A VCPO samples. The NMR parameters extracted from the spectra are expected to shed light on the protonation states of vanadate oxygen atoms under different conditions and bring new insights into the reaction mechanism of VHPO.

REFERENCES

1) Wittung-Stafshede, P. *Acc. Chem. Res.* **2002** *35* (4), 201-208

2) Messerschmidt, A.; Bode, W.; Cygler, M. Handbook of Metalloproteins. New York: John Wiley & Sons, Inc, 2006.

3) Thompson, K., Orvig, C. *Coord. Chem. Rev*. **2001**, *219-221*, 1033-1053.

4) McNeill, J.H.; Heyliger, C.E.; Tahiliani, A.G. *Science*.**1985,** *227*, 1474-1477.

5) Collier, R.W. *Nature.* **1984,** *309,* 441-444.

6) De Boer, E.; Wever, R. *J. Biol. Chem*. **1998,** *263* (25), 12326.

7) Butler, A. *Curr. Opin. Chem. Biol.* **1998,** *2*, 279-285.

8) Messerschmidt, A.; Wever, R. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 392-396.

9) Messerschmidt, A.; Prade, L.; Wever, R. *Biol. Chem.* **1997**, *378*, 309-315.

10) Hemrika, W.; Renirie R.; Macedo-Ribeiro, S.; Messerschmidt, A.; Wever, R. *J. Biol. Chem.* **1999**, *274* (34), 23820-23827.

11) Weyand, M.; Hecht, H. J.; Kiess, M.; Liaud, M. F.; Vilter, H.; Schomberg, D. J. *Mol. Biol.* **1999,** *293* (3), 595-611.

12) Hasan, Z.; Renirie, R.; Kerkman, R.; Ruijssenaars, H.; Hartog, A.; Wever, R. *J. Biol. Chem.* **2006,** *281* (14), 9738-9744.

13) Pooransingh, N.; Pomerantseva, E.; Ebel, M.; Jantzen, S.; Rehder, D.; Polenova, T. *Inorg. Chem.* **2003**, *42*, 1256−1266.

14) Jakobsen, H.J.; Skibsted, J.; Bildsøe, H.; Nielsen, N.C. *J. Magn. Reson.* **1989,** *85*, 173-180.

15) Abragam, A.; Boutron, F. *C. R. Hebd. Séances Acad. Sci.* **1961**, *252*, 2404.

16) Smith, M. E.; van Eck, E. R. H. *Prog. Nucl. Magn. Reson. Spectrosc.* **1999**, *34*, 159−201.

17) Schmidt-Rohr, K.; Spiess, H. W. <u>Multidimensional Solid-state NMR and Polymers.</u> Harcourt Brace & Company: London, San Diego, New York, Boston, Sydney, Tokyo, 1999.

18) Rehder, D. <u>Multinuclear NMR</u>. New York: Plenum, 1989; p. 488−493.

19) Pooransingh-Margolis, N.; Renirie, R.; Hasan, Z.; Wever, R.; Vega, A. J.; Polenova, T. *J. Am. Chem. Soc*. **2006,** *128* (15) 5190-5208.

20) Lahaye, D.; Groves, J.T. *J. Inorg. Biochem*. **2007**, *101*(11-12), 1786-1797.

21) Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254

22) Hager, L.P.; Morris, D. R.; Brown, F.S.; Eberwein, H. *J. Biol. Chem.* **1966**, *241*, 1769-1777.

23) Bak, M.; Rasmussen, J. T.; Nielsen, N. C. *J. Magn. Reson.* **2000**, *147*, 296−330.

24) Shoji, A.; Ando, S.; Kuroki, S.; Ando, I.; Webb, G. A. *Annu. Rep. NMR Spectrosc.* **1993**, *26*, 55−98.
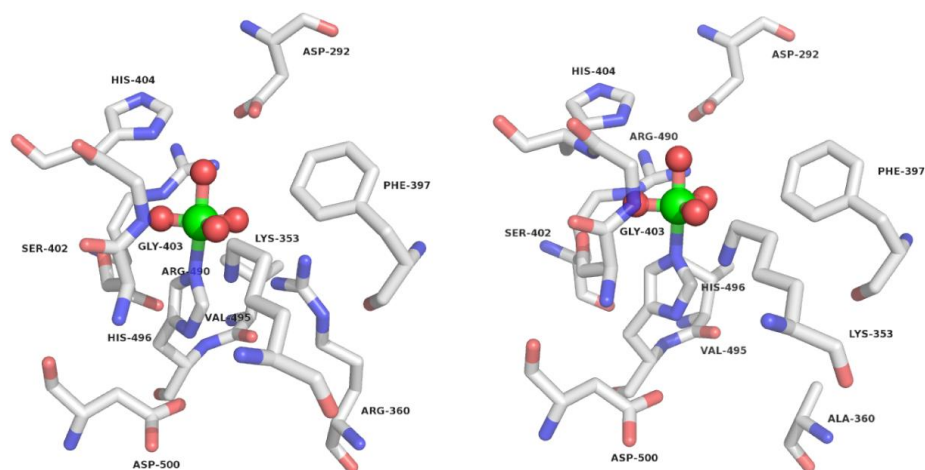
APPENDIX

