Coordination Geometry of Cadmium at the Zinc and Copper Sites of Superoxide Dismutases: A Study Using Perturbed Angular Correlation of γ-Rays from Excited ^{111}Cd

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^{111}Cd time-differential perturbed γ–γ angular correlation (PAC) has been used to investigate the Zn site in yeast and bovine copper and zinc-containing superoxide dismutases by substitution of the zinc ions with excited $^{111}Cd^{2+}$ ions. The PAC spectra obtained from the enzymes in aqueous solution reveal a single coordination geometry of $^{111}Cd^{2+}$, showing that the coordination of $^{111}Cd^{2+}$ to the Zn site in the two subunits is identical. Furthermore, the PAC spectra of the yeast and bovine enzymes show that the Zn sites are very similar in the two enzymes. The PAC experiments show a clear difference depending on whether the copper ion is in the oxidized or the reduced state. In the latter case the results resemble those obtained for derivatives with no metal ion at the Cu site. Hence, the coordination geometry of the Zn site in these two situations must be similar, and it is very unlikely that the imidazole ring of His61 bridges the two metal ions in the reduced enzyme. The PAC spectrum of $^{111}Cd^{2+}$ ions at the Zn site with copper(II) ions at the Cu site is in agreement with that predicted by applying the angular overlap model (AOM) to the known crystal structure of the bovine enzyme, with known nuclear quadrupole interactions for the ligands involved. Furthermore, results from experiments with copper in the reduced state show that reduction of the copper ion causes a significant change at the Zn site. An explanation for this conformational change has been proposed by computer modelling. The PAC experiments also show that it is possible to incorporate cadmium ions into the Cu site in the absence of copper ions, and the result has also been interpreted in terms of the AOM.

Introduction

Copper and zinc-containing superoxide dismutases (superoxide: superoxide oxidoreductase, EC 1.15.1.1) are enzymes which catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The enzyme is found in the cytoplasm of the eukaryotic cells and has been identified in plants, bacteria, animals and fungi.

The crystal structure of the oxidized form of the copper- and zinc-containing superoxide dismutase (Cu_2Zn_2SOD) from bovine erythrocytes is known to within 2 Å resolution.[1] It is a dimer, consisting of two identical subunits kept together by non-covalent bonds. Each subunit consists of 151 amino acids with a subunit molecular mass of about 16 kg mol^{-1}. The enzyme crystallizes with two dimers per unit cell, and the structures of the four different subunits have been deposited in the Brookhaven National Data Bank.[2]

The copper ion is coordinated to four histidines (His44, -46, -61 and -118) in a distorted tetrahedral complex, whereas the zinc ion is coordinated to three histidines and one aspartate (His61, -69, -78 and Asp81) in a tetrahedral complex. The copper and zinc ions are located 6.3 Å apart, bridged by an imidazolate from His61 that coordinates to both metal ions in the oxidized enzyme.[3,4]

The copper ion is essential for the catalytic activity of the enzyme. The catalytic cycle involves alternate reduction and oxidation of the copper ion during reaction with the superoxide ion according to reactions (I) and (II). Both

$$\text{Enzyme-Cu(II)} + O_2^- \rightarrow \text{Enzyme-Cu(I)} + O_2 \quad \text{(I)}$$

$$\text{Enzyme-Cu(I)} + O_2^- + 2H^+ \rightarrow \text{Enzyme-Cu(II)} + H_2O_2 \quad \text{(II)}$$

reactions have second-order rate constants of ca. 3×10^9 M^{-1} s^{-1}.[5,6] The reaction rate seems to be controlled by diffusion and only limited by the availability of the superoxide ion. However, precollision electrostatic guidance could increase the availability of the O_2^-, thereby making the reaction faster than would be accounted for by diffusion alone.[7–9]

The detailed reaction mechanism for the enzymatic reaction, as put forward in several papers,[3,10,11] implies a break-

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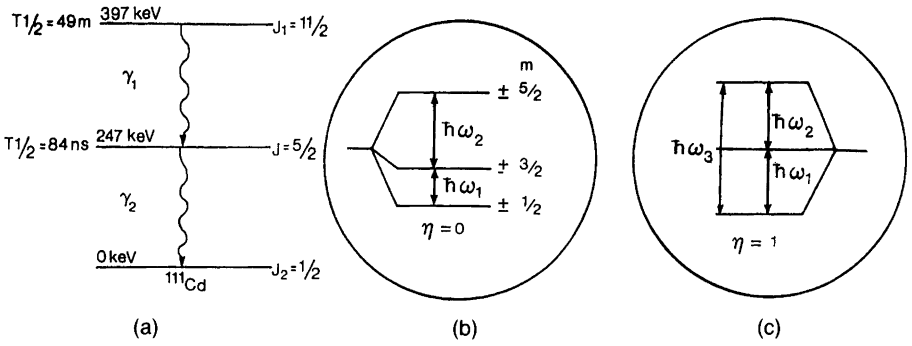

Fig. 1. (a) Schematic presentation of the decay of the 397 keV state of ^{111}Cd. (b) The energy splitting for the intermediate state in an axial symmetric electric field gradient ($\eta = 0$; $\omega_2 = 2\omega_1$; $\omega_3 = \omega_1 + \omega_2$). (c) The energy splitting for the intermediate state in an electric field gradient with $\eta = 1$ ($\omega_2 = \omega_1$; $\omega_3 = \omega_1 + \omega_2$).

age of the copper–His61 bond, when the copper ion is reduced, together with a movement of the imidazole ring from His61 towards the solvent. His61 is assumed to keep its bond to the zinc ion during this process. Extended X-ray absorption fine structure (EXAFS) experiments on the bovine enzyme, probing copper and zinc, respectively, indicate that the copper ion binds to fewer ligands in its reduced form than in its oxidized form.[12] In contrast to these findings, a PAC study on ^{111}Cd derivatives of yeast Cu_2Zn_2SOD suggested that His61 in the active enzyme in solution does not bridge the two metal ions.[13] Another abnormality concerning the yeast enzyme is that experiments with reconstitution of the apoSOD with Co^{2+} have suggested that the Zn sites in the two subunits of yeast Cu_2Zn_2SOD are different.[14] This result is supported by a three-dimensional structure of the yeast enzyme constructed by computer-graphics techniques using the bovine enzyme atomic coordinates as template.[15] The non-equivalence of the two subunits in the yeast enzyme has, however, recently been questioned on the basis of ^{1}H NMR on Co^{II}-substituted yeast Cu_2Zn_2SOD.[16]

The aim of the present study of Cu_2Zn_2SOD has been to investigate the discrepancy between the characteristics of the yeast enzyme[13–16] and those of the bovine enzyme.[1,2] In particular, the conclusions of the earlier PAC study on the yeast enzyme have been investigated. As PAC spectra of $^{111}Cd^{2+}$ compounds are sensitive to the angular arrangement of the ligands, and as substitution of Zn^{2+} by Cd^{2+} conserves enzyme activity, PAC spectroscopy should offer important information about the equivalence of the metal binding sites of the two subunits, together with changes in the geometry of the Zn site that accompany changes in the oxidation state of copper. The PAC investigations are in the present study expanded to include the bovine enzyme, together with various new combinations of metal substitutions of the two enzymes. Furthermore, the recent application of the angular overlap model (AOM) to the calculation of electric field gradients is used to investigate in more detail the coordination chemistry of cadmium ions bound to SOD and its relevance to the native enzyme.

