The Copper Stoichiometry and Oxidation State of Dopamine-β-Monooxygenase. ^1H and ^{19}F NMR Studies

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The stoichiometry of copper bound to dopamine-β-monooxygenase (DβM) has been investigated by ^1H NMR. The longitudinal relaxation rates of water protons were measured as a function of Cu(II) added to the apoenzyme. The results clearly indicate 8 Cu per tetramer, in agreement with similar experiments on different DβM preparations. ^{19}F NMR was applied to determine the position of the redox equilibrium in the native protein using 0.5 M NaF as indicator. A reasonable interpretation of these measurements had to invoke 8 rather than 4 Cu per tetramer. The enzyme was found to exist almost exclusively in the oxy form.

Dedicated to Professor Olav Foss on his 70th birthday

Dopamine-β-monooxygenase is a copper-containing enzyme which catalyzes the reaction:

$$\text{Dopamine} + \text{ascorbate} + O_2 \rightarrow \text{noradrenaline} + \text{dehydroascorbate} + H_2O.$$

The general equation for the hydroxylation reaction is:

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O.$$

The enzyme has a molecular weight of about 290 000 and is assumed to consist of four identical subunits.[1] The copper content in the native protein is still a matter of dispute, the question being whether 4 or 8 mol of Cu are bound per mol of enzyme tetramer. In order to understand the catalytic mechanism of the enzyme it is of vital importance to determine the number of copper atoms at the active sites.

Skotland *et al.*, using liquid chromatography,[2] dialysis techniques,[3] and more recently a Cu(II)-specific electrode,[4] claim that the tetramer contains 4 Cu(II). Blackburn *et al.* propose, on the basis of EPR techniques in combination with CN^- and azide inhibition studies of the enzyme, an active site involving two four-coordinated copper atoms per subunit.[5] This is consistent with a recent NMR study reported by Ash *et al.*,[6] in which relaxation rates ($1/T_1$) of water protons were measured as a function of the amount of copper added to the enzyme. The titration curve showed a discontinuity in the range 7–8 Cu per tetramer.

Both the Cu(II)-specific electrode measurements and the NMR results look quite convincing. One possible explanation for the discrepancy may be found in the different methods of preparing the enzyme. DβM is a glycoprotein and exists in two different molecular forms: an amphiphilic form and a hydrophilic form. The enzyme used by Ash *et al.* in the NMR experiment was purified from bovine chromafine granules prepared by the method of Smith and Winkler.[7] The final product contained 2.1 Cu per tetramer as determined by atomic absorption spectroscopy. No attempt was made to prepare the apoenzyme. Skotland *et al.*, following a somewhat different purification procedure,[9] also prepared the apoenzyme for their titration experiment with the Cu-specific electrode. The apoenzyme was produced by dialysis against EDTA[9] and had less than 0.2 Cu per tetramer as determined after wet ashing.

In the present paper we report NMR studies on samples of holo- and apoenzymes prepared by the Skotland method.[8] In this way we practically eliminate the possibility of the discrepancies be-

ing due to the use of different sample preparations. At the outset it is difficult to rationalize why the Cu-specific electrode measurements disagree completely with the reported spectroscopic results.

Theory

The addition of a paramagnetic ion to water produces an increase in the relaxation rates of the solvent water protons. If a macromolecule to which the paramagnetic ion binds is added to the solution, a further enhancement of water proton relaxation is observed. This proton relaxation enhancement (PRE) effect may be explained in the following way:

In water, proton relaxation occurs by a dipole–dipole mechanism, with the interaction being modulated by the tumbling motion of the molecule. The addition of a paramagnetic ion to water introduces the electron–proton interaction as the predominant relaxation mechanism. The large magnetic moment associated with the unpaired electron of the paramagnetic ion leads to a very efficient relaxation.

In this ionic water solution, three correlation times modulating the dipolar interaction have to be considered: the tumbling motion relaxation time (t_r), the electron spin relaxation time (t_s), and the lifetime of a water molecule in the coordination sphere (t_m). The contributions of these correlation times to the overall correlation time (t_c) may be expressed as:

$$t_c^{-1} = t_s^{-1} + t_r^{-1} + t_m^{-1}.$$

If t_m is very long on the NMR time-scale no information will be transmitted from the metal ion to the bulk solvent during the course of a relaxation measurement and consequently no enhancement will be observed. Only in "fast exchange" will an average effect on the bulk relaxation be noticeable. The addition of a macromolecule to the water solution changes t_r, which is now expected to be much larger. For metal ions attached to a macromolecule the overall correlation time, t_c, may be about two orders of magnitude greater than for free ions. The larger t_c leads to a further relaxation enhancement.

