was obtained on warming the sample to 175 K. This spectrum has the same doublet and singlet features that were observed with a C_{60}/C_{70} mixture, confirming that the spectral features discussed above are due to the interaction of Ag atoms with C_{60} rather than any other carbon cluster present in the mixed sample.

Reaction of natural silver (51% ^{107}Ag and 49% ^{109}Ag) with C_{60} in cyclohexane at 77 K gave the EPR spectrum shown in Figure 3a and has two isotropic doublets from trapped Ag atoms with a_{109} = 1947 ± 5 MHz and g = 2.0017 ± 0.0005 and a_{107} = 1690 ± 5 MHz and g = 2.0017 ± 0.0005 as well as a complex central feature. Each of the lines of the Ag doublets had satellites that were attributed to forbidden proton "spin-flip" lines from simultaneous flipping of the unpaired electron and the protons of the cyclohexane matrix.[17] On warming this sample to 165 K two doublets became apparent with the parameters a_{109} = 263 ± 5 MHz and g = 1.9913 ± 0.0005 and a_{107} = 228 ± 5 MHz and g = 1.9913 ± 0.0005 (Figure 3b). The ratio of these hfi, 1.154, was close to the ratio of the magnetic moments of ^{109}Ag and ^{107}Ag, and they were attributed to the formation of species A, AgC_{60}, from natural Ag atoms. In addition the lines corresponding to species B and C were also present.

All the spectral lines assigned to AgC_{60} disappeared in the temperature range 175–185 K, and all samples when warmed to 280 K gave identical EPR spectra consisting of a single isotropic line with g = 2.0023 ± 0.0002.

As a result of the successful preparation of AgC_{60} by low-temperature matrix-isolation techniques we are investigating the reaction of C_{60} with other metal atoms in inert hydrocarbon matrices on a rotating cryostat. Preliminary results suggest that Au atoms give an EPR spectrum that can be assigned to AuC_{60} while Cu atoms do not appear to give CuC_{60}. The results of these investigations will be published in a subsequent paper.

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Vanadate Interactions with Bovine Cu,Zn-Superoxide Dismutase As Probed by ^{51}V NMR Spectroscopy

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Abstract: The interaction of various vanadate anions with native and chemically modified derivatives of bovine Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was studied by ^{51}V NMR spectroscopy to obtain information on the substrate selectivity of Cu,Zn-SOD. Using quantitative ^{51}V NMR measurements and speciation analysis, we determined the concentrations of the rapidly equilibrating vanadate derivatives that form in solutions containing Cu,Zn-SOD. As the concentration of native Cu,Zn-SOD increased, the total visible vanadium concentration decreased and the intensity of a broad signal assigned to a vanadate–protein (V-SOD) complex increased. Because of the quadrupolar nature of the ^{51}V nucleus, most of the bound vanadium was invisible and only 15–30% of the V-SOD complex was visible in the ^{51}V NMR spectra. Significant changes in the line widths of the ^{51}V NMR resonances of vanadate anions were observed. Several quantitative models were applied to the intensity changes to determine which anions formed V-SOD complexes. Native Cu,Zn-SOD bound four vanadium atoms per subunit (or eight vanadium atoms per enzyme molecule). Bovine Cu,Zn-SOD has the greatest affinity for the vanadate tetramer and forms two specific complexes, V_4·SOD and $(V_4)_2$·SOD. The affinity constants for these vanadate–protein complexes are 2×10^7 M^{-1} and 5×10^6 M^{-1}, respectively. The affinities of the vanadate dimer and monomer for native Cu,Zn-SOD were less than that of the vanadate tetramer but more than that of phosphate. Addition of vanadate to native Cu,Zn-SOD was also monitored by difference UV/vis spectroscopy. No changes in absorbance maxima were observed, indicating that vanadate does not bind directly to Cu(II) in the enzyme. When Cu,Zn-SOD was modified at arginine-141, the vanadate tetramer still bound to the enzyme with drastically reduced affinity; much weaker interactions were observed between the vanadate tetramer and Cu,Zn-SOD modified at lysines-120 and -134. Our data suggest that the vanadate tetramer binds to the two lysine residues in the solvent channel of Cu,Zn-SOD. The vanadate tetramer is the first large anion that is reported to have such a high affinity for native bovine Cu,Zn-SOD.

Introduction

Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is an abundant protein in most eukaryotic cells, with high water solubility and thermal stability.[2] Apo-SOD (the metal-free protein) can bind several metal ions, in place of the native Cu^{2+} and Zn^{2+} ions, in distorted square-planar and tetrahedral configurations.[3,4] Despite extensive biophysical studies of Cu,Zn-SOD, the relationship between its physical properties and its physiological role in vivo is not well understood.[5,6] The proposed enzymatic function of Cu,Zn-SOD is based on the observation that this protein catalyzes superoxide disproportionation.[6]

The putative substrate of Cu,Zn-SOD, superoxide, is an anion. Anion-binding studies of Cu,Zn-SOD have provided key infor-

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mation as to how superoxide interacts with this metalloprotein. Moreover, the specificity of Cu,Zn-SOD for various anions may be related to alternative or additional functions for this metalloprotein. EPR and optical spectroscopy data[2] have indicated that small anions such as CN^-, N_3^-, and halides bind to Cu^{2+} at the active site of oxidized Cu,Zn-SOD. These small anions bind in an equatorial position after displacing one of the histidyl imidazole copper ligands. Positive charges on the amino acid side chains in the solvent channel of native Cu,Zn-SOD play an important role in facilitating anion binding to the protein and in guiding superoxide to the copper site. These conclusions were based on the sensitivity of SOD activity[7,8] and of anion-binding affinity[8-10] to ionic strength. X-ray diffraction studies indicated that the positively charged side chains of Arg-141, Lys-120, and Lys-134 are located 5, 12, and 13 Å away from the copper site.[11,12] The binding affinities of CN^- and N_3^- for the native (oxidized) enzyme were decreased upon chemical modification of the arginine[8,9] or lysine[10] residues. It is therefore believed that these residues, in addition to the Cu^{2+} ion, provide anion-binding sites.

Phosphate binds to Cu,Zn-SOD in a site other than the Cu^{2+} site.[8,13] This conclusion is based on the observation that the visible and EPR spectra due to the Cu^{2+} ion in the protein were unchanged at high phosphate concentrations. ^{31}P NMR relaxation studies conducted on both native and arginine-modified Cu,Zn-SODs[13] showed that phosphate binds to the native protein via the guanidinium group of Arg-141.

Vanadate is a strong inhibitor of several phosphate-dependent enzymes, including phosphatases,[14] ATPases,[15] and ribonucleases.[16] The reactivity and biological activities of vanadium are likely to depend on the active vanadium derivatives. The vanadate anion responsible for inhibition of phosphatases and ATPases is usually monomeric vanadate.[14,17,18] However, recent reports have provided evidence that vanadate oligomers including the dimer, tetramer, and decamer have biological activity and bind specifically to various proteins.[18-25] Although vanadate solutions contain many vanadate anions, only one or two of these interact with proteins. The dimer, for example, interacts with the acid phosphatase in human seminal fluid,[18] with glucose-6-phosphate dehydrogenase,[19] and with glycerol-3-phosphate dehydrogenase.[20] The tetramer appears to interact with the enzymes 6-phosphogluconate dehydrogenase,[21] glucose-6-phosphate dehydrogenase,[19] and glycerol-3-phosphate dehydrogenase.[20] The tetramer may also be the vanadate species responsible for the photoactivated cleavage of the flagellar ATPase dynein[22] and of myosin subfragment 1.[23] The decamer has been found both to inhibit enzymes, including hexokinase[24] and phosphofructokinase,[24] and to bind to Ca^{2+}-ATPase.[25]

Because phosphate binds to Cu,Zn-SOD[8,13] and since monomeric vanadate is a structural analogue of phosphate, we investigated and report here the results of vanadate binding to Cu,Zn-SOD. We studied the specific interactions between the enzyme and vanadate and found that the vanadate-binding sites are not the same as those for phosphate. Aqueous vanadate solutions contain several vanadate anions that differ in vanadium content, shape, and charge, and each anion is likely to have different affinities for Cu,Zn-SOD. We used ^{51}V NMR spectroscopy to examine the interactions of bovine Cu,Zn-SOD with solutions of vanadate containing the monomer, dimer, and tetramer. Because ^{51}V has a quadrupolar nucleus with a nuclear spin of $^7/_2$, ^{51}V NMR studies of vanadate interactions with proteins require special experimental precautions[25-27] and careful speciation analysis of rapidly equilibrating vanadate species.[18-21,28,29] We describe the interactions of the oxidized forms of native and chemically modified bovine Cu,Zn-SOD with vanadate anions. The vanadate tetramer is the first large anion found to show high affinity for native bovine Cu,Zn-SOD.