PAC spectroscopy. The technique of perturbed angular correlation of γ-rays has been known since the mid 1950s. It provides a means of measuring the interaction of a nucleus with an electric field gradient or a magnetic field at the site of the nucleus, and is widely used to study solid-state problems of various kinds. A review of the technique and its applications to chemistry has been given by Lerf and Butz.[17] The application of PAC spectroscopy to biological systems has been reviewed by Bauer.[18]

The 397 keV excited state of ^{111}Cd decays via two γ-rays to the ground state [Fig. 1(a)]. For ^{111}Cd-nuclei in randomly oriented molecules the probability density of detecting the second γ-ray at an angle θ and a time t, relative to the emission of the first γ-ray, is given by eqn. (1), where τ_N

$$W(\theta,t) = \frac{\exp(-t/\tau_N)\{1 + A_2G_2(t)[3/2\cos^2(\theta) - 1/2]\}}{4\pi\tau_N} \quad (1)$$

is the lifetime of the intermediate level and A_2 an amplitude factor. The time-dependent factor $G_2(t)$ contains the information available about the interaction of the nucleus with its surroundings. For a static interaction it can be shown that $G_2(t)$ contains time-dependent terms of the form $\cos[2\pi(E_n - E_{n'})t/h]$, where E_n and $E_{n'}$ denote energies of different intermediate levels. The Fourier transform of $G_2(t)$ thus gives the energy splittings. For ^{111}Cd in proteins the perturbing interaction is the interaction of the electric quadrupole moment of the nucleus with the electric field gradient originating from the charge distribution of the surroundings. This interaction is called the nuclear quadrupole interaction (NQI).

The intermediate level of the ^{111}Cd decay has the nuclear spin $I = 5/2$, in which case a non-vanishing NQI gives rise to a splitting of the energy into three energy levels [Figs. 1(b) and 1(c)]. Consequently, there are three different energy differences giving rise to three frequencies in the PAC spectrum. From the magnitudes of these frequencies, V_{zz} and η can be determined. V_{zz} is defined as the largest component of the electric field gradient and η is the

asymmetry parameter, defined as $\eta = (V_{xx} - V_{yy})/V_{zz}$ where V_{zz}, V_{yy} and V_{xx} are the components of the electric field gradient tensor in the coordinate system where the tensor is diagonal, and $| V_{zz} | \geq | V_{yy} | \geq | V_{xx} |$. The electric field gradient can be decribed by V_{zz}, η and the orientation of the coordinate system in which the tensor is diagonal. For randomly oriented molecules, however, only $\omega_0 = 12\pi | V_{zz}eQ | /40h$ and η can be measured.

The above discussion is valid for molecules that do not change their orientation. For superoxide dismutase in aqueous solution at room temperature the rotational correlation time is of the order of $1/\omega_0$. This will wipe out the oscillations, and it is therefore necessary to slow down the rotational diffusion. Therefore, we have measured the NQI of $^{111}Cd^{2+}$ in SOD in 52 % (w/w) solution of sucrose at 4 °C.

Experimental

Abbreviations. The following abbreviations will be used: SOD, superoxide dismutase; E, empty metal binding site; MES, 2(*N*-morpholino)ethanesulfonic acid; tris, tris(hydroxymethyl)aminomethane; PAC, perturbed angular correlation of γ-rays; NQI, nuclear quadrupole interaction; CD, circular dichroism; AOM, angular overlap model. All numbering of amino acids refers to the sequence of bovine Cu_2Zn_2SOD.[2]

Chemicals. The sources used were as follows. Carlbiotech: lyophilized yeast Cu_2Zn_2SOD (lot 96877). Fluka: 1,10-phenanthroline, pro analysi; diethylenetriaminepentaacetic acid, pro analysi. Merck: sodium dithionite; 100 % acetic acid, pro analysi; zinc(II) chloride, pro analysi; cadmium(II) sulfate, reinst; pyrogallol, pro analysi; tris, pro analysi. Riedel: copper(II) acetate, rein krist. Serva: sucrose, reinst. Sigma: 2(*N*-morpholino)ethanesulfonic acid; chelating resin, sodium form; lyophilized bovine Cu_2Zn_2SOD, (lot 18F-9323). Pharmacia: Sephadex G-25, fine. All other reagents employed were of analytical purity.

Determination of protein concentration. Protein concentrations were determined from the absorption at 259 nm. The molar absorbance of the yeast enzyme at 259 nm is 12×10^3 M^{-1} cm^{-1} for the native Cu_2Zn_2SOD and 4.9×10^3 M^{-1} cm^{-1} for the apo-enzyme using a molecular mass of 31.9 kg mol^{-1}.[19] The molar absorbance of the bovine enzyme at 259 nm is 9.84×10^3 M^{-1} s^{-1} for the native Cu_2Zn_2SOD and 3.68×10^3 M^{-1} cm^{-1} for the apo-enzyme.[20]

Circular dichroism measurements. Circular dichroism spectra were measured using a CNRS-Roussel Jouan Dichrographe model III instrument, which was calibrated with $(+)_{589}$tris(ethylenediamine)cobalt(III) chloride ($\Delta\varepsilon_{492\ nm}$ = 1.91 M^{-1} s^{-1}).

Determination of superoxide dismutase activity. The superoxide dismutase activity of protein samples was measured according to the method of Marklund and Marklund[21] with minor modifications. The specific activity of the native yeast superoxide dismutase measured after gel filtration of the commercial product is determined by this assay to be 10 100 unit mg^{-1}, where one unit is defined as the amount of superoxide dismutase activity able to decrease the change in absorption by 50 % in 1 ml assay volume. All assays were performed at 25 °C in a thermostatted cell.

Metal determination. Quantitative multi-trace elemental analysis was performed by means of energy-dispersive X-ray fluorescence spectroscopy, using the 'infinitely' thin sample method.[22] Neutron activation was performed at the Risø Research Centre.

Preparation of metal-free buffers. Prior to use buffers and eluents were passed through a column of chelating resin (10 cm×0.9 cm i.d.) at a flow rate < 100 ml h^{-1}.

Preparation of pure ^{111}Cd in its 397 keV excited state from ^{108}Pd. Enriched ^{108}Pd (98 %) was deposited on graphite by electrolysis. 10 mg of ^{108}Pd were dissolved in 150 μl concentrated HBr and 50 μl concentrated HNO_3. After evaporation of excess acid over a water bath the sample was redissolved in 150 μl conc. HBr and 1000 μl conc. NH_3. The mixture, left overnight until a clear solution was obtained, was used for electrolysis in a device designed to give a 1 cm diameter circular spot of ^{108}Pd. An applied voltage of 2 V gave a current decreasing from 20 to 8 mA, and ca. 80 % depletion of the solution (measured by the increased mass of the target). The electrolysis was carried out in a bath consisting of a cylinder clamped to the graphite target. The target was the cathode, and a Pt wire was used as the anode.

The graphite bar with the circular spot of ^{108}Pd was placed in the inner part of a cyclotron and irradiated with a ^4He beam of 35 μA for 1.5 h. For 10 mg ^{108}Pd on the graphite an activity of ca. 400 MBq of ^{111}Cd was obtained.

