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A WATER ^{17}O NMR STUDY OF THE pH DEPENDENT PROPERTIES OF SUPEROXIDE DISMUTASE

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SUMMARY: ^{17}O NMR studies of ^{17}O enriched water solutions containing superoxide dismutase have been performed between pH 7.5 and 11.7. Whereas T_1 measurements do not reveal any interaction between ^{17}O and the paramagnetic copper center, the linewidth results appreciably increased with increasing pH with an apparent pK_a of 11.3. Comparison with ^1H NMR relaxation studies allows to interpret the present data as due to binding to the copper ion of an OH^- anion at high pH. The binding position should be of "equatorial" type, not involving the binding position of the coordinated water.

INTRODUCTION

Bovine erithrocyte superoxide dismutase is a dimeric enzyme containing both copper(II) and zinc(II) metal ions in each subunit (1).

Whereas the zinc(II) ion is of structural type, the copper(II) ion is essential for the catalytic process and is accessible to solvent molecules (2). X-ray investigations have shown that the copper(II) ion is bound to four histidine nitrogens, one of them belonging to an imidazolate ion which is bridging the two metal ions; the four nitrogens give rise to a distorted planar arrangement (3).

The ESR spectra show an $A_{||}$ value of 143×10^{-4} cm^{-1}, (4) which is typically in the range of tetragonal five coordinated copper(II) complexes (5). This interpretation is consistent with the electronic spectra which show a broad absorption at 14.7×10^3 cm^{-1} (6).

^1H NMR relaxation data of water solutions containing the enzyme have definitely estabilished that water interacts with copper(II)

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(7,8). Rotilio et at. found that the ^{1}H NMR relaxation rates increase with increasing pH, with a pK_a of about 11.5 (9). This behaviour was taken as indicative of a $CuOH_2 \rightleftharpoons CuOH^-$ equilibrium. Indeed, water bound to metals is known to display a pK_a quite smaller than 14. The hydroxide proton was believed to experience higher nuclear relaxation rates with respect to a coordinated water owing to a shorter Cu-H distance.

Later, Boden et al. suggested that the increase in ^{1}H T_1^{-1} relaxation rate with pH could be due to an addition of an OH^- group to the copper cromophore containing the water molecule (10). They also suggested that the hydroxide group could substitute a coordinated histidine residue.

We have recently shown that ^{17}O NMR of $H_2^{17}O$ solutions of copper containing enzymes can be a sensitive tool for detecting the binding of ^{17}O containing species to the paramagnetic center (11). We felt therefore that an ^{17}O NMR study would contribute to shed light on the problem, and possibly to better understand the conditions under which ^{17}O NMR parameters are useful in the investigation of the interaction with the paramagnetic centers.

MATERIALS AND METHODS

Bovine erithrocyte superoxide dismutase was obtained as a lyophylized powder from Sigma, purified and checked as previously reported (12). Enzyme solutions were concentrated by ultradialysis up to about 2×10^{-3} M. Enzyme concentrations were calculated from the absorbance values of the copper d-d transition ($\varepsilon_{680} = 300\ M^{-1}\ cm^{-1}$ per dimeric unit (6)). Reduced enzyme was obtained by addition of sodium dithionite and checked through the disappearance of the d-d transition. The electronic spectra were recorded on a Cary 17 D spectrophotometer in the absorbance range 0-0.1.

NMR samples were prepared by adding 20% enriched $H_2^{17}O$ to the enzyme solutions, to final enrichments of 2-5%. NMR measurements were performed with a CXP Bruker spectrometer operating at 8.13 MHz. T_1 values were calculated from a best fitting treatment of the peak heights obtained through the inversion recovery method. T_2 values were obtained from the experimental linewidths, appropriately reduced for the line broadening introduced by exponential weighting of the free induction decay, through the relationship $T_2 = (\pi \Delta\nu)^{-1}$.

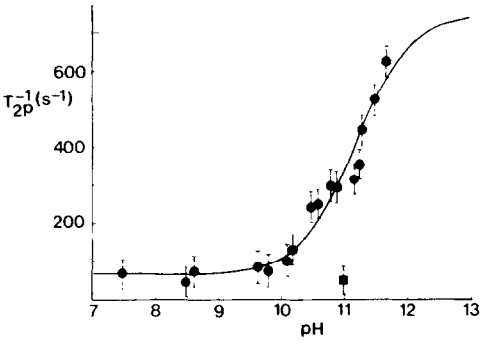

Figure 1. T_{2p}^{-1} values of $H_2{}^{17}O$ in 1.33×10^{-3} M dimeric superoxide dismutase solutions as a function of pH (●) and in the presence of stoichiometric amounts of cyanide (■). The best-fit curve for a single acid base equilibrium is also shown.

