DOPAMINE β-MONOOXYGENASE: ELECTRON PARAMAGNETIC RESONANCE AND OXIDATION–REDUCTION PROPERTIES OF THE ENZYME-BOUND COPPER

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1. Introduction

Dopamine β-monooxygenase (dopamine β-hydroxylase; EC 1.14.17.1) contains enzyme-bound copper which is essential for enzymatic activity [1]. EPR spectroscopy has shown that the copper is reduced by ascorbate [2–4], and proposed models for the mechanism of catalysis in the hydroxylation of dopamine implicate the copper in electron transfer from ascorbate to O_2 [1,5]. Direct experimental evidence for oxidation and reduction of the enzyme-bound copper during each catalytic cycle has not yet been presented, however.

Although ascorbate is belived to be the physiological electron donor for dopamine β-monooxygenase, other reducing agents will also function [1,6]. Among these is hexacyanoferrate (II) ion [7], and this observation suggested that the electron accepting group (presumably copper) on dopamine β-monooxygenase must have a redox potential which is close to the standard potential for the $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ couple (+ 400 ± 10 mV under the conditions used; see also [8]). As this requires reconsideration of the proposed mechanisms of hydroxylation [1,5], a determination of the redox potential of the enzyme-bound copper seemed relevant to discussions on the catalytic mechanism of dopamine β-monooxygenase. Using EPR spectroscopy to monitor the Cu(II) content, we have performed equilibrium potentiometric titrations of dopamine β-monooxygenase. The results differ significantly from the data in [4], where redox potentials in reaction mixtures were not measured. In addition, experimental data on the O_2 induced oxidation of enzyme-bound Cu(I) in the presence of the substrate tyramine, are presented.

2. Methods

Dopamine β-monooxygenase was purified from bovine adrenal medulla by the method in [9], with the modification that Triton X-100 was omitted from the extraction buffer. Enzyme activities were measured as in [9] with $K_4Fe(CN)_6$ as reductant. Concentrations of this protein were calculated from the absorbance at 280 nm ($E^{1\%}_{280\ nm}$ = 12.4; see [10]). Copper was determined with the method in [11].

Cu(II) concentrations were estimated from EPR spectra corrected for background by measuring peak to peak amplitudes at $g_\perp$ and by computation of double integrals calibrated against a Cu(II)EDTA solution. The two methods gave a linear correlation and the half-reduction potentials obtained were essentially the same. Since the amplitude method gave less spread of data at the low concentrations of protein used, only those results are presented. EPR spectra at 95°K were obtained with a Varian 4502 spectrometer with 100 kHz field modulation. Spectra processing

was performed on a Varian 620i computer. Redox titrations in 20 mM phosphate buffer, pH 7.0, were performed using buffered $K_3Fe(CN)_6$ (0.04 M or 0.1 M) and ascorbate (0.02 M) solutions to vary the potential. Quinhydrone, 20 μM, was used as a redox mediator with the exceptions of points above 360 mV in the aerobic case. EPR backgrounds from titrants and mediator were found to be negligible. Redox potentials were measured, as in [12], at 25°C using a platinum electrode with a calomel electrode as reference. Equilibration for 6–9 min was required to reach a stable potential. In the anaerobic experiment samples were transferred by the pressure of deoxygenated argon through a glass tube into argon flushed EPR tubes.

3. Results

In agreement with [4] we found that all of the copper in dopamine β-monooxygenase was EPR detectable after oxidation with excess $K_3Fe(CN)_6$. There was good agreement between the copper content determined chemically and that obtained from the EPR spectrum of the fully oxidized enzyme. Our values for copper content [9] indicate 4 copper atoms/protein molecule (290 000 daltons) when corrected for the revised protein determination [10]. The enzyme as isolated, however, contained only a fraction, which varied between 30% and 50%, of its total copper content in the form of Cu(II), as detected by EPR (fig.1). The effect of oxidation by $K_3Fe(CN)_6$

