Circular Dichroism of Holo- and Apoprotocatechuate 3,4-Dioxygenase from *Pseudomonas aeruginosa*†

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ABSTRACT: Circular dichroism studies have been carried out on both apo- and holoprotocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*, in the absence and presence of competitive inhibitors, protocatechualdehyde and 4-nitrocatechol. The apo- and holoenzyme showed identical spectra in the ultraviolet region between 200 and 250 nm (peptide back bone region), but the low intensity negative bands at 330 and 480 nm of the holoenzyme were completely absent in the apoenzyme. On the side chain region, the positive ellipticity peaks of the holoenzyme change into a lower intensity and broader band indicating the participation of aromatic amino acid residues in the primary binding of iron ion. Under anaerobic conditions, spectral changes were evident in the side chain region for the binary complexes of both the holo- and the apoenzyme with protocatechuate. The presence of iron in the holoenzyme results in an increase in positive ellipticity between 290 and 320 nm. Either with or without the iron, the enzyme protein binds protocatechuate and has a greater positive circular dichroism increase at 240–260 nm. CD difference spectra indicate that the modes of binding to form the binary complexes of holo- or apoenzyme with either substrates or competitive inhibitors are different. The bound iron ion stimulates binding. Spectral changes of the holoenzyme in the aromatic region were also observed in different pH environments of lower enzymatic activity. It is still not established whether these aromatic residues play an active or passive role in the binding of iron and/or substrates and inhibitors.

Protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-oxidoreductase, EC 1.13.11.3) is a non-heme trivalent iron-containing enzyme that catalyzes the transformation of protocatechuic acid to β-carboxy-*cis,cis*-muconic acid. The dioxygenases from *Pseudomonas aeruginosa, Acinetobacter calcoaceticus,* and *Thiobacillus* have similar molecular weights (ca. 700,000) and consist of eight subunits (Stanier and Ingraham, 1954; Ornston, 1966; Fujisawa and Hayaishi, 1968; Fujisawa et. al., 1971, 1972a,b; Fujiwara and Nozaki, 1973; Fujisawa and Uyeda, 1974; Peisach et al., 1972; Wells, 1972; Hou et al., 1975a,b). The enzyme forms stable binary complexes with substrates such as protocatechuic acid, catechol, or 3,4-dihydroxyphenylacetic acid under anaerobic conditions, and transient ternary complexes under aerobic conditions. Stable binary complexes are also formed with inhibitors such as protocatechualdehyde, 3,4-dihydroxyacetophenone, or 4-nitrocatechol in the presence or absence of oxygen.

The mode of binding of the iron to the enzyme protein is still obscure, although reaction mechanisms have been proposed based on analyses of the enzyme-bound iron by electron spin resonance (ESR) spectroscopy (Peisach et al., 1972). Sulfhydryl residues, arranged in a tetrahedron around the metal, are responsible for all of the ligands of iron. However, in a recent report, Fujiwara and Nozaki (1973) indicated that the number of sulfhydryl groups unmasked by the removal of iron is less than one per atom of iron removed.

Circular dichroism is a powerful tool for studying protein configuration. We have studied the circular dichroism of holoprotocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* (Zaborsky et al., 1975). With holoenzyme, however, many questions remain unanswered; for example: the mode of binding of the iron to the enzyme protein; the effect of the bound iron on the binding between enzyme and substrates; and the primary binding function of the enzyme protein to the substrates or inhibitors. In order to approach these problems, we prepared iron-free apoprotocatechuate 3,4-dioxygenase and studied its circular dichroism (CD) spectra.

