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Circular Dichroism of Catechol 1,2-Dioxygenase from Acinetobacter Calcoaceticus

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Circular dichroism (CD) spectra of catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* exhibit three positive ellipticity bands between 240 and 300 nm (250, 283, and 292 nm), two negative bands at 327 and 480 nm, and a low-intensity positive band at 390 nm. The fractions of helix, β-form, and unordered form of the enzyme are 8, 38, and 54%, respectively. The circular dichroic bands at 327 and 480 nm and a part of the positive bands at 292 and 390 nm are associated with enzyme activity. Significant changes in absorption and CD spectra of the enzyme were observed when the temperature of the enzyme preparation was increased to 47°C, coinciding with the sharp decrease in enzyme activity observed at this temperature.

Catechol 1,2-dioxygenase (catechol:oxygen 1,2-oxidoreductase, EC 1.13.11.1) (pyrocatechase), a nonheme trivalent iron-containing enzyme, catalyzes the cleavage of the aromatic ring of catechol to *cis*, *cis*-muconic acid with the incorporation of two atoms of molecular oxygen into the substrate. The enzyme purified from *Pseudomonas arvilla* (*1*) has a molecular weight of 90 000 and contains 2 g-atoms of trivalent iron and two subunits per molecule. The enzyme purified from *Brevibacterium fuscum* (*2*), however, has a molecular weight of 60 000, containing 1 g-atom of trivalent iron and one subunit per molecule. Circular dichroism (CD) studies of these two isofunctional enzymes indicated that their CD spectra are quite similar to each other at above 300 nm and below 250 nm. However, the CD bands between 250 and 300 nm are markedly different. Most of the bands in this region are positive in the *Pseudomonas* enzyme, whereas most of them are negative in the *Brevibacterium* enzyme. Recently, Fujiwara *et al.* (*3*) reported that in addition to its intradiol cleavage ability, the *Pseudomonas* enzyme also cleaves 3-substituted catechol between the carbon 2 and 3 positions. The *Brevibacterium* enzyme, however, does not have this extradiol cleavage ability.

In our continuing effort to understand the nature of oxygenase, particularly the nonheme, trivalent, iron-containing dioxygenase, we have been investigating the properties of protocatechuate 3,4-dioxygenase (PCD) from *Pseudomonas* and *Acinetobacter* (*4–9*). Recently, we have purified pyrocatechase from *A. calcoaceticus* (*10*). This dioxygenase has a molecular weight of 81 000 and contains 2 g-atoms of trivalent iron and two subunits per molecule. The substrate specificity, pH stability, amino acid composition, and immunological properties of these isofunctional enzymes differ from each other. *Acinetobacter* pyrocatechase has extradiol cleavage ability, but the reaction

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ratio of intradiol/extradiol cleavage is different from that of *Pseudomonas* pyrocatechase (*11*). In order to learn more about the nature of the enzyme and to compare with its isofunctional enzymes, we studied the CD spectra of pyrocatechase from a new source, *A. calcoaceticus*.

Homogeneous pyrocatechase, with a specific activity of 20 U/min/mg of protein, was prepared from *A. calcoaceticus* ADP-96, as previously described (*10*). CD results were expressed in terms of the mean residue ellipticity, $[\theta]$, in units of degrees · square centimeters per decimole (based on two subunits per molecule). Repetitive scanning of the samples was performed.