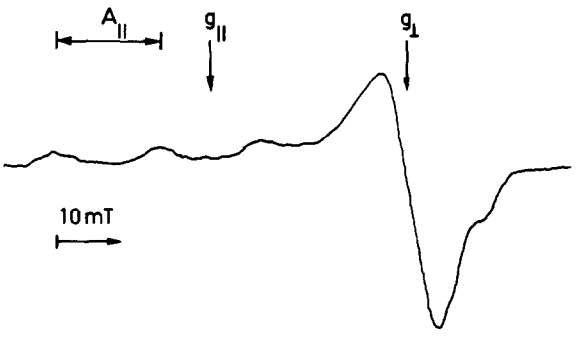

Fig.1. EPR spectrum of bovine dopamine β-monooxygenase, native form, (8.5 μM) pH 7.0. The spectrum was recorded at 95 K. Microwave frequency 9.05 GHz, modulation amplitude 1.2 mT; microwave power 4.0 mW.

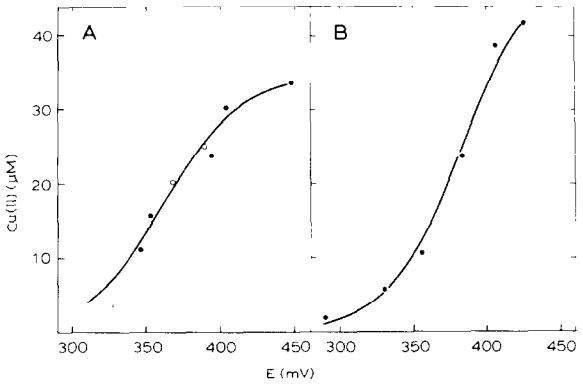

Fig.2. Redox titrations of dopamine β-monooxygenase, at pH 7.0, in the presence (A) and absence (B) of air. Preparation of samples and measurement of EPR spectra were as in section 2. Cu(II) concentrations were estimated from peak to peak amplitudes at $g_\perp$. (●) Oxidative titrations; (○) reductive titrations.

strongly suggests that the balance of copper in dopamine β-monooxygenase is bound as Cu(I).

Redox titrations of the copper in dopamine β-monooxygenase both in the presence and absence of O_2 are shown in fig.2. The curves were fitted to the experimental data according to the Nernst equation with $n = 1$ and the agreement is reasonably good. However, a higher value of n cannot be excluded. The half-reduction potentials (E_{m7}) were found to be + 360 ± 15 mV (aerobic titration) and + 385 ± 15 mV (anaerobic titration), and thus they are not significantly different.

Enzyme-bound copper reduced by stoichiometric amounts of ascorbate was oxidized slowly by O_2. In one experiment, enzyme containing 56 μM total copper (15 μM Cu(II)) was reduced by 5 μM ascorbate to give 6.1 μM Cu(II). After 40 min at 25°C in air the Cu(II) content had increased to 6.7 μM. When tyramine was added the oxidation was faster (fig.3). The half-life of this reaction was ~20 min. Addition of 0.12 mM fusaric acid, an inhibitor of dopamine β-monooxygenase, increased the half-life to more than 200 min, and the rate of oxidation was only slightly increased by 0.2 M acetate, an activator of the enzyme. Oxidation of Cu(I) in dopamine β-monooxygenase as isolated (no ascorbate added) was also stimulated by tyramine. O_2 was necessary for the tyramine-stimulated increase in the Cu(II)-signal, as there was no increase when

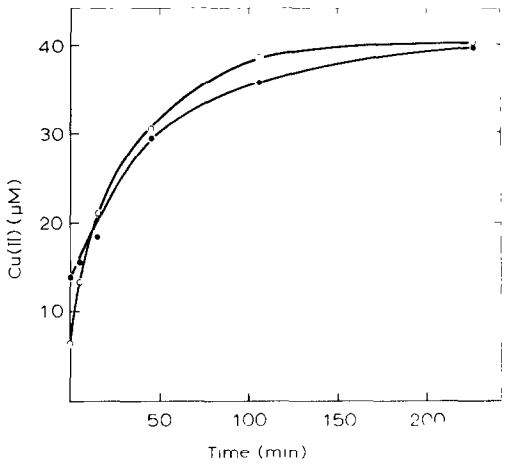

Fig.3. Oxidation of Cu(I) in dopamine β-monooxygenase in the presence of tyramine and O_2. The enzyme (7.5 mg/ml) in 20 mM potassium phosphate buffer, pH 7.0, was incubated in EPR tubes at 25°C with 0.5 mM tyramine, and the time course of oxidation was monitored with EPR after freezing in liquid nitrogen. The tubes were then rapidly thawed, and the incubation at 25°C was continued. The time scale indicates the time of exposure to 25°C only. (○) The enzyme was first reduced with 5μM ascorbate; (●) the enzyme was not reduced by ascorbate.

ascorbate-reduced enzyme was incubated anaerobically with tyramine for 30 min. Control experiments showed that there was no loss of enzymatic activity during these experiments.