Materials and Methods

Crystalline protocatechuate 3,4-dioxygenase with a specific activity of 65 μmol per min per mg of protein was prepared from *P. aeruginosa* grown with *p*-hydroxybenzoate as the sole carbon source as previously described (Fujisawa and Hayaishi, 1968). The holoenzyme appears to be homogeneous by both acrylamide gel electrophoresis and ultracentrifugation (Hou et al., 1975a). Iron-free apoprotocatechuate 3,4-dioxygenase was prepared by prolonged anaerobic dialysis against a buffer solution containing both *o*-phenanthroline and sodium dithionite (Fujiwara and Nozaki, 1973). The apoenzyme thus prepared was washed thoroughly with 0.05 *M* Tris-HCl buffer (pH 8.5) under anaerobic conditions through a AMICON XM 300 membrane, in order to remove the unreacted *o*-phenanthroline and *o*-phenanthroline–iron complex. The apoenzyme solution was clarified by centrifugation. The apoenzyme used in these studies has the following characteristics: a molecular size of 19 S; homogeneity on acrylamide gel electrophoresis; identical mobility on the acrylamide gel with holoenzyme; 1.06 g-atoms of iron/molecule, that is 0.13 g-atom or 2.32 mmol of iron/subunit (eight identical subunits/molecule of enzyme); enzyme activity of about 0.3 units/mg of protein, about 0.2% of the holoenzyme; show ability to be reconstituted by known methods (Fujiwara and Nozaki, 1973).

Protocatechuic acid was obtained from the Sigma Chemical Co., St. Louis, Mo.; protocatechualdehyde was purchased from Aldrich Chemical Co., Milwaukee, Wis.; 4-nitrocatechol was a product of Aldrich Chemical Co., Inc.

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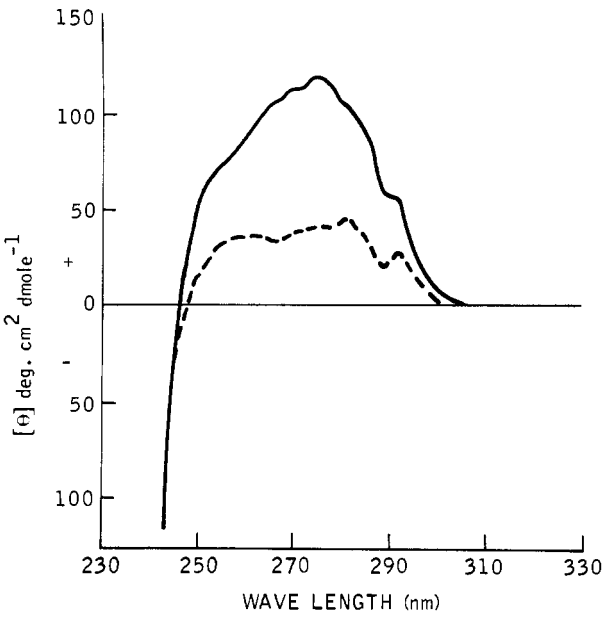

FIGURE 1: CD spectra of holo- and apoprotocatechuate 3,4-dioxygenase from 230 to 350 nm in 50 m*M* Tris-HCl buffer (pH 8.5). Spectra were recorded at 25° with a 10-mm path-length cell. (—) Holoenzyme, 0.75 mg/ml. (- - -) Apoenzyme, 0.80 mg/ml.

(Milwaukee, Wis.). Catechol was obtained from Matheson Coleman and Bell, East Rutherford, N.J.

CD spectra were recorded on a Durrum-Jasco J-20 spectropolarimeter with conditions described previously (Zaborsky et al., 1975). CD results are expressed in terms of the mean residue ellipticity, $[\theta]$, in units of deg cm^2 dmol^{-1}.

Iron analyses were carried out with a flameless atomic absorption spectrophotometer.

Results and Discussion

CD of Apoprotocatechuate 3,4-Dioxygenase. In order to estimate the contribution of the bound iron to the CD spectrum, the CD of the apoenzyme was measured. Apoprotocatechuate 3,4-dioxygenase was colorless and did not show CD absorption above 300 nm. Therefore, the negative CD bands at 327 and 480 nm of the holoenzyme are clearly the result of chelation between the iron ion and the amino acid residues of the protein. This agrees with the previous conclusion from spectrophotometric, electron spin resonance spectroscopic, and chemical analyses, that the trivalent iron bound to the enzyme is responsible for the visible absorption band and functions as an integral part of the enzyme (Fujisawa and Hayaishi, 1968; Fujisawa et al., 1971, 1972a,b; Peisach et al., 1972). In the vicinity of the peptide absorption bands, no appreciable difference between holo- and apoenzyme was observed. This indicates that there is no significant change in the peptide backbone even after removal of iron from the enzyme protein. In the region of side chain chromophores of the protein, the CD bands decreased markedly but not completely (Figure 1). This suggested that a part of the ellipticity in the region 250–300 nm is associated with iron. Absorption spectra at uv region of both holo- and apoprotocatechuate 3,4-dioxygenase are identical. The possible participation of sulfhydryl groups in the binding of iron is still unclear (Peisach et al., 1972; Fujiwara and Nozaki, 1973). However, our CD results indicate that some of the aromatic amino acid residues directly or indirectly participate in the binding of iron.

