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DOPAMINE β-MONOOXYGENASE: REDUCED ENZYME NEEDS ADDITIONAL REDUCTANT FOR COMPLETION OF THE CATALYTIC CYCLE

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SUMMARY

The enzyme-bound copper of dopamine β-monooxygenase was reduced by ascorbate and the enzyme separated from excess reductant by gel filtration. The reduced enzyme did not hydroxylate substrate at a rate consistent with the turnover time. This observation supports a sequential mechanism.

INTRODUCTION

Dopamine β-monooxygenase (dopamine β-hydroxylase; EC 1.14.17.1) is a copper-containing glycoprotein which catalyzes the conversion of dopamine to noradrenaline, and also the β-hydroxylation of many analogues of phenylethylamine, e.g. tyramine (for reviews see refs. 1,2). Friedman and Kaufman (3) have proposed that each enzyme tetramer of 290 000 daltons contains one active site with two Cu(II) and reported that enzyme-bound copper reduced by ascorbate was able to hydroxylate an approximately equivalent amount of substrate aerobically in the absence of excess reductant. Furthermore, Goldstein et al. (4) have interpreted their kinetic results in support of such a ping-pong mechanism. On the other hand, Ljones and Flatmark (5) have pointed out that these kinetic results cannot be taken as evidence for such a mechanism, as the parallel initial velocity patterns in double-reciprocal plots can be due to a nearly irreversible reaction between ascorbate and the electron

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accepting group(s) on the enzyme (presumably Cu(II)). Moreover, with $Fe(CN)_6^{4-}$ as the electron donor the kinetic patterns were intersecting, supporting a sequential mechanism. Our measurement of a redox potential of +370 mV for the enzyme-bound copper (6) provides further evidence in support of this view.

We have recently found, by using EPR spectroscopy, that enzyme-bound Cu(I) is oxidized in the presence of O_2 and the substrate tyramine at a rate which is much lower than expected from the turnover time of the enzyme (6). In further support of a sequential mechanism, we now report that reduced enzyme, in the presence of O_2 and tyramine, but in the absence of a reducing agent, will not hydroxylate tyramine to octopamine at a rate consistent with catalysis.

METHODS

Dopamine β-monooxygenase was purified from bovine adrenal medulla as described earlier (7), except that Triton X-100 was omitted in the extraction buffer. Concentrations of purified enzyme was estimated by assuming an absorbance of 1.24 at 280 nm (10 mm light path) for a solution of 1 mg/ml (8). Octopamine was determined using high-performance liquid chromatography with fluorometric detection (9). Copper was determined with the method of Wharton and Rader (10).

RESULTS AND DISCUSSION

Studies using EPR spectroscopy have shown that all the Cu(II) of dopamine β-monooxygenase can be reduced by ascorbate, and that only approximately 1% of the Cu(I) is oxidized by exposure to air for 40 min at 25°C in the absence of substrate (6). The enzyme-bound copper in the preparation used in the present study (35 nmol copper in 4.2 mg of protein dissolved in 1.5 ml 20 mM potassium phosphate, pH 7.0) was reduced by 1.0 mM ascorbate. The solution was then applied to a column of Sephadex G-50 (1.5 x 8 cm), equilibrated at room temperature with anaerobic buffer (evacuated and flushed with N_2) containing 20 mM

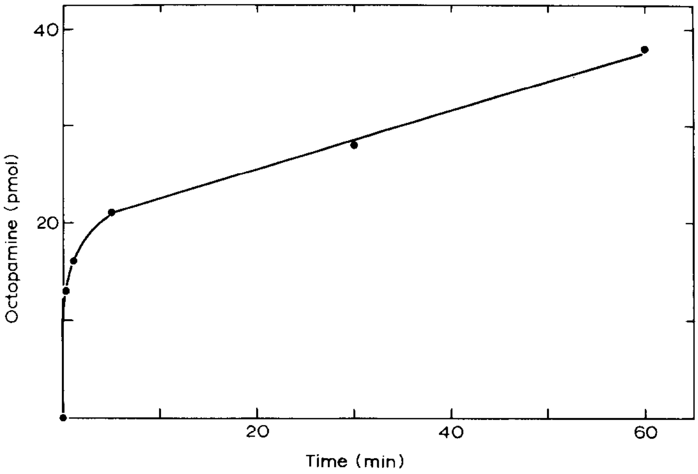

Figure 1. Time course of octopamine formation from 220 pmol reduced enzyme tetramer in the presence of excess oxygen and tyramine. The value at zero time was obtained by adding ethanol containing 1.0 mM fusaric acid to the enzyme before addition of tyramine.