Isolation of ^{111}Cd from ^{108}Pd. All glassware was rinsed in aqua regia and washed with metal-free water. Plastic tubes were used without special rinsing. The ^{108}Pd on the graphite bar was, after activation in the cyclotron, dissolved by eight succesive applications of 100 μl of conc. HNO_3 on top of the palladium spot. Each 100 μl portion was removed after ca. 30 s and transferred to a 25 ml Duran beaker. By this procedure > 70 % of the ^{111}Cd activity was finally transferred to the beaker. The nitric acid was evaporated by boiling, and 800 μl of conc. hydrochloric acid was added and evaporated (to obtain $PdCl_4^{2-}$). To the solution containing the chlorocomplexes of Pd(II) and ^{111}Cd(II) was added 1 ml metal-free water. The solution was then passed through an anion-exchange column of Amberlite CG400 (5 cm×0.4 cm i.d.) which was washed with 0.1 M HCl followed by metal-free water. Most of the red–brown $PdCl_4^{2-}$ was attached to the first few millimetres of the column, as judged by the violet colour produced upon binding of the palladium complex to the anion-exchange

gel. The eluate containing $^{111}Cd^{2+}$ ions was collected and the ion exchange repeated. A single step gave a reduction factor of 1000, which was determined by using ^{109}Pd as a tracer. The ^{109}Pd was produced by activating ^{108}Pd by exposing it to a beam of deuterons at 12 MeV. The 88 keV transition was then followed on a GeLi detector. If we assume that repeating the passage of the $^{111}Cd^{2+}$ solution through a second anion-exchange column would give an additional reduction factor of 1000, then the amount of palladium would have been reduced to 10^{-10} mol. The single ion-exchange procedure used in earlier experiments[13] gave a palladium content of about 0.2–0.9 molar equivalent relative to the amount of SOD used. About 4 ml of the eluate, after the second ion exchange, with the majority of the ^{111}Cd activity (> 80 %) were collected and gently evaporated to dryness (to avoid fixation of $^{111}Cd^{2+}$ to the Duran glass), thereby removing excess HCl. The $^{111}CdCl_2$ was immediately redissolved in 50 mM MES buffer (pH 6.0). This sample contained very pure $^{111}Cd^{2+}$ as measured by the activity. After gel filtration of the $^{111}Cd^{2+}$-substituted SOD, no other activity than ^{111}Cd and ^{107}Cd (due to ^{104}Pd from the target) could be detected within the first 24 h. Isotope determination was made using a GeLi detector for ^{109}Pd and a NaI detector for ^{111}Cd.

Preparation of apoSOD. ApoSOD was prepared by dialysis (Servapor 44145, 16 mm) of the native enzyme (ca. 10 mg ml^{-1}) against 100 vol. 25 mM 1,10-phenanthroline (pH 2.9) (adjusted with HCl) for 24 h at room temperature.[19] 1,10-Phenanthroline was subsequently removed by repeated dialysis against metal-free buffer. Metal analysis by X-ray fluorescence analysis showed that no transition metals were present in amounts larger than 0.02 molar equivalent in apoSOD prepared in this manner.

Metal substitution with $^{111}Cd^{2+}$ and sample preparation. In the case of $Cu(II)_2{}^{111}Cd_2SOD$ and $Cu(I)_2{}^{111}Cd_2SOD$ the samples were prepared by adding 2.1 molar equiv. of Cu^{2+} ions [2.5 mM copper(II) acetate] to the apoSOD (3-4 mg ml^{-1}) in 50 mM MES, pH 6.0 and incubating overnight. The $^{111}Cd^{2+}$ solution prepared as described was mixed prior to use with 2.1 molar equiv. Cd^{2+} ions (cadmium sulfate, 2.5 mM). The resulting solution was adjusted to neutrality by titration with 0.1 M NaOH. This $^{111}Cd^{2+}$ solution was transferred to the Cu_2E_2SOD solution and incubated for 10 min. In one experiment the ^{111}Cd solution was added to the apo-enzyme (of yeast SOD) before the addition of Cu^{2+}, with no alteration of the resulting enzyme as judged from PAC. Preparation of $^{111}Cd_2Zn_2SOD$ was made in a similar way, with Zn^{2+} ions (2.5 mM zinc chloride) instead of Cu^{2+} ions. The copper-free cadmium derivatives $E_2{}^{111}Cd_2SOD$ and $^{111}Cd_2{}^{111}Cd_2SOD$ were made by adding 2 or 4 equiv. of Cd^{2+} ions mixed with excited $^{111}Cd^{2+}$ directly to apoSOD and incubating the mixture for 10 min. The cadmium-substituted proteins were desalted on a Sephadex G-25 column (15 cm×0.9 cm i.d.) in order to remove any possible excess of non-bound metal ions. The elution profile was determined by measuring the 246 keV γ-transition in excited ^{111}Cd. The two fractions containing the major part of the protein were pooled, and 1.2 ml of this protein solution were added to 1.30 g of sucrose, to give a 52 % (w/w) solution of sucrose ready for the PAC experiment. The reduced form, i.e. $Cu(I)_2Cd_2SOD$, was prepared by reduction of $Cu(II)_2{}^{111}Cd_2SOD$ with 20 µl 300 mM sodium dithionite prior to addition of sucrose. All samples were cooled to 4 °C during the PAC experiment.

Experimental PAC setup. Details of the electronics will be given elsewhere.[23] The PAC spectrometer consists of four γ-ray detectors, each consisting of a BaF_2 crystal mounted on a photomultiplier. The four detectors are arranged at fixed angles of 0, 90, 180 and 270°. The coincidences of a 150 keV γ-ray in one detector with a 247 keV γ-ray in another detector are measured as a function of time. These time spectra contain the function $W(\theta,t)$ plus background due to accidental coincidences [eqn. (1)]. Four combinations of $W(180°,t)$ and four combinations of $W(90°,t)$ were measured. After background subtraction the function $A_2G_2(t)$ was found using eqn. (2). This is the spectrum

$$A_2G_2(t) = 2[W(180°,t)-W(90°,t)]/[W(180°,t)+2W(90°,t)] \quad (2)$$

shown in all illustrations. The BaF_2 crystals were 2 inches in diameter and 2 inches long. The distance to the source was 5 cm. The finite solid angle reduced the amplitude A_2 from 0.18 to about 0.12. The sample had a volume ranging from 30 µl to 2 ml. Changing the volume had a negligible effect on the value of A_2. The final spectrum had a time resolution between 1.54 and 2.50 ns.

Data analysis. For slowly rotating molecules ($\omega_0\tau_R >> 1$) $G_2(t)$ takes the form of eqn. (3),[18] where τ_R is the correlation time of the rotational diffusion. The four amplitudes a_i depend on η, and the three angular frequencies ω_i depend on ω_0 and η.[18] For fast rotational diffusion ($\omega_0\tau_R << 1$) the oscillations are lost and $G_2(t)$ takes the form of eqn. (4).

$$G_2(t) = \exp(-t/\tau_R)\left(a_0 + \sum_{i=1,3} a_i \cos(\omega_i t)\right) \quad (3)$$

$$G_2(t) = \exp(-k\omega_0{}^2\tau_R t);\ k = 2.8(1 + \eta^2/3) \quad (4)$$

In the case of $\omega_0\tau_R \approx 1$ no analytical expression can be given, but $G_2(t)$ can be calculated as described by Dattagupta.[24] In the case of ^{111}Cd present in two different complexes $G_2(t)$ becomes the sum of the two different perturbation factors weighted by their relative populations. The effect of the time resolution was taken into account by multiplying each cosine by the term $\exp[-0.09025 \times \omega_i^2(\Delta t)^2]$, where Δt is the time resolution of the spectrometer. Thus the function used for analyzing the spectra was eqn. (5) ($\omega_0\tau_R >> 1$), where the summation over j is

$$A_2G_2(t) = \sum_j A_{2j} \exp(-t/\tau_R) \times \left(a_{0j} + \sum_{i=1,3} a_{ij} \cos(\omega_{ij}t) \exp[-0.09025\omega_{ij}^2(\Delta t)^2]\right) \quad (5)$$

carried out over the different sets of NQI(ω_0,η) present in the spectrum. From the spectrum one to three sets of NQI(ω_0,η) were determined. The rotational diffusion time τ_R was determined from a $Cu(II)_2Cd_2SOD$ spectrum and used in all other analyses. In each case the baseline was allowed to vary; however, it did not not show any significant deviation from zero.

