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STUDIES ON MECHANISM OF INACTIVATION OF CATECHOL 1,2-OXYGENASE BY ELECTRON SPIN RESONANCE

HIROSHI WATARI, TERUKO NAKAZAWA* AND TOSHIO YAMANO

Department of Physico-chemical Physiology, Osaka University Medical School, Osaka, Department of Medical Chemistry, Kyoto University, Faculty of Medicine, Kyoto and Department of Biochemistry Osaka University, Medical School, Osaka (Japan)

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SUMMARY

An ESR spectrum of inactive catechol 1,2-oxygenase (catechol:O_2 1,2-oxidoreductase, EC 1.13.1.1, formerly known as pyrocatechase) was measured and found to be an anisotropic one. These g-values were estimated to be $g_x = 3.87$, $g_y = 4.41$ and $g_z = 4.07$, whereas that of active catechol 1,2-oxygenase is $g = 4.28$, which was degenerated. Comparing the theoretical g-values, which were calculated by AASA *et al.*, with the experimental ones, the E- and D-terms in spin-Hamiltonian were estimated to be 0.19 cm^{-1} and 0.021 cm^{-1}, respectively. From theoretical considerations, the inactivation mechanism seems to be the displacement of one of ligands on iron.

INTRODUCTION

Catechol 1,2-oxygenase (catechol:O_2 1,2-oxidoreductase, EC 1.13.1.1, formerly known as pyrocatechase) was found by HAYAISHI, KATAGIRI AND ROTHBERG[1] in *Pseudomonas fluorescence* and this enzyme catalyses the ring fission of catechol to form *cis,cis*-muconic acid by an oxygenation reaction. Catechol 1,2-oxygenase has two iron atoms per molecule (molecular weight 80 000–100 000) and it was considered that Fe^{2+} is essential for the enzymatic activity[2]. Recently, ESR spectra of active catechol 1,2-oxygenase were studied by NAKAZAWA *et al.*[3], and a sharp signal at $g = 4.28$ was observed. This result indicated that Fe^{3+} is contained in the active state of catechol 1,2-oxygenase, because this signal ($g = 4.28$) is known to be characteristic of the high spin state of Fe^{3+} in the center of a tetrahedron[4]. Such a sharp signal was observed in the following cases; Fe^{3+} in glass[4,5], Fe^{3+}-transferrin[6], Fe^{3+}-conalbumin[6] and Fe^{3+}-fructose[7]. A lowering of the symmetry of the ligand field from tetrahedron brought about a broadening of the ESR spectrum and a shift in g-values, as calculated by AASA *et al.*[6].

It is assumed that the ligands of Fe^{3+} correlate to the enzymatic activity of

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catechol 1,2-oxygenase and that ESR study would be a powerful tool for analysis of the inactivation mechanism, since small differences in energy levels of ferric *d*-orbitals can be detected by ESR. In this paper, we report an ESR spectrum of inactive catechol 1,2-oxygenase, and discuss the strength of interaction of a ligand field on Fe^{3+} with respect of its *D*- and *E*-terms in spin -Hamiltonian,using *g*-values from this spectrum.

MATERIALS AND METHODS

Catechol 1,2-oxygenase was extracted by sonication from *Pseudomonas arvilla* and purified by the procedure reported recently[8]. Enzymatic activity of catechol 1,2-oxygenase was measured spectrophotometrically by the increase in absorbance at 260 mμ, which is due to *cis,cis*-muconic acid produced by the enzymatic reaction[8]. Protein concentration was determined spectrophotometrically from the absorbance at 280 and 260 mμ (ref. 9).

Measurements of ESR were carried out with a Varian V–4500 spectrometer (100 kcycles magnetic field modulation) equipped with a variable temperature accessory. Temperature was measured by a thermocouple. For the measurement of magnetic field, a Hall effect element was used; an ESR signal was put in at the X-axis and an output voltage of the Hall effect element in at the Y-axis of a Y–X recorder, simultaneously. The microwave frequency used was measured by a cavity wavemeter. Principal *g*-values were estimated by KNEUBÜHL's method[10]. The absorption spectrum was measured by a Cary spectrophotometer Model 15 at 24°.

