Cadmium-113 Nuclear Magnetic Resonance Studies of Cadmium-Substituted Derivatives of Bovine Superoxide Dismutase†

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ABSTRACT: We have prepared the following cadmium-113-substituted derivatives of bovine superoxide dismutase and recorded the nuclear magnetic resonance (NMR) spectrum of the cadmium: 2Cd(II), in which Cd(II) is presumed to bind to the Zn(II) site and the copper site is unoccupied, and 2Cd(II)–2Cu(I), which is analogous to the reduced form of the native protein. NMR transitions were observed at 310 ppm downfield from $Cd(ClO_4)_2$ for 2Cd(II) and at 320 ppm for the 2Cd(II)–2Cu(I)-containing proteins. In each case the observed line width was 27 ± 2 Hz. The following conclusions were drawn. (a) The very small chemical-shift difference between the two derivatives indicates that the Cd(II) binding site is very similar in both samples. It follows from this result and previous work that the imidazolato bridge is protonated on the Cu side upon reduction of the Cu ion from the II to I valence state. (b) The extremely narrow line width of the resonance in both forms suggests a virtual identity of Cd(II) bound to both subunits of the molecule. (c) The relaxation time, T_1 = 1.2 s, is caused by approximately equal contributions from chemical-shift anisotropy and dipolar interactions with nearby protons.

Superoxide dismutases, which are found in both aerobic and strictly anaerobic organisms (Michelson et al., 1977), are a diverse class of proteins which possess Fe, Mn, or Cu as cofactors necessary for catalysis of the reaction

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

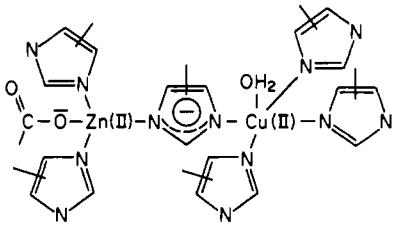
The protein isolated from bovine erythrocytes has received the greatest attention and is the subject of this paper. This protein has a molecular weight near 32 000 and is made up of two identical subunits, each of which binds a Zn(II) and a Cu(II). See Fee (1977) for a review.

Richardson et al. (1977a,b) have determined the structure of bovine superoxide by X-ray crystallography to a resolution of 3.0 Å. The metal binding site is shown in Scheme I. The two metals are ~6–7 Å apart. The Cu(II) resides in a nearly square-planar arrangement of imidazole ligands, and a water molecule appears to act as an axial ligand directed toward the exterior of the protein (Gaber et al., 1972). One of the imidazoles has lost both its protons and forms a bridge to the Zn(II). The Zn(II) resides in an irregular tetrahedron of ligand atoms supplied by three histidines and a carboxyl group.

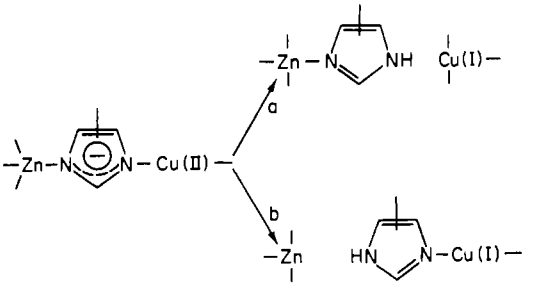
During catalysis of superoxide dismutation the Cu ion cycles between the II and I valence states (Klug-Roth et al., 1973; Fielden et al., 1974). A number of experiments (Fee & Di-Corleto, 1973) support the contention (Fee, 1977) that the bridge between the two metals is broken concomitant with the uptake of a proton when Cu(II) is reduced and re-formed with the release of a proton when Cu(I) is oxidized.