Experimental Section

Chemicals. Bovine liver Cu,Zn-SOD was purchased as a lyophilized powder from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, succinic anhydride, xanthine, xanthine oxidase (grade I), horse heart cytochrome *c* (type III), and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were supplied by Sigma. Potassium phosphate dibasic trihydrate, ammonium vanadate, and $VOCl_3$ were purchased from Aldrich. Deuterium oxide (99.8%) was obtained from Cambridge Isotope Laboratories. All of these chemicals were used without further purification.

Modification of Proteins, Enzyme Activity, and Protein Concentration. Bovine Cu,Zn-SOD was modified chemically at Arg-141 with phenylglyoxal as previously described.[30] Lysine residues were succinylated with succinic anhydride.[31] The arginine- and lysine-modified proteins were purified by dialysis against distilled water and lyophilized. The SOD activities of the modified protein derivatives were approximately 15% of that of native protein, as measured by the xanthine oxidase/cytochrome *c* assay.[32] It was previously shown by using polyacrylamide gel electrophoresis[7,31] that arginine- and lysine-modified proteins with approximately 15% residual SOD activity exhibit only one band. No residual bands in polyacrylamide gel slabs stained for SOD activity corresponding to native protein were observed.[7,31] Thus, the modified protein samples in our study were not contaminated with native protein and the residual 15% SOD activity was inherent to the fully modified proteins in agreement with previous reports.[7,30,31] Protein concentrations were determined by measurement of the absorbances at 680 nm (ϵ = 300 M^{-1} cm^{-1}) for the native and lysine-modified proteins or at 660 nm (ϵ = 220 M^{-1} cm^{-1}) for the arginine-modified protein. Protein concentrations (based on molecular weights of 31 200, 31 500, and 32 800 for native, arginine-modified, and lysine-modified proteins, respectively) were also determined by weighing the lyophilized powders to be dissolved. The two methods generated protein concentrations that agreed within 5%.

Preparation of NMR Samples. A stock solution of 0.010 M ammonium vanadate was prepared and diluted to a final concentration of 2.0 mM. Cu,Zn-SOD was added in powder form to 2.0 mM vanadate solutions, resulting in the enzyme concentrations reported in the titration studies of native and chemically modified proteins (Figures 1 and 4). Samples were buffered with 0.10 M HEPES, 20 or 60 mM phosphate, pH 7.4. Measurements of pH were made with an Orion pH meter. The pH values quoted were not corrected for deuterium isotope effects. NMR samples contained 15% (v/v) D_2O.

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UV/Vis Measurements. Difference UV/vis spectra were recorded on a IBM 9420 spectrophotometer using tandem cuvettes with solutions containing 0.20 mM native Cu,Zn-SOD and vanadate (2.0–20 mM) in 0.10 M HEPES, pH 7.4.

NMR Spectroscopy. ^{51}V and ^{31}P NMR measurements were conducted at 79.0 and 121.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer (Loyola University). ^{51}V NMR measurements were also carried out at 131.5 MHz on a Bruker AM-500 NMR spectrometer (Colorado State University). The samples were placed in 10-mm NMR tubes. The NMR measurements were obtained at the ambient probe temperature (23 ± 1 °C) unless noted otherwise.

For quantitative ^{51}V measurements, the sweep width was 72993 Hz, the accumulation time 0.15 s, the pulse angle 90°, the number of transients 2000, and the relaxation delay 1.6 s. Additional parameters, including spectral intensity, phasing, and integration parameters, were kept constant. ^{51}V chemical shifts are reported relative to an external reference of $VOCl_3$ (0 ppm). The reported ^{51}V line widths represent the widths of the resonances at half-height (in Hz) after subtraction of 25 Hz used in line broadening. Relative peak areas of ^{51}V signals were obtained by means of the integration routines included in the software provided by the manufacturers for the Varian VXR-300 and the Bruker AM-500 NMR spectrometers.

^{51}V spin–lattice (T_1) relaxation times were measured on the Bruker AM-500 NMR spectrometer by using the modified Freeman–Hill procedure described previously.[28,29,33] ^{31}P T_1 relaxation times were measured on the Varian VXR-300 NMR spectrometer using the inversion recovery pulse sequence.[13]

Data Analysis. The concentrations of various vanadate derivatives, including the monomer at −559 ppm, the dimer at −574 ppm, the tetramer at −580 ppm, and the pentamer at −589 ppm, as well as the presumed V-SOD signal (a wide peak overlapping the dimer and tetramer), were calculated by integration of the 1D ^{51}V NMR spectra. We used control samples (prepared in triplicate) containing a known total vanadate concentration (without enzyme) to quantify the observable vanadium species by integrating the vanadate anion resonances. From the mole fraction of each species in the control samples and from the known total vanadate concentration, we calculated the concentrations of the individual anions. The control samples also allowed us to quantify the samples to which protein was added. By using the settings defined by the control samples, we were able to calculate the extent of disappearance of the vanadium signal in the protein–vanadate samples. The total integration of the protein–vanadate sample was compared to the total integration of the vanadate control samples and yielded the total observable vanadate concentration in the former sample. The concentration of each vanadate derivative was then calculated from the mole fraction of each vanadate derivative in the protein–vanadate sample and from its total observable vanadium content.

Aqueous solutions of vanadate contain various concentrations of vanadate monomer, dimer, tetramer, and pentamer, depending on the overall vanadium concentration, pH, ionic strength, and temperature.[17,34,35] Because the vanadate anions in solution are at equilibrium, the equilibria are described by eqs 1–4.[17,34,35] Consequently, the concentrations of the vanadate oligomers (dimer and tetramer) are related to the concentration of monomeric vanadate as described by eqs 5 and 6.

$$2V_1 \underset{}{\overset{K_{12}}{\rightleftharpoons}} V_2 \quad (1)$$

$$4V_1 \overset{K_{14}}{\rightleftharpoons} V_4 \quad (2)$$

$$5V_1 \overset{K_{15}}{\rightleftharpoons} V_5 \quad (3)$$

$$2V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (4)$$

$$[V_2] = K_{12}[V_1]^2 \quad (5)$$

$$[V_4] = K_{14}[V_1]^4 \quad (6)$$

Using the oligomer concentrations determined for the control samples (see above), we calculated K_{12} to be 270 M^{-1} and K_{14} to be 6.5×10^8 M^{-3}. From these K_{12} and K_{14} values, the expected concentrations of the dimer and tetramer can be calculated on the basis of the observed monomer concentrations, as shown in eqs 5 and 6. In samples containing both vanadate and Cu,Zn-SOD, vanadate anions (V_x) interact with the enzyme, and equilibria as shown in eq 7 are established.

$$nV_x + \text{SOD} \rightleftharpoons (V_x)_n\text{·SOD} \quad (7)$$

In the equilibrium stated in eq 7, x can be 1, 2, 4, or 5, and n is the number of vanadate-binding sites on the enzyme. For the pH range and vanadate concentrations used in this investigation, we found no evidence using ^{51}V NMR spectroscopy for the presence of a trimer ($x = 3$). The absence of observable trimer at 2.0 mM vanadate is consistent with previous reports[34] and prevents the analysis of trimer binding to protein because the appropriate affinity constant cannot be measured under these conditions. Because of the quadrupolar nature of the vanadium nucleus and the large molecular weight of Cu,Zn-SOD, the resonance for the $(V_x)_n$·SOD species is observable only at reduced intensity by using ^{51}V NMR spectroscopy.[25-27] The total concentration of the protein-bound vanadate species (V-SOD) therefore will include the observable $(V_x)_n$·SOD species and the vanadium species that is no longer visible by ^{51}V NMR spectroscopy.

Errors. The concentrations of the vanadate anions calculated from 1D ^{51}V NMR spectra are reproducible within 2–3%. The overall concentration of total observable vanadate, and thus the concentration of invisible vanadate–protein complex, are accurate within 10–15%. The concentrations of vanadate oligomers in solutions containing high concentrations of Cu,Zn-SOD are determined with less precision than are those of the control samples, because the vanadate dimeric and tetrameric signals are not resolved from each other and from the observable $(V_x)_n$·SOD signal. Therefore, we used the vanadate monomer resonance to calculate the concentrations of both the dimer and tetramer, as well as the expected difference between the calculated and observed concentrations of vanadate oligomers. We estimate that the concentration of the calculated $(V_x)_n$·SOD derivative is accurate with about 30% uncertainty.