RESULTS

Optical spectrum of inactivated catechol 1,2-oxygenase

When active catechol 1,2-oxygenase was stored at −20° for about 6 months, the activity decreased to about 1%. Fig. 1 shows absorption spectra of active and

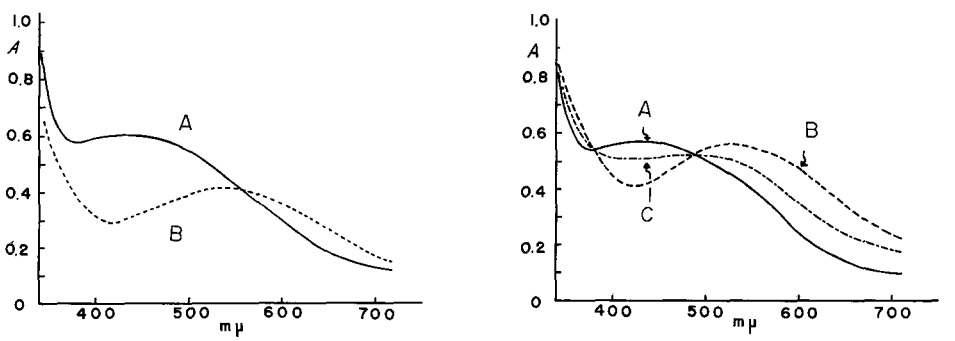


Fig. 1. Absorption spectra of catechol 1,2-oxygenase. A. Active enzyme. Specific activity, 21.7 units/ml. Protein concn., 12 mg/ml. B. Stored inactive enzyme. Specific activity, 0.15 units/ml. Protein concn., 10.8 mg/ml.

Fig. 2. Absorption spectrum of catechol 1,2-oxygenase treated by sodium mersalyl. A. Active enzyme. B. Inactive enzyme. C. Reactivated enzyme after reduced glutathione addition. Enzyme activities are shown in Table II.

TABLE I

EFFECT OF SODIUM MERSALYL ON CATECHOL 1,2-OXYGENASE

Protein concn., 12 mg/ml; buffer, 0.05 M Tris–HCl (pH 8.0).

	Specific activity (units/ml)	
Active enzyme	22.8	(100%)
plus sodium mersalyl (0.48 μmole) (incubated 25 min)	1.48	(6.5%)
plus glutathione (1 μmole) added to the enzyme treated with sodium mersalyl (incubated 10 min)	13.3	(58%)