This communication is in part concerned with the question of whether the Zn–N or the Cu–N coordinate bond is broken on reduction (Scheme II). Already two lines of evidence suggest that it is the Cu–N bond that is broken, i.e., path a in Scheme II. First, the optical spectrum of the 2Co(II) protein [Co(II) in the Zn(II) site and the Cu site unoccupied]

Scheme I



Scheme II



is very similar to that of the 2Co(II)–2Cu(I)–protein [Cu(I) in the Cu site] (Moss & Fee, 1975; McAdam et al., 1977). One can infer from this result that the Cu–N bond is broken in 2Co(II)–2Cu(I)–protein, resulting in an isolation of the Cu(I) binding site from the Co(II) site. The second line of evidence comes from X-ray absorption studies (Blumberg et al., 1978) which reveal that the Zn(II) undergoes only minor structural perturbations upon reduction of Cu(II), consistent with the Zn(II)–N bonds remaining intact.

In this study, $^{113}Cd(II)$ has been substituted for Zn(II), allowing direct observation of the cadmium nucleus by NMR spectroscopy. Using this technique, we have examined the NMR properties of various cadmium derivatives of superoxide dismutase. One of our conclusions is that the Cd(II)–N bond is not broken upon reduction of the Cu(II).

Materials and Methods

Bovine superoxide dismutase was isolated from erythrocytes according to the method of McCord & Fridovich (1969). Enriched ^{113}CdO (96% enrichment) was obtained from Oak

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Ridge National Laboratories and converted to the chloride salt by using metal-free HCl. Copper sulfate was obtained commercially and used without further purification. Doubly distilled water was used throughout, and all other chemicals were of reagent grade.

The preparation of apoprotein was carried out by using published methods (Fee, 1973), and the Cd derivatives were prepared by adding the desired metals directly to dilute apoprotein (1–3 mg/mL) in a 0.1 M acetate buffer at pH 5.5 and allowing this solution to stand overnight at room temperature. The spectral properties of the 2Cd(II)–2Cu(II)–protein were identical with those described by Beem et al. (1974), and substitution levels of 90% were obtained. Protein concentrations were determined by using the method of Lowry et al. (1951); copper content of the protein sample was measured by using $\epsilon_{680} = 150\ M^{-1}$ (McCord & Fridovich, 1969); and cadmium content was determined by standard atomic absorption techniques. All samples were ~2 mM in ^{113}Cd.

The preparation of 2Cd(II)–2Cu(I)–dismutase from the oxidized protein was carried out under strictly anaerobic conditions in a Kewaunee inert atmosphere glovebox. First, the 2Cd(II)–2Cu(II)–dismutase solution was rendered oxygen free by purging the sample with N_2. Solid sodium dithionite was then added directly to the enzyme solution until the characteristic blue-green color of the oxidized protein was completely blanched. The reduced protein was then extensively dialyzed against the appropriate buffer, also under rigidly oxygen-free conditions, to remove the dithionite. Oxygen-free D_2O was added to the sample, either by direct addition or by dialysis, to provide a deuterium lock for the NMR experiments. After dialysis the sample and the surface of the dialysis bag occasionally exhibited a pink discoloration. The nature of this material is not known; however, reoxidation of the sample caused the pink color to disappear; the presence of this colored material had no effect on the outcome of the experiments. The pH of the protein solution (uncorrected for deuterium isotope effects and henceforth referred to as pH*) was measured inside the inert atmosphere box by using a Corning Model 109 general purpose pH meter. The pH* of the solutions was adjusted by using either 1 M H_3PO_4 or 1 M NaOH with stirring. The reduced sample was finally transferred to an 18-mm NMR tube which was sealed with a rubber stopper, silicone grease, and Parafilm. The inert atmosphere box was maintained at greater than atmospheric pressure so that a positive pressure was present in the NMR tube during data accumulation. After the collection of NMR data, the sample was reoxidized by the admission of air into the solution; the optical properties of the reoxidized protein were identical with the original 2Cd(II)–2Cu(II) preparation.