Table I. Chemical Shifts and Line Widths of Monomeric (V_1), Dimeric (V_2), and Tetrameric (V_4) Forms of Vanadate in the Presence of Native Cu,Zn-SOD in 0.10 M HEPES, pH 7.4, at 23 ± 1 °C[a,b]

	V_1	V_2	V_4
	No SOD		
δ/ppm	−559	−574	−580
$\Delta\nu_{1/2}$/Hz	96	110	99
	0.030 mM Native SOD		
δ/ppm	−559	−574	−579
$\Delta\nu_{1/2}$/Hz	105	188	202
	0.060 mM Native SOD		
δ/ppm	−560	−574	−579
$\Delta\nu_{1/2}$/Hz	120	193	344
	0.10 mM Native SOD		
δ/ppm	−559	−574	−579
$\Delta\nu_{1/2}$/Hz	122	200[c]	410[c]
	0.13 mM Native SOD		
δ/ppm	−559	−573	−579
$\Delta\nu_{1/2}$/Hz	157	250[c]	525[c]
	0.16 mM Native SOD		
δ/ppm	−559	−574	−579
$\Delta\nu_{1/2}$/Hz	190	269[c]	650[c]
	0.20 mM Native SOD		
δ/ppm	−559	−574	−579
$\Delta\nu_{1/2}$/Hz	247	400[c]	1675[c]

[a] Experimental conditions for recording the spectra are described in the Experimental Section. [b] The reported line widths represent the widths of the resonances at half-height after subtracting 25 Hz in line broadening. We estimate that the line widths are at least accurate within 10 Hz. [c] The dimeric and tetrameric resonances of vanadate were not baseline resolved. The line widths reported at these protein concentrations were estimated by deconvolution of the total peak into two resonances corresponding to the dimeric and tetrameric resonances. For partially resolved resonances, we estimate that the line widths are accurate within 30 Hz.

Results

Interactions of Vanadate with Native Cu,Zn-SOD. Addition of native bovine Cu,Zn-SOD (in the concentration range 0.030–0.50 mM) to 2.0 mM vanadate in 0.10 M HEPES, pH 7.4,

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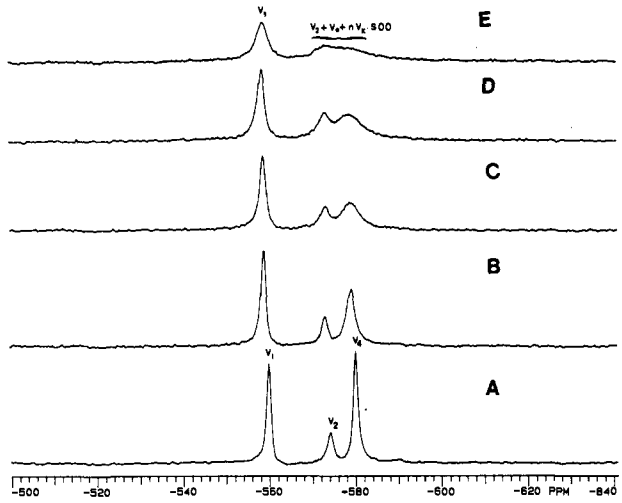

Figure 1. 79.0-MHz ^{51}V NMR spectra of 2.0 mM vanadate in 0.10 M HEPES, pH 7.4, at 23 °C in the absence (A) and in the presence of 0.030 (B), 0.10 (C), 0.20 (D), and 0.50 mM (E) native bovine Cu,Zn-SOD.

Table II. Concentrations (mM) of Vanadate Species in the Presence of Increasing Concentrations of Cu,Zn-SOD at pH 7.4 and 23 °C in 0.10 M HEPES Containing 2.0 mM Total Vanadium[a,b]

[SOD]	$[V_1]$	$[V_2]$	$[V_4]$	$[V_x\text{-SOD}]$	$[V_x\text{-SOD}]_{vis}$
0.000	0.762	0.153	0.233	0.000	0.000
0.030	0.703	0.130	0.165	0.377	0.057
0.060	0.685	0.123	0.153	0.457	0.047
0.10	0.608	0.097	0.090	0.838	0.144
0.13	0.513	0.069	0.045	1.17	0.281
0.15	0.497	0.065	0.040	1.21	0.284
0.20	0.437	0.050	0.024	1.37	0.339
0.50	0.360	0.034	0.011	1.53	0.286

[a] $[V_1]$ is calculated from integrations in the ^{51}V NMR spectra. We estimate the uncertainty on this concentration to be below 10%. $[V_2]$ and $[V_4]$ are calculated as described in the Experimental Section, and $[V_x\text{-SOD}]_{vis}$ is calculated as the difference between the observed intensity from −574 to −580 ppm and the calculated concentrations of dimer and tetramer. $[V_x\text{-SOD}]$ is determined as $[V_{tot}] - [V_1]_{obs} - 2[V_2]_{calc} - 4[V_4]_{calc}$ and expresses the total concentration of V atoms in the protein–vanadate complex. $[V_x\text{-SOD}]_{invis}$ can be calculated as $[V_x\text{-SOD}] - [V_x\text{-SOD}]_{vis}$. [b] Although the accuracy of the measurements only warrants two significant figures, we report concentrations in some cases to three decimal places so that the readers may reproduce our calculations of vanadate concentrations and affinity constants.

at 23 ± 1 °C caused significant decreases in the intensities of the dimeric and tetrameric vanadate ^{51}V NMR resonances. Much smaller changes were observed for the monomeric resonance (Figure 1). The chemical shifts of the vanadate monomer, dimer, and tetramer did not change significantly upon addition of native Cu,Zn-SOD (Table I). Addition of 0.030–0.10 mM native Cu,Zn-SOD to 2.0 mM vanadate yielded significant increases (from 202 to 410 Hz) in the line width of the vanadate tetramer resonance (Table I). Much smaller increases were observed for the line widths of the monomer and dimer resonances in this range of concentrations of Cu,Zn-SOD. Table II shows the concentrations of vanadate derivatives at various Cu,Zn-SOD concentrations.

The ^{51}V T_1 values for the monomer, dimer, and tetramer species for 2.0 mM vanadate in 0.10 M HEPES, pH 7.4, were 14, 13, and 5.4 ms, respectively. Upon addition of 0.030 mM native Cu,Zn-SOD, the T_1 values for the monomer, dimer, and tetramer vanadate anions were 14, 13, and 5.4 ms, respectively. Thus, no changes in the spin–lattice (T_1) relaxation times were observed for the monomer, dimer, or tetramer vanadate upon addition of native Cu,Zn-SOD. The viscosity of 2.0 mM vanadate in 0.1 M HEPES, pH 7.4, was measured with a Brookfield Cone Plate viscometer and found to be 1.2 cP. The viscosity of the 2.0 mM vanadate solution did not change significantly upon addition of native Cu,Zn-SOD (up to 0.15 mM). The observed line width changes in the presence of native Cu,Zn-SOD are therefore consistent with changes in the kinetics between vanadate derivatives.

To confirm that free vanadate anions in solution were in rapid exchange with the V-SOD complex, we recorded low-temperature ^{51}V NMR spectra. When the temperature was decreased from 23 to 4 °C, the line width of the monomeric resonance of a 2.0 mM vanadate control solution (no SOD) increased from 96 to 127 Hz; changes for the dimer and tetramer resonances were within the experimental uncertainty. These results suggest that indeed the vanadate monomer is in rapid exchange with the higher oligomers, as observed previously,[28] although part of these line width changes are caused by changes in the T_1 values at the two temperatures. In the presence of 0.030 mM native Cu,Zn-SOD, a similar drop in temperature caused increases of approximately 13, 41, and 150 Hz in the line widths of the vanadate monomer, dimer, and tetramer resonances, respectively. Thus, at 4 °C the line widths of the vanadate tetramer and dimer in the presence of native Cu,Zn-SOD were broader than those observed at 23 ± 1 °C for the vanadate–SOD sample or those at 4 ± 1 °C for the vanadate control samples. These observations are consistent with the interpretation that at 4 °C the vanadate tetramer and dimer could be in exchange with a protein–vanadate complex.

The ^{51}V NMR spectra (Figure 1) of samples containing 2.0 mM vanadate and higher native Cu,Zn-SOD concentrations (0.13–0.20 mM) showed that the line widths of both the tetrameric and the dimeric vanadate resonances become considerably wider (Table I). In contrast, a significantly smaller increase in the line width of the monomer resonance was observed. The vanadate monomer appeared to interact with native Cu,Zn-SOD only at protein concentrations ≥0.20 mM.

The changes in intensities and line widths of the ^{51}V resonances of the vanadate tetramer and dimer were not observed when Cu,Zn-SOD was absent in the solutions. Inactivation of Cu,Zn-SOD by either denaturation[36] or removal of metal ions[37] led to protein derivatives that are significantly different from the native protein on several counts, including structure and anion affinity. We found that addition of apo or zinc-only derivatives to vanadate solutions caused smaller changes in intensities and line widths of the ^{51}V resonances of the vanadate tetramer and dimer than equivalent concentrations of native Cu,Zn-SOD (unpublished observations). Thus, addition of metal-depleted protein derivatives to vanadate solutions does not verify the observations we have made with native Cu,Zn-SOD. We have however examined chemically modified holoproteins and found that they can interact with vanadate as described below. We conclude that the observed changes in intensities and line widths of the ^{51}V NMR resonances of vanadate solutions are caused by the presence of native Cu,Zn-SOD.