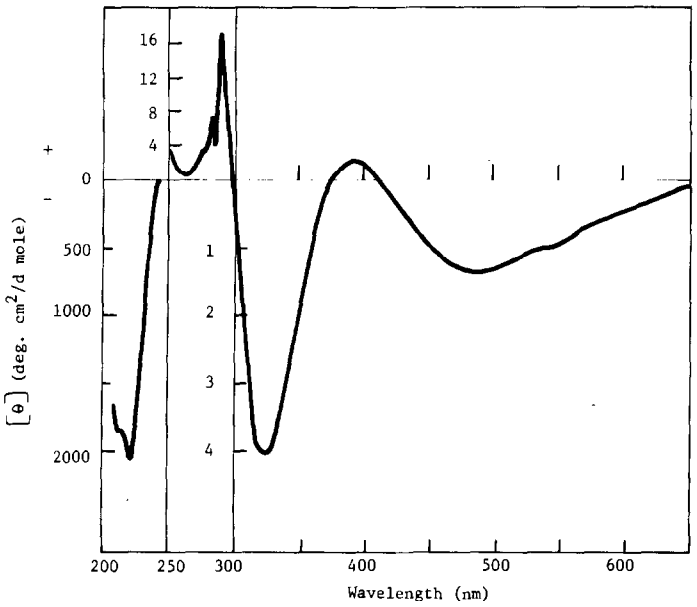

FIG. 1. Circular dichroism spectra of pyrocatechase from *Acinetobacter calcoaceticus*. CD spectra were recorded on a Durrum–Jasco J-20 spectropolarimeter. The following conditions were employed for the wavelength ranges of 200–250, 240–350, and 300–650 nm, respectively: scanning speed (nm/min): 2.5, 5, 10; time constant (sec): 1,1,1; scale (m/cm): 2,2,2; cell length (cm): 0.05,1,1; protein concentration (mg/ml): 0.97,0.97,14; in 0.05 *M* Tris–HCl buffer, pH 7.5. The temperature of the solution in the cell was kept constant at 25°C.

The CD spectrum of *Acinetobacter* pyrocatechase is shown in Fig. 1. In the visible region, the enzyme shows a moderate negative band at 327 nm and a weak intensity broad negative band around 480 nm. These were strikingly similar to those of isofunctional enzymes from *Pseudomonas* and *Brevibacterium*. The negative 480-nm band, which was also found among the isofunctional enzymes of PCD, was attributed to the state of Fe^{3+} coordination. A weak positive CD band was observed unexpectedly at 390 nm. This is different from that of the isofunctional enzyme from *Pseudomonas arvilla* but is similar to that of the isofunctional enzyme from *Brevibacterium fuscum*. The nature of this band is not clear; however, a similar phenomenon was reported for PCD. The CD band of PCD isolated from *Acinetobacter calcoaceticus* centers at 380 nm and does not extend into the positive ellipticity, whereas PCD isolated from *Pseudomonas aeruginosa* does have a positive band at 380 nm (*5*). In the region of side

chain chromophores of the protein, the CD spectrum is resolved into a strong (292 nm) and two weak CD bands (283 and 250 nm), markedly different from the behavior of its isofunctional enzymes. *Pseudomonas* pyrocatechase has strong positive CD bands at 292, 285, 378 nm and a weak band at 258 nm. The bands at 292 and 283 nm are possibly due to tryptophyl or tyrosyl residues, or both. The positive band at 250 nm (which increased significantly at a higher pH) may be due to the ionized tyrosyl residues (*12*). In the vicinity of the peptide absorption band, a strong negative CD band at 222 nm is observed. Based on the value of the molar ellipticity at 222 and 210 nm using the approximation method of Chen *et al.* (*13*) for estimating the fraction of three forms, *Acinetobacter* pyrocatechase is 8% α-helix, 38% β-form, and 54% unordered form. This is a more than threefold lower α-helix content than the isofunctional enzymes from *Brevibacterium* and *Pseudomonas* [calculated using the method of Chen *et al.* (*13*)].