All ^{113}Cd NMR spectra were obtained on a highly modified Varian XL 100-15 spectrometer equipped with Gyro-Observe. Some of the experiments were carried out by using a frequency synthesizer mode of operation to be described elsewhere (A. R. Garber and P. D. Ellis, unpublished experiments). All experiments involved the use of a home-built multinuclear 18-mm NMR probe (Byrd & Ellis, 1977). This probe requires 5 mL of sample to obtain a ^{113}Cd NMR spectrum. All ^{113}Cd chemical shifts were referenced to an external sample of 0.1 M $Cd(ClO_4)_2$ in 50:50 H_2O/D_2O. A *positive* shift denotes resonances to *lower* shielding. Spin–lattice relaxation times (T_1) were measured by using the progressive saturation pulse sequence:

$$-(90°-\tau-)_n$$

where τ is the recycle time (including data acquisition) and 90° refers to the angle of the perturbing pulse (Freeman & Hill, 1971). No field gradient pulse was employed in these experiments due to the broadness of the line; i.e., $T_2^* \ll T_1$. The resulting data were fitted to the following equation by using a nonlinear least-squares program:

$$A(\tau) = A_0 \exp(-\tau/T_1)$$

where $A(\tau)$ is the intensity of the resonance obtained by using the pulse interval and A_0 is the intensity obtained for $\tau \geq 5T_1$. The use of the nonlinear program obviates the necessity of determining A_0. The nuclear Overhauser effect, NOE, was determined by using the pulse sequence described above with $\tau = 7T_1$. The "enhanced" spectrum was obtained with the proton decoupler on at all times except during data acquisition. For minimization of heating effects and line broadening, as well as decoupler interference, the NOE suppressed spectrum was obtained in the same manner. In the latter case, however, the decoupling frequency was offset 20000 Hz from the proton chemical-shift range and the noise modulation was turned off. The NOE was calculated by using the equation

$$\text{NOE} = A(\text{"enhanced"})/A(\text{"suppressed"})$$

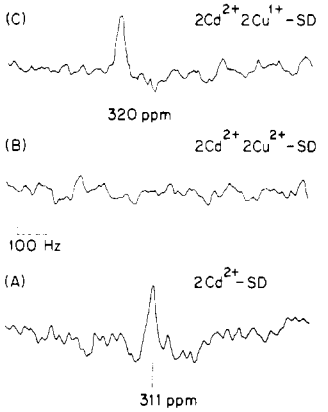

FIGURE 1: ^{113}Cd NMR spectra of bovine superoxide dismutase derivatives. See text for details of solution conditions. (A) ^{113}Cd NMR spectrum of 2Cd(II)–superoxide dismutase. The spectrum was obtained on a 5000-Hz spectral window by using 45° flip angle with a 0.8-s recycle time. 5000 transients were accumulated. The spectrum shown is a 2000-Hz expansion of the transformed spectrum. One resonance appears at 311 ppm. (B) ^{113}Cd NMR spectrum of 2Cd(II)–2Cu(II)–superoxide dismutase. This spectrum was obtained under the same conditions as those employed in (A). (C) ^{113}Cd NMR spectrum of 2Cd(II)–2Cu(I)–superoxide dismutase. This spectrum was obtained under the same conditions employed in (A). One resonance appears at 320 ppm.

Table I: Cadmium-113 Chemical Shifts for Various Metal Derivatives of Superoxide Dismutase

derivatives[a]	^{113}Cd chemical shift[b]	line width[c]	pH*/buffer
2Cd(II)	311	27	5.5/0.1 M acetate
2Cd(II)–2Cu(II)	*d*	*d*	
2Cd(II)–2Cu(I)	320	27	4.7 or 8.0/0.1 M phosphate, 5.5/0.1 M acetate

[a] The designation *m* metal refers to the number of metals per dimer of protein. [b] Chemical shifts are in parts per million with respect to 0.1 M $Cd(ClO_4)_2$. A positive value denotes resonances to lower shielding. [c] Line widths are expressed in hertz. [d] No signal observed.

Results

The NMR spectra of three derivatives of ^{113}Cd-substituted superoxide dismutase were obtained: 2Cd(II), 2Cd(II)–

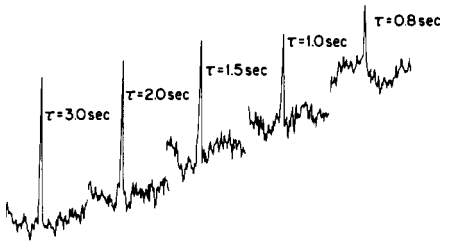

FIGURE 2: ^{113}Cd NMR spectra obtained in a progressive saturation T_1 experiment on Cd(II)–superoxide dismutase. T_1 was found to be 1.2 s.