The concentrations of the vanadate monomer (V_1), dimer (V_2), and tetramer (V_4) were measured by integration of the ^{51}V NMR spectra. The calculated concentrations of V_2 and V_4 were also estimated from the observed concentration of V_1 according to eqs 5 and 6 (see Experimental Section). Because the calculated concentrations of V_2 and V_4 were smaller than the observed concentrations at −574 and −580 ppm (corresponding to V_2 and V_4, respectively), it is likely that visible V-SOD complexes are observed in the ^{51}V NMR spectra (see Figures 1D,E, and 4B). The total intensity of these visible V-SOD complexes is about 15–30% of the intensity of V-SOD complexes expected as determined by the disappearance of observable vanadium atoms as vanadate oligomers. Thus, the total concentration of protein-bound vanadate will include both the visible V-SOD signal (from −574 to −580 ppm) and an invisible portion (see Table II).

The number of V atoms bound to Cu,Zn-SOD was determined by measuring the concentrations of total V–SOD complexes at a constant vanadate concentration and varying protein concentrations. The total concentration of V-SOD complexes, including

(36) Roe, J. A.; Butler, A.; Scholler, D. M.; Valentine, J. S.; Marky, L.; Breslauer, K. J. *Biochemistry* **1988**, *27*, 950.
(37) Lippard, S. J.; Burger, A. R.; Ugurbil, K.; Pantoliano, M. W.; Valentine, J. S. *Biochemistry* **1977**, *16*, 1136.

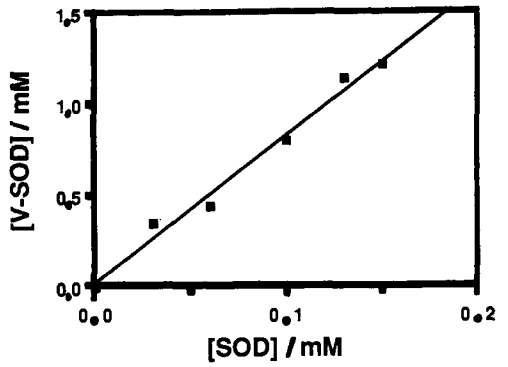

Figure 2. Total vanadate–protein concentrations (the sum of observable and invisible vanadate–protein complexes obtained by ^{51}V NMR spectroscopy) plotted against the total concentration of native Cu,Zn-SOD. The slope of the line reflects the number of vanadium atoms associated with the native dimeric Cu,Zn-SOD enzyme.

the concentrations of visible $(V_x)_n$·SOD and invisible V_x·SOD, was linearly proportional to Cu,Zn-SOD concentration (Figure 2). The slope of the curve (=8) represents the number of vanadium atoms bound to each SOD molecule. Thus, we conclude that native Cu,Zn-SOD has eight vanadium atoms associated with each enzyme molecule. Since bovine Cu,Zn-SOD is a dimer with two equivalent subunits,[38] we presume that four vanadium atoms are associated with each subunit. At protein concentrations of 0.15 mM or higher, vanadate cannot saturate the enzyme, probably because under these conditions vanadate oligomer concentrations are very low. At low Cu,Zn-SOD concentrations and 2.0 mM vanadate concentration, only the line width of the vanadate tetramer resonance increases as the protein concentration increases. From these observations, we expect that one vanadate tetramer or two vanadate dimers interact with native Cu,Zn-SOD at low enzyme concentrations (≤0.10 mM) and 2.0 mM vanadate.

Using the approach described by Gresser and Tracey[39] for phosphoglycerate mutase and in general terms by Segel,[40] we determined the nature and number of vanadate anions in the V-SOD complex under conditions of varying vanadate concentrations and constant protein concentration. Using this data analysis, it is possible to examine which vanadate derivatives bind to native Cu,Zn-SOD. We will first discuss this analysis when V_4 binds. Then we will show the implications of binding V_2 and V_1. The results of all these analyses will then be compared with the experimental data shown in Figure 2, which is based on a different approach to study this problem. A series of ^{51}V NMR spectra were recorded for solutions containing 0.0157 mM native Cu,Zn-SOD in 0.10 M HEPES at pH 7.4 and 23 ± 1 °C and 0.050–0.60 mM total vanadate concentration. The concentrations of the vanadate derivatives measured in this series of experiments are shown in Table III. A plot of total V-SOD complex concentration as a function of vanadate monomer concentration is sigmoidal, suggesting that more than one vanadate binds to Cu,Zn-SOD. A double-reciprocal plot of the monomer data shown in Table III is nonlinear. However, a plot of $1/[\text{V-SOD}]$ as a function of $1/[V_1]^4$ is linear (Figure 3). From the vertical intercept in Figure 3, the limiting stoichiometry of eight V atoms per molecule of Cu,Zn-SOD was derived. These results confirm that the vanadate tetramer binds to Cu,Zn-SOD and that two tetramers bind per Cu,Zn-SOD molecule, as suggested previously by the results shown in Figure 2. A simple model to rationalize the linear plot shown in Figure 3 involves noncooperative binding of V_4 to each of the two subunits of Cu,Zn-SOD, as shown in eq 8.

$$\text{SOD} + 2V_4 \overset{K_1}{\rightleftharpoons} V_4\text{·SOD} + V_4 \overset{K_2}{\rightleftharpoons} (V_4)_2\text{·SOD} \qquad (8)$$

(38) Ming, L.-J.; Banci, L.; Luchinat, C.; Bertini, I.; Valentine, J. S. *Inorg. Chem.* **1988**, *27*, 728.
(39) Stankiewicz, P. J.; Gresser, M. J.; Tracey, A. S.; Hass, L. F. *Biochemistry* **1987**, *26*, 1264.
(40) Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, 1975.

Table III. Concentrations (mM) of Vanadate Species in the Presence of 0.0157 mM Cu,Zn-SOD at pH 7.4 and 23 °C in 0.10 M HEPES Containing Varying Total Vanadium Concentrations[a,b]

$[V_{tot}]$	$[V_1]$	$[V_2]$	$[V_4]$	$[V_x\text{·SOD}]$
0.050	0.0450	0.001	0.000	0.005
0.075	0.0648	0.001	0.000	0.008
0.100	0.0790	0.001	0.000	0.019
0.125	0.0930	0.002	0.000	0.030
0.175	0.123	0.003	0.000	0.046
0.225	0.153	0.0044	0.000	0.063
0.300	0.190	0.0076	0.000	0.095
0.325	0.205	0.0095	0.000	0.101
0.350	0.217	0.0125	0.000	0.108
0.375	0.236	0.0131	0.000	0.112
0.400	0.253	0.0165	0.000	0.114
0.425	0.270	0.0185	0.000	0.118
0.450	0.285	0.0221	0.000	0.121
0.500	0.302	0.0245	0.0056	0.126
0.550	0.330	0.0320	0.0080	0.122
0.600	0.360	0.0375	0.010	0.123

[a] $[V_1]$ is calculated from the integrations in the ^{51}V NMR spectra. We estimate that the uncertainty in the $[V_1]$ concentrations is below 10%. Since the visible V-SOD complex resonance overlaps with the V_2 and V_4 resonances, we have used $[V_1]$ to calculate $[V_2]$ and $[V_4]$. $[V_x\text{·SOD}]$ is calculated as $[V_{tot}] - [V_1] - 2[V_2]_{calc} - 4[V_4]_{calc}$ and expresses the total concentration of V atoms in the protein–vanadate complex. [b] Although the accuracy of the measurements only warrants two significant figures, we report concentrations to three decimal places so that the readers may reproduce our calculations of vanadate concentrations and affinity constants.

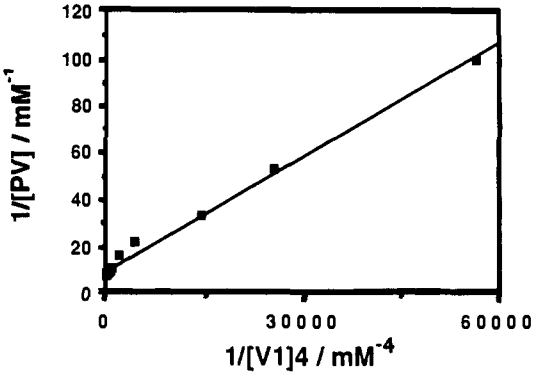

Figure 3. Double-reciprocal plot of the concentration of total vanadate–protein complex against the fourth power of the concentration of vanadate monomer (data from Table III).

Equations 9 and 10 define the two V-SOD complex dissociation constants K_1 and K_2.

$$K_1 = \frac{[\text{SOD}]_f[V_4]}{[V_4\text{·SOD}]} \qquad (9)$$

$$K_2 = \frac{[V_4\text{·SOD}][V_4]}{[(V_4)_2\text{·SOD}]} \qquad (10)$$

From the slope and the vertical intercept in Figure 3, an intrinsic dissociation constant of 1×10^{-7} M and the apparent number of 8 V atoms bound to a native Cu,Zn-SOD molecule were obtained. The dissociation constant corresponds to values for the experimental dissociation constants K_1 and K_2 of 0.5×10^{-7} M and 2×10^{-7} M, respectively. These values differ from each other because of the statistical factor deriving from the occurrence of two identical binding sites.