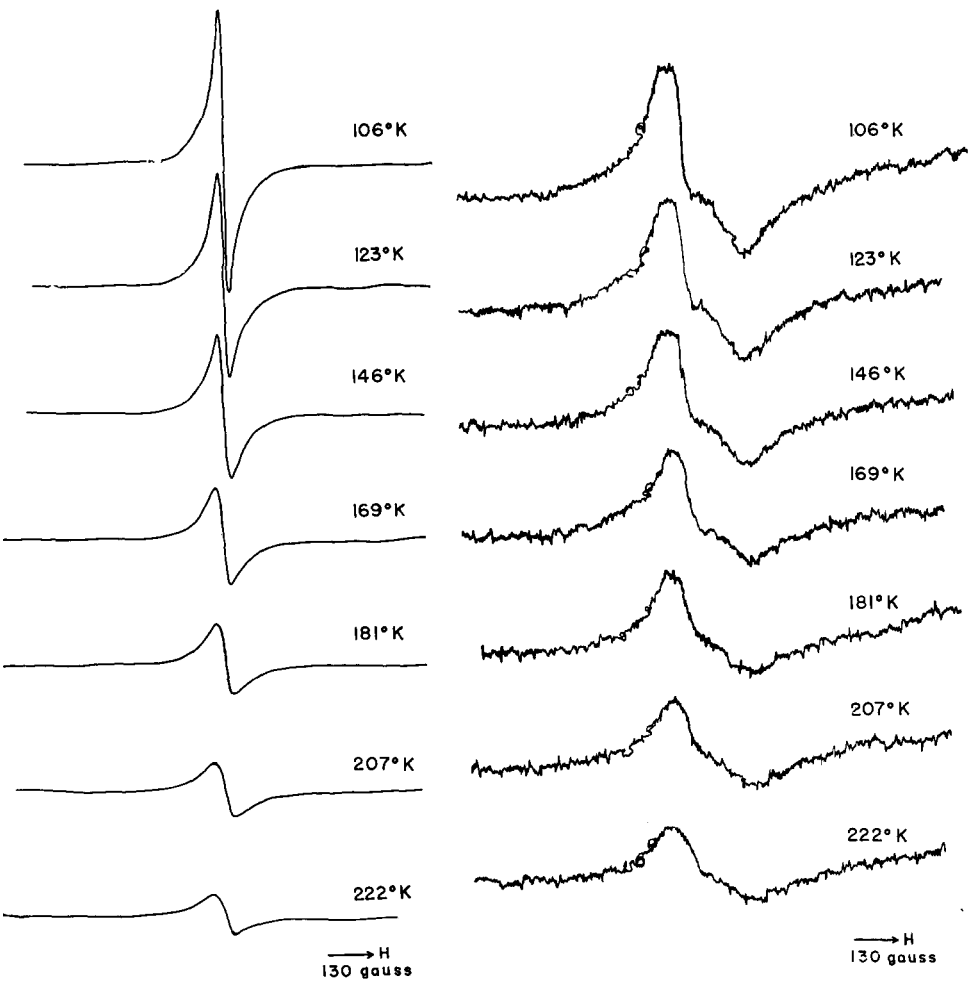


Fig. 3. ESR spectra of active catechol 1,2-oxygenase at various temperatures. Specific activity, 21.7 units/ml. Protein concn., 24 mg/ml. Magnetic field modulation, 15 gauss. Microwave power, 10 db attenuation. Sensitivity of spectrometer, 100 times.

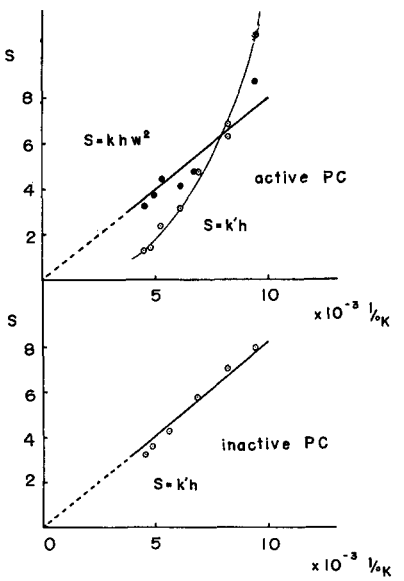
Fig. 4. ESR spectra of stored inactive catechol 1,2-oxygenase at various temperatures. Specific activity, 0.15 units/ml. Protein concn., 21.5 mg/ml. Sensitivity of spectrometer, 800 times. Other experimental conditions same as in Fig. 3.

inactive catechol 1,2-oxygenase. Activity of the stored enzyme was not recovered by the addition of reduced glutathione.

By adding sodium mersalyl to active enzyme, 93% of the activity was lost and a spectral change occurred as shown in Fig. 2. Absorption spectra of inactivated preparation of catechol 1,2-oxygenase obtained by storage at −20° or by treatment with sodium mersalyl resembled one another. However, these differed from each other with respect to reactivation by reduced glutathione. The enzyme treated with sodium mersalyl was reactivated up to 58% by reduced glutathione, as shown in Table I, whereas the stored enzyme was not. Reactivated enzyme showed a spectrum which was similar to that of untreated catechol 1,2-oxygenase (Fig. 2c).

ESR spectra of catechol 1,2-oxygenase

Active catechol 1,2-oxygenase showed a signal at $g = 4.28$, as previously reported[3]. The signal height decreased with increase in temperature as shown in Fig. 3. With stored inactive catechol 1,2-oxygenase, a different signal from that of active enzyme was observed, as shown in Fig. 4. The ESR signal of inactive enzyme also showed temperature dependence. In Fig. 5, signal heights of both native and inactivated enzymes are shown as the ordinate versus the reciprocal of the temperature as the abcissa. A linear relation was observed in the latter case (Fig. 5b) only. However, if signal height was substituted by signal intensity, which is proportional



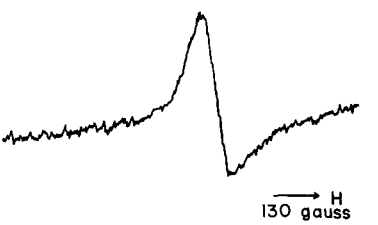


Fig. 5. Curves of signal intensity against reciprocal of temperature. Upper curve, active catechol 1,2-oxygenase (PC) (a); lower curve, inactive catechol 1,2-oxygenase (b). h, signal height; w, signal width. Signal intensity is expressed either by signal height ($S = k'h$) or by signal height multiplied by the square of signal width ($S = khw^2$).