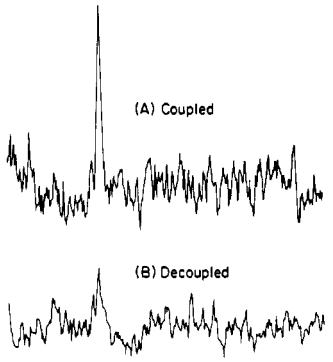

FIGURE 3: Determination of the nuclear Overhauser enhancement factor for 2Cd(II)–superoxide dismutase: (A) is the proton-coupled spectrum and (B) is the proton-decoupled spectrum.

2Cu(II), 2Cd(II)–2Cu(I) (Figure 1). Some typical spectral parameters are given in Table I. For one of these derivatives, 2Cd(II), a detailed study of the relaxation properties was made. The results of progressive saturation and NOE experiments are shown in Figures 2 and 3, respectively.

Theory and Discussion

Relaxation Properties of 2Cd(II)–Superoxide Dismutase. In any systematic treatment of a molecular system by Fourier transform NMR methods, it is essential to have a working knowledge of the spin–lattice relaxation time, T_1, of the nucleus of interest. It is well-known that this parameter is a strong function of molecular structure and the motional dynamics of the system. At the very least, a knowledge of T_1 is essential for optimum data collection rates. Cadmium–metalloproteins are no exception; however, it is worth noting that a single T_1 and NOE experiment can represent a significant amount of instrument time, ~1 week on our XL-100. In this section of the paper we will discuss the measurement and interpretation of the ^{113}Cd T_1 in ^{113}Cd(II)-substituted superoxide dismutase and its associated NOE.

There are several mechanisms available to an $I = {}^1/_2$ nuclide for spin–lattice relaxation (Farrar & Becker, 1971). However, when this spin is in a metalloprotein, in a more or less ionic environment, there are basically two principle relaxation mechanisms available: heteronuclear dipole–dipole (usually with protons) and chemical-shift anisotropy (CSA). Under ideal conditions these two mechanisms may be separated by a knowledge of the NOE for the spin of interest. The experimental data used to deduce the value of T_1 (1.2 s) and the NOE (0.66) are depicted in Figures 2 and 3, respectively. From the amount of noise present in these spectra we estimate that the error associated with these numbers is ~20%. This error will not invalidate the overall conclusions that one can draw from these experiments.

From simple geometrical models ("Interatomic Distances Supplement", 1965) one can calculate a distance of 2.8 Å between a hydrogen and the cadmium atom in a cadmium–histidine complex. If one makes the assumption that two hydrogens per histidine are equivalent (with respect to their distance to the cadmium), then there will be six hydrogens that could provide a dipolar relaxation pathway for the cadmium spin. It is expected that the reorientational correlation time for the ^{113}Cd bound to a protein of 33 000 daltons will be in the range 10–100 ns. Using these values of the correlation time and the geometrical model discussed above, one can predict the dipolar T_1 from standard equations (Farrar & Becker, 1971). These values increase from ~2.5 s for a correlation time of 10 ns to ~17 s for a correlation time of 100 ns. The experimental value is 1.2 s. The T_1 minimum occurs at a correlation time of 6 ns, with a corresponding value for T_1 of 2.2 s. Therefore, one is forced to conclude that if the geometrical arguments presented are valid, then the spin–lattice relaxation is not dominated by dipolar processes but rather that CSA processes are also contributing to the T_1.

The amount of dipole vs. CSA processes can be estimated by a knowledge of the NOE. The NOE in this type of spin system is not subject to a geometrical conjecture concerning the structure of the active site. The NOE for a given pair of heteronuclear dipolar coupled spins (such as ^{113}Cd coupled to n ^{1}H's) is only a function of the correlation time describing the motion of the internuclear vectors between the spins of interest. If the spin–lattice relaxation mechanism was dominated by CSA processes, then the NOE would be equal to 1. The largest algebraic value of the NOE, $1 + n$, was dominated by dipolar processes and is equal to 0.837 for a system out of the region of extreme narrowing. The experimental value of 0.66 strongly suggests that the two relaxation pathways (CSA and dipole–dipole) are about equally as efficient in providing spin–lattice relaxation for the ^{113}Cd nucleus in superoxide dismutase. The value of 0.837 for the NOE represents the limiting value for a system with a correlation time in excess of 50 ns. If the correlation time for superoxide dismutase is shorter, then the predicted NOE would be algebraicly smaller. This would imply a greater importance of the CSA process in the mechanism for spin–lattice relaxation. Hence, the 50:50 mixture represents a lower limit for the amount of CSA relaxation.

