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THE Zn-SITE IN BOVINE COPPER, ZINC SUPEROXIDE DISMUTASE STUDIED BY ^{111}Cd PAC

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The active site in bovine copper, zinc superoxide dismutase (Cu_2, Zn_2SOD) has been studied by ^{111}Cd time differential Perturbed Angular Correlation (PAC) on enzyme with Zn^{2+} replaced by excited $^{111}Cd^{2+}$. The PAC spectra obtained for both the oxidized and the reduced form of Cu_2Cd_2SOD show no asymmetry between the two Zn-sites in the dimeric enzyme. The spectra further reveal that a significant change has taken place at the Zn-site in the reduced form compared to the oxidized form.

Semi-empirical calculations based on the Angular Overlap Model (AOM) and coordinates from the crystal structure of the native enzyme are in agreement with the experimental PAC data of the oxidized enzyme. The results indicate that Cd^{2+} coordinates in the same manner as Zn^{2+} and that the crystal structure of SOD is valid for the enzyme in solution. The PAC spectrum of the reduced enzyme can be explained by extending the AOM calculations to the enzyme in the reduced form and assuming that the imidazol ring of His61 is no longer bridging the copper and cadmium ions in the reduced state.

KEY WORDS: Superoxide dismutase, zinc, cadmium, active site, structure, spectroscopy.

INTRODUCTION

Zinc has been shown to be an essential part of SOD and elucidation of the substructure of the Zn-site during the catalytical cycle is thus important. The first step in the enzymatic cycle of Cu_2Zn_2SOD is the reduction of copper(II) to copper(I). The theory for the enzymatic reaction, as put forward in the literature,[1] implies that the copper and zinc ions are bridged by the imidazole ring of His61 in the oxidized state and that the bond from His61 to copper(II) is released upon copper reduction with concurrent protonation of the His61 nitrogen not coordinated to the zinc ion. It is assumed that His61 keeps binding to the zinc ion during this process. This model for the catalytical activity is mainly based on the high resolution crystal structure of the enzyme[2] but is in agreement with most experimental work.

The first indication of the histidine bridge between the copper(II) ion and the zinc ion came from EPR on enzyme where zinc ions were replaced by cobalt(II) ions.[3] EPR showed a magnetic coupling between the copper and cobalt ions. The coupling was later assigned to be due to a bridging ligand His61, based on the crystal structure of the bovine SOD.[2,4] Numerous other investigations carried out on metal substituted enzyme have also been indicative of a bridging ligand.[5] Studies on the native bovine enzyme are more scarce due to the few spectroscopic techniques available. Investigations using EXAFS have been carried out with the native enzyme in solution and in

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the freeze-dried state. The EXAFS studies indicate that the copper ion binds to fewer ligands in its reduced form compared to its oxidized form.[6] These investigations are thus indicating an imidazole bridging the copper(II) and zinc ions in the oxidized state and that the copper imidazole bond is released upon reduction of copper(II) to copper(I). Other studies on the yeast SOD including ^{3}H exchange[7] and ^{111}Cd PAC[8] have, however, indicated that the imidazole ring is not bridging under all circumstances in the oxidized enzyme and studies by Dunbar *et al.*[9] indicate that there is asymmetry between the two subunits in the dimeric enzyme. These results for the yeast enzyme have been questioned on the basis of NMR on cobalt substituted yeast SOD[10] and the results from the ^{111}Cd PAC experiments on yeast SOD have recently been shown to depend on the sample preparations.[11]

Despite the strong evidence for the model describing the function of $Cu(II)_2Zn$ SOD there is still lacking information of the geometry of the Zn-site for the enzyme in solution. We report here a spectroscopic investigation using PAC spectroscopy on bovine SOD where Zn^{2+} is substituted by excited $^{111}Cd^{2+}$. The PAC technique is sensitive to the angular distributions of the ligands in contrast to EXAFS. This method is thus a good supplement to EXAFS investigations. The pitfall in this approach is as always in metal substituted enzymes whether or not the coordination of Cd^{2+} differs from the coordination of Zn^{2+} in native SOD. However, the coordination of Cd^{2+} is expected to be very similar to the coordination of Zn^{2+}. This point of view is supported by the full catalytic activity and high stability of $Cu(II)_2Cd_2SOD$. The circular dichroism spectra of $Cu(II)_2Zn_2SOD$ and $Cu(II)_2Cd_2SOD$ are nearly identical, which also indicates that Cd^{2+} and Zn^{2+} have very similar coordination properties when coordinated to the Zn-site in SOD.

MATERIALS AND METHODS

Sample Preparation

Bovine Cu_2Zn_2SOD (lot 18F-9323) was from Sigma. All other reagents employed were of analytical purity.