Fig. 6. ESR spectrum of inactive enzyme treated with sodium mersalyl. Specific activity, 1.48 units/ml. Protein concn., 12 mg/ml. Sensitivity of spectrometer, 800 times. Temp., 169 °K. Other experimental conditions same as in Fig. 3.

to signal height multiplied by the square of signal width, a straight line was obtained in the former case, shown in Fig. 5a by the dotted line.
The ESR spectrum of enzyme that was treated with sodium mersalyl showed a similar spectrum to the stored inactive catechol 1,2-oxygenase, as shown in Fig. 6. A red shift of absorption spectrum of the inactivated catechol 1,2-oxygenase corresponded to the change in ESR signal. In the case of stored inactive enzyme, 1% of the activity remained, and it is considered that the ESR spectra shown in Fig. 4 are those of the mixture of 99% of inactive and 1% of the active enzymes.

g-Value determination of inactive catechol 1,2-oxygenase

The ESR spectra of Fig. 4 probably are the mixed spectra, as mentioned above. Since the active enzyme has a sharp signal at $g = 4.28$, the signal due to the inactive enzyme can be obtained by substracting the signal of the active enzyme from that of the mixture. In Fig. 7, the dotted line represents the true signal of the inactive enzyme. From this spectrum, g-values of the inactive enzyme were estimated by the method of KNEUBÜHL[10], as $g_x = 3.87$, $g_y = 4.41$ and $g_z = 4.07$, as shown in Fig. 7a. The dotted line of Fig. 7a was integrated and the integrated curve is shown in Fig. 7b.

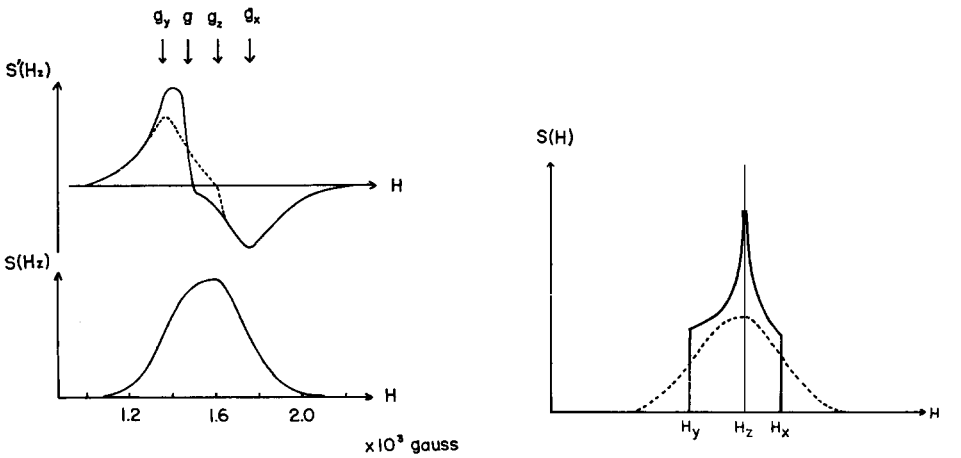


Fig. 7. Determination of g-values of inactive catechol 1,2-oxygenase (106 °K). g_x, g_y and g_z are those of inactive enzyme. g is that of active enzyme ($g = 4.28$). Solid line indicates ESR signal of mixed enzyme. Integration of dotted line was carried out as shown in lower figure (b).

Fig. 8. Theoretical ESR absorption curve of inactive catechol 1,2-oxygenase (solid line). Dotted line indicates real absorption curve, in which gaussian function with 150 gauss of half line-width is superimposed near H_x, H_y and H_z semiqualitatively.

Assuming that the D-term in spin-Hamiltonian is not zero, and $2\sqrt{7}\,D \gg g\beta H$, D, AASA *et al.*[6] calculated theoretical g-values by using third order perturbation. Comparing these theoretical g-values with the experimental ones, the E- and D-terms were calculated to be $E = 0.19$ cm^{-1} and $D = 0.021$ cm^{-1}, respectively.
A theoretical ESR absorption curve for inactive catechol 1,2-oxygenase in the amorphous state was drawn by substituting the particular values of magnetic field

strength in the shape function, calculated by KNEUBÜHL[10]. These particular values of H_x (1680 gauss), H_y (1477 gauss) and H_z (1601 gauss) corresponded to g_x (3.87), g_y (4.41) and g_z (4.07), respectively. Fig. 8 shows the derived theoretical absorption curve. As calculated, the ideal curve does not assume finite line-width; in order to approach the real ESR absorption curve with an ideal curve as starting point, a Gaussian line-width function should be superimposed upon the ideal curve near H_x, H_y and H_z. The half line-width of the Gaussian function was taken as about 150 gauss, and the Gaussian function was drawn semi-qualitatively as a dotted line in Fig. 8. This real curve showed a good resemblance to the experimental integrated curve in Fig. 7b.