Analysis of the ^{113}Cd NMR of 2Cd(II)–, 2Cd(II)–2Cu(II)–, and 2Cd(II)–2Cu(I)–Superoxide Dismutase. This section details the analysis of ^{113}Cd NMR spectra of the various metal derivatives of bovine superoxide dismutase. Before beginning this analysis, however, it is prudent to review the known ^{113}Cd NMR data for relevant model complexes of cadmium and of metalloproteins. Attention will be directed to the relationship between the chemical shift and the environment of the ^{113}Cd(II) nucleus, as well as the effects of external variables such as anions and pH on these shifts.

From several reports in the literature the following generalizations are made: octahedral systems have the smallest chemical shifts (most shielded) followed by progressively larger chemical shifts in tetrahedral and pentacoordinate complexes. Within each of these groups, nitrogen ligands deshield the resonance more than oxygen atoms. Sulfur ligands tend to cause a pronounced deshielding of the cadmium nucleus.

Presently known ^{113}Cd chemical-shift data for metalloproteins are summarized and referenced in Table II. The most shielded ^{113}Cd chemical shifts reported to date for metalloprotein are for the lectin concanavalin A. This protein exhibits three ^{113}Cd resonances of which those at −125 and 43 ppm are assigned to the known S2 and S1 sites, respectively. (The third resonance at 68 ppm corresponds to "free" cadmium in solution.) These sites have been shown by X-ray crystallographic

Table II: Cadmium-113 Chemical Shifts of Various Metalloproteins

metalloprotein	approximate metal coordination	metal site ligands	pH sensitive	counterion sensitive	^{113}Cd chemical shift (ppm)[a]
concanavalin A	S2: coordinate	6 oxygens	no	no	−125 to −132[b]
	S1: 6-coordinate	5 oxygens, 1 nitrogen	no	no	43[b]
parvalbumin	6-coordinate	6 oxygens			−90 to −100[c]
carboxypeptidase A	4-coordinate	2 nitrogens, 1 oxygen, substrate donor atom (usually oxygen)	yes	yes	240,[d] 217[d]
carbonic anhydrase	4- or 5-coordinate[e]	3 nitrogens, substrate donor atom (usually oxygen)	yes	yes	145–241[f–h] 410[g]
alkaline phosphatase	4-coordinate	3 nitrogens, substrate donor atom (usually oxygen)	yes	yes	55–170[f–h]
superoxide dismutase	4-coordinate	3 nitrogens, 1 oxygen	no	no	310–330[i]
metallothionein	possibly 4-coordinate	sulfur			614, 624, 643, 668[j–l]

[a] Chemical shifts are in parts per million with respect to external 0.1 M $Cd(ClO_4)_2$. A positive value denotes resonances to lower shielding. [b] Bailey et al. (1978). In this reference the assignment of the S1 and S2 sites was in error. The correct assignments are given here (A. R. Palmer, P. D. Ellis, W. D. Behnke, D. B. Bailey, and A. D. Cardin, unpublished experiments). [c] Drakenberg et al. (1978). [d] D. B. Bailey and P. D. Ellis (unpublished experiments). Both entries correspond to the ^{113}Cd chemical shift of cadmium-substituted carboxypeptidase A in the presence of the inhibitor *dl*-benzyl succinate. The chemical shift of 240 ppm corresponds to a solution at pH 8 in a ClO_4^- buffer, whereas the 217-ppm shift corresponds to a pH 8 solution in the presence of Cl^- buffer. The line widths in these experiments are 90 and 230 Hz, respectively. Further examination of this system is clearly needed. Work along these lines is currently in progress in our laboratories. [e] Haffner & Coleman (1973). [f] Armitage et al. (1976). [g] Sudmeier & Bell (1977). [h] Armitage et al. (1978). [i] Present work. [j] Suzuki & Maitani (1978). [k] Sadler et al. (1978). [l] J. D. Otvos and I. M. Armitage (personal communication).

studies to have slightly distorted octahedral symmetry with the majority of the first coordination sphere atoms being the oxygen of amino acid residues or water (Edelman et al., 1972). The concanavalin A protein resonances do not exhibit an anion dependency.