The circular dichroism of holo- and apopyrocatechase, another non-heme trivalent iron dioxygenase that catalyzes the oxidation of catechol to *cis,cis*-muconic acid, has been reported (Nakazawa et al., 1968). It is interesting to compare the CD spectra of these two closely related intradiol dioxygenases. The number of aromatic amino acid residues per each subunit of protocatechuate 3,4-dioxygenase (eight subunits) and pyrocatechase (two subunits) are as follows: phenylalanine, 30.8, 20.0; tyrosine 23.5, 13.2; tryptophan 19.7, 2.7, respectively. The CD bands of apopyrocatechase at the aromatic region are markedly reduced, especially from 270 to 295 nm. The peaks at 258, 278, 285, and 292 nm of holopyrocatechase still can be observed clearly in its apo form although the magnitude is much smaller. The CD peaks at 255, 269, and 275 nm of holoprotocatechuate 3,4-dioxygenase, however, cannot be recognized in its apo form except for the 292-nm peak. A new 282-nm peak appeared and the whole region became broader with a lower magnitude of ellipticity.

The function of free sulfhydryl groups in these two enzymes is different. Protocatechuate 3,4-dioxygenase retained full activity after the free sulfhydryl groups of the enzyme were titrated with *p*-chloromercuribenzoate (Fujisawa and Hayaishi, 1968; Hou et al., 1975a,b). In pyrocatechase, no enzyme activity remained after the same treatment (Nagami, 1972). This indicates that the free sulfhydryl groups (from cysteine) of protocatechuate 3,4-dioxygenase do not participate in the enzymatic reaction.

The role of iron in the dioxygenases such as protocatechuate 3,4-dioxygenase, pyrocatechase, and metapyrocatechase is different from its role in dioxygenases such as 3,4-dihydroxyphenylacetate 2,3-dioxygenase and steroid oxygenases. In the former type of dioxygenases, the number of iron atoms coincides with the number of subunits, and it has been suggested that iron participates in catalytic function. In the latter type of dioxygenases, it has been suggested that only one atom of iron participates in the catalytic function and the rest are involved in the association of subunits.

Effect of Substrates on CD. In the previous paper, we reported that in the presence of the organic substrate and in the absence of air, the CD spectrum of the holoprotocatechuate 3,4-dioxygenase shifts its maximum from 275 to 267 nm (Zaborsky et al., 1975). A difference CD spectrum of holoenzyme–protocatechuate complex minus holoenzyme is shown in Figure 2. The CD spectrum of the iron-free apoenzyme–protocatechuate complex, however, shows increases in intensity throughout the whole side chain region with a maximum increase around 240–255 nm. A difference CD spectrum of apoenzyme–protocatechuate complex minus apoenzyme is shown in Figure 3. From Figures 2 and 3 CD increases between 255 and 270 nm and 290 and 320 nm can be mainly attributed to the presence of iron. It is also clear that the iron-free apoenzyme has the ability to bind with substrate. In the presence of bound iron, additional participation of the aromatic amino acid residues in binding occurred. This is in contrast to apopyrocatechase, where no CD change in the side chain region could be observed upon addition of organic substrate (Nakazawa et al., 1968).

The stable binary complex of holoprotocatechuate 3,4-dioxygenase with a poor substrate, catechol (relative reaction rate of 0.4%), can be detected spectrophotometrically at 450 nm (Fujisawa et al., 1972a). However, under anaerobic conditions the addition of catechol to either holo or iron-free apoenzyme resulted in no detectable CD changes in the aromatic side chain region. This indicates that there is too