DISCUSSION

Inactivation of catechol 1,2-oxygenase occurs through storage at −20° or by treatment with sodium mersalyl. Thus inactivated catechol 1,2-oxygenase preparations showed similar changes in their absorption spectra as well as in ESR signal. There is assumed to be a relation between the optical and ESR spectral changes, because these concomitant changes both accompany the inactivation of catechol 1,2-oxygenase. However, theoretical treatment of molecular orbitals between iron ion and ligands are still too obscure to support an adequate discussion of the optical spectra of catechol 1,2-oxygenase. Mechanisms of catechol 1,2-oxygenase inactivation through storage or through treatment with sodium mersalyl seem to be almost the same, even though a reactivation by reduced glutathione is observed only in the case of the mersalyl treatment. Perhaps if storage of the enzyme were examined thoroughly, then reactivation by glutathione would be found.

The estimation of the g-values of inactive catechol 1,2-oxygenase according to KNEUBÜHL's method[10] may include some errors. Another possible error may arise from the large line-width. Even at liquid N_2 temperature, a half line-width is over 150 gauss, enough to exceed the intervals between the magnetic fields of H_x and H_z or H_z and H_y. When the temperature was increased in ESR measurements of inactive catechol 1,2-oxygenase, the position of the absorption maxima which corresponded to the magnetic field strength of H_x and H_y did not change, as shown in Fig. 4. When the signal heights at each temperature, without considering width, were plotted as cited in Fig. 5b, a linear relationship was obtained. From these facts, a large line-width seems not to affect the estimation of g-values. The real absorption curve (dotted curve of Fig. 8), drawn by using the experimental g-values, corresponded well to the experimental integration curve (Fig. 7b). Taking these points into consideration, the experimental g-values are considered to be correct.

The valency of iron contained in the active catechol 1,2-oxygenase was found to be Fe^{3+} by ESR measurements[3] and by measurement of magnetic susceptibility[11]. AASA *et al.*[6,7] calculated anisotropic g-values, using third order perturbation when $2\sqrt{7}\,E \gg g\beta H$, D ($\neq 0$). The same method of calculation of anisotropic g-values can be applied to the Fe^{3+} in the inactive catechol 1,2-oxygenase. It is worthwhile to mention that the value of D is comparatively large, compared with that of E. The E- and D-terms of spin-Hamiltonian in a high spin state of Fe^{3+} were calculated theoretically by KOTANI[12,13]. $D = 0$ means that the energy separation between d_{xz}- and d_{xy}-orbitals and between d_{xy}- and d_{yz}-orbitals are equal. A three-dimensional

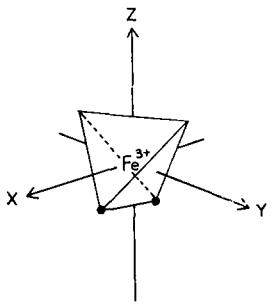


Fig. 9. Tertiary structural model of active catechol 1,2-oxygenase. Black circle indicates ligands with *minus* charges. Fe^{3+} is positioned in the center of the tetrahedron.

model for active catechol 1,2-oxygenase could be drawn as shown in Fig. 9. Fe^{3+} is at the center of a tetrahedron; two of four vertices are occupied by ligands with *minus* charge, probably SH-residues of enzyme; the other two are vacant. However, as the theoretical treatment for this model has not been obtained, this model may be changed to a more precise one. In our model, two ligands are positioned in the X–Z plane and the Z-axis is perpendicular to the line which binds the two ligands. It is understood that the wave functions of the d_{xz}-orbital are contracted and those of d_{yz} expanded in order for two *minus* charges to exist. In the case of inactive catechol 1,2-oxygenase, the D-term was not zero, therefore the energy separation of $d\gamma$-orbitals is not the case of $D = 0$, but the d_{xy}-orbital position is somewhat higher than the middle of d_{yz}- and d_{xz}-orbitals. One of the ligands changes its position to the X- or Y-direction, away from Fe^{3+}. Presumably one of the SH-residues distorts the original position. For an explanation of the change of SH-residue, there appear to be two possibilities. One is a conformational change of protein, as a result of which the SH-residue moves. The other is the direct movement of the SH-residue. By considering the effect of mersalyl on the enzyme, the second possibility seems quite probable. However, after a greater resolution of primary and tertiary structure is obtained, more precise information will be obtained, and the analysis of the mechanism of the enzyme action could be clarified.

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