Protein concentrations were determined from UV-absorption. The superoxide dismutase activity of protein samples was measured according to Marklund and Marklund.[12] Buffers and eluents were prior to use passed through a column of Chelex 100. Metal analysis by X-ray fluorescence spectroscopy showed that all metal ions with charge of 2+ or higher were removed by this procedure.

ApoSOD was prepared by dialysis of the native enzyme (approx. 10 mg ml^{-1}) against 100 vol. 25 mM 1,10-phenanthroline, pH 2.9 for 24 hours at room temperature. 1,10-phenanthrolin was subsequently removed by repeated dialysis against 100 vol 30 mM sodium acetate pH 5.0, followed by a change to 50 mM 2(N-morpholino)ethanesulfonic acid (MES), pH 6.0. The apoSOD obtained in this manner was essentially free of copper and zinc ions and showed a superoxide dismutase activity of less than 1% of the native enzyme.

$Cu(II)_2{}^{111}Cd_2SOD$ was prepared by adding 2.1 molar equiv. of Cu^{2+} ions to the apoSOD (approx. 4 mg ml^{-1}) in 50 mM MES pH 6.0, and the solution was left to incubate overnight. The radioactive sample of $^{111}Cd^{2+}$ was prior to use diluted with 50 mM MES pH 6.0 and mixed with 2.1 molar equiv. Cd^{2+} ions. This $^{111}Cd^{2+}$ solution was transferred to the copper-protein and was allowed to incubate for 10 minutes

before further treatment. All samples of metal substituted SOD were desalted on Sephadex G-25 prior to the PAC experiment to remove any possible excess of non-bound metal ions. The elution profile was determined using the 246 keV gamma transition in excited ^{111}Cd. The main protein fractions were collected and pooled and sucrose was added to give a 52% (w/w) solution of sucrose ready for the PAC experiment. $Cu(I)_2{}^{111}Cd_2SOD$ was prepared by reduction of $Cu(II)_2{}^{111}Cd_2SOD$ with sodium dithionite prior to addition of sucrose. All samples were thermostated to 4°C during the PAC experiment.

PAC Spectroscopy

A ^{111}Cd PAC experiment investigating the Zn-site in SOD requires the substitution of Zn^{2+} with $^{111}Cd^{2+}$ in its 396 keV excited state. The excited ^{111}Cd with a halflife of 49 minutes decays through an intermediate state and is thus emitting two gamma rays. The probability of emission of the second gamma ray as a function of the time difference between the two gamma rays is recorded. This is done for gamma rays emitted at 90° and 180° relative to the direction of the first gamma ray. The PAC spectrum is then the result of dividing the time spectrum for 180° with the time spectrum for 90°. A detailed description of the experimental setup and the application of PAC can be found elsewhere.[13]

The PAC spectrum is influenced by the Nuclear Quadrupole Interaction (NQI) via the electric field gradient at the position of the nucleus arising from the surrounding charge distribution. The electric field gradient tensor $V_{ab} = (\partial/\partial b)E_a$, where E_a is the a'th component of the electric field and $a,b = x,y,z$, can be expressed in terms of the molecular charge distribution around the ^{111}Cd nucleus as

$$V_{aa} = -e\int \psi^*(\bar{r})\psi(\bar{r})(3\alpha^2 - 1)/r^3\, dV + \sum_i z_i\,(3\alpha_i^2 - 1)/r_i^3; \qquad (1)$$

$$V_{ab} = -e\int \psi^*(\bar{r})\psi(\bar{r})\; 3\alpha\beta/r^3\, dV + \sum_i z_i\; 3\alpha_i\beta_i/r_i^3;$$

where the first term represents the electronic charge around the cadmium nucleus and the second term represents the charge distribution from the surrounding muclei. $\psi(\bar{r})$ is the total wavefunction describing the cadmium complex, a, and b refer to cartesian coordinates, α and β refer to direction cosines i.e. $\alpha_i = \cos(\theta_i)$ where θ_i is the angle between the a-axis and the ith ligand, z_i and r_i are the charge and the distance to the ith surrounding charge components, respectively. Since the molecules in the PAC experiments described here are randomly oriented, we can only determine the diagonal elements of the electric field gradient tensor. In the coordinate system where V_{ab} is diagonal and $|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|$, the two parameters determined are $\omega_0 = 12\pi|V_{zz}eQ|/40h$ and $\eta = (V_{xx} - V_{yy})/V_{zz}$ since $V_{xx} + V_{yy} + V_{zz} = 0$ (Laplace equation for a nonspherical charge distribution). Q is the nuclear quadrupole moment of the nucleus.