The majority of the proteins that have been investigated by ^{113}Cd NMR have a four-coordinate metal site, although in the case of carbonic anhydrase five coordination is possible (Armitage et al., 1978). The ^{113}Cd-substituted metalloenzyme carboxypeptidase A exhibits no resonance unless an inhibitor such as β-phenyl propionate is added, in which case a resonance at 134 ppm occurs in the presence of chloride anion. The crystal structure of this system shows the Cd(II) to be in a roughly tetrahedral environment formed by oxygen atoms from two glutamic acid residues and a histidyl nitrogen with the fourth site open to solvent. In the above cases, this fourth site is occupied either by water or chloride anion (Reeke et al., 1967).

Another metalloenzyme which has been investigated by ^{113}Cd NMR is Cd(II)-substituted human carbonic anhydrase B, which Sudmeier & Bell (1977) and Armitage et al. (1978) have studied. In the absence of anions, the latter investigators report a chemical shift for the resonance of 146 ppm while the former report 228 ppm for the same system. The nature of this discrepancy is not clear but may possibly be due to the amount of dissolved CO_2 present in the samples and/or different isozymes of the protein (J. L. Sudmeier, personal communication). The X-ray structure of this system shows the metal site to be a distorted tetrahedron composed of three histidyl nitrogens with the fourth site open to solution (Kannan et al., 1975). In the absence of anions, this site is occupied by H_2O or OH^-, depending on the pH. Further, the use of a variety of simple halide anions in the fourth site demonstrated a chemical-shift dependence of the Cd(II) resonance in this system, with an observed variation of ~100 ppm, and addition of cyanide to this enzyme appears to form a relatively more covalent Cd(II) species with a chemical shift of 410 ppm (Sudmeier & Bell, 1977). The ^{113}Cd chemical shifts of Cd(II)-substituted alkaline phosphatase and bovine carbonic anhydrase B have also been determined. In the absence of competing effects, these metalloenzymes have chemical shifts of 117 and 214 ppm, respectively. The crystal structures of these systems have not yet been determined.

The ^{113}Cd NMR spectrum of 2Cd(II)–dismutase (Figure 1A) consists of one resonance with a line width of 27 Hz. The appearance of only one resonance indicates that if the Cd(II) sites in the two subunits differ, then this difference only manifests a ^{113}Cd chemical-shift difference of less than 10 Hz (0.5 ppm). Given the known sensitivity of the ^{113}Cd chemical shifts to environmental changes, this result suggests that the metal sites in the two subunits are identical. The presence of the resonance at 310 ppm is in line with the metal site being either four or possibly five coordinate. The crystallographic study of this system reveals that the Zn(II) site is four coordinate, with three of the ligands due to histidyl nitrogens and the fourth site occupied by an oxygen from an aspartate residue (Richardson et al., 1975a,b). It should be noted that this chemical shift is 90–150 ppm more deshielded than human carbonic anhydrase B, which has the same ligand atoms (when no anions are present) and a roughly similar geometry at the Cd(II) site as the dismutase. This deshielding effect is in all probability due to the greater covalent character of the aspartate–cadmium bond in dismutase compared to the water–cadmium bond in carbonic anhydrase or to a slightly different local symmetry about the cadmium.