If the majority of the V-SOD complex is assumed to exist in the form of $(V_4)_2$·SOD, we can calculate the formation constant $K_{(V_4)_2\text{·SOD}}$ directly from the ^{51}V NMR spectra. $K_{(V_4)_2\text{·SOD}}$ is defined by (11). Such an assumption allows us to explore the possibility

$$K_{(V_4)_2\text{·SOD}} = \frac{[(V_4)_2\text{·SOD}]}{[\text{SOD}]_f[V_4]^2} \qquad (11)$$

for a cooperative mode of vanadate binding. At a Cu,Zn-SOD protein concentration of 0.060 mM, the vanadate tetramer concentration was 0.153 mM, the vanadate–protein complex concentration (expressed as V atoms of $[(V_4)_2$·SOD]) was 0.457 mM, and the free Cu,Zn-SOD concentration ($[SOD]_f$) was calculated to be 0.0029 mM. These values yield a $K_{(V_4)_2SOD}$ of 8×10^8 M^{-2}. If indeed most of the V-SOD complex was in the form $(V_4)_2$·SOD, the $K_{(V_4)_2SOD}$ would approximate $1/(K_1K_2)$. Since there is a large difference (on the order of 10^5) between these constants, the cooperative description implied by eq 11 is not the proper model. We conclude that the tetramer is more likely to bind in a noncooperative manner to native Cu,Zn-SOD.

At 0.15 mM total Cu,Zn-SOD concentration, both the dimer and tetramer resonances showed increased line broadening, suggesting the possibility that both vanadate oligomers exchanged with a V-SOD complex under these conditions. The treatment described above for the tetramer was therefore also carried out with the dimer. The double-reciprocal plot of [V-SOD] as a function of $1/[V_1]^2$ is also linear. Although such linearity suggests that the dimer may bind to the enzyme, the values of the slope and intercept of the dimer-based plot will help establish whether such an interpretation is likely. From the vertical intercept, a limiting stoichiometry of 14–16 V atoms per Cu,Zn-SOD molecule was obtained and not the 8 V atoms as obtained from the treatment shown in Figure 2. From these results, an intrinsic dissociation constant of 3×10^{-5} M was determined for the dimer. If the vanadate dimer were to bind, it would be a significantly weaker anion binder than the tetramer. This treatment suggests that the dimer may bind to Cu,Zn-SOD. However, the large number of dimers (7 or 8) that must bind to each Cu,Zn-SOD molecule makes this alternative interpretation less likely in view of the stoichiometry of the V atoms bound to Cu,Zn-SOD (Figure 2) and what is known about anion-binding sites of Cu,Zn-SOD.[7-13]

The above treatment suggests that the dimer is only weakly binding to native Cu,Zn-SOD. However, the dimer resonance significantly broadens at high protein concentrations. For example, at 0.15 mM total Cu,Zn-SOD concentration, the dimer resonance showed significant line broadening. In this solution, 0.065 mM dimer was present and the tetramer concentration was reduced to 0.04 mM. Since the V-SOD concentration was 1.2 mM, the Cu,Zn-SOD molecule had almost depleted the tetramer concentration. An alternative explanation for the broadening of the dimer is that when the tetramer comes off the protein it immediately hydrolyzes to form a dimer. In this case, no direct binding between dimer and vanadate–protein complex occurs but the conversion of a short-lived tetramer into a dimer gives the appearance that direct exchange between dimer and V-SOD complex occurs on the NMR time scale. Analogous line broadening of the dimer signal is not expected when high concentrations of tetramer are present in solution because the equilibrium does immediately drive the hydrolysis of V_4 to V_2. Extensive conversion will only occur in solutions with low V_4 concentrations. The conversion of vanadate dimer into tetramer followed by immediate capture of V_4 can also explain why a solution containing very little V_4 and Cu,Zn-SOD still manages to form some $(V_4)_2$·SOD complex.

By using eq 12, we estimated the affinity constant of monomeric vanadate per subunit of native Cu,Zn-SOD, as the V_1·SOD subunit, from the ^{51}V NMR spectra at protein concentrations of

$$K_{V_1\cdot SOD} = \frac{[V_1\cdot SOD]}{[SOD]_f[V_1]} \qquad (12)$$

0.20 and 0.50 mM. The affinity constant was of the order of 10^3 M^{-1}. We arbitrarily assumed a stoichiometry of 1:1 for the monomer–SOD subunit complex so that a comparison with the phosphate–protein complex (with a 1:1 stoichiometry per subunit)[13] could be made. The lower affinity of Cu,Zn-SOD for vanadate monomer was seen in the series of ^{51}V NMR spectra leading to the data in Figure 3 and Table III. At low vanadate concentrations, only the vanadate monomer is observable in the ^{51}V NMR spectrum. The presence of Cu,Zn-SOD increases the line width of the monomer resonance by about 5–10 Hz. However, as the total vanadate concentration increases, the dimer and tetramer resonances appear in the ^{51}V NMR spectrum. The line widths of the dimer and tetramer resonances increase immediately after their first appearance. The preferential line width increases of the dimer and tetramer resonances therefore occur whether or not protein or vanadate is present in excess.

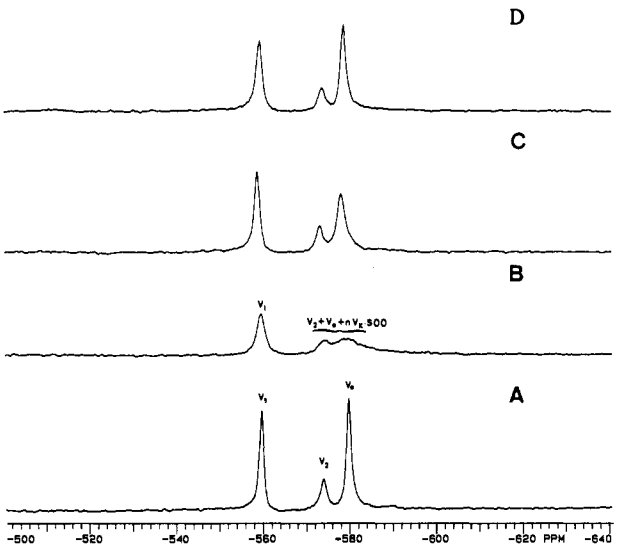

Figure 4. 79.0-MHz ^{51}V NMR spectra of 2.0 mM vanadate in 0.10 M HEPES, pH 7.4, at 23 °C in the absence (A) and presence of 0.15 mM native Cu,Zn-SOD (B), 0.15 mM arginine-modified Cu,Zn-SOD (C), and 0.15 mM lysine-modified Cu,Zn-SOD (D).

Table IV. Chemical Shifts and Line Widths of Monomeric (V_1), Dimeric (V_2), and Tetrameric (V_4) Forms of Vanadate in the Presence of Arginine-Modified and Lysine-Modified Cu,Zn-SOD in 0.10 M HEPES, pH 7.4, at 23 ± 1 °C[a,b]

	V_1	V_2	V_4
	No SOD		
δ/ppm	−560	−574	−580
$\Delta\nu_{1/2}$/Hz	102	121	85
	0.15 mM Arg-Modified SOD		
δ/ppm	−560	−574	−580
$\Delta\nu_{1/2}$/Hz	125	185	202
	0.15 mM Lysine-Modified SOD		
δ/ppm	−560	−574	−580
$\Delta\nu_{1/2}$/Hz	102	148	110

[a] Experimental conditions for recording the spectra are described in the Experimental Section. [b] Same as for Table I.

Samples containing vanadate at various concentrations and 0.20 mM native Cu,Zn-SOD in 0.10 M HEPES, pH 7.4, were examined by difference UV/vis spectroscopy in tandem cuvettes from 300 to 900 nm. When up to 100 equiv of vanadate was added, no changes were observed in the position and intensity of the absorbance maximum corresponding to the d–d transition of native Cu,Zn-SOD. This observation supports the conclusion that vanadate anions do not bind directly to the Cu^{2+} site in the enzyme.

Interactions of Vanadate with Modified Derivatives of Cu,Zn-SOD. The possibility that vanadate binds to the amino acid residues arginine-141, lysine-120, and lysine-134 in native bovine Cu,Zn-SOD was examined by using chemically modified derivatives of Cu,Zn-SOD. ^{51}V NMR spectra of samples containing 2.0 mM vanadate and no protein (Figure 4A), 0.15 mM native Cu,Zn-SOD (Figure 4B), and 0.15 mM arginine-modified (Figure 4C) and 0.15 mM lysine-modified (Figure 4D) protein derivatives were recorded. The line widths of the monomer did not change significantly in the presence of arginine-modified Cu,Zn-SOD. However, the line width of the tetramer resonance, and to a smaller extent that of the dimer resonance, increased as the concentration of arginine-modified Cu,Zn-SOD increased (Table IV).