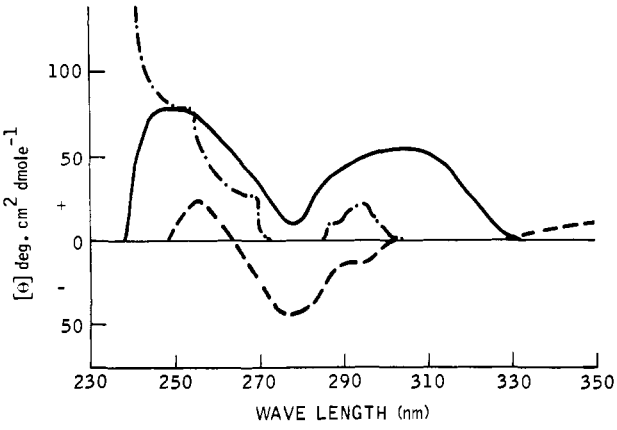

FIGURE 2: Difference CD spectra of holoprotocatechuate 3,4-dioxygenase-substrates or -inhibitors complex minus holoenzyme. CD spectra were studied under anaerobic conditions as described in the text. (—) Holoenzyme-protocatechuate complex minus holoenzyme. Two milliliters of enzyme solution (0.78 mg/ml, 1.78×10^{-8} mol based on eight subunits/molecule) was mixed with 50 μl of protocatechuic acid (2.03 mg/ml, 6.60×10^{-7} mol). (- - -) Holoenzyme-protocatechualdehyde complex minus holoenzyme; 1.78×10^{-8} mol of holoenzyme and 6.6×10^{-7} mol of protocatechualdehyde were used. (-·-) Holoenzyme-4-nitrocatechol complex minus holoenzyme; 1.78×10^{-8} mol of holoenzyme and 6.6×10^{-7} mol of 4-nitrocatechol were used.

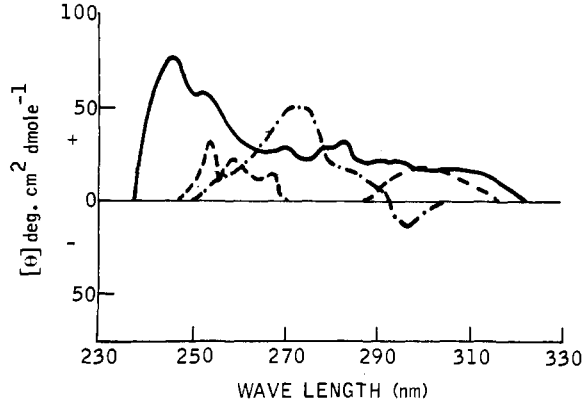

FIGURE 3: Difference CD spectra of apoprotocatechuate 3,4-dioxygenase-substrates or -inhibitors complex *minus* apoenzyme. CD spectra were studied under anerobic conditions as described in the text. Apoenzyme (1.69×10^{-8} mol) (based on eight subunits/molecule) in 2 ml of 50 m*M* Tris-HCl buffer (pH 8.5) was mixed with 50 μl (6.60×10^{-7} mol) of substrates or inhibitors. (—) Apoenzyme-protocatechuate complex minus apoenzyme; (- - -) apoenzyme-protocatechualdehyde complex *minus* apoenzyme; (-·-) apoenzyme-4-nitrocatechol complex minus apoenzyme.

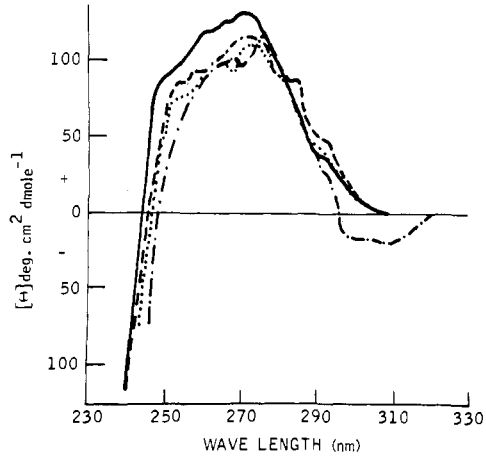

FIGURE 4: The effect of pH on the ultraviolet CD of holoprotocatechuate 3,4-dioxygenase. The CD spectrum of the enzyme (0.80 mg/ml) was recorded in 0.05 *M* Tris-HCl (pH 8.0) (—); 0.05 *M* potassium phosphate (pH 6.0) (- - -); 0.05 *M* carbonate buffer (pH 10.0) (-·-), and 0.1 *N* NaOH (···) with a 10-mm light path cell at 25°. The specific enzyme activities under these pH buffers are: 0.1 *N* HCl, 0 unit; pH 6.0, 9 units; pH 8.0, 65 units; pH 10.0, 39 units; 0.1 *N* NaOH, 0 unit.

weak or no binding at all between the aromatic amino acid residues and the poor substrate.