No resonance was observed from the 2Cd(II)–dismutase derivative [cf. also Armitage et al. (1976)]. This result is to be expected as the paramagnetic Cu(II) atom is 6–7 Å from the Cd(II) sites and would be expected to broaden the ^{113}Cd resonance beyond detection [cf. Cass et al. (1977)]. The reduced copper–protein is of interest for several reasons. First, the *number* of resonances that are observed will indicate the number of chemically different Cd(II) sites in the enzyme. Second, the chemical shifts of this system will indicate the amount of perturbation placed on the Cd(II) sites by the Cu(I). Third, the dependence of this system on pH and buffer conditions will indicate the accessibility of this site to solute molecules. Figure 1C depicts the ^{113}Cd NMR spectrum of 2Cd(II)–2Cu(I)–dismutase. Only one resonance is observed with a chemical shift of 320 ppm. Again, this result indicates that any difference between the two Cd(II) sites in the protein

generates a <0.5-ppm chemical shift. Thus, as with the 2Cd(II)–protein, the two sites are considered equivalent.

The observation of a small chemical shift between the Cu-free protein and the 2Cd(II)–2Cu(I)–protein immediately suggests that only minor changes occur at the Cd coordination site on the binding of Cu(I) to the Cu site. Mechanism a of Scheme II requires the breaking of Cu(I)–imidazole bond to provide the necessary proton uptake while mechanism b has the Zn(II)–imidazole bond breaking. If the correct mechanism were a and the Cd(II) system is analogous to the Zn(II) system, the ^{113}Cd NMR properties of the reduced protein would be similar to those of the copper-free system. On the other hand, if mechanism b were correct, the Cd(II) species would be required to break the Cd–N bond which either would not be replaced (which is unlikely) or would be replaced by water or some component from the solution. In any case, the ^{113}Cd NMR properties of the reduced system would be dramatically different from those of the copper-free protein. On this basis, the ^{113}Cd NMR results indicate that mechanism a is the more likely. This point can be strengthened by obtaining the ^{113}Cd NMR data with a variety of pH and buffer conditions. The following systems were therefore used: (1) 0.1 M acetate buffer, pH* 5.5, (2) 0.1 M phosphate buffer, pH* 8.0, and (3) 0.1 M phosphate buffer, pH* 4.7. Under all of these conditions there were no changes observed in the ^{113}Cd NMR spectrum of the 2Cd(II)–2Cu(I)–protein. An experiment was also carried out in which the dithionite was not dialyzed away from the protein. Again, no change was noted in the chemical shift of the protein. Several lines of evidence now support the contention that the imidazole bridge between Zn and Cu is broken at the Cu side upon reduction. The Cu(I), which remains very tightly bound to the protein (Fee & Briggs, 1975), would thus appear to be coordinated to three atoms donated by the protein and to have at least one position open to the solvent (Fee & Ward, 1976).

The certainty of this conclusion now allows speculative comment on the role of the bridging imidazole in the catalysis process. Hodgson & Fridovich (1975) conjectured that during catalysis the bridge breaks at Cu, and a proton is then taken up from the solvent as was shown by Fee & DiCorleto (1973). This proton is subsequently donated to Cu(I)-bound O_2^- followed by electron transfer from Cu(I) to the superoxide to form Cu(II)–O–O–H (Fee & Ward, 1976) and to re-form the bridge. The present work in conjunction with other studies provides a rational structural basis for such a mechanism but does not provide any evidence for its occurrence. However, McAdam et al. (1977) have recently demonstrated that the breaking and re-reforming of the bridge occur at a rate equal to the overall rate of catalysis which was earlier shown to be diffusion controlled (Fielden et al., 1974). Thus, while this mechanism appears possible, two important facts must be considered in asking how protons are donated to O_2^- in the second step of catalysis (Fee & Ward, 1976). (a) Free aquo Cu ions are ~4× more efficient catalysts of superoxide dismutation than Cu–dismutase (Rabani et al., 1973); thus, no special structural requirements are required for proton transfer. (b) Removing the Zn from the protein lowers activity by at most a factor of 2 at pH 7.8 (Fee & Briggs, 1975), but activity is unaffected below pH 7 (Valentine, personal communication). The question is raised as to whether a protein-mediated proton-transfer mechanism is necessary for this catalytic process.