AOM calculations. The calculation of the NQI, i.e. ω_0 and η, for cadmium complexes was performed within the angular overlap model (AOM).[25,26] The application of the AOM to NQI calculations is based on a set of assumptions of which the two most important are that the angular momentum of the metal orbitals is limited to a value of 1 and that the contributions from different ligands are additive. This means that the NQI can be calculated from partial NQI parameters for the ligands involved. The electric field gradient scaled to the angular frequency observed in a PAC experiment can, within the AOM, be written as eqn. (6),[26]

$$\omega_{ab} = \sum_i \omega_0(i) (3/2\ \alpha_i\beta_i - 1/2\ \delta_{ab}) \quad (6)$$

where a, $b = x$, y or z are Cartesian coordinates, α_i, $\beta_i = x_i$, y_i or z_i are directional cosines from the z-axis of the ith ligand to the a and b directions, respectively, and $\omega_0(i)$ is a partial NQI for the ith ligand. In the principal coordinate system this expression is diagonal, and ω_0 and η are defined by eqns. (7a) and (7b), choosing $|\omega_{zz}| \geq |\omega_{yy}| \geq |\omega_{xx}|$.

$$\omega_0 = |\omega_{zz}| \quad (7a)$$

$$\eta = (\omega_{xx} - \omega_{yy})/\omega_{zz} \quad (7b)$$

From eqns. (6) and (7) we see that the NQI parameters are capable of discriminating both the type and the geometry of the ligand sphere. As partial NQIs, published parameters were used; for the relevant ligands these are as follows: carboxylate oxygen from aspartate = 245 ± 5 Mradian s^{-1}; imidazole nitrogen from histidines = 95 ± 4 Mradian s^{-1}; oxygen of water = 207 ± 10 Mradian s^{-1},[26] The partial NQI parameter of the bridging imidazolate was assumed to be 95 Mradian s^{-1}. For comparison, the estimated partial NQI of a unit charge at a distance 2.3 Å is 131 Mradian s^{-1}.[26]

Integral PAC experiments. Integral PAC spectra were obtained as in a normal PAC experiment except for a much shorter period (ca. 15 min). The integral was calculated from eqn. (8).

$$I = \int_{t=0}^{150\text{ ns}} W(180°,t)\,dt \Big/ \int_{t=0}^{150\text{ ns}} W(90°,t)\,dt \quad (8)$$

The integral measurements on $^{111}Cd^{2+}$ were performed in a buffer without sucrose. In the case of $^{111}Cd^{2+}$ bound at the Cu or Zn site in SOD, we have $\omega_0\tau_R \approx 1$. In this case the effect of the electric field gradient changing its orientation is that the nuclei 'forget' their original spin orientation, and the second γ-ray is emitted isotropically. This results in a value of I close to 1. If, however, the cadmium ions are free in the buffer the rotational correlation time will be much smaller than $1/\omega_0$. Consequently, the electric field gradient is averaged out, giving $G_2(t) \approx 1$, and the full anisotropy is achieved, resulting in a value of I close to $1+(3/2)A_2$.[18]

Results

The method used to remove Cu^{2+} and Zn^{2+} ions from the native Cu_2Zn_2SOD yielded apoSOD with < 1 % SOD activity compared to the native enzyme. 1,10-Phenanthroline has a large molar absorption with maximum at 264 nm, and consequently any remnant metal chelator will show up as a band at this wavelength. The ultraviolet absorption spectrum of neither apoSOD nor the reconstituted Cu_2Zn_2SOD exhibited any disturbance due to protein-bound 1,10-phenanthroline. Addition of stoichiometric amounts of Cu^{2+} and Zn^{2+} gave a reconstituted enzyme having 95–105 % SOD activity compared to native Cu_2Zn_2SOD, regardless of the sequence of copper and zinc. There was no significant effect of the buffer used (30 mM sodium acetate, pH 5.0; 30 mM sodium acetate, pH 6.0; mM MES, pH 6.0; 10 mM tris-HCl, pH 8.0). The timescale of reconstitution was investigated by absorption spectroscopy and showed that the changes in the absorption spectra of apoSOD or E_2Zn_2SOD upon addition of Cu^{2+} ions, or of Cu_2E_2SOD upon addition of Zn^{2+}, occurred very quickly, i.e. within the mixing time (< 1 min) at room temperature, followed by minor changes for about 15 min.

The binding properties of Cd^{2+} ions to apoSOD and derivatives. The binding of Cd^{2+} ions to yeast apoSOD was investigated by addition of Cd^{2+} ions, labelled with traces of radioactive $^{111}Cd^{2+}$ ions, to the apo-enzyme in 50 mM MES, pH 6.0, followed by desalting on Sephadex G-25. In Fig. 2(a) is shown the chromatogram of a sample containing 4 molar equiv. of Cd^{2+} with $^{111}Cd^{2+}$ as tracer. It is evident that all Cd^{2+} ions are bound to the enzyme in this case. However, if the same experiment is carried out with 8 molar equiv. of Cd^{2+}, it is evident that only approximately half of the Cd^{2+} is bound to the enzyme [Fig. 2(b)]. From these experiments it is clear that the apo-protein can bind no more than four Cd^{2+} ions or one Cd^{2+} ion per metal binding site. Further investigations of the binding properties of Cd^{2+} were made by the addition of Cd^{2+} mixed with $^{111}Cd^{2+}$ to yeast E_2Zn_2SOD and yeast Cu_2E_2SOD. The results of these experiments are shown in Figs. 2(c) and 2(d).

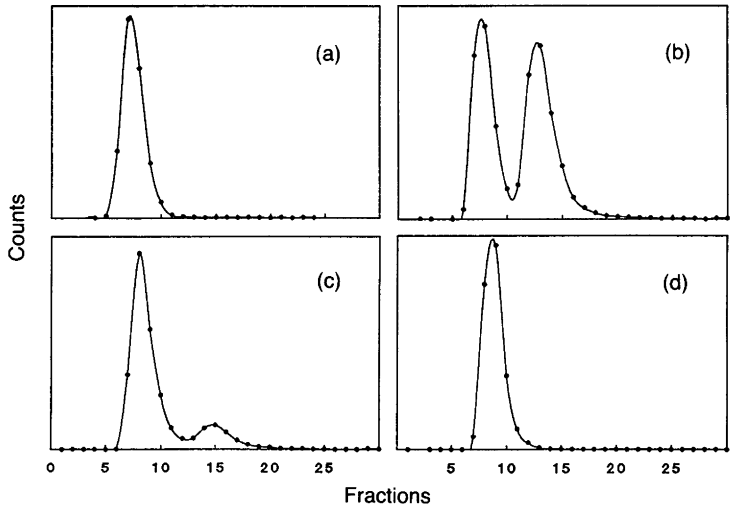

Fig. 2. Sephadex G-25 chromatograms. Elution profiles of yeast SOD derivatives obtained after addition of: (a) 4.2 ± 0.2 molar equiv. $^{111}Cd^{2+}$ to apoSOD, (b) 8.0 ± 0.4 molar equiv. $^{111}Cd^{2+}$ to apoSOD, (c) 2.1 ± 0.1 molar equiv. $^{111}Cd^{2+}$ to E_2Zn_2SOD and (d) 2.1 ± 0.1 molar equiv. $^{111}Cd^{2+}$ to $Cu(II)_2E_2SOD$. The measured activity (counts) of ^{111}Cd in each fraction is corrected for exponential decay using a half-life for excited ^{111}Cd of 49 min.