4. Discussion

From our measurements we find no reason to believe that the copper ions should represent an inhomogenous population with regard to their redox behavior. All the 4 copper ions present in each enzyme molecule are paramagnetic in the fully oxidized enzyme. The shoulder in the high-field part of the EPR-spectrum, as seen for the native enzyme in fig.1, varied somewhat between different preparations, but its absolute intensity remained substantially unaffected during the redox experiments and should not influence the results. Attempts to clarify its origin by means of difference spectra have so far not been successful. If it is due to Cu(II) it most likely represents less than 10% total copper. The spectrum of enzyme-bound Cu(II), obtained by oxidation in the presence of tyramine and O_2 was not qualitatively different from the spectrum of the native enzyme. In contrast with [4] we could not detect any superhyperfine structure in the $g_\perp$ region.

Previous stoichiometric [1] and kinetic [5] experiments have been interpreted as evidence of a mechanism of hydroxylation in which a cluster of 2 copper atoms per active site of dopamine β-monooxygenase accepts 2 electrons before O_2 and substrate bind (a ping-pong mechanism). It was shown in [7] that the kinetic experiments in [5] cannot be taken as evidence for such a mechanism, as the apparent agreement with a ping-pong mechanism is due to the nearly irreversible reaction between ascorbate and oxidized groups on the enzyme (presumably Cu(II)). Our determination of the redox potential of the copper in dopamine β-monooxygenase lends further support to this view, since the half-reduction potential is reasonably close to the standard potential of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple [8]. The assumption that the copper in dopamine β-monooxygenase participates in electron transfer is thus compatible with the observation that $Fe(CN)_6^{4-}$ supports high rates of hydroxylation [7]. In contrast, the report [4] that the copper in dopamine β-monooxygenase has a half-reduction potential of + 310 mV, i.e., ~100 mV more negative than that of the $Fe(CN)_6^{4-}$ couple, is difficult to reconcile with this function for the enzyme-bound copper.

$K_3Fe(CN)_6$ was selected as the oxidant in potentiometric titrations partly due to its appropriate potential range and partly due to the finding that oxidation of the enzyme by excess (1 mM) $K_3Fe(CN)_6$, followed by its removal by ultrafiltration, did not affect the enzymatic activity. The discrepancy between our value and that obtained [4] for the half-reduction potential is probably not due to interaction between $K_3Fe(CN)_6$ and the protein, since the point at + 290 mV in the anaerobic experiment, taken with quinhydrone but no $K_3Fe(CN)_6$ present, resulted in almost completely reduced enzyme copper (fig.2). A half-reduction potential of + 310 mV should give significantly more, or about 30%, of the copper in the oxidized form.

Further evidence against the ping-pong mechanism comes from our present observation that Cu(I) in dopamine β-monooxygenase is oxidized slowly in the

presence of O_2 and tyramine, at a rate which is much lower than what is indicated by the turnover time under these conditions. In the case of the ping-pong mechanism the reduced enzyme should contain both of the 2 electrons/active site needed for rapid completion of the catalytic cycle. Thus, the results presented in fig.3 support a sequential mechanism with only 1 electron available/active site, such that the catalytic cycle cannot be completed without excess reductant. An obvious alternative explanation is that excess ascorbate serves as a modifier and is necessary for rapid turnover, but this seems unlikely in light of the high rate of hydroxylation with $Fe(CN)_6^{4-}$ and the generally low specificity for electron donors in this monooxygenase reaction. Tyramine stimulates the rate of oxidation compared with O_2 alone, but the low rate suggests that this effect is due to other reasons than a completion of the normal catalytic cycle.

Acknowledgements

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