RESULTS

The ^{17}O NMR spectra of $H_2{}^{17}O$ enriched solutions of native and dithionite-reduced superoxide dismutase have been recorded as a function of pH. The differences in ^{17}O transverse relaxation rates (T_{2p}^{-1}) observed in solutions of native and reduced enzyme at the same concentration and pH are directly related to the paramagnetic effect of the copper(II) center; their pH dependence is reported in Figure 1. Above pH 11.7 the results are not reproducible, owing to the progressive irreversible denaturation of the enzyme; such effect, previously observed by Rotilio at al. (9) and Boden et al. (10) leads to an abrupt reduction of the T_{2p}^{-1} values to almost zero. Addition of stoichiometric amounts of cyanide at pH 11 also results in almost complete quenching of the paramagnetic effect (Figure 1). At variance with the T_2^{-1} results, the longitudinal relaxation rates are not appreciably affected by the paramagnetic center in the pH range 7.5-11.7. T_1 values of 2.9 ± 0.2 ms were measured for both pure and reduced enzymes as well as for the cyanide-inhibited samples.

The T_2^{-1} data (Figure 1) parallel the proton relaxation rate data previously reported (9,10); they can be satisfactorily fitted to a single acid-base equilibrium with pK_a of 11.3 ± 0.3, which

compares well with the value of 11.5 proposed by Rotilio et al.(9). A major difference between the ^{1}H and ^{17}O NMR results is however apparent: while the proton relaxation rate enhancements are approximately doubled on passing from neutral to alkaline pH, the ^{17}O T_{2p}^{-1} values show a ten-fold increase in the same pH range. The effect measured at neutral pH is indeed very small, and arises from linewidth differences of about 10-15 Hz; such differences are obtained from linewidths of more than one hundred Hz and are therefore affected by a large percent error, as shown by the error bars in Figure 1.

The temperature dependence of the ^{17}O linewidth has also been investigated in the range 5-35 °C at several pH values. The linewidths of $H_2{}^{17}O$ solutions containing both the native and the reduced enzymes markedly decrease with increasing temperature all over the pH range, analogously to what happens for pure $H_2{}^{17}O$. On the other hand, above pH 9 the linewidth differences are temperature independent indicating that the equilibrium between ^{17}O containing species interacting with the copper(II) center and in the bulk solution is fast on the NMR time scale. Below pH 9 the paramagnetic linewidths increase with decreasing temperature, indicating that the exchange is still fast, although slower than at high pH values. This rules out the possibility that the small effect measured at neutral pH be due to exchange controlled mechanisms (13).

DISCUSSION

The above results show that the ^{17}O NMR linewidth is sensitive to the interaction of the solvent with the copper(II) center in the active site of superoxide dismutase. The nature of the interaction is likely to be the Fermi contact coupling between the nucleus and the unpaired electron, which can only arise from direct binding to the metal ion of an ^{17}O containing species. Indeed, T_{2p}^{-1} values much larger than T_{1p}^{-1} are indicative of dominant contact contributions operative in the former relaxation process, since dipolar contributions

are expected to be nearly equal for the two mechanisms in the present experimental conditions.† Large contact contributions to T_{2p}^{-1} in copper(II) containing systems are not unexpected, owing to the long electronic relaxation times, τ_e, usually displayed by the above ion (11,14,15); in the present enzyme system the reported τ_e values are in the range 10^{-9} - 10^{-8} s (7,10).

The pH dependence of T_{2p}^{-1} can be related to the binding at the metal of an ^{17}O containing group, the pK_a for the interaction being about 11.3. Such a group can reasonably only be the hydroxide ion. The problem remains of the small paramagnetic effect on the ^{17}O NMR linewidth at pH <9, where ^{1}H NMR relaxation data indicate the presence of a copper coordinated water molecule. Since the paramagnetic effect on ^{17}O nuclei is related to the efficiency of contact coupling mechanisms, a possible explanation is that the copper(II) cromophore in the enzyme has an essentially square pyramidal structure with water in an apical position. In fact, in tetragonal copper(II) complexes the unpaired electron resides mainly in the $d_{x^2-y^2}$ orbital, and is not expected to be efficiently coupled via contact mechanisms with axial ligand nuclei (16); an essentially square pyramidal copper(II) cromophore in superoxide dismutase is indeed consistent with the X-ray data and with recent spectroscopic results (12,17). Within this frame the hydroxide ion would be bound in the equatorial position, as proposed by Boden et al. (10), and would thus experience much larger paramagnetic effects through contact contributions.