It is evident that in both cases the enzyme can bind approximately 2 molar equiv. of Cd^{2+}. Analogous experiments with the bovine enzyme revealed behaviour equal to that of the yeast enzyme. These experiments show clearly that Cd^{2+} ions can bind to both the Cu site and the Zn site in 50 mM MES, pH 6.0, but no non-specific binding of Cd^{2+} occurs under the same conditions. Cu_2Cd_2SOD obtained in this manner had about 70 % SOD activity compared to the native enzyme, whereas copper-free cadmium derivatives had < 2 % SOD activity compared to the native enzyme.

The near-ultraviolet circular dichroism spectrum of yeast Cu_2Cd_2SOD is identical to that of reconstituted Cu_2Zn_2-SOD. These are in turn substantially different from both apoSOD and Cu_2E_2SOD. Although the spectra are not fully understood, this indicates that Cd^{2+} is coordinated to the Zn site in a manner similar to Zn^{2+}.

Integral PAC experiments. The binding properties of $^{111}Cd^{2+}$ to aposSOD and various metal-substituted SODs were investigated by performing integral PAC experiments. If it is assumed that all $^{111}Cd^{2+}$ is either bound to SOD or free, then the fraction of $^{111}Cd^{2+}$ that is bound can be determined by eqn. (9), where I(free), I(bound) and

$$\text{Percentage of protein-bound } ^{111}Cd^{2+} = \frac{I(\text{measured}) - I(\text{free})}{I(\text{bound}) - I(\text{free})} \times 100\% \qquad (9)$$

I(measured) are found from integral PAC experiments using eqn. (8). The results of these experiments are shown in Table 1, and they indicate, like the chromatography on Sephadex G-25, that four $^{111}Cd^{2+}$ ions can bind to apoSOD and that the Cd^{2+} ion can bind to both the Zn site and the Cu site. It is also clear that Cd^{2+} ions cannot significantly displace Cu^{2+} and Zn^{2+} in native and reconstituted Cu_2Zn_2SOD, at least on a timescale of a few hours.

Table 1. Binding of $^{111}Cd^{2+}$ to various derivatives of Cu_2Zn_2SOD in 50 mM MES, pH 6.0, studied by integral PAC experiments.

Experiment[a]	Protein-bound $^{111}Cd^{2+}$/%[b]
4 $^{111}Cd^{2+}$ + E_2E_2SOD	93 ± 6
2 $^{111}Cd^{2+}$ + Cu_2Zn_2SOD (native)	−1 ± 5
2 $^{111}Cd^{2+}$ + Cu_2Zn_2SOD (reconstituted)	−3 ± 5

[a]Each measurement took approximately 15 min. [b]Calculated according to eqn. (9) with I(free) = 1.172 ±0.001 and I(bound) = 1.022 ± 0.006.

Table 2. Binding of $^{111}Cd^{2+}$ to various derivatives of Cu_2Zn_2SOD in 50 mM MES, pH 6.0, as a function of time studied by integral PAC experiments.

Experiment	Time /min[a]	Protein-bound $^{111}Cd^{2+}$/%[b]
$^{111}Cd_2{}^{111}Cd_2SOD$ + $2Cu^{2+}$	0[c]	93 ± 6
	20	73 ± 6
	52	64 ± 5
	84	61 ± 6
($2^{111}Cd$, 2Pd)SOD + 2 Cu^{2+}	0[c]	99 ± 7
	18	65 ± 5
	114	51 ± 7

[a]Time gives the period elapsed from the addition of Cu^{2+} to the beginning of the measurement. (Each measurement took about 15 min.) [b]Calculated according to eqn. (9) with I(free) = 1.172 ± 0.001 and I(bound) = 1.022 ± 0.006. [c]Measured before addition of Cu^{2+}.

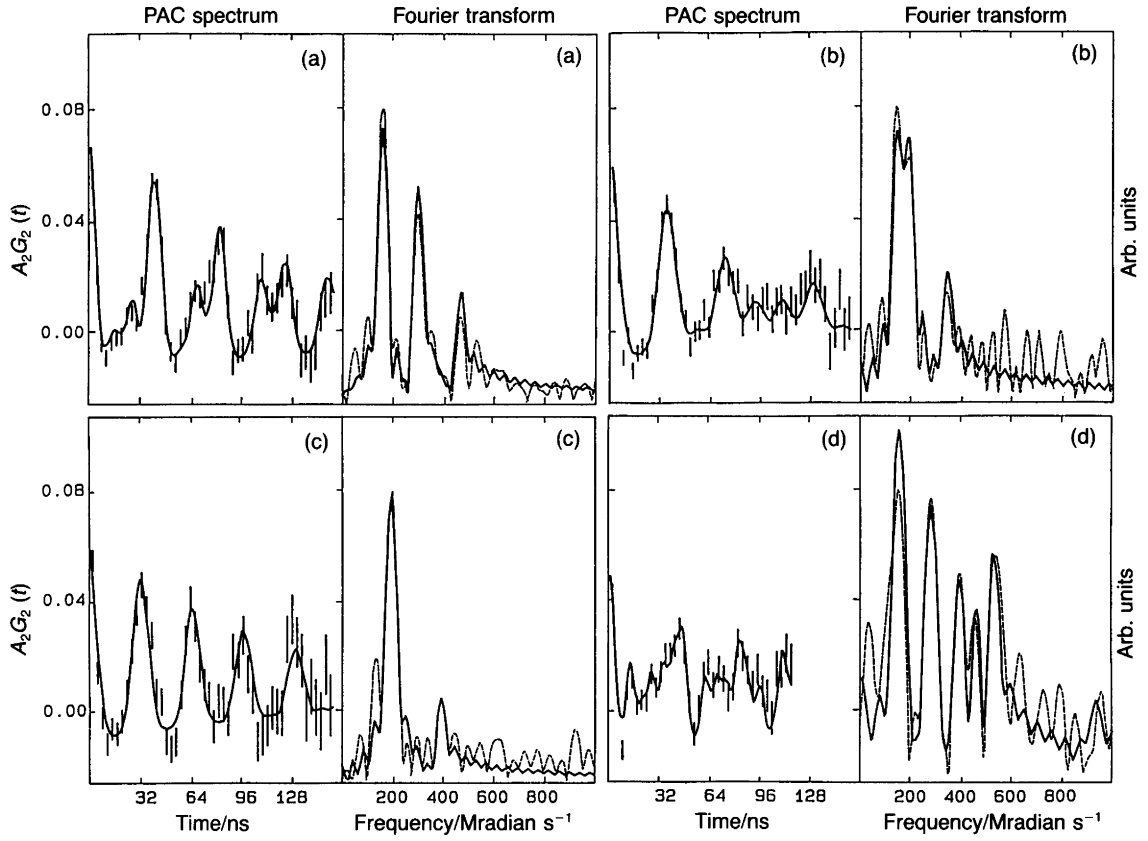

Fig. 3. PAC spectra of metal-substituted SOD from yeast. (a) $Cu(II)_2{}^{111}Cd_2SOD$, (b) $Cu(I)_2{}^{111}Cd_2SOD$, (c) $^{111}Cd_1SOD$ and (d) $^{111}Cd_2{}^{111}Cd_2SOD$. The assignment stated is the major species present in the sample.