potassium phosphate, pH 7.0. The collected fraction of reduced enzyme with the highest protein content (1.4 mg/ml) was incubated at 25°C in the presence of O_2 in air and 0.5 mM tyramine (from Koch-Light, England). The reaction was stopped at different time intervals by withdrawing aliquots of 75 µl and adding them to an equal volume of ethanol containing 1.0 mM fusaric acid (from ICN, U.S.A.). Samples of 100 µl were injected into the liquid chromatograph for the assay of octopamine. From Fig. 1 it is seen that 16 pmol octopamine is formed after 1 min and 36 pmol after 1 h from 220 pmol enzyme tetramer. There was complete recovery of octopamine and no interference from binding to enzyme after addition of ethanol, as shown by control experiments with added standard octopamine in the presence and absence of enzyme.

As a control, the activity of the enzyme was measured at the end of the experiment (after the gel filtration and incubation for 1 h), and the specific activity was found to be 3.1 µmol $min^{-1}mg^{-1}$ in the assay system of Ljones _et al_. (7) using

$Fe(CN)_6^{4-}$ as the electron donor, i.e. approximately 65% of the highest activity reported. The activity of the enzyme in 20 mM potassium phosphate, pH 7.0, containing 0.5 mM tyramine and 1.0 mM $Fe(CN)_6^{4-}$ was 0.08 µmol $min^{-1}mg^{-1}$, which corresponds to a turnover time of 10 s, assuming two active sites per tetramer. Since the dimer of the human enzyme, isolated from serum (11,12) as well as from pheochromocytoma (13,14), has been shown to have catalytic activity, there is reason to believe that there are at least two active sites per tetramer in the bovine enzyme. This means that the amount of octopamine formed after 1 min is less than 5% of that expected in the case of a ping-pong mechanism. The initial rapid formation of some octopamine in the experiment may be due to binding of some ascorbate to the protein, as the protein and free ascorbate were well separated on the Sephadex column.

In an experiment where all the enzyme was reduced by 1 mM $Na_2S_2O_4$ (which was shown by EPR to cause complete reduction of the enzyme-bound copper), and the excess of reducing agent was oxidized by O_2, the octopamine formation followed a similar time course as that shown in Fig. 1, except for the lack of an initial rapid phase (data not shown). This experiment was, however, more difficult to interprete, as the oxidation product of 1 mM $Na_2S_2O_4$ (i.e. 2 mM $NaHSO_3$) inhibited the enzyme by about 85%. Nevertheless, the catalytic rate under these inhibitory conditions was sufficiently high to allow us to conclude that the rate of octopamine formation from reduced enzyme was much less than expected in the case of a ping-pong mechanism.

The amount of octopamine formed in the presence of O_2 and tyramine (Fig. 1) is much less than the amount of Cu(I) oxidized under similar experimental conditions (6), although both processes are much slower than the catalytic rate. A possible explanation for the slow formation of octopamine, which is consistent with a sequential mechanism and a storage capacity of only one electron per active site, could be that there is an exchange of electrons between different sites, thus providing the two electrons needed for hydroxylation. The higher rate of Cu(I) oxidation could be due to auto-oxidation stimulated by tyramine but uncoupled from hydroxylation (6).

Friedman and Kaufman (3) have reported aerobic hydroxylation of about 1 nmol phenylethylamine per nmol reduced enzyme tetramer in 5 min after removal of excess ascorbate by ascorbate oxidase. This observation appears to be in conflict with our present results. Nevertheless, we feel that Friedman and Kaufman's data may be compatible with our model, in which the reduced enzyme contains only one of the two necessary electrons per active site for completion of the catalytic cycle, for the following reasons: (a) The results given in their Table IV, when considered in light of the stability of reduced dopamine β-monooxygenase towards auto-oxidation (6), suggest that ascorbate oxidase is not capable of rapid and complete oxidation of ascorbate in the presence of dopamine β-monooxygenase, and (b) they did not measure hydroxylation at points in time earlier than 5 min. Thus, their experiments did not prove that the hydroxylation was caused by rapid completion of a catalytic cycle, instead of by a combination of a rapid hydroxylation due to residual ascorbate and a slower reaction possibly due to electron transfer between different active sites.

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