EVIDENCE FROM THE ACCELERATION OF CYTOCHROME *c* REDUCTION FOR THE FORMATION OF ASCORBATE FREE RADICAL BY DOPAMINE β-MONOOXYGENASE

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1. Introduction

Dopamine β-monooxygenase (dopamine β-hydroxylase; EC 1.14.17.1) catalyzes the reaction:

Dopamine + ascorbate + O_2 →

noradrenaline + dehydroascorbate + H_2O

This is believed to be the physiologically important reaction in mammalian tissues, but the purified enzyme is also an efficient catalyst for similar hydroxylations with a number of alternative substrates. Dopamine can be replaced by several other derivatives of phenylethylamine, with tyramine frequently being used for assays of the enzyme. Ascorbate can be replaced by other two-electron (2 e^-) donors, such as catechol, or by the one-electron (1 e^-) donor $Fe(CN)_6^{4-}$. The path of electron transfer is believed to involve the enzyme-bound copper (reviewed [1]).

Classification of ascorbate as a 2 e^-donor in enzymic oxidations describes the overall stoichiometry correctly. It leaves open, however, the question of whether:

(i) Each ascorbate ion transfers 2 e^- to the enzyme and is released as dehydroascorbate; or
(ii) Each ascorbate ion transfers 1 e^- and is released as the unstable ascorbate free radical (semidehydroascorbate), followed by nonenzymic dismutation of two radicals to give one ascorbate and one dehydroascorbate.

Most discussions of the mechanism of dopamine β-monooxygenase have assumed alternative (i), but we have discussed alternative (ii) [1]. Compelling evidence for 1 e^- mechanisms (alternative (ii)) in the cases of ascorbate oxidation by ascorbate oxidase and by peroxidase has been presented [2]. The experimental methods used were EPR spectroscopy [3] and a scavenger method which employed oxidized cytochrome *c* as the scavenger of radicals [4]. Ascorbate free radical reduces cytochrome *c* more rapidly than the fully reduced ascorbate, and hence an acceleration of cytochrome *c* reduction upon the addition of oxidizing enzyme was interpreted as evidence for free radical formation in the enzymic oxidations.

Although the question of 1 e^- or 2 e^- transfer in the oxidation of ascorbate by dopamine β-monooxygenase must be answered for correct interpretation of the kinetics and mechanism of this enzyme, the problem had not yet been addressed experimentally. We now report evidence obtained with the cytochrome *c* scavenger method that dopamine β-monooxygenase also employs a 1 e^- mechanism, releasing ascorbate free radical as the product of enzymic oxidation. Our experimental evidence includes measurements with ascorbate oxidase for the purpose of comparing with a standard reaction [2], and the results reveal close correspondence between these two enzymes in the cytochrome *c* reduction assay.

2. Materials and methods

The water-soluble form of dopamine β-monooxygenase was purified from bovine adrenal medulla [5,6]. Cytochrome *c*, type VI from horse heart, catalase from bovine liver (C-100), and superoxide

dismutase, type I from bovine blood, were obtained from Sigma. Ascorbate oxidase from squash was obtained from Boehringer Mannheim. Fusaric acid (5-butyl picolinic acid) was obtained from ICN Pharmaceuticals. Absorbance changes were measured with a Cary 219 recording spectrophotometer. Rates were calculated using these values for extinction coefficients: ϵ for ascorbate (pH 6.0) at 265 nm: 14 $mM^{-1} \cdot cm^{-1}$ [7], and $\Delta\epsilon$ (reduced minus oxidized) for cytochrome *c* at 550 nm: 22 $mM^{-1} \cdot cm^{-1}$ [8].

3. Results and discussion

Addition of dopamine β-monooxygenase markedly accelerates the reduction of ferricytochrome *c* in the presence of ascorbate (fig.1). The time-course of this reduction (b) coincides with the time-course of ascorbate oxidation (a) i.e., the reduction of cytochrome *c* ceases when all the ascorbate has been oxidized in the enzyme reaction. A similar experiment with ascorbate oxidase is also depicted in fig.1, which reveals that the ratios between cytochrome *c* reduction and ascorbate oxidation are quite similar for these two enzymes under our experimental conditions. The variation in shapes between the time-courses for the two enzymes is explained by their different Michaelis constants for ascorbate.