The integral experiment was also used to investigate the metal displacement in various ^{111}Cd-substituted SODs by measuring the change in the amount of bound $^{111}Cd^{2+}$ as a function of time after addition of copper. The results are given in Table 2. The experiments show that addition of Cu^{2+} to $^{111}Cd_2{}^{111}Cd_2SOD$ results in a displacement of $^{111}Cd^{2+}$ from the enzyme.

The experiments with Pd^{2+} were made in order to investigate the binding properties of $^{108}Pd^{2+}$, since $^{108}Pd^{2+}$ ions are a possible cause of contamination in the preparation of $^{111}Cd^{2+}$. The results show that $^{111}Cd^{2+}$ in ($2^{111}Cd$, 2Pd)SOD is displaced from the protein upon addition of 2 molar equiv. of Cu^{2+}. We interpret this as being due to exchange between Cd^{2+} and Cu^{2+} ions. However, this will take place only if all metal binding sites are occupied prior to addition of Cu^{2+}. Thus we have indirect evidence that Pd^{2+} ions bind to one or both metal binding sites. This information is noteworthy, since it emphasizes the importance of removing all traces of Pd^{2+} during the purification of $^{111}Cd^{2+}$.

PAC spectra. The PAC spectra of yeast SOD are shown in Fig. 3 and those of bovine SOD in Fig. 4. The left-hand side

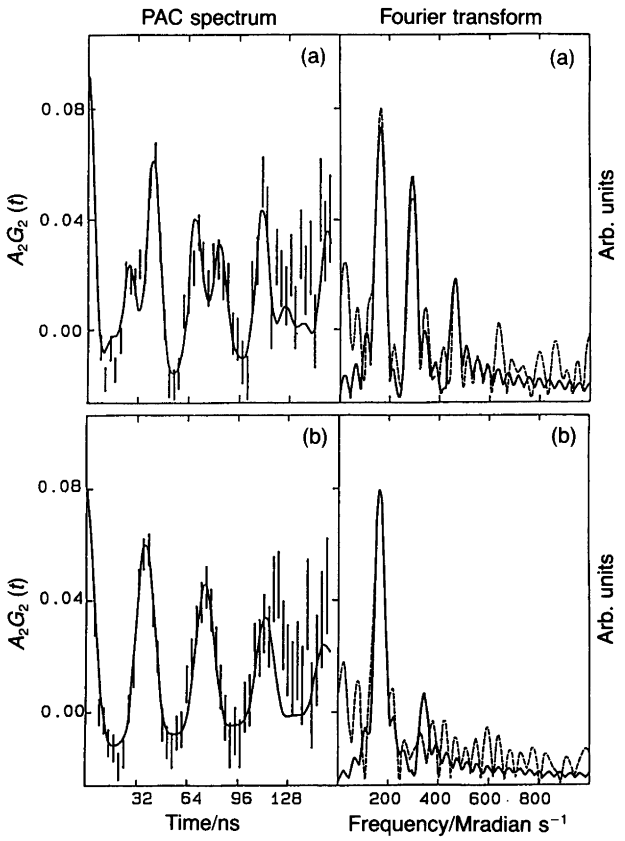

Fig. 4. PAC spectra of metal-substituted SOD from bovine erythrocytes. (a) $Cu(II)_2{}^{111}Cd_2SOD$ and (b) $Cu(I)_2{}^{111}Cd_2SOD$.

shows the divided spectrum $A_2G_2(t)$ together with the fitted curve. The right-hand side shows the fourier transform of both the fitted (——) and the experimental points (------). In the figure, four channels have been added, although all analyses have been performed on the raw spectra. For Fig. 3(a), 3(b) and 3(d) the spectra show the sum of two experiments. The results of analysis of the spectra are given in Tables 3 and 4. The measured NQIs are given in Table 3 together with their proposed assignment. Table 4 shows the relative populations of the different ^{111}Cd derivatives.

AOM analysis. The AOM analysis for both sites was carried out on the four different subunits in the unit cell of bovine Cu_2Zn_2SOD, together with calculations on an averaged structure derived from the coordinates of the four different subunits.[2] The results, together with the uncertainties originating from the uncertainty of the partial field gradients, are given in Table 5. For the copper site the position of the water molecule published in the Brookhaven Data Bank is too far from the copper ion to coordinate. Studies published later, however, show that a water

Table 3. Measured NQIs of excited $^{111}Cd^{2+}$ derivatives of Cu_2Zn_2SOD.[a]

Site[b]		Yeast		Bovine	
Cu	Zn	ω_0	η	ω_0	η
Cu(II)	^{111}Cd	149.5 ± 0.4	0.25 ± 0.01	147.2 ± 0.6	0.36 ± 0.01
Cu(I)	^{111}Cd	101.1 ± 0.7	0.79 ± 0.02	96.1 ± 0.9	0.92 ± 0.03
E	^{111}Cd	111.9 ± 0.8	0.90 ± 0.02	not measured	
Cd	^{111}Cd	145.5 ± 0.9	0.27 ± 0.03	not measured	
^{111}Cd	Cd	280 ± 2	0.68 ± 0.01	not measured	
^{111}Cd	Zn	259 ± 2	0.65 ± 0.01	not measured	
Previous result from Ref. 13:					
Cu(II)	^{111}Cd	144 ± 3	0.19 ± 0.07		
Cu(I)	^{111}Cd	96 ± 3	0.82 ± 0.08		
^{111}Cd	Pd/Cd?	273 ± 5	0.69 ± 0.03		

[a]All ω_0 values are given in Mradian s^{-1}. Experimental uncertainties are the results of conventional fitting procedures assuming parabolic χ^2 surfaces. [b]Site gives the proposed assignment of the excited $^{111}Cd^{2+}$ position (see text).

Table 4. Measured relative populations (in %) of excited ^{111}Cd derivatives of yeast Cu_2Zn_2SOD as determined by analysis of their relative amplitudes, A_2, in the PAC spectra (see text).

Experiment	$E_2{}^{111}Cd_2SOD$	$Cd_2{}^{111}Cd_2SOD$	$^{111}Cd_2Cd_2SOD$	$^{111}Cd_2Zn_2SOD$
1 $^{111}Cd^{2+}$ + apoSOD	76 ± 4	16 ± 5	8 ± 5	
2 $^{111}Cd^{2+}$ + apoSOD	49 ± 5	27 ± 7	24 ± 7	
4 $^{111}Cd^{2+}$ + apoSOD		58 ± 3	42 ± 4	
2 $^{111}Cd^{2+}$ + E_2Zn_2SOD		32 ± 4	27 ± 5	41 ± 5

Table 5. AOM calculations of NQIs based on X-ray crystallographic data.[a]

Subunit	ω_0			η		
	Zn site	Cu site[b]	Cu site[c]	Zn site	Cu site[b]	Cu site[c]
Orange	184	160	147	0.05	0.90	0.33
Yellow	142	256	157	0.26	0.96	0.27
Blue	141	192	146	0.24	0.76	0.15
Green	134	104	153	0.30	0.71	0.15
Average	139 ± 5	121 ± 8	153 ± 7	0.20 ± 0.02	0.74 ± 0.09	0.17

[a]The average structure is the average geometry of the four individual subunits found in the unit cell. The uncertainty given for the average structure is that arising from the uncertainty in the NQIs. All ω_0 values are given in Mradian s^{-1}. [b]Assuming one water molecule coordinating to $^{111}Cd^{2+}$ at the Cu site (see text). [c]Assuming no water molecule coordinating to $^{111}Cd^{2+}$ at the Cu site.

molecule is weakly coordinated to copper in the crystal structure at a distance of 2.8 Å from the Cu^{2+} ion.[3] In our AOM analysis of the Cu site the NQI was calculated in both the situation where the four histidines are coordinated to Cd^{2+} alone and in the situation where a water molecule is coordinated in the direction given by the water molecule in the published structure.[2] Assuming this direction of the coordinating water molecule only gives a very crude estimate of the NQI at the copper site. The uncertainty in the determination of the position of the water molecule can probably explain the very large range in calculated NQI of this site in the four different subunits (Table 5).