The PRE technique can be used to study several important aspects of metal-macromolecule interaction,[10,11,12] In the present paper the relaxation enhancement will be used as a titration indicator to yield information on metal binding. Other, more elaborate applications of the method, yielding information on molecular motion and ion coordination number, have been reported.[12,13] However, the derivation of these parameters rests on a less firm theoretical basis.[11]

Experimental

Materials. Dopamine-β-monooxygenase was supplied both as holo- and apoenzymes by Skotland. The procedure for purifying the enzyme is described elsewhere.[14] A solution of 3.92 mg of ultra-pure holoenzyme in 0.5 ml of 20 mM sodium phosphate buffer (pH = 7.0, A_{280} = 2.31) was used. Two samples of apoenzymes were furnished: one in Na-phosphate buffer and one in MES-buffer, with A_{280} of 4.53 and 4.30, respectively. The apoenzyme was prepared by dialysis against EDTA.[8] The concentrations of holoenzyme and apoenzyme were estimated assuming an absorbance of 1.24 at 280 nm (10 mm light path) for a solution containing 1 mg ml^{-1}. The metal salts and the ascorbate were analytical grade reagents purchased from Merck.

NMR measurements. Proton and fluorine NMR

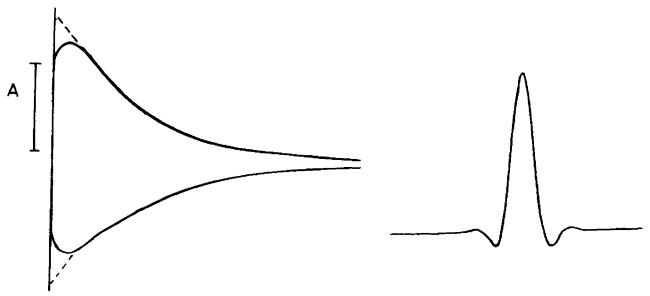

Fig. 1. Free Induction Decay (FID) and corresponding FT signal for the proton resonance of H_2O in a 5 mm sample tube.

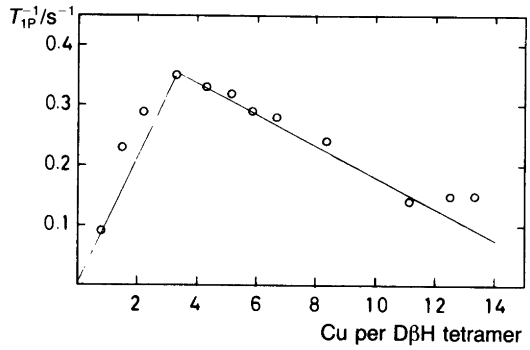

Fig. 2. Paramagnetically induced spin-lattice relaxation (T_{1P}^{-1}) vs. added $CuSO_4$. The measurements were made at 25 °C in 20 mM MES-buffer, with dopamine-β-monooxygenase concentration 12 μM; pH = 6. Precipitation starts at about 4 Cu per tetramer.

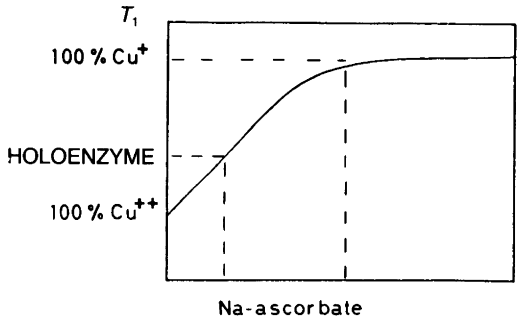

Fig. 4. Principle for determining the position of redox equilibrium in the holoenzyme using ^{19}F NMR. An equivalent amount of ascorbate reduces paramagnetic divalent copper to diamagnetic monovalent copper.

spectra were recorded at 90 MHz on a Bruker instrument operating in the Fourier transform mode. The free induction decay over 1000 Hz band-width contained 8*K* data points. Spin-lattice relaxation times (T_1) were determined using a 180-τ-90 pulse sequence. Usually, 15 τ-values were used and the peak intensities were fitted by a single exponential decay curve using a three-parameter least-squares program.