While this work was in progress, a report appeared by Armitage et al. (1978) concerning similar studies of superoxide dismutase. These workers found that the copper-free system exhibited a chemical shift of 179 ppm. Further, upon reduction of the 2Cd(II) preparation with dithionite, a chemical shift to 7 ppm was observed, with a large increase in the line width of the resonance. These observations are diametrically opposed to our own results. The possibility of differences due to pH and buffer composition must be ruled out on the basis of our own work. Further, the results of Armitage et al. (1978) suggest that a large change occurs in the Cd(II) site between the copper-free protein and the reduced copper system. This change is incompatible with the mechanism proposed for proton insertion by both ourselves and Armitage. Also, the results shown for other systems indicate that the Zn(II) site is extremely stable and not likely to exhibit such pronounced differences in the ^{113}Cd NMR. On these bases, we must conclude that our results are more indicative of the native system than those of Armitage. The difference in results may be due to the method by which the enzyme systems were prepared. For example, the work referenced by Armitage describing the preparation of the apoprotein does not include the perchlorate dialysis to remove tightly bound EDTA (Fee, 1973), the presence of which may produce erroneous results.

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Resolution and Identification of Iron-Containing Antigens in Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: Iron-containing antigens present in membrane vesicles prepared from *Escherichia coli* ML 308-225 were analyzed by crossed immunoelectrophoresis following growth of the organism in the presence of ^{59}Fe. Seven discrete antigens (or antigen complexes) are detected by autoradiography, and six contain primarily nonheme iron. Three immunoprecipitates are positively identified as NADH dehydrogenase (EC 1.6.99.3), NADPH dehydrogenase (EC 1.6.99.1), and glutamate dehydrogenase (EC 1.4.1.4) based on activity stains for these enzymes. Two other immunogens containing nonheme iron correspond to antigens no. 22 and 37 in the crossed immunoelectrophoresis reference pattern of Owen & Kaback [Owen, P., & Kaback, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3148]. In addition, the immunoprecipitate corresponding to Braun's lipoprotein contains tightly bound iron.

Iron-containing proteins are important components of the respiratory chain of microorganisms (Orme-Johnson, 1973; Haddock & Jones, 1977). However, resolution of the individual iron-containing redox proteins comprising the aerobic respiratory chain of *Escherichia coli*, for example, has not been achieved nor has a clear notion of the functional organization of the redox carriers emerged. Most of the cytochromes and iron–sulfur proteins involved in aerobic electron transfer are membranous and refractory to extensive purification (Hendler & Burgess, 1972, 1974). Moreover, analysis of membrane proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis usually results in loss of intrinsic biological activity and also nonheme iron (Clegg & Skyrme, 1973).

The use of crossed immunoelectrophoresis (CIE;[1] Johansson & Hjerten, 1974; Owen & Salton, 1975, 1977; McLaughlin & Meerovitch, 1975; Smyth et al., 1976, 1978; Alexander & Kenny, 1977, 1978) represents an important innovation in the analysis of microbial membrane components. In a recent study (Owen & Kaback, 1978), a high-resolution CIE reference pattern comprised of over 50 discrete antigens was established for *E. coli* membrane vesicles, and many of the immunogens were identified by zymogram techniques that rely on retention of intrinsic biological activity (Owen & Kaback, 1979a). Furthermore, quantitative immunoadsorption was utilized to study the topological distribution of antigens across the plane of the membrane (Owen & Kaback, 1978, 1979b), and it was demonstrated that vesicles prepared in the manner described (Kaback, 1971; Short et al., 1975) retain essentially the same polarity and configuration as the membrane of the intact cell. In view of the obvious advantages of CIE, we have undertaken an immunological investigation of the iron-containing antigens in membrane vesicles prepared from cells of *E. coli* grown aerobically on the nonfermentable carbon source succinate. In this paper, seven such antigens are clearly resolved and four are identified.

Experimental Procedures

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium A (Davis & Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate) and $^{59}FeSO_4$ (32.5 mCi/mg) at a final concentration of 1 mCi/L. Membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975), frozen in liquid nitrogen, and maintained at or below −70 °C. The prepara-

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[1] Abbrevations used: CIE, crossed immunoelectrophoresis; EDTA, ethylenediaminetetraacetic acid.

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