NUCLEAR MAGNETIC RESONANCE STUDIES ON THE INTERACTION BETWEEN SALICYLATE AND CERULOPLASMIN

Toshiaki IMOTO, Kazuyuki AKASAKA and Hiroyuki HATANO
Department of Chemistry, Faculty of Science, Kyoto University, Kyoto, Japan

Received 5 April 1971

1. Introduction

Ceruloplasmin, a blue copper-containing protein with a molecular weight of about 160,000 obtained from mammalian plasma [1], shows oxidase activity to aromatic polyamines and polyphenols [2]. The oxidase activity has been thought to be associated with a reversible valence change between the cupric and the cuprous state in an approximately half of its total 8 copper atoms, whereas the rest to be in the cuprous state [3]. Curzon and coworkers have found that the oxidase activity is inhibited by various substances [4], which may be classified into two groups, one consisting of small anions such as azide, cyanide, thiocyanate, fluoride and chloride, and the other a series of substances which have the structural unit $>C=C<_{COO^-}$, such as fumarate, crotonate and also some benzoate derivatives. The inhibition mechanism of the former group has been thought to involve a direct interaction with the cupric ions of ceruloplasmin [5–7]. However, little has been known about the inhibition mechanism by compounds belonging to the latter group, particularly concerning whether or not these compounds bind directly to the cupric ions of ceruloplasmin. To investigate this point, salicylate (*o*-hydroxybenzoate), an inhibitor belonging to the latter group, has been chosen in the present work, and its interaction with ceruloplasmin has been studied by the high resolution nuclear magnetic resonance (NMR) method. Experimental results have indicated that salicylate does not bind directly to the cupric ions of ceruloplasmin, in spite of the evidence that their reduction with a substrate is considerably inhibited by the presence of salicylate.

2. Experimental

Human ceruloplasmin was prepared from Cohn Fraction IV by the method of Deutsch [8]. The preparations used were about 88 to 92% pure, as determined from the absorbance ratio at 610 nm and 280 nm. Ceruloplasmin concentrations were determined spectrophotometrically with $A^{1\%\ 1\ \mathrm{cm}}_{610\ \mathrm{nm}} = 0.68$ [8]. Analytical grade sodium salicylate was obtained from Nakarai Chemicals Co. Ltd. and was used without further purification.

NMR signals from the aromatic ring protons of salicylate were taken with a Varian HA-100 spectrometer in D_2O solution, pD 5.90, buffered by 0.1 M sodium acetate and containing 50 μM EDTA. Since the resonance lines became overlapped when broadened, the width was determined by a computer simulation by assuming Lorentian line shapes. The resonance lines from para-proton H(4) of salicylate were chosen for this purpose.

3. Results

In the absence of ceruloplasmin, a fairly well resolved NMR spectrum from aromatic protons of salicylate was obtained at 25° (fig. 1a). When ceruloplasmin was added to the solution of salicylate, all the proton signals became broadened rather uniformly (fig. 1b, 1c). The line width increased almost proportionally with increasing ceruloplasmin concentration (fig. 2), and became even broader as the temperature was lowered. This is expected when salicylate is exchanged rapidly between binding sites on cerulo-

North-Holland Publishing Company – Amsterdam

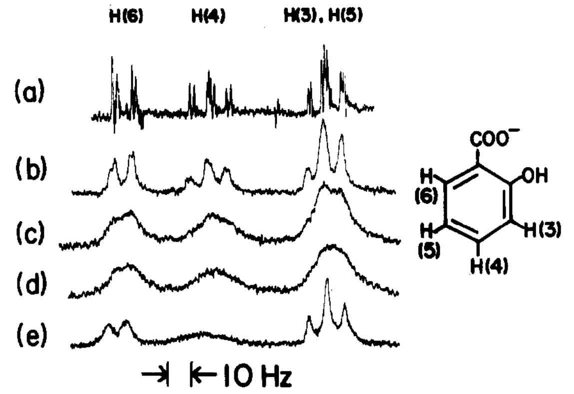

Fig. 1. Effect of ceruloplasmin and of free cupric ion on aromatic proton signals in the 100 MHz NMR spectrum of salicylate. The spectrum was taken at 25° with 50 mM salicylate in the presence of (a) nothing, (b) 34.4 μM ceruloplasmin, (c) 138 μM ceruloplasmin, (d) 138 μM ceruloplasmin and excess of ascorbic acid, and (e) 167 μM $CuSO_4$. The solvent was D_2O containing 0.1 M sodium acetate and 50 μM EDTA (pD 5.9), except (e) for which only D_2O was used.