A standard 5 mm NMR sample tube was used initially. However, severe distortion of the FID was observed (Fig. 1). This phenomenon is especially pronounced for samples of high proton concentration, but may also be seen in T_1 experiments with samples of moderate proton content. Several experimental modifications were tried in order to alleviate the problem. First, the gain of the amplifier was reduced in order to eliminate the possibility that the phenomenon was related to saturation of the detector. Secondly, the delay time between pulses and acquisition was varied. Neither of these changes had any effect on the amount of distortion. Finally, by reducing the sample diameter from 5 mm to 1 mm an undistorted signal was obtained. Evidently, field inhomogeneity and/or diffusion becomes critical with samples of high proton content even when normal 5 mm sample tubes are used. Consequently, all proton relaxation measurements were carried out on samples contained in 1 mm

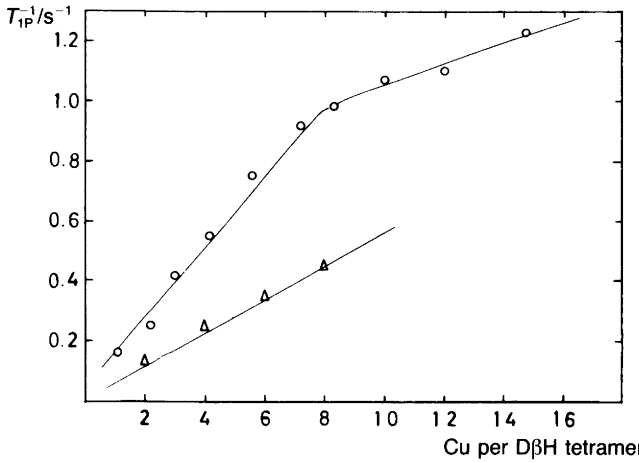

Fig. 3. T_{1P}^{-1} for solvent water protons vs. added $CuCl_2$. Dopamine-β-monooxygenase plus MES-buffer (○), and MES-buffer alone (△).

glass capillary tubes supported in a standard 5 mm NMR tube, the lock substance being placed in the outer tube. In order to ensure proper mixing during titration, the capillary had to be inverted and gently centrifuged after each addition of metal salt.

In the first trial run the paramagnetism-induced relaxation rates changed in a manner which indicated onset of precipitation of the protein at a certain metal concentration (Fig. 2). Further tests showed the formation of precipitate to be time-dependent. In subsequent runs, NMR tubes each containing the same amount of protein were used. Only the apoenzyme showed signs of instability in solution.

Results and discussion

A plot of the paramagnetic contribution to the spin-lattice relaxation rate (T_{1P}^{-1}) of solvent water protons vs. added $CuCl_2$ is shown in Fig. 3. The two curves correspond to solutions containing buffer and enzyme, and buffer alone. A comparison of the slopes of the curves demonstrates clearly the PRE effect associated with the solvent-protein interaction. The titration curve for the enzyme solution shows a discontinuity at about 8 Cu per DβM tetramer. This result is consistent with the stoichiometry obtained by Ash *et al.*[6] In their experiment, a holoenzyme containing approximately 2.1 Cu per tetramer was used as starting material for the titration. In preliminary work by the same group[14] a second deflection point at a Cu:DβM ratio of 4:1 was observed. This feature is not mentioned in the subsequent publication.[6]

In our NMR experiment the precision is not high enough to verify or rule out the existence of a separate binding constant for the first four copper ions which is only slightly larger than that for the next four ions. The results obtained using a Cu-specific electrode[4] clearly indicate four high-affinity binding sites (K_f = 11) and further binding sites of lower affinity (K_f = 5–7). At the moment it is difficult to rationalize the differences between the NMR and the electrode results.

The principle for determining the position of redox equilibrium in the holoenzyme by ^{19}F NMR is shown schematically in Fig. 4. The solutions contain 0.5 M F^- ions added as NaF. The relaxation time (T_1) is monitored as a function of added ascorbate. The two titration curves plotted in Fig. 5 represent the reduction profiles of holoenzyme and reactivated apoenzyme, respectively, the latter being reactivated with 4 Cu per tetramer. In order to explain the course of reduction one has to assume a larger content of Cu in the holoenzyme than in the reactivated apoenzyme. T_1-values for a second preparation of apoenzyme reactivated with 8 Cu per tetramer (marked as triangles) are consistent with a scheme in which the holoenzyme contains 8 Cu per tetramer. The plotted data indicate that the holoenzyme exists almost exclusively in the oxy form.