Discussion

We report a spectroscopic investigation using PAC spectroscopy on bovine and yeast Cu_2Zn_2SOD where Zn^{2+} is substituted with excited $^{111}Cd^{2+}$. The PAC technique is, unlike EXAFS, sensitive to the angular distribution of the ligands, and is thus a good supplement to EXAFS for obtaining information concerning the structure and function of the active site in Cu_2Zn_2SOD. The main difference in the chemical properties of Zn^{2+} and Cd^{2+} can be ascribed to their different ionic radii, which are 0.97 Å for Cd^{2+} and 0.74 Å for Zn^{2+}. The larger radius for Cd^{2+} will probably have some influence on the coordination of Cd^{2+} compared to Zn^{2+}. However, the facts that Cu_2E_2SOD can bind only 2 molar equiv. of Cd^{2+} and that the UV and visible CD spectra of $Cu(II)_2Zn_2SOD$ and $Cu(II)_2Cd_2SOD$ are identical indicate that the coordination of Cd^{2+} at the Zn site is similar to the coordination of Zn^{2+}. This is further supported by the fact that the cadmium derivative has almost full catalytic activity. It may therefore be expected that the results concerning the structure of the Zn site reported in this study will also be valid for the native enzyme.

Addition of Cu^{2+} to Cd_2Cd_2SOD results in a release of $^{111}Cd^{2+}$ from the protein (Table 2), indicating that Cd^{2+} binds to the Cu site. This is important with respect to the interpretation of the measured NQIs. Since apoSOD can bind only 4 molar equiv. of Cd^{2+} in MES buffer at pH 6.0, no non-specific binding of $^{111}Cd^{2+}$ occurs.

Interpretation of the PAC spectra. For $Cu(II)_2Cd_2SOD$ and $Cu(I)_2Cd_2SOD$ [Fig. 3(a) and 3(b)] analysis according to eqn. (5) with only one NQI gave a satisfactory χ^2 (Table 3). This shows that Cd^{2+} has a single-coordination geometry. For Cd_2Cd_2SOD [Fig. 3(d)] the experiments clearly show two NQIs, one close to the NQI of $Cu(II)_2Cd_2SOD$ and one high-frequency component being interpreted as Cd^{2+} ions in the Cu site with Cd^{2+} ions in the adjacent Zn site. This interpretation is supported by results from chromatography on Sephadex G-25, showing that up to 4 molar equiv. of Cd^{2+} can bind to the apoSOD (Fig. 2). For E_3Cd_1SOD the spectrum could be adequately analyzed with only one NQI; however, analysis with three NQIs gave a smaller χ^2 (Table 3). The major NQI is interpreted as Cd^{2+} in the Zn site, with no metal ion at the Cu site. It is characteristic that this NQI is very similar to the NQI of $Cu(I)_2Cd_2SOD$. In the case of 2 molar equiv. Cd^{2+} and no other added metal ions, the spectra show three NQIs. The relative populations (Table 4) in these experiments with only Cd^{2+} ions are in accordance with the assumption that Cd^{2+} can bind to the Cu site only if the adjacent Zn site is occupied. In the case of Cd_2Zn_2SOD the spectra show three different NQI values, two of them identical to the two measured on Cd_4SOD, and a third close to the high-frequency component. The latter is interpreted as Cd^{2+} ions in the Cu site with a Zn^{2+} ion at the adjacent Zn site.

The result that only one NQI is present in Cu_2Cd_2SOD appears to be in conflict with a previous PAC spectral analysis[13] which showed two NQIs with about equal amplitudes for the Cu(II) and the copper-free derivatives. In the cited paper[13] Cd^{2+} was added subsequent to the addition of $^{111}Cd^{2+}$. We also used this procedure in the present experiment, but the analysis still showed that only one NQI was present. The two NQI values detected earlier[13] are in fact almost identical to the two observed for yeast Cd_2Cd_2SOD. The single NQI with 149.5 Mradian s^{-1} detected in the present paper for $Cu(II)_2Cd_2SOD$ is, within the uncertainty, identical to one of the two NQIs detected earlier. The PAC experiment performed on Cd_2Zn_2SOD also gives an NQI with $\omega_0 = 259$ Mradian s^{-1} very close to the second NQI detected earlier. As Cu^{2+} preferentially binds to the Cu site,[27] it is safe to assign the NQI of 149.5 Mradian s^{-1} and $\eta = 0.25$ to $^{111}Cd^{2+}$ at the Zn site of $Cu(II)_2Cd_2SOD$ and the NQI of 259 Mradian s^{-1} and $\eta = 0.65$ to $^{111}Cd^{2+}$ at the Cu site, with Zn^{2+} at the adjacent Zn site. This inevitably leads to the conclusion that the previous results[13] were caused by having Cd^{2+} at both the Zn site and the Cu site, except for the reduced enzyme. This also infers that the copper-free cadmium enzyme referred to by Bauer *et al.*[13] did not (in spite of the absence of copper) have the Cu site empty. Therefore, any bridge between the two metal ions could still be present and, as seen from Table 3, the NQIs of $^{111}Cd^{2+}$ at the Zn site of $Cu(II)_2Cd_2SOD$ and Cd_2Cd_2SOD are practically identical. This was misinterpreted as indicating that Cu^{2+} had no influence on the NQI of $^{111}Cd^{2+}$ in the Zn site, and it was erroneously concluded that His61 did not bridge Cu^{2+} and $^{111}Cd^{2+}$. On the other hand, both the present experiments and the former ones[13] give the same results on the reduced enzyme. The reason for the double occupancy in the former PAC study on SOD is best explained by the presence of a higher metal content than 2 molar quiv. before addition of Cu^{2+}. This could either be an excess of Cd^{2+} or pollution by any other metal with binding properties for the Zn site. However, neutron activation analysis of lyophilized samples of native and apoSOD used in the former PAC experiment[13] showed a content of 1.6 molar eqiv. zinc in the native enzyme and 6×10^{-3} molar equiv. zinc in the apo-enzyme. As mentioned in the Experimental section, 0.2–0.9 molar equiv. Pd^{2+} could possibly be present in the earlier experiments, because only a single passage through the ion-exchange column was performed. It is evident from the integral experi-

ments (Table 2) that Pd^{2+} does have an affinity for the enzyme, otherwise no Cd^{2+} would exchange upon addition of Cu^{2+} to ($2^{111}Cd$, 2Pd)SOD. This hypothesis can also explain why reduction with dithionite reveals a ^{111}Cd derivative with only one NQI. Dithionite is likely to reduce Pd(II) to Pd(0), and since the latter is unlikely to coordinate to the enzyme, it will be released upon reduction. Release of Pd from the Zn site will cause migration of Cd^{2+} from the adjacent Cu site to the Zn site, because Cd^{2+} preferentially binds to the Zn site, and does not bind to the Cu site unless the Zn site is occupied. Furthermore, the NQIs of $^{111}Cd^{2+}$ at the Zn site with reduced copper at the Cu site and empty Cu site, respectively, are very close (Table 3), and are not easily resolved by PAC spectroscopy. Thus, the previous PAC results[13] may be explained by the presence of about 1 molar equiv. of Pd^{2+}, as this would give derivatives with Cd^{2+} in both the Cu and Zn sites.