RESULTS

PAC spectra of $Cu(II)_2Cd_2SOD$ and $Cu(I)_2Cd_2SOD$ from bovine erythrocytes were analysed with one NQI each described by a value for ω_0 and η. In addition the rotational tumbling was taken into account by the rotational diffusion time, τ_R. Also

TABLE I
Measured nuclear quadropole interactions of excited $^{111}Cd^{2+}$ derivatives of bovine Cu_2Zn_2-SOD. See text for further information.

Cu-site	Zn-site	ω_0 [Mrad/s]	η
Cu(II)	$^{111}Cd^{2+}$	147.2 ± 0.6	0.36 ± 0.02
Cu(I)	$^{111}Cd^{2+}$	96.1 ± 2.0	0.92 ± 0.06

the base line was set free. Analysis with only one set of NQI parameters gave in both cases a satisfying value of χ^2. Further details concerning PAC data analysis can be found elsewhere.[13] The results of the analysis of the different PAC spectra of the bovine enzyme are given in Table I.

DISCUSSION

PAC Experiments and Simple Conclusions

The PAC experiments reveal that only one NQI is present in the PAC spectra of bovine $Cu(II)_2Cd_2SOD$. This shows that no asymmetry is present in solution between the Zn-site in the two subunits in the oxidized state. PAC measurements on reduced bovine $Cu(I)_2Cd_2SOD$ can also be explained by one NQI showing that also in the reduced state are the two subunits equal. However, the NQI for the oxidized and reduced states are quite different. This reveals that a significant change in geometry is taking place at the Zn-site.

PAC and the Angular Overlap Model

Exact calculation of the NQI for cadmium complexes is not yet possible because of the large number of electrons present in the system. However, it has been shown that semi-empirical calculations of NQI parameters can be made using an Angular Overlap Model.[14,15] The basic assumptions of the AOM are that the energy change of the metal orbitals can be described by perturbation theory, that the contributions from different ligating atoms are additive, and that only a single angular momentum for the metal orbitals is involved. The usefulness of this theory applied to PAC is based upon the fact that the main contribution to the electric field gradient arises from ligand donation of σ-bond electrons to empty 5p-orbitals on Cd^{2+}. Consequently each ligand results in a given occupation of cadmiums $5p_x$, $5p_y$ and $5p_z$ orbitals depending on the orbital overlap between the ligand σ-bond orbital and the $5p$ orbitals on Cd^{2+}.

A more rigorous treatment of the AOM applied to NQI calculations can be found in the literature[14,15] and results in the following equations for the NQI tensor

$$\omega_{aa} = \sum_i [3F(p_a, L_i)\, F(p_a, L_i) - 1]\omega_i/2; \qquad (2)$$

$$\omega_{ab} = \sum_i 3F(p_a, L_i)\, F(p_b, L_i)\, \omega_i/2$$

Where $a,b = x,y,z$, $F(p_a, L_i)$ is the angular overlap factor for orbital p_a with the ligand L_i and ω_i is the partial NQI parameter for ligand L_i's contribution to the NQI in case of maximal overlap with the p_z orbital. It is thus possible to obtain $\omega_0 = \omega_{zz}$ and $\eta = (\omega_{xx} - \omega_{yy})/\omega_{zz}$ after diagonalising the 3 × 3 matrix obtained from equa-

TABLE II

Polar coordinates for the ligating atoms in the four different subunits in the unit cell as found from X-ray crystallography[16] together with calculated NQIs for $^{111}Cd^{2+}$ in bovine $Cu(II)_2Cd_2SOD$. The uncertainties in the ω_0 and η values arising from the uncertainties in the partial NQI parameters is 7 Mrad/s and 0.02 respectively.

Ligand	Orange subunit		Yellow subunit		Blue subunit		Green subunit	
	θ	ϕ	θ	ϕ	θ	ϕ	θ	ϕ
His69	0	0	0	0	0	0	0	0
His78	130.0	0	119.7	0	122.4	0	116.4	0
His61	111.1	221.7	110.8	231.0	105.4	221.4	109.2	218.2
Asp81	78.7	125.3	104.9	107.3	91.3	121.1	90.1	111.4
ω_0 [Mrad/s	184		142		141		134	
η	0.05		0.26		0.24		0.30	

tion (2). The angular overlap factors $F(p_a, L_i)$ for p-orbitals are $F(Px, L_i) = \sin(\theta_i)\cos(\phi_i)$, $F(p_y, L_i) = \sin(\theta_i)\sin(\phi_i)$ and $F(p_z, L_i) = \cos(\theta_i)$ expressed in polar coordinates of the ligating atom.