The line widths and intensities of the monomer, dimer, and tetramer resonances did not change significantly in the presence of 0.15 mM lysine-modified Cu,Zn-SOD (Figure 4D and Table

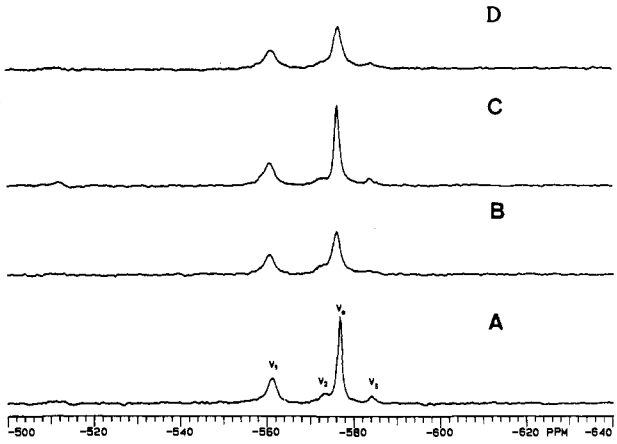

Figure 5. 79.0-MHz ^{51}V NMR spectra of 2.0 mM vanadate in 20 (A, B) or 60 (C, D) phosphate, pH 7.4, at 23 °C in the absence (A, C) and in the presence (B, D) of 0.15 mM native Cu,Zn-SOD.

Table V. Chemical Shifts and Line Widths of Monomeric (V_1), Dimeric (V_2), and Tetrameric (V_4) Forms of Vanadate in the Presence of Native Cu,Zn-SOD in either 20 mM or 60 mM Phosphate, pH 7.4, 0.30 M KCl at 23 ± 1 °C[a,b]

	V_1	V_2	V_4
No SOD + 20 mM P_i			
δ/ppm	-561	-573	-578
$\Delta\nu_{1/2}$/Hz	186	250	107
0.15 mM Native SOD + 20 mM P_i			
δ/ppm	-561	-573	-578
$\Delta\nu_{1/2}$/Hz	191	285[c]	154[c]
No SOD + 60 mM P_i			
δ/ppm	-561	-573	-578
$\Delta\nu_{1/2}$/Hz	225	275[c]	105[c]
0.15 mM Native SOD + 60 mM P_i			
δ/ppm	-560	-573	-579
$\Delta\nu_{1/2}$/Hz	205	275[c]	180[c]

[a] Experimental conditions for recording the spectra are described in the Experimental Section. [b] Same as for Table I. [c] Same as for Table I.

IV). This suggests that the vanadate anions do not interact significantly with this modified protein.

Interactions of Vanadate and Native Cu,Zn-SOD in the Presence of Phosphate. Phosphate binds to native Cu,Zn-SOD at arginine-141.[8,13] Therefore, we examined the influence of phosphate on the interaction between vanadate anions and native Cu,Zn-SOD (Figure 5 and Tables V and VI). The anion-binding affinity of native Cu,Zn-SOD[8] and the vanadate anion distribution[28] are both dependent on ionic strength. Thus, 0.30 M KCl was added in the phosphate/vanadate competition experiments to maintain ionic strength constant irrespective of the concentrations of vanadate and phosphate used. At this higher ionic strength, a pentamer resonance at −584 ppm was observed. The line widths of the monomer resonance of a 2.0 mM vanadate control sample (no protein) in 20 or 60 mM phosphate, pH 7.4, were 90 and 129 Hz wider than in 0.1 M HEPES (Tables I and V). These observations are consistent with the formation of a vanadate–phosphate anhydride.[41] We also observed similar increases in the line widths of the dimer resonance in the presence of phosphate. However, the line widths of the vanadate tetramer resonance were constant within experimental uncertainty in the presence or absence of phosphate. Upon addition of 0.15 mM native Cu,Zn-SOD to 2.0 mM vanadate in 20 mM phosphate, the resonance for the vanadate tetramer was broadened considerably more than the resonances for the monomer and dimer (Figure 5 and Table V). We conclude that, in 20 mM phosphate and 2.0 mM vanadate, the vanadate tetramer interacted mainly with native Cu,Zn-SOD.

(41) Gresser, M. J.; Tracey, A. S.; Parkinson, K. M. *J. Am. Chem. Soc.* **1986**, *108*, 6229.

Table VI. ^{31}P Spin–Lattice (T_1) Relaxation Times of Phosphate in the Presence and Absence of Native and Chemically Modified Cu,Zn-SODs[a]

	T_1/s[b,c]
20 mM phosphate	8.6
20 mM phosphate + 2.0 mM vanadate	7.2
20 mM phosphate + 0.15 mM native SOD	1.9
20 mM phosphate + 2.0 mM vanadate + 0.15 mM native SOD	2.2
20 mM phosphate + 0.15 mM Arg-modified SOD	2.6
20 mM phosphate + 2.0 mM vanadate + 0.15 mM Arg-modified SOD	2.9
20 mM phosphate + 0.15 mM Lys-modified SOD	2.9
20 mM phosphate + 2.0 mM vanadate + 0.15 mM Lys-modified SOD	2.8

[a] The pH of all solutions was 7.4. [b] The reported T_1 values are the average of measurements conducted in three separately prepared samples, with the exception of the lysine-modified samples, for which we carried out measurements in four separately prepared samples. [c] We found that the reported relaxation values are at least accurate within 10%.

Phosphate may not be able to compete significantly with vanadate binding to native Cu,Zn-SOD unless a large excess of phosphate relative to vanadate is used. When 60 mM phosphate buffer, pH 7.4, was used, the resonance for the vanadate tetramer was once again broadened considerably more than those for the monomer and dimer (Figure 5, spectra C and D). The relative changes in line widths and intensities of the vanadate anion resonances for the same concentration of native Cu,Zn-SOD were (within experimental error) approximately the same in 20 or 60 mM phosphate.

We have also examined the influence of phosphate on the interaction between vanadate anions and Cu,Zn-SOD by ^{31}P spin–lattice (T_1) relaxation times (Table VI). The addition of 2.0 mM vanadate to 20 mM phosphate, pH 7.4 (no protein), led to a decrease in T_1 values. This observation is consistent with the increases in line widths reported above and is due to the formation of a vanadate–phosphate anhydride.[41] Solutions containing 20 mM phosphate and 0.15 mM native or chemically modified Cu,Zn-SODs gave shorter T_1 values than solutions containing only phosphate because of paramagnetic relaxation induced by the Cu^{2+} ion at the active site of the protein. The T_1 values for 20 mM phosphate in the presence of 0.15 mM protein followed the order native < arginine-modified < lysine-modified SOD. The shorter ^{31}P T_1 values observed for native SOD relative to its arginine-modified derivative are in agreement with previous distance calculations based on ^{31}P relaxation data.[13] The relatively long ^{31}P T_1 values observed with lysine-modified SOD are presumably related to the high negative charge of this protein derivative and its drastically reduced anion affinity.[10] The addition of 2.0 mM vanadate to 0.15 mM native or arginine-modified Cu,Zn-SOD in 20 mM phosphate caused a significant increase in ^{31}P T_1 values. By contrast, no significant change in T_1 values was observed upon addition of vanadate to 0.15 mM lysine-modified SOD in 20 mM phosphate.

Discussion

The line width changes for the ^{51}V NMR resonances of vanadate anions in the presence of native Cu,Zn-SOD (Figure 1 and Table I) indicate that the tetramer interacts most strongly with Cu,Zn-SOD. No significant changes in solution viscosity or in spin–lattice (T_1) relaxation times were observed for the monomer, dimer, or tetramer vanadate upon addition of native Cu,Zn-SOD, supporting the interpretation that the observed line width changes are related to kinetics between vanadate derivatives. The observed line width changes are consistent with exchange between vanadate anions and a V-SOD complex. In the 0.030–0.20 mM Cu,Zn-SOD concentration range, both the tetramer and the dimer show line broadening. The vanadate monomer resonance showed the least broadening in the presence of native Cu,Zn-SOD. At low temperatures, the line widths of the

dimer and tetramer in the presence of native Cu,Zn-SOD were broader than at room temperature, in part because of decreased kinetics and changes in T_1 values.

The calculated concentrations of dimer and tetramer were smaller than the observed concentrations in the chemical shift range from −574 to −580 ppm (Table II). The difference is due to the formation of an NMR-visible V-SOD complex. The 15–30% visibility of the V-SOD complexes probably occurs because, of four possible transitions, only the $+^1/_2 \rightarrow -^1/_2$ transition is observable. The observed intensity of the V-SOD complex is in the range predicted theoretically (19%)[42] and in agreement with previous observations.[26,27]

Cu,Zn-SOD is a dimer containing two active sites. By determining the concentrations of V-SOD complexes in the Cu,Zn-SOD concentration range 0.030–0.16 mM, we found that an average of four vanadium atoms bind per Cu,Zn-SOD subunit (Figure 2). Each active site can in principle bind vanadate monomers, dimers, tetramers, or pentamers. Binding of four vanadium atoms to each subunit in native Cu,Zn-SOD could in principle involve (a) four monomers, (b) two monomers and one dimer, (c) two dimers, or (d) one tetramer. These possibilities were examined by using the approach described previously.[39,40] The binding of two vanadate tetramers provides the most likely explanation for the linear correlation between the reciprocal of the V-SOD complex concentration and the reciprocal of $[V_4]$, $[V_2]^2$, or $[V_1]^4$ (Figure 3). Analogous treatments with the reciprocal of $[V_1]^2$ or $[V_1]$ did not yield protein–vanadate complexes of the observed stoichiometry of eight V atoms for each native Cu,Zn-SOD molecule. At high vanadate tetramer concentrations, this anion interacts strongly with Cu,Zn-SOD whereas the dimer exchanges significantly with Cu,Zn-SOD at lower vanadate oligomer concentrations. Exchange between V-SOD and the dimer may take place indirectly via the tetramer, which is presumably formed from at least one dimer.[28] The appearance of exchange between the dimer and the V-SOD resonances at lower vanadate oligomer concentrations may occur because the tetramer is not present in sufficient concentration to show exchange broadening directly. The presented conclusion is based on the changes in the line widths of the ^{51}V NMR resonances and the analysis that showed that the vanadate tetramer is the species that binds to Cu,Zn-SOD.