The fact that the PAC spectrum of both $Cu(II)_2{}^{111}Cd_2SOD$ and $Cu(I)_2{}^{111}Cd_2SOD$ could be analyzed with a single NQI infers the immediate conclusion that there is no difference between the two subunits. (Each subunit should have contributed with a unique NQI if they were different.) Furthermore, since the NQI of $E_2{}^{111}Cd_2SOD$ is close to the NQI from $Cu(I)_2Cd_2SOD$, but very different from the NQI from $Cu(II)_2Cd_2SOD$, there is no evidence for the absence of a bridging imidazolate in $Cu(II)_2Cd_2SOD$.

Coordination geometry of $^{111}Cd^{2+}$ at the Zn site. The NQI, with $\omega_0 = 147.2$ Mradian s^{-1} and $\eta = 0.36$ assigned to $^{111}Cd^{2+}$ at the Zn site of bovine $Cu(II)_2Zn_2SOD$, has been compared to the NQI calculated within the AOM based on the geometry of Zn^{2+} at the Zn site.[2] From Table 5 it is seen that the AOM prediction of the NQI based on the structure from X-ray diffraction data is close to the observed NQI from the $Cu(II)_2Cd_2SOD$ derivative. The implication is that the coordination geometry of Zn^{2+} in the crystalline state must be close to the geometry of Cd^{2+} at the Zn site in solution. Note that this does not preclude longer metal–ligand distances for the geometry of Cd^{2+} compared to the geometry of Zn^{2+}, such as have been observed with EXAFS,[28] since the NQI parameter for different metal–ligand distances represents a scaling of the ω_0 parameters. This is inherent in the semi-empirical nature of the AOM. The good agreement between the observed and calculated NQIs means, furthermore, that the imidazole ring from His61 must be coordinating to Cd^{2+} in a manner similar to that found in the crystalline state, i.e. the imidazolate bridges the Cu^{2+} and Cd^{2+} ions in solution.

The experimental NQI for the bovine $Cu(II)_2Cd_2SOD$ derivative does not match perfectly the NQI calculated for the average structure found from the X-ray diffraction data. This could be due to the disregarding of any difference in the partial NQI for the bridging relative to the non-bridging histidine ligands. However, it should be noted also that there is a considerable range in the NQI from subunit to subunit as determined from the X-ray structure (Table 5). The yeast enzyme has experimental NQI values which come very close to the NQI for the bovine enzyme, showing that the zinc sites are almost identical for the two enzymes.

The close resemblance of the NQIs obtained on $Cu(I)_2{}^{111}Cd_2SOD$ and $E_2{}^{111}Cd_2SOD$ (Table 3) agrees well with the theory that His61 is not bridging in the reduced enzymes, as His61 cannot bridge when copper is absent. Since the experimental data and the AOM calculations are in such good agreement for the oxidized enzyme, it would seem reasonable to assume that the AOM can also be applied to the enzyme in its reduced state. Therefore, we have explored the possibilities of adapting the geometry of the Zn site in such a way that the calculated NQI could meet the observed NQI. In the search for solutions, the following assumptions were made: His69 and His78 are unaffected by the reduction or removal of Cu^{2+}, and Asp81 continues to coordinate with only one oxygen. This coordinating oxygen was, however, allowed some movement by rotation of the carboxylate group around the CH_2–COO^- bond. Selection of the rotational movement was made after investigating the spatial freedom of the coordinating oxygen by computer graphics based on the X-ray diffraction data.[2] The effect of reducing or removing the copper ion was assumed to have its major impact on the coordination of His61. Moreover, His61 was assumed to keep coordinating to Cd^{2+}, with its bond to Cd^{2+} in the plane of the imidazole ring, and without changing the angles between this bond and the bonds in the imidazole ring. This assumption limits the possible positions of the coordinating nitrogen considerably. Investigation of the geometry shows, however, that this last assumption can be fulfilled by rotation of the imidazole ring of His61 around the C(α)–C(β) bond in the side chain.

In the case of one aspartate and three histidines it is possible to show that the AOM predicts a global minimum of $\omega_0 = \omega_0(\text{Asp}) - 3/2\ \omega_0(\text{His}) = 102.5 \pm 7.8$ Mradian s^{-1}. This minimum ω_0 will be achieved only if the three coordinating nitrogen atoms of His61, His69 and His78 form bonds to Cd^{2+} that are at right angles to the bond between Cd^{2+} and the oxygen atom of Asp81, resulting in a trigonal pyramid with the oxygen from Asp81 in the apex. In such a conformation the angles between the coordinating nitrogens in the base-plane will mainly affect the value of η. As this minimum ω_0 just meets the measured ω_0 of bovine $Cu(I)_2{}^{111}Cd(II)_2SOD$, any solution satisfying the AOM calculation must imply that the imidazole ring of His61 has moved closer to the coordinating oxygen from Asp81 as compared to the oxidized enzyme, in which His61 has an angle of 105° to Asp81. A more detailed discussion of these AOM calculations, together with the coordinates of the coordinating atoms fulfilling the above conditions, is given by Bjerrum *et al.*[29]

The proposed geometry of Cd^{2+} in $Cu(I)_2Cd_2SOD$ is a solution restricted by the above-mentioned conditions. It does not take into account major changes in the coordination of the ligands, such as a Cd–N bond from His61 being significantly out of the plane formed by the imidazole

ring, as suggested by Tainer *et al.*[3] This changes the contribution of the ligand to the NQI considerably, as it might lead to a significant amount of π-bonding, violating the assumptions of the AOM calculations.

Coordination geometry of $^{111}Cd^{2+}$ at the Cu site. An AOM calculation of ω_0 and η at the Cu site was also made. However, in this case some discrepancies might occur, since the coordination geometry of Cd^{2+} is less likely to resemble that of Cu^{2+} than that of Zn^{2+}. Also some imprecision arises owing to the uncertainty of the position of the coordinating water molecule. From Table 5 it is clear that assuming that only the four histidines coordinate results in NQIs far from the measured NQI assigned to $^{111}Cd^{2+}$ at the Cu site. However, if a coordinating water is assumed, the calculated range encompasses the experimental NQI assigned to be $^{111}Cd^{2+}$ at the Cu site with Zn^{2+} ions at the Zn site. If small distortions (10°) of the position of the imidazole from His61 are introduced in the average structure and the position of the water molecule is allowed some spatial freedom, then a range of solutions emerges. The small difference between the NQI of $^{111}Cd_2Zn_2SOD$ and the NQI of $^{111}Cd_2Cd_2SOD$ is probably due to size effects of Cd^{2+} ions at the Zn site, although this apparently does not change the geometry of the Zn site much. The fact that it is possible to find AOM solutions for the observed high-frequency NQI gives further support to the interpretation of this NQI as assignable to Cd^{2+} at the Cu site.

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