Figure 1. Geometries of the vanadium chloroperoxidase active site in the wild type protein (left) and the R360A mutant (right). The figure was prepared in PyMol55 using the original PDB coordinates (1IDQ and 1VNF). (Figure from unpublished work in the Polenova laboratory)
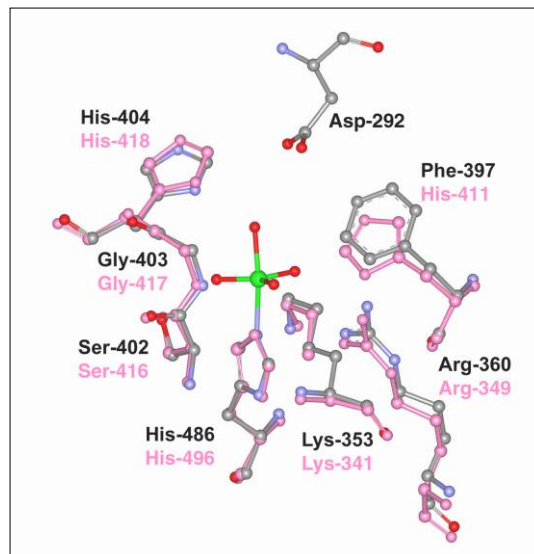
Figure 2. Active site residues for vanadium chloroperoxidase (gray) and vanadium bromoperoxidases (pink).[19]

Table 1. Results of the isolation and purification of the wild type protein. Yields were determined using the Bradford assay concentration values and the final volume of the sample. The specific activity was calculated using the results of the MCD assay and the Bradford assay.

Mutant	Date of Expression	Yield (mg)	Specific Activity (units/mg)
WT	6/1/2007	7	n/a
WT	6/13/2007	53	0.14 ± 0.021
WT	4/15/2008	12	n/a
WT	6/16/2008	19	1.3 ± 0.31
WT	10/15/2008	47	7.9 ± 0.27

Table 2. Results of the isolation and purification of R360A and R490A. Yields were
determined using the Bradford assay concentration values and the final volume of
the sample. The specific activity was calculated using the results of the MCD assay
and the Bradford assay.

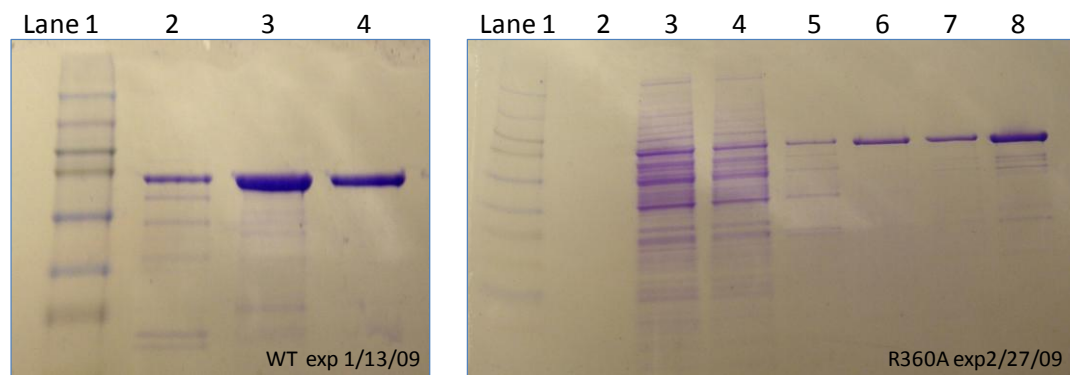| Mutant	Date of Expression	Yield (mg)	Specific Activity (units/mg)
R360A	6/28/2007	56	2.1 ± 0.049
R360A	11/3/2007	9	0.37 ± 0.16
R490A	4/30/2007	10	n/a
R490A	7/6/2007	66	2.8 ± 0.20
R490A	7/27/2007	42	n/a