From equation (2) it is clear that application of the AOM model to the Zn-site in SOD requires knowledge of the type of ligands and their spatial arrangement i.e. their polar coordinates around the Cd^{2+} together with the partial NQI parameters ω_i for each ligand. The experimental parameter ω_i has been obtained for a series of ligands derived from PAC studies on cadmium complexes with known geometry.[15] The partial NQI parameters are for the relevant ligands: aspartate coordinating with one oxygen = 245 ± 5 Mrad/s; imidazole of histidine = 95 ± 4 Mrad/s and water = 207 ± 10 Mrad/s. The coordinates for the four ligands around Zn^{2+} given in Table II have been extracted from the high resolution structure of bovine SOD in the BROOKHAVEN databank.[16]

The NQI obtained from AOM calculations on the four different subunits are given in Table II. An average structure was also constructed and its values of ω_0 and η are given in Table III.

The AOM calculations on the Zn-site of the four different subunits in Table II reveal that the orange unit gives calculated NQI parameters that substantially differ from the values of the other three subunits which, however, have nearly identical NQI parameters. The large deviation for the orange unit is probably due to either an uncertainty in the coordinates from X-ray crystallography or an actual difference in

TABLE III

AOM calculations of NQI parameters for Cd^{2+} in bovine $Cu(II)_2Cd_2SOD$ and $Cu(I)_2Cd_2SOD$. The coordinates for the ligating atoms in the oxidized form are mean coordinates for all four subunits based on the crystal structure, whereas the coordinates for the reduced form are determined by modelling calculated NQI parameters to the experimental NQI parameters in Table I (see text).

Ligand	$Cu(II)_2Cd_2SOD$		$Cu(I)_2Cd_2SOD$	
	θ	ϕ	θ	ϕ
His69	0	0	0	0
His78	122.1	0	122.1	0
His61	109.1	223.1	125.3	180.3
Asp81	91.3	116.3	90.0	90.0
ω_0 [Mrad/s]	139		102	
η	0.21		0.33	

the coordiantion of Zn^{2+} for this subunit in the crystalline state. The geometry around zinc for the other three subunits are more alike. The values of the calculated NQI parameters for the yellow, the blue, the green and the averaged structure (Table II and III) are seen to be in perfect agreement with the measured NQI parameters for the enzyme in solution (Table I). The close resemblance of the calculated and measured NQI parameters for $Cu(II)_2Cd_2SOD$ is thus in good agreement with the theory that His61 is bridging Cu^{2+} and Zn^{2+} when the enzyme is in solution. The coordinates from the crystal structure seem to give a good description of the active site for the native enzyme in solution.

The positive result for the oxidized enzyme made it reasonable that AOM calculation could discriminate between a bridging or non-bridging His61 in the reduced state and furthermore that such calculations could give information of the direction of the movement of the His61 imidazole ring. Two approaches to the geometry of the Zn-site in the reduced state were investigated. In the first approach only the four amino acid ligands were assumed to coordinate to Cd^{2+}, in the other an additional water molecule was introduced in the direction of the solvent. It was further assumed that the change of the measured NQI upon reduction, primarily reflects a new position of the bridging His61. The coordinating oxygen of aspartate was however allowed some movement as this part of the structure seems rather flexible. No movement of the other two coordinating histidines were introduced. All movements of the nitrogen of His61 were carried out in such a way that the angle between the backbone carbon of His61, the zinc atom and the ligating nitrogen was allowed only minor alterations. This was done to ensure that no major stress was applied to the imidazole ring of His61.

These calculations revealed that no reasonable solution could be obtained with an additional water ligand, despite that the position of the water molecule was allowed great flexibility. For the three histidines and one aspartate it can be shown that a theoretical minimum field gradient ω_0 is achieved, when all three coordinating nitrogens form right angles to the coordinating oxygen. Table III shows such a solution for the average structure, where the predicted NQI is:$\omega_0 = 102$ Mrad/s and $\eta = 0.33$. This solution is the result of a 27° movement of the coordinating oxygen around the Zn-site. This roughly corresponding to a rotation of the carboxylate group around the carbon-carbon bond in the side chain. The coordinating nitrogen of His61 is moved somewhat more, about 42°, around the Zn-site or approximately 1.5 Å. However, computer modelling based on the X-ray diffraction data[16] has not revealed any objections to such a movement from the surrounding structure. The same procedure applied to the four different subunits gave each time $\omega_0 = 102$ Mrad/s but η ranging from 0.10 to 0.75 demonstrating the large freedom in η. As the measured $\omega_0 = 96$ Mrad/s is within the uncertainty of the theoretical minimum, the AOM calculations imply that any solution to the reduced form consisting of three histidines and one aspartate must lie close in geometry to the one suggested in Table III. This geometry can only be achieved if the imidazole ring of His61 is moved away from the protein surface and closer to the coordinating oxygen of Asp81. This movement of the imidazole ring implies also that the copper-His61 bond must be released upon reduction.

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