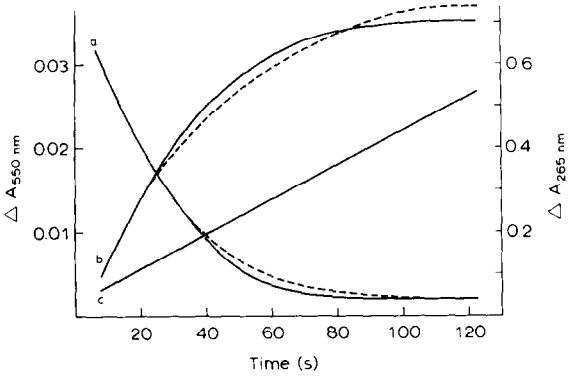

Fig.1. Ascorbate oxidation and cytochrome *c* reduction in the presence of dopamine β-monooxygenase or ascorbate oxidase. Reaction mixtures contained: 10 mM 2(*N*-morpholino)-ethanesulfonic acid (MES), 1 mM tyramine, 2 μM $CuSO_4$, 80 mM sodium acetate (an activator of dopamine β-monooxygenase), 8 μM ferricytochrome *c*, 125 μg/ml catalase, 50 μM ascorbate. Total vol. 1 ml; temp. 25°C; pH 6.0; lightpath 1 cm. The time courses depict ascorbate oxidation measured at 265 nm (a) and cytochrome *c* reduction measured at 550 nm in the presence (b) or absence (c) of oxidizing enzyme. Enzymes used for curves (a,b): 52 μg/ml of dopamine β-monooxygenase, unbroken curves; 0.23 units/ml of ascorbate oxidase, broken curves where they do not coincide with the curves for dopamine β-monooxygenase. The ascorbate oxidation in the absence of oxidizing enzymes was 9% of the oxidation in (a).

Experiments similar to that in fig.1 were performed with varying concentrations of oxidizing enzymes (fig.2). The results again clearly indicate the same ratios between cytochrome *c* reduction and ascorbate oxidation for dopamine β-monooxygenase and ascorbate oxidase. The data also give a reasonably good fit to the expected linear relationship between the rate of cytochrome *c* reduction and the square root of the rate of ascorbate oxidation [4]. This relationship is predicted because of the second-order rate law for the radical dismutation, and because dismutation is the dominant pathway for removal of the radical at our reaction conditions. The data in fig.1 indicate that only 1.7% of the radical formed reacts with cytochrome *c*. The % reacting with cytochrome *c*

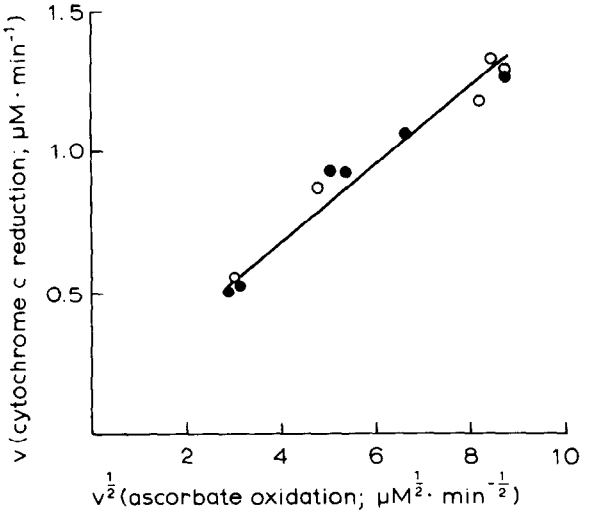

Fig.2. The relationship between the initial rates of cytochrome *c* reduction and ascorbate oxidation in the presence of different amounts of dopamine β-monooxygenase (○) or ascorbate oxidase (●). Experimental conditions were as described in the legend to fig.1. The values for the highest rates of ascorbate oxidation for both enzymes were taken from fig.1. The values on the ordinate are given as the differences between the rates of cytochrome *c* reduction in the presence and absence of oxidizing enzyme.

could be increased by using higher levels of cytochrome *c*, but this would also increase experimental error due to the higher rate of nonenzymic reaction between ascorbate and cytochrome *c*.

The rate of cytochrome *c* reduction in the absence of oxidizing enzyme (fig.1(c)) was rather high, partly due to the inclusion of 2 μM $CuSO_4$ in the reaction mixture. The $CuSO_4$ was nevertheless retained to give maximal stimulation of dopamine β-monooxygenase catalysis [9].