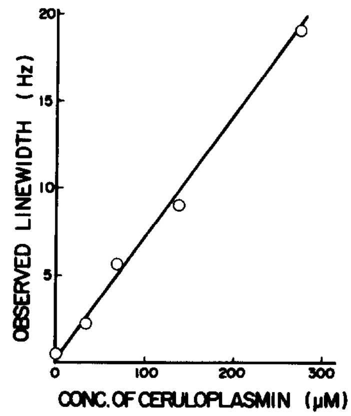

Fig. 2. Observed line width of aromatic proton signals of salicylate as a function of ceruloplasmin concentration. The width was determined from the computer simulation of resonance lines from the para-proton H(4) of salicylate by assuming Lorentian line shapes.

plasmin and the bulk solution so that the transverse relaxation of salicylate protons is dominated by an effective correlation time in the bound state.

To examine whether the observed line width includes the paramagnetic contribution from cupric ions associated with ceruloplasmin, excess ascorbic acid was added to the mixture of salicylate and ceruloplasmin. Although, under this condition, all cupric ions were completely reduced to the cuprous state [9], no significant change occurred in the observed line width (fig. 1d).

In contrast to the uniform broadening in the presence of ceruloplasmin, the NMR spectrum of salicylate in the presence of free cupric ions was found to show a preferential broadening of the H(4) proton (fig. 1e), indicating that a salicylate–Cu^{2+} complex was formed in which the unpaired electron is transmitted preferentially to the carbon(4) atom through delocalized π-orbitals of salicylate.

4. Discussion

For the present situation in which salicylate is exchanged rapidly between binding sites on ceruloplasmin and the bulk solution, the observed line width $\Delta\nu_{obs}$ of NMR signals from salicylate protons will consist mainly of the following terms:

$$\Delta\nu_{obs} = \Delta\nu_{n,dipole} + \Delta\nu_{e,dipole} + \Delta\nu_{e,contact} \qquad (1)$$

Here, the first term represents the intra- and intermolecular dipolar contribution from other nuclei (mostly protons), whereas the second and the third terms represent the paramagnetic contribution from ceruloplasmin cupric ions, through the anisotropic dipolar interaction, and through the contact and pseudocontact interaction, respectively. The contribution from other effects, such as anisotropic chemical shift, spin-rotation interaction and quadrupole relaxation may be neglected in the present case.

Since the preferential broadening of salicylate proton signals as observed in the salicylate-Cu^{2+} mixture does not occur in the presence of ceruloplasmin, $\Delta\nu_{e,contact}$ in eq. (1) due to the direct transfer of unpaired electrons to the salicylate protons from cupric ions is considered negligible in the salicylate–ceruloplasmin complex. The fact that no significant change in the electron spin resonance spectra of ceruloplasmin was observed by addition of salicylate [10], is consistent with the above interpretation.

On the other hand, the observation that the line width does not change by the reduction of the cupric ions of ceruloplasmin indicates that the contribution of $\Delta\nu_{e,dipole}$ is also negligible within experimental error, which is estimated to be less than 1.0 Hz in the presence of 138 μM of ceruloplasmin. This result leads one to a rough estimate of the lower limit for the distance r between a salicylate proton and and electron spin of a cupric ion in ceruloplasmin from the theoretical expression [11].

$$\Delta\nu_{e,dipole} \cong \frac{7}{20\pi} \gamma_H^2 \gamma_e^2 \hbar^2 \left(\frac{1}{r}\right)^6 \tau_c \cdot R$$

$$(\omega_H^2 \tau_c^2 < 1, \omega_e^2 \tau_c^2 \gg 1) \qquad (2)$$

where γ_H, γ_e, ω_H, ω_e, and R represent the gyromagnetic ratio of proton, that of electron spin, the resonance frequency of nuclear spin, that of electron spin and the ratio of the ceruloplasmin concentratior to the salicylate concentration, respectively. The effective correlation time in the bound state is related, in this case, to the rotational correlation time τ_r, the electron spin relaxation time τ_e and the life time of the complex τ_h as

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_h} + \frac{1}{\tau_e} .$$

As, in the present situation, τ_r and τ_h will be long compared with τ_e for cupric ions ($\sim 10^{-9}$ sec) [12, 13], τ_c will be determined by τ_e. Numerical calculation based on eq. (2) indicates that a salicylate proton is separated by at least 6 Å from the electron spin of a ceruloplasmin cupric ion.