Figure 3. SDS-PAGE Gel electrophoresis results for wild type (left) and R360A (right). For the wild type gel, Lane 1: Molecular Marker, Lane 2: wild type after DNA precipitation, Lane 3: wild type after FPLC, Lane 4: wild type after final dialysis. For the R360A gel: Lane 1: Molecular Marker, Lane 3: R360A after cell lysis, Lane 4: R360A after second centrifugation, Lane 5: R360A after DNA precipitation, Lane 6: R360A after gravity column, Lane 7: R360A after FPLC, Lane 8: R360A after final dialysis.
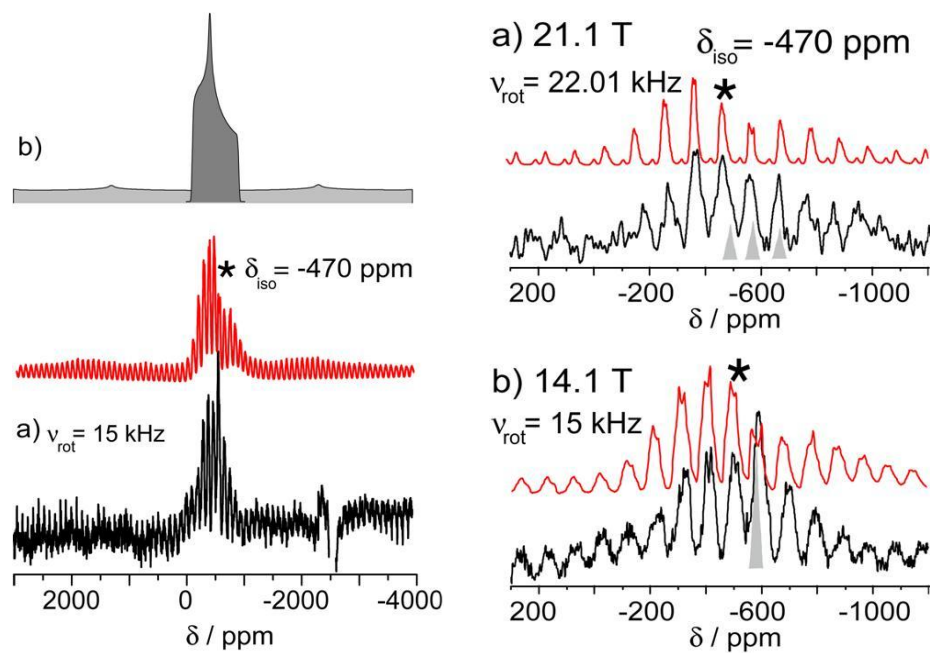
Figure 4. The ^{51}V solid-state MAS NMR spectra of 12 mg of R360A VCPO mutant protein obtained at pH 6.3. Spectra were acquired at 21.1 T and 14.1 T and are expanded to show the central transition region. Experimental spectra are shown in black and calculated spectra are shown in red.
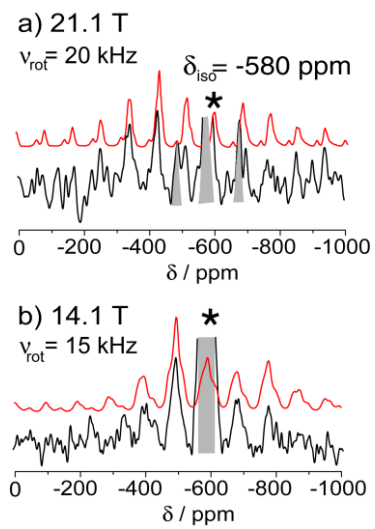
Figure 5. ^{51}V solid-state MAS NMR spectra of 11 mg of wild type VCPO protein prepared at pH 6.3 acquired at 21.1 T and 14.1 T. Experimental spectra are shown in black and calculated spectra are shown in red.
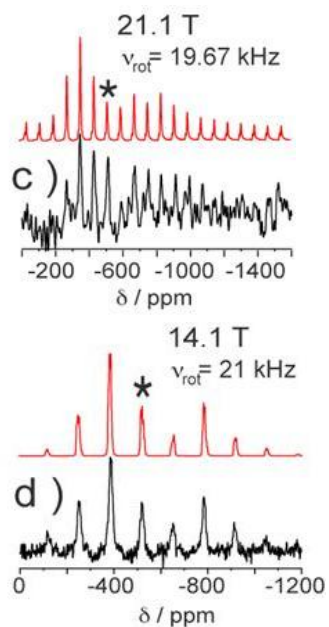
Figure 6. The [51]V solid-state MAS NMR spectra of 13 mg of wild type VCPO prepared at pH 8.3. Expansions of the experimental (black) and calculated (red) central transition region of the spectra obtained at 21.1 T and 14.1 T.

Table 3. Experimental ^{51}V solid-state NMR parameters for wild type at pH 8.3, wild type at pH 6.3 and R360A at pH 6.3. All parameters were extracted from the experimental spectra using SIMPSON software.

Parameter	WT pH 8.3	WT pH 6.3	R360A pH 6.3
δ_{iso} (ppm)	-507 ± 2	-580 ± 10	-470 ± 10
δ_{σ} (ppm)	-530 ± 10	-380 ± 30	-420 ± 10
η_{σ}	0.1 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
C_Q (MHz)	10.0 ± 0.2	15 ± 1	18 ± 1
η_Q	0.5 ± 0.05	0.8 ± 0.3	0.5 ± 0.1