Fusaric acid, an inhibitor of dopamine β-monooxygenase, at 30 μM completely abolished the effect of the enzyme on both ascorbate oxidation and cytochrome *c* reduction.

To investigate the possibility that cytochrome *c* reduction went by way of superoxide anion rather than ascorbate free radical, we included a control experiment with superoxide dismutase. Addition of this enzyme at 200 μg/ml to a reaction mixture otherwise identical to that described in the legend to fig.1, gave no inhibition of the dopamine β-monooxygenase stimulated reduction of cytochrome *c*.

From the results in fig.1 and 2, together with the established evidence for ascorbate free radical as the product in the ascorbate oxidase reaction [2–4], we conclude that dopamine β-monooxygenase also oxidizes ascorbate to the free radical. The final product, dehydroascorbate, must then arise from nonenzymic dismutation of the free radical.

A small EPR contribution, attributed to ascorbate free radical, has been observed in frozen mixtures of dopamine β-monooxygenase and ascorbate [10]. The contribution was, however, smaller in complete hydroxylation mixtures, and thus its significance to enzymically formed ascorbate free radical seems doubtful.

Several alternatives for the involvement of the enzyme-bound copper in dopamine β-monooxygenase catalysis have been presented. It was proposed [11,12] that reduction of 2 Cu/active site by 1 ascorbate molecule is the first step, followed by release of dehydroascorbate. However, we have reported evidence in conflict with this type of mechanism [9,13–15], leading us to propose that reduction by ascorbate of 1 Cu/active site is the first step, followed by transfer of this first electron to bound O_2 before the same copper atom accepts a second electron [1]. The evidence for 1 e^- transfer and production of ascorbate free radical certainly presents no conflict with our proposed type of mechanism. The alternative mechanism with 2 Cu/active site is, however, more in line with the mechanism of tyrosinase, which contains 2 Cu/active site [16] and is a 2 e^- transfer enzyme, producing the fully oxidized forms rather than the free radical (semiquinone) forms of its catechol substrates [17].

References

[1] Skotland, T. and Ljones, T. (1979) Inorg. Perspect. Biol. Med. 2, 151–180.
[2] Yamazaki, I. (1977) in: Free Radicals in Biology (Pryor, W. A. ed) vol. 3, pp. 183–218, Academic Press, New York.
[3] Yamazaki, I. and Piette, L. H. (1961) Biochim. Biophys. Acta 50, 62–69.
[4] Yamazaki, I. (1962) J. Biol. Chem. 237, 224–229.
[5] Ljones, T., Skotland, T. and Flatmark, T. (1976) Eur. J. Biochem. 61, 525–533.
[6] Skotland, T. and Flatmark, T. (1979) J. Neurochem. 32, 1861–1863.
[7] Karayannis, M. I., Samios, D. N. and Gousetis, C. P. (1977) Anal. Chim. Acta 93, 275–279.
[8] Flatmark, T. (1964) Acta Chem. Scand. 18, 1517–1527.
[9] Skotland, T. and Ljones, T. (1979) Eur. J. Biochem. 94, 145–151.
[10] Blumberg, W. E., Goldstein, M., Lauber, E. and Peisach, J. (1965) Biochim. Biophys. Acta 99, 187–190.
[11] Friedman, S. and Kaufman, S. (1965) J. Biol. Chem. 240, 4763–4773.
[12] Goldstein, M., Joh, T. H. and Garvey, T. Q. (1968) Biochemistry 7, 2724–2730.
[13] Ljones, T. and Flatmark, T. (1974) FEBS Lett. 49, 49–52.
[14] Ljones, T., Flatmark, T., Skotland, T., Petersson, L., Bäckström, D. and Ehrenberg, A. (1978) FEBS Lett. 92, 81–84.
[15] Skotland, T., Ljones, T. and Flatmark, T. (1978) Biochem. Biophys. Res. Commun. 84, 83–88.
[16] Jolley, R. L., Evans, L. H., Makino, N. and Mason, H. S. (1974) J. Biol. Chem. 249, 335–345.
[17] Mason, H. S., Spencer, E. and Yamazaki, I. (1961) Biochem. Biophys. Res. Commun. 4, 236–238.