TABLE 1

THE EFFECT OF TEMPERATURE ON THE CD AND ABSORPTION SPECTRA OF PYROCATECHASE FROM *Acinetobacter calcoaceticus*[a]

Temperature (°C)	$-[\theta]_{500}$	$[\theta]_{390}$	$-[\theta]_{327}$	$[\theta]_{292}$	$E_{440} \times 10^{-2}$
5–25	1.4	0.25	4	18	28.6
30	1.4	0.25	4	18	27.7
35	1.4	0.25	4	18	26.0
40	1.0	0.25	3	18	23.0
47	0	0.22	0	7	6.0
50	0	0.19	0	7	0

[a] A thermostatic cell compartment was used to keep the temperature of the solution in the cell at the temperature indicated. Enzyme concentration was 14 mg/ml in 0.05 *M* Tris–HCl buffer, pH 7.5. Other conditions were the same as in Fig. 1. *E* denotes the molar absorptivity. $[\theta]$ is the mean residue ellipticity in degrees · square centimeters per decimole at the wavelength indicated in the suffix. Repetitive scanning of the samples was performed.

The reaction rates of a fully active holo enzyme at temperatures of 6, 11, 17, 20, 25, 30, 35, 37, 40, 47, and 50°C were 3.6, 6.7, 9, 13, 15, 18, 21, 23, 19, 0, and 0 units, respectively (1 unit of enzyme activity was defined as 1 μmol of product formed/min/mg of protein). The activation energy calculated from the Arrhenius plots of the reaction rates vs the reciprocal of the absolute temperature is 2.83 kcal.

The effects of temperature on CD and absorption spectra of *Acinetobacter* pyrocatechase are summarized in Table 1. Significant changes were observed at 47°C both in absorption and CD spectra in the visible region. The positive CD band at 390 nm, however, decreased only slightly. In the aromatic region, no CD change was observed under these temperatures except for the CD spectrum obtained at 47°C or above. The intensity of CD was not changed in the far uv region at the temperature examined. The relationship between the enzyme activity and the intensity of the CD bands was studied quantitatively with a partially deactivated enzyme, obtained by heat inactivation at 47°C for various time periods. A linear relationship between the enzyme activity and the intensity of the molar ellipticity was observed for the 292-, 327-, 390-

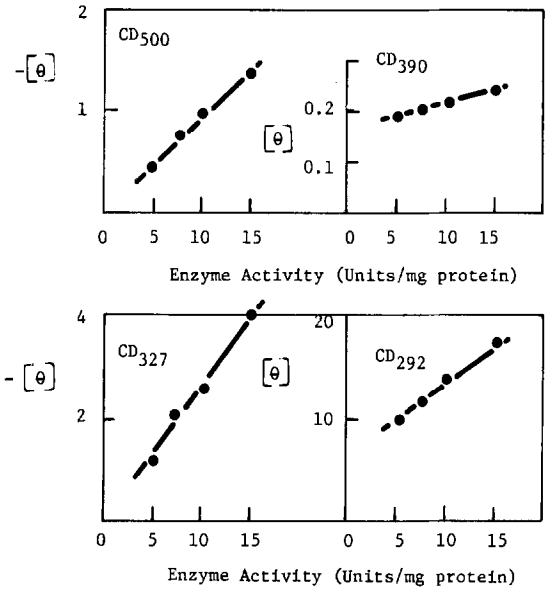

FIG. 2. The correlation between enzyme activity and CD at 292, 327, 390, and 500 nm. Enzyme solutions (14 mg/ml for >300 nm and 0.97 mg/ml for 292 nm) in 0.05 *M* Tris–HCl buffer pH 7.5 were inactivated in a water bath at 47°C for various periods of time. The enzyme solutions were cooled down to room temperature, and then the enzyme activity and ellipticity were measured.

and 480-nm bands (Fig. 2). Since the correlation lines in $[\theta]_{292}$ and $[\theta]_{390}$ do not pass through the origin on the graph, the CD bands at both 292 and 390 nm appear to include some portions indifferent to the enzyme activity.

The CD spectra of the isofunctional enzymes of nonheme trivalent iron-containing dioxygenases, pyrocatechase and PCD, have identical bands at around 480 nm and in the peptide backbone region. The differences in CD spectra in other regions reflect the differences in substrate specificity and intradiol/extradiol cleavage ability among these dioxygenases.

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