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Free radical in Michaelis complex of D-amino acid oxidase

In the course of our study on the mechanism of enzyme action, we succeeded in crystallizing the Michaelis complex of D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3) under anaerobic condition[1]. Both the faint purple-coloured crystal and the purple-coloured mother liquid showed the same absorption band at 500–600 mμ. On aeration, the crystal was converted into an equimolar amount of the holoenzyme and pyruvate accompanying by the formation of H_2O_2. These findings led us to suppose that the complex consisted of the semiquinoid form of the holoenzyme and the activated substrate. To test for the possible presence of free radicals in the complex, an electron-spin-resonance (ESR) study has been carried out on both the crystal and the mother liquid.

The ESR spectra were measured using a X-band EPR spectrometer Varian type V 4500 with 100-kcycles field modulation. The measurements were made in the same way as KUBO *et al.*[2]. For the measurements, a capillary type cell (Fig. 1) was devised and made from quartz glass.

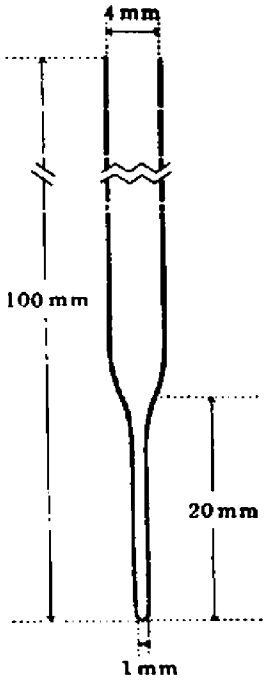

Fig. 1. Capiliary type cell for ESR measurement.

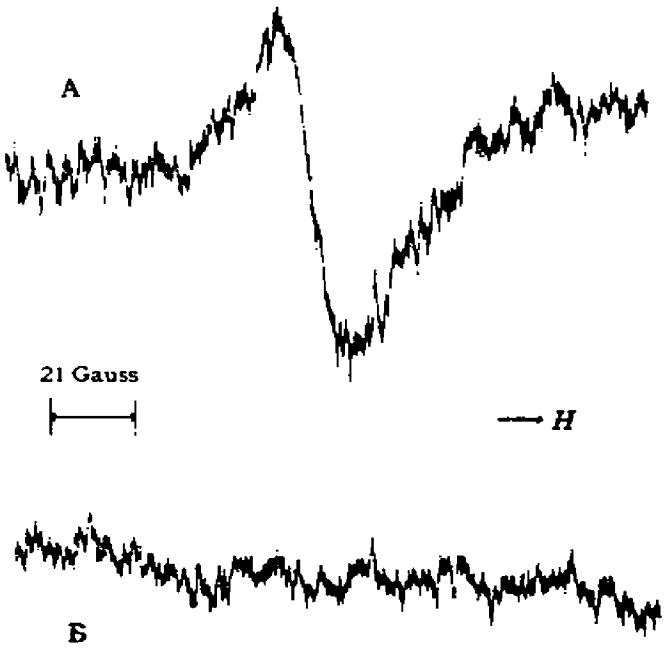

Fig. 2. Free-radical signal of the crystal of the Michaelis complex of D-amino acid oxidase at 25°. A, the crystal of the Michaelis complex; B, the mother liguid (see ref. 1).

The crystals of the Michaelis complex were prepared using D-alanine as substrate as reported previously[1]. The crystal suspension was injected into the cell and centrifuged at 3000 × *g* for 15 min. By the centrifugation, the crystals were packed in the capillary part of the cell. ESR measurement was performed at room temperature (25°) under the following conditions: modulation, 100 kcycles, 6 Gauss; sensitivity, 2000; power, 5 db; response, 0.3 sec; magnetic-field scanning speed, 42 Gauss/min; chart speed, 4 cm/min.

As shown in Fig. 2 A, a typical signal was observed with crystal, the *g* value being 2.004.

The crystals were then removed and the mother liquid was injected into the capillary part of the cell. No ESR signal was demonstrable (Fig. 2 B). Elevating the field modulation to be 100 kcycles, 15 Gauss, however, revealed a typical signal with the same *g* value as that of the crystal.

These results suggest that both the crystal and the mother liquid contain a free radical. Considering this fact together with the former results[1], it is concluded that the crystal is a complex of the semiquinoid form of the holoenzyme and the activated or partially modified substrate; *viz.*, an intermediate complex formed during the enzymic catalysis, which was theoretically assumed by MICHAELIS AND MENTEN[3].

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The reaction kinetics of respiratory enzymes studied by electron-spin-resonance absorption

We have already reported on the free-radical signals observed during reactions catalysed by fungal glucose oxidase (EC 1.1.3.4) and pig-heart succinate dehydrogenase (EC 1.3.99.1)[1]. In this paper the reaction kinetics of these enzymes are described.

As an acceptor methylene blue was used, with 1–100 mM β-glucose and 10 to 100 mM succinate as substrate. The experimental conditions of the ESR apparatus were: X-band, low-frequency (380 cycles/sec) field modulation (modulation width: 0.22–0.8 Gauss) and ambient air temperature (27°). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0).

Abbreviation: ESR, electron-spin resonance.