Effect of Inhibitors on CD. Upon addition of protocatechualdehyde, one of the most potent competitive inhibitors for protocatechuate 3,4-dioxygenase, to the holoenzyme under either aerobic or anaerobic conditions, CD changes occur in both the side chain and visible regions (Zaborsky et al., 1975). A difference CD spectrum of holoenzyme-protocatechualdehyde complex minus holoenzyme is shown in Figure 2. Increases in CD bands were observed at 248–263 nm and above 300 nm. However, more significant decreases were observed between 264 and 300 nm with a maximum at 278 nm. Upon addition of protocatechualdehyde to the apoenzyme under the same conditions, CD changes were observed only in the side chain region. A difference CD spectrum of apoenzyme-protocatechualdehyde minus apoenzyme is shown in Figure 3. Slight increases at 248–269 nm and 287–316 nm indicate binding between the aromatic amino acid residues or disulfide groups of the iron-free apoenzyme and the competitive inhibitor.

By equilibrium analysis, Fujiwara and Nozaki (1973) observed that 4-nitrocatechol, a nonmetabolizable competitive inhibitor, binds with either holo- or apoprotocatechuate 3,4-dioxygenase. They assume that 1 mol of inhibitor binds with one active site. By circular dichroism, we are able to demonstrate the differences between these bindings in the aromatic side chain region. Difference CD spectra of holoenzyme-4-nitrocatechol complex minus holoenzyme or apoenzyme-4-nitrocatechol complex minus apoenzyme are shown in Figures 2 and 3. For the holoenzyme complex, CD increases are in the region below 270 nm and also at 285–300 nm, with no changes at 270–285 nm. For the apoenzyme complex CD increases are seen at 250–293 nm with a maximum at 273 nm, but decrease at 293–300 nm. This also indicates that there is binding between the enzyme protein and 4-nitrocatechol.

From Figures 2 and 3, it is clear that the mode of binding of holo- or apoenzyme to substrates or inhibitors is quite different. This might be a good indication about the nature of the strict substrate specificity of protocatechuate 3,4-dioxygenase.

Effect of pH on Ultraviolet CD. The effect of pH on the CD of holoprotocatechuate 3,4-dioxygenase over the wavelength region 230–330 nm was analyzed (Figure 4). The specific enzyme activity at each pH is also shown in the figure. At pH 6.0, the CD bands between 248 and 283 nm decreased somewhat. In 0.1 *N* HCl, almost all positive bands in this region disappeared (not shown in the figure). At pH 10, the CD bands at 248 nm become negative, and also decrease in magnitude at the following wavelengths: 250 nm (by two-thirds), 260 nm (by one-fourth), and 270 nm (by one-ninth). A new negative ellipticity between 295 and 320 nm is also observed. On a more alkaline solution, i.e., in 0.1 *N* NaOH, CD band decreases were also observed between 240 and 280 nm. These results are quite different from those with pyrocatechase (Nakazawa et al., 1968). Here, in 0.1 *N* NaOH solution, the CD bands between 270 and 300

nm disappear with an appearance of a new intensity positive band at 250 nm. This is in agreement with the enzyme stability differences between these two dioxygenases under different pH conditions (Fujisawa and Hayaishi, 1968; Hou et al., 1975a,b; Kojima et al., 1967).

It is difficult at this time to draw a definite conclusion about the mode of binding of iron to the amino acid residues of the non-heme protein, or the binding of substrates or inhibitors to the holo- or apoenzyme, based on the evidence presented here or available elsewhere. However, it becomes clear from our CD studies that some of the aromatic amino acid residues participate in the iron binding. It is also clear that the primary binding site for substrate involves the amino acid residues of the iron-free enzyme protein, and that the bound iron ion stimulates these bindings.

Amino acids sequences and further chemically oriented studies are needed in order to identify precisely the groups that are essential for binding and/or catalysis in this dioxygenase.

Acknowledgment

The author thanks Ms. Marjorie O. Lillard and Mr. Andre Felix for their excellent technical assistance and Dr. Allen I. Laskin for his continued encouragement and assistance in preparing this manuscript.

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