A simple ionization of the coordinated water molecule to a hydroxide ion would hardly be consistent with such a large increase

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† According to the Solomon-Bloembergen model the ^{17}O T_{2p}^{-1}/T_{1p}^{-1} ratio for essentially dipolar relaxation mechanisms in a 1.4 T magnetic field would range from 1.17 to 1.34, for correlation times ranging from 10^{-9} to 10^{-8} s. Larger correlation times cannot be operative since they would exceed the rotational correlation time for a macromolecule of the size of superoxide dismutase. On the other hand, the upper limit for T_{1p}^{-1}, as judged from the uncertainty in the longitudinal relaxation measurements, is some 30-fold smaller than the observed T_{2p}^{-1} values.

in T_{2p}^{-1} values, even if the decrease in Cu-O distance is taken into account. On the other hand, addition of OH^- in equatorial position would leave the axial coordinated water molecule and would satisfactorily account for both the ^1H and ^{17}O NMR data.

The cyanide ion gives rise to a square planar complex when bound to copper(II) in superoxide dismutase (4,17). Consistently with the ^1H (10) and ^{17}O NMR results, the cyanide ligand is capable of displacing the OH^- ion from coordination. Direct cyanide-hydroxide competition was also suggested on the basis of the decrease of the cyanide affinity constant at high pH (10).

A comment is due on the exchange rate of the coordinated hydroxide ion in the present system. From the ^{17}O T_{2p}^{-1} values at high pH and from the known concentration of enzyme a lower limit for the exchange rate of the OH^- group can be placed at about 10^7 s^{-1}; this value is rather high, but still within the diffusion limit (6×10^{11} M^{-1} s^{-1} (18)) for OH^- concentrations higher than 10^{-4} - 10^{-3} M.

REFERENCES

1. Bannister, J. V., and Hill, H. A. O. (1980) Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase, Elsevier, New York.
2. Rotilio, G., Rigo, A., Viglino, P., and Calabrese, L. (1977) in: Superoxide and Superoxide Dismutase, Michelson, A. M. et. al. eds., pp. 207-214, Academic Press, London.
3. Richardson, J. S., Thomas, K. A., Rubin, B. H., Richardson, D. C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349-1353.
4. Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L., and Mondovì, B. (1972) Biochemistry 11, 2187-2192.
5. Bencini, A., Bertini, I., Gatteschi, D., and Scozzafava, A., (1978) Inorg. Chem. 17, 3194-3197.
6. Mc Cord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
7. Gaber, P. B., Brown, R. D., Koenig, S. M., and Fee, J. A. (1972) Biochim. Biophys. Acta 271, 1-5.
8. Koenig, S. M., and Brown, R. D. (1973) Ann. N. Y. Acad. Sci. 222, 752-763.
9. Terenzi, M., Rigo, A., Franconi, C., Mondovì, B., Calabrese, L., Rotilio, G. (1974) Biochim. Biophys. Acta 351, 230-236.
10. Boden, N., Holmes, M. C., Knowles, P. F. (1979) Biochem. J. 177, 303-309.

11. Bertini, I., Canti, G., and Luchinat, C. (1981) Inorg. Chim. Acta 56, in press.
12. Bertini, I., Luchinat, C., and Scozzafava, A. (1980) J. Am. Chem. Soc. 102, 7349-7353.
13. Swift, T. J. (1973) in: NMR of Paramagnetic Molecules, La Mar, G. N. et al.,eds, pp. 53-83, Academic Press, New York.
14. Espersen, W. G., and Martin, R. B. (1976) J. Am. Chem. Soc. 98, 40-44.
15. Bertini, I., and Scozzafava, A. (1981) in : Metal Ions in Biological Systems, Sigel, H., ed., Marcel Dekker, New York, Vol. XII, in the press.
16. Lewis, W. B., Alei, M. Jr., and Morgan, L. O. (1966) J. Chem. Phys. 45, 4003-4013.
17. Bertini, I., Borghi, E., Luchinat, C. and Scozzafava, A., submitted.
18. Eigen, M., and De Maeyer, L. (1963) in: Technique of Organic Chemistry, Weissberger, A. ed., pp. 1034-1053, John Wiley and Sons, New York, Vol. VIII, part. II.