The observation of significant line broadening between vanadate dimer and protein complex deserves some discussion. Two interpretations are possible: the first is that the dimer does bind to native Cu,Zn-SOD, and the second is that the dimer just appears to bind to the protein. When the tetramer concentration becomes sufficiently small, free SOD and the dimer may directly form the V-SOD complex, or alternatively the dimer may generate a vanadate derivative which then binds to the protein. We favor the direct dimer binding interpretation less because the quantitative analysis indicates that seven or eight dimers should then bind to Cu,Zn-SOD. This stoichiometry does not correspond to the observed stoichiometry of eight V atoms bound to the protein shown in Figure 2. Furthermore, such binding seems less reasonable on the basis of what is known about the number and nature of anion-binding sites of Cu,Zn-SOD.[7-13]

The dissociation constants for the V_4·SOD and $(V_4)_2$·SOD vanadate–protein complexes were calculated to be 0.5×10^{-7} M and 2×10^{-7} M, respectively. The difference in numerical value between these constants is due to statistical factors. These dissociation constants are smaller than the dissociation constants determined for phosphoglycerate mutase (PGM): 2×10^{-6} M (for the V_2·PGM complex) and 8×10^{-6} M (for the $(V_2)_2$·PGM complex).[39] Comparison of the reported affinity constant of 34 M^{-1} for phosphate[13] with the analogous crude affinity constant for the vanadate monomer (on the order of 10^3 M^{-1}) shows that the vanadate monomer may have a higher affinity for Cu,Zn-SOD than phosphate. The monomer may bind to Cu,Zn-SOD because a small line width increase is observed even when protein is added to vanadate solutions containing no vanadate oligomers (Table III). However, it is possible that this line width change is observed because the vanadate monomer is the only observable species in the ^{51}V NMR spectra at low vanadate concentrations. In contrast, the vanadate tetramer binds significantly more tightly to Cu,Zn-SOD with dissociation constants of 0.5×10^{-7} M (for the V_4·SOD complex) and 2×10^{-7} M (for the $(V_4)_2$·SOD complex). The smallest anion dissociation constant previously reported for Cu,Zn-SOD was for CN^- (5×10^{-6} M).[8] Thus, the vanadate tetramer binds more tightly to Cu,Zn-SOD than CN^-, which is known to bind the protein via the Cu^{2+} ion.[2,9] The Michaelis–Menten constant K_m for Cu,Zn-SOD measured in the presence of saturating concentrations of its putative substrate, superoxide, was of the order of 3.5×10^{-3} M.[43] Thus, the dissociation constants of the complexes formed between the vanadate tetramer and Cu,Zn-SOD are smaller than the dissociation constant of the enzyme–superoxide complex, suggesting that the vanadate tetramer binds to Cu,Zn-SOD even more tightly than superoxide. Therefore, the very high affinity of Cu,Zn-SOD for the vanadate tetramer is not only unprecedented but very surprising. The high affinity of Cu,Zn-SOD for such a large anion may be related to additional functions, other than superoxide dismutation, for this metalloprotein.

The absorbance spectra of native Cu,Zn-SOD upon vanadate binding showed no changes and rule out direct binding of vanadate to the Cu^{2+} ion. We examined the nature of the vanadate-binding sites in native Cu,Zn-SOD by using protein derivatives specifically modified at selected amino acid residues. The chemical modifications of Cu,Zn-SOD were carried out as in previous studies that suggested that Arg-141 or Lys-120 and Lys-134 were likely candidates for anion binding.[9,10,30,31] Three possible modes of vanadate binding are shown in Figure 6. Parts a and b of Figure 6 show the binding of the vanadate tetramer between the lysine residues, Lys-120 and Lys-134. The vanadate tetramer has the shape of a cup, with 7.2 Å as the longest distance between oxygens and 5.9 Å as the distance between the other two oxygens on the same V atoms.[44] The shortest oxygen distance is 2.6 Å.[44] The tetramer has the possibility of binding to Cu,Zn-SOD with the cup inward, as seen in Figure 6a, or with the cup outward, as in Figure 6b. Other binding mode possibilities, including changes in the tetramer conformation upon binding, are possible but have not been shown in this figure. Figure 6c illustrates the possibility that the tetramer binds to all three residues, Lys-120, Lys-134, and Arg-141. The dimensions of the tetramer[44] and of the solvent channel of Cu,Zn-SOD[11,12] are such that it is not likely that the tetramer can penetrate further into the solvent channel. Although the binding modes in Figure 6 are not the only possibilities, they do suggest likely interaction modes of the vanadate tetramer with bovine Cu,Zn-SOD.

The binding of the vanadate tetramer to the arginine-modified Cu,Zn-SOD was less than that to the native protein (Figure 4 and Table IV). This is in agreement with the known decreased anion affinity of arginine-modified Cu,Zn-SOD.[8,9] In arginine-modified Cu,Zn-SOD, the lysine residues are available for vanadate binding. These considerations suggest that the vanadate tetramer interacts with the native protein at sites other than arginine-141. We conclude that the change in the line width of the tetramer resonance reflects the interaction of the tetramer with the lysine residues in the solvent channel of the protein and that these residues are the main binding sites when vanadate interacts with Cu,Zn-SOD. The binding of the vanadate tetramer to the lysine-modified Cu,Zn-SOD was less than that to the arginine-modified Cu,Zn-SOD (Figure 4 and Table IV). These findings indicate that the line width changes in the tetramer resonance are specific for native bovine Cu,Zn-SOD. Lysine-modified Cu,Zn-SOD at similar protein concentrations only induce small changes in the tetramer line width. The lack of binding of the tetramer to lysine-modified Cu,Zn-SOD is consistent with tetramer binding to the amino groups of Lys-120 and Lys-134 in the solvent channel of native Cu,Zn-SOD.

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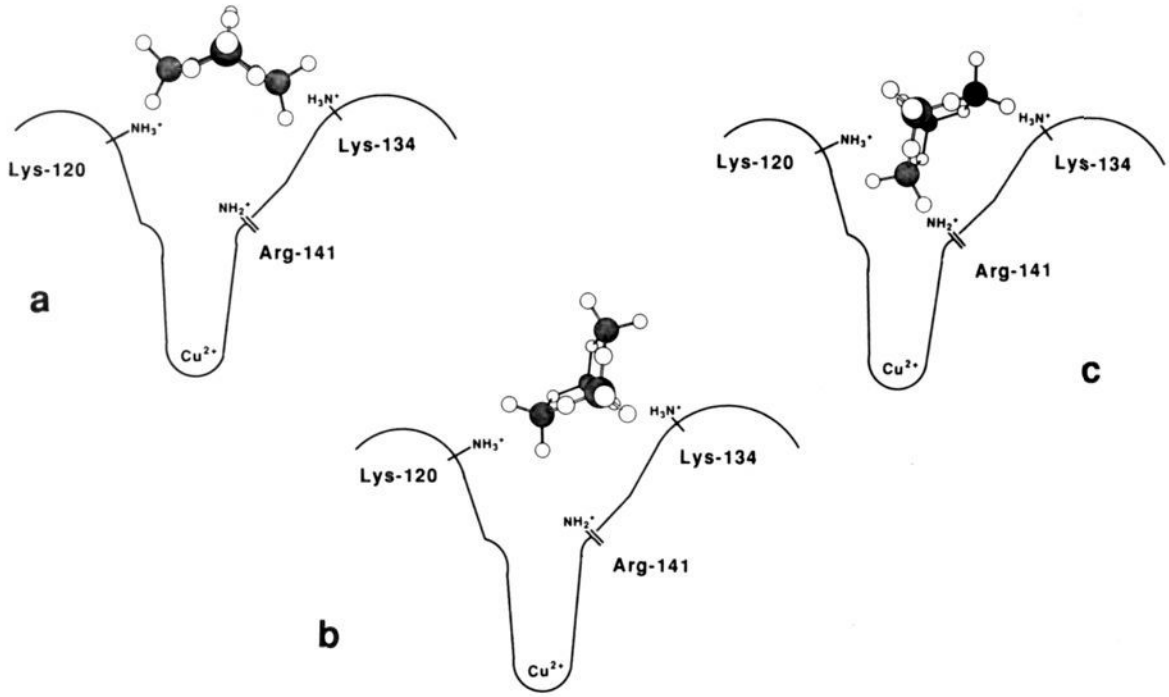

Figure 6. Three possible binding modes of the vanadate tetramer in the solvent channel of bovine Cu,Zn-SOD: (a) tetramer in inward cup shape mode spanning Lys-120 and Lys-134; (b) tetramer in outward cup shape mode spanning Lys-120 and Lys-134; (c) tetramer spanning Lys-134 and Arg-141.