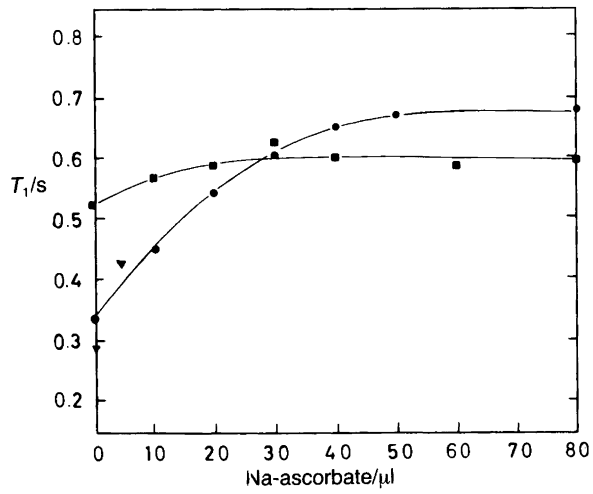

Fig. 5. T_1 relaxation times vs. added ascorbate: (●) holoenzyme, (■) 4 Cu reactivated apoenzyme, (▼) 8 Cu reactivated apoenzyme.

Theoretically, the curves for holoenzyme and the reactivated apoenzyme should converge at a common ^{19}F relaxation rate. The observed discrepancy may be accounted for by invoking slight precipitation of the more labile apoenzyme. The formation of precipitate prohibited a complete titration run for the sample reactivated with 8 Cu per tetramer.

In conclusion, our proton and fluorine NMR results are consistent with a dopamine-β-monooxygenase stoichiometry of 8 Cu per tetramer. This is also in agreement with previous NMR and ESR experiments.[5,6] At the moment it is difficult to reconcile the data obtained with spectroscopic methods and those obtained with a Cu-specific electrode. Chemically, a model involving two Cu atoms per subunit seems more reasonable since a two-electron reaction has to be accomplished. A binuclear, peroxy-bridged copper site has been proposed to participate in the redox cycle.[1] Other results indicate that only one of the two coppers interacts directly with oxygen,[15] accomplishing electron transfer from the second copper through an active site residue. This mechanism could explain the absence of magnetic coupling in the ESR spectrum and, furthermore, imply slightly different binding constants for the two types of copper ions in the active site.

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References

1. Friedman, S. and Kaufman, S. *J. Biol. Chem. 240* (1965) 4763.
2. Skotland, T. and Flatmark, T. *Eur. J. Biochem. 132* (1983) 171.
3. Skotland, T., Peterson, L., Backstrøm, D., Ljones, T., Flatmark, T. and Ehrenberg, A. *Eur. J. Biochem. 103* (1980) 5.
4. Syvertsen, C., Gaustad, R. and Ljones, T. *Rev. Port. Quim. 27* (1985) 262.
5. Blackburn, N. J., Mason, H. S. and Knowles, P. F. *Biochem. Biophys. Res. Commun. 95* (1980) 1275.
6. Ash, D. E., Papadopoulos, N. J., Colombo, C. and Villafranca, J. J. *J. Biol. Chem. 259* (1984) 3395.
7. Smith, A. D. and Winkler, H. *Biochem. J. 103* (1967) 480.
8. Ljones, T., Skotland, T. and Flatmark, T. *Eur. J. Biochem. 61* (1976) 525.
9. Skotland, T. and Ljones, T. *Eur. J. Biochem. 94* (1979) 145.
10. Dwek, R. A. *Adv. Mol. Relax. Proc. 4* (1972) 1.
11. Burton, D. R., Forsen, S. and Karlstrøm, G. *Prog. NMR Spectrosc. 13* (1979) 1.
12. Koenig, S. H. and Brown, R. D. *J. Magn. Reson. 61* (1985) 426.
13. Kushnir, T. and Navon, G. *J. Magn. Reson. 56* (1984) 373.
14. Villafranca, J. J. In: Spiro, T. G., Ed., *Copper Proteins,* Wiley, New York 1981, pp. 264–289.
15. Ahn, N. and Klinman, J. P. *Biochemistry 22* (1983) 3096.

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