Thus the broadening should mainly be attributable to the dipolar interaction with other nuclei (mostly protons), which is given by

$$\Delta\nu_{n,dipole} = \frac{1}{4\pi} \gamma_H^4 \hbar^2 \sum_i \left(\frac{1}{r'_i}\right)^6 n \cdot R \cdot \tau_r$$

$$(\omega_H \tau_r \gg 1) \qquad (3)$$

where r'_i and n represent the distance between the proton under consideration and the ith nucleus interacting with it, and the number of binding sites on a ceruloplasmin molecule, respectively. If we assume that the intramolecular contribution from the nearest neighbor protons dominates in the broadening of the para-proton resonance, the straight line of fig. 2 gives $n \cdot \tau_r = 7.8 \times 10^{-6}$ sec. If we assume that salicylate binds rigidly to ceruloplasmin, τ_r will be dominated by the rotational correlation time of ceruloplasmin itself. If we take, as the lower limit of τ_r, the slower component of the rotational correlation time (2.6×10^{-6} sec) calculated from Perrin's equation [14] for the prolate ellipsoid-shaped ceruloplasmin [15], this places the lower limit for the value of n as 3. The existence of more than several binding sites on ceruloplasmin may well be expected in view of the experimental evidence that the protein consists of at least eight subunits [16], although the specific identification of the binding site cannot be made from the present experiment only.

The results obtained in the present work suggest a great difference in the inhibition mechanism between salicylate and other small anions. The most interesting point is that salicylate does not interact directly with the cupric ions associated with ceruloplasmin, in contrast with the small anions, although salicylate does inhibit the reduction of the cupric ions of ceruloplasmin with a substrate *N,N*-dimethyl-*p*-phenylenediamine, as revealed by our separate experiment with a stopped-flow technique [10].

Acknowledgements

We wish to express our thanks to Dr. O. Nakagawa for the kind offer of Cohn Fraction IV of human serum plasma, to Professor K. Hiromi and to Mr. and Mrs. T. Manabe for helpful discussions and comments, and to Mr. S. Shibata for assistance in carrying out computer simulations.

References

[1] C.G. Holmberg and C.B. Laurell, Acta Chem. Scand. 2 (1948) 550.
[2] C.G. Holmberg and C.B. Laurell, Acta Chem. Scand. 5 (1951) 476.
[3] L. Broman, B.G. Malmström, R. Aasa and T. Vänngård, J. Mol. Biol. 5 (1962) 301.
[4] G. Curzon and B.E. Speyer, Biochem. J. 105 (1967) 243.
[5] G. Curzon, Biochem. J. 100 (1966) 295.

[6] C.B. Kasper, J. Biol. Chem. 243 (1968) 3218.

[7] L.E. Andréasson and T. Vänngård, Biochim. Biophys. Acta 200 (1970) 247.

[8] H.F. Deutsch, Arch. Biochem. Biophys. 89 (1960) 225.

[9] W.E. Blumberg, J. Eisinger, P. Aisen, A.G. Morell and I.H. Scheinberg, J. Biol. Chem. 238 (1963) 1675.

[10] T. Imoto, K. Akasaka, K. Hiromi and H. Hatano, Bulletin of the 9th annual meeting of the Biophysical Society of Japan (1970) p. 184.

[11] A. Abragam, The Principles of Nuclear Magnetism (Clarendon Press, Oxford, 1961).

[12] A.S. Mildvan and M. Cohn, Advances in Enzymol. 33 (1970) 1.

[13] W.B. Lewis, M. Alei, Jr. and L.O. Morgan, J. Chem. Phys. 44 (1966) 2409.

[14] F. Perrin, J. Phys. radium [7] 5 (1934) 497.

[15] C.B. Kasper and H.F. Deutsch, J. Biol. Chem. 238 (1963) 2325.

[16] W.N. Poillon and A.G. Bearn, Biochim. Biophys. Acta 127 (1966) 407.