Phosphate is known to bind to native Cu,Zn-SOD primarily via the guanidinium group of arginine-141.[13] We therefore examined the binding of vanadate to native Cu,Zn-SOD in the presence of 20 and 60 mM phosphate buffer (Figure 5 and Table V). Experiments conducted in phosphate buffer have the additional complication arising from formation of vanadate–phosphate anhydrides and thus show much wider line widths for the vanadate monomer.[41] At low phosphate concentrations, the tetramer was found to be the major species that interacted with the native protein. If phosphate were to compete with vanadate for the same protein binding sites, less vanadate would be expected to be bound at higher phosphate concentrations. This was not observed, suggesting that there is little competition between vanadate and phosphate for binding sites in native Cu,Zn-SOD at both 20 and 60 mM phosphate. In summary, vanadate, unlike phosphate, binds primarily to native Cu,Zn-SOD as a tetramer at a site other than Arg-141, as suggested by Figure 6a,b.

^{51}V NMR spectra allow us to probe bulk vanadate binding to Cu,Zn-SOD in contrast to ^{31}P T_1 measurements, which are sensitive to vanadate binding to Arg-141. To further support the conclusion that the vanadate tetramer binds to the two lysine residues in the solvent channel of Cu,Zn-SOD, we conducted ^{31}P T_1 measurements of 20 mM phosphate solutions containing 0.15 mM native and chemically modified proteins in the presence and absence of 2.0 mM vanadate (Table VI). While vanadate was able to compete with phosphate for binding sites in native and arginine-modified SOD, no appreciable competition was observed in the presence of lysine-modified SOD. The magnitude of ^{31}P T_1 values in protein samples is controlled by paramagnetic relaxation induced by the Cu^{2+} ion at the active site of Cu,Zn-SOD. Paramagnetic relaxation is proportional to $1/r^6$ where r is the distance between the paramagnetic Cu^{2+} ion and the ^{31}P nucleus of phosphate. Competition between vanadate and phosphate for Arg-141 is easily detected by ^{31}P T_1 measurements because Arg-141 is located at a short distance (5 Å)[11,12] from the Cu^{2+} ion, thus enhancing paramagnetic relaxation. Because the lysine residues are at least 12 Å away from the Cu^{2+} center,[11,12] the contribution of paramagnetic relaxation toward the ^{31}P nucleus of phosphate bound to lysine residues would be expected to be approximately 200 times smaller. Thus, ^{31}P T_1 measurements are more sensitive than ^{51}V NMR measurements to interactions occurring within a short distance of the Cu^{2+} ion at the active site of the protein. A significant increase of approximately 10% in ^{31}P T_1 values was observed upon addition of 2.0 mM vanadate to 0.15 mM native or arginine-modified SOD (Table VI). Thus, the increases in ^{31}P T_1 values observed upon addition of vanadate to native or arginine-modified SOD are most likely due to competition between the vanadate tetramer and phosphate for Arg-141. In lysine-modified SOD, Arg-141 is available for interaction with the vanadate tetramer, and yet no evidence for such binding was obtained by ^{51}V NMR (Figure 4) or ^{31}P T_1 relaxation measurements (Table VI), suggesting preferential binding of the vanadate tetramer to the lysine residues rather than to Arg-141. These observations are consistent with binding of the vanadate tetramer to lysine residues of the type shown in Figure 6a,b and not of the type shown in Figure 6c.

Chemical modification of Arg-141 with phenylglyoxal maintains the overall charge of the protein. Nevertheless, the introduction of this bulky substituent at Arg-141 results in reduced anion affinity.[8,9] This reduction in anion affinity is not as drastic as that induced by succinylation of lysine residues.[9,10,45] Succinylation of lysine residues causes the overall charge in the protein to become more negative, resulting in reduced anion affinities for lysine-modified Cu,Zn-SOD.[10,45] Thus, the reduced binding of the vanadate tetramer to lysine-modified Cu,Zn-SOD cannot be construed as unequivocal evidence that the lysine residues are the binding site for the tetramer.[46] It is possible that succinylation of lysine residues seriously alters the configuration of the protein in and around the appropriate binding pockets and that interaction with vanadate anions is thus prevented.[45] Similarly, the reduced vanadate binding to arginine-modified Cu,Zn-SOD does not necessarily imply that the arginine-141 residue is not involved in binding of the vanadate tetramer to the native enzyme.[45] Steric hindrance alone may lead in part to reduced vanadate affinity of modified proteins relative to native Cu,Zn-SOD. However, the characteristic residual 15% SOD activity of arginine- and lysine-modified proteins used in this study are an indication that they were completely modified as proven previously by polyacrylamide gel electrophoresis.[7,31] Thus, the reduced vanadate

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affinity of the modified proteins is not due to a contamination of native protein that escaped chemical modification. Chemical modification of essential lysine and arginine residues is likely to reduce the affinity of Cu,Zn-SOD for vanadate in analogy to the activity reduction observed upon chemical modification.[30,31] In general, inability of the modified protein to bind vanadate strongly suggests that the particular modified residue is essential for binding vanadate.

The vanadate tetramer $V_4O_{12}^{4-}$ at pH 7.0 carries a charge of −4. Of the three possible binding modes shown in Figure 6, we expect that the tetramer spans the solvent channel of native Cu,Zn-SOD in such a way that both lysines are in close contact with the tetramer (Figure 6a,b). The vanadate dimer at pH 7.0 carries charges of −2 and −3 in $H_2V_2O_7^{2-}$ and $HV_2O_7^{3-}$. Because the two dimeric species do not fit as well in the solvent channel, it is reasonable to expect that the vanadate dimer binds less tightly. However, the fact that native Cu,Zn-SOD has some affinity for all three vanadate anions shows that this protein has a high affinity for many different types of anions. The strong dependence of the anion-binding affinity of native Cu,Zn-SOD on ionic strength[8] is consistent with our observations of variable affinities for several vanadate anions.

An alternative mechanism of vanadate binding is covalent attachment of vanadate to the enzyme. Such vanadate interactions could involve anhydride linkages to the carboxylate side chains of glutamate or aspartate residues. Alternatively, such covalent interactions could involve ester linkages to the hydroxyl side chains of serine or tyrosine residues or amide linkages to the amine side chains of lysine and arginine residues. Precedents for the formation of complexes of these amino acid residues with vanadate under these conditions have been reported.[17,47] Formation of a covalent linkage between a protein and the vanadate tetramer has not been reported previously. However, analogous esters and anhydrides have been reported between the vanadate monomer or dimer and small molecules.[29,48,49] It has been suggested that the negatively charged amino acid residues, in particular glutamate and aspartate residues, play a role in ligand binding to human Cu,Zn-SOD.[50,51] Vanadate interactions with native Cu,Zn-SOD may be explored further by comparison of the vanadate binding behavior of native protein with that of mutants of yeast[52] or of human Cu,Zn-SOD.[50,51]

The inhibition of SOD activity by vanadate has previously been studied.[53] We have repeated these SOD activity measurements using the xanthine oxidase–cytochrome *c* assay.[32] In our hands, nonlinear assay curves resulted upon addition of 2.0 mM vanadate. We also observed this nonlinear pattern in controls in the absence of native Cu,Zn-SOD. Presumably, vanadate undergoes redox chemistry under the assay conditions. Thus it will be difficult to determine which vanadate species are inhibitors of SOD activity of Cu,Zn-SOD without first characterizing the chemical reactions occurring under the assay conditions in the presence and absence of superoxide. The affinity of Cu,Zn-SOD for the vanadate tetramer may be relevant for the reported vanadate-stimulated oxidation of pyridine nucleotides.[54,55]

The vanadate tetramer is the first large anion that showed high affinity for native Cu,Zn-SOD. The affinity of the vanadate tetramer for bovine Cu,Zn-SOD surpasses the affinity of the tetramer for other proteins including 6-phosphogluconate dehydrogenase,[21] glucose-6-phosphate dehydrogenase,[19] glycerol-3-phosphate dehydrogenase,[20] myosin,[23] and phosphoglycerate mutase.[39] Moreover, the affinity of the vanadate tetramer for bovine Cu,Zn-SOD also surpasses the affinity of this enzyme for other anions, including the substrate superoxide.[2,7-13,43] Future binding studies of bovine Cu,Zn-SOD with other large anions, including the vanadate pentamer, heteropolyanions, and polyphosphates, may assist in characterizing which types of large anions can be accommodated within the solvent channel of native Cu,Zn-SOD.

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