Magnetic and natural circular dichroism of L-tryptophan 2,3-dioxygenases and indoleamine 2,3-dioxygenase. I. Spectra of ferric and ferrous high spin forms

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 4, Issue of February 25, pp. 2519-2525, 1983
Printed in U.S.A.

Magnetic and Natural Circular Dichroism of L-Tryptophan 2,3-Dioxygenases and Indoleamine 2,3-Dioxygenase

I. SPECTRA OF FERRIC AND FERROUS HIGH SPIN FORMS*

(Received for publication, March 3, 1982)

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The ferric form of L-tryptophan 2,3-dioxygenases from both *Pseudomonas acidovorans* (ATCC 11299b) and rat liver showed magnetic CD spectra ascribable to a high spin protohemoprotein at neutral pH, whereas the ferric indoleamine 2,3-dioxygenase from rabbit intestine exhibited a spectrum due to a mixture of high and low spin states under comparable conditions. Upon addition of L-tryptophan, the spectra of the former enzymes changed to another type of high spin spectra, while the latter showed a marked increase in the low spin component. From these findings and effects of pH on the spectra, it is suggested that the sixth ligand of ferric L-tryptophan 2,3-dioxygenases is water at a neutral pH and that for the ferric indoleamine 2,3-dioxygenase is a strong field ligand such as an imidazole nitrogen. The mixed spin state observed for the latter enzyme was ascribable to a thermal equilibrium between high and low spin states as judged by low temperature spectroscopy.

With the ferrous form, the Soret magnetic CD spectra of these enzymes were all similar, giving those of a typical high spin ferrous protohemoprotein, whereas visible spectra were different from one another, suggesting differences in the electronic structure of the heme and its vicinity. The natural CD spectra of both ferric and ferrous forms of each enzyme showed negative Cotton effects in the Soret region. Their intensities were different from one another, presumably due to some differences in the interaction of the heme with nearby aromatic amino acid residue(s).

L-Tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase are protohemoproteins which catalyze the oxygenative ring cleavage of either L-tryptophan or its analogues to form a corresponding derivative of L-formylkynurenine (1, 2). L-Tryptophan 2,3-dioxygenases from both *Pseudomonas acidovorans* and rat liver have been shown to be tetrameric proteins containing 2 mol of protoheme and are specific for the L-isomer of tryptophan (3-5). On the other hand, indoleamine 2,3-dioxygenase from rabbit intestine has been shown to be a monomeric protein with 1 mol of protoheme. It has a broad substrate specificity toward a variety of indoleamine compounds including L- and D-tryptophan and serotonin (1, 6). The heme in these enzymes has been shown to be the oxygen-binding site (7, 8) and no other metallic cofactor such as copper was found associated with the three enzymes (6, 9-11).

In order to understand the reaction mechanisms of the heme-containing dioxygenases, we have undertaken magnetic and natural circular dichroism studies on the electronic structure of the heme and its vicinity in these dioxygenases.[1] In contrast to other physicochemical methods such as EPR and NMR, the magneto-optical and optical methods are applicable to dilute solutions of both ferric and ferrous hemoproteins at an ambient temperature, and thus have provided valuable information concerning the electronic structures of several other hemoproteins (12-20). The results described in this paper serve as the first description of MCD[2] and CD spectra of the heme-containing dioxygenases and permitted us to compare their ligand-binding and spin equilibrium properties with those of other types of hemoproteins. MCD and CD spectra of ferric and ferrous low spin forms of these enzymes including an oxygenated form of indoleamine 2,3-dioxygenase are described in an accompanying paper (21).

EXPERIMENTAL PROCEDURES

Enzymes—Pseudomonas and liver L-tryptophan 2,3-dioxygenases were prepared from *P. acidovorans* (ATCC 11299b) and livers of male Wistar rats previously received the combined administration of L-tryptophan and hydrocortisone, respectively (22). Indoleamine 2,3-dioxygenase was prepared from rabbit small intestine according to the method of Shimizu *et al.* (6) with the following modifications. After the step of the first Sephadex G-100 column (Step 6 in Ref. 6), the enzyme fraction was concentrated to about 1 ml by ultrafiltration (Amicon CF25) and was then applied to a column of Sepharose 4B conjugated with 5-methyl-DL-tryptophan (carboxyl group-free) which

* This investigation was supported in part by grants from the Waksmann Foundation, Japan, and the Keio University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Recipient of Grant for Scientific Research 543005 from the Ministry of Education, Science, and Culture, Japan.

[1] See Miniprint at end of accompanying paper (21).

[2] The abbreviations used are: MCD, magnetic circular dichroism; Hb, hemoglobin; Mb, myoglobin.

had been equilibrated with 10 mM potassium phosphate buffer, pH 6.0. The conjugation of Sepharose 4B was carried out by the method of Sjöholm and Ljungstedt (23). Large amounts of other contaminating proteins which were adsorbed on the column were removed by this procedure without significant loss of the enzymic activity. Additional purification steps described by Shimizu *et al.* (6) (isoelectric focusing and the second Sephadex G-100 column) were not employed because no contamination of other hemoproteins was detected in the preparations after the Sepharose chromatography.

The purified dioxygenases thus obtained were in ferric states with R_z values (an absorbance ratio of the Soret to 280 nm bands) of 1.3, 1.1, and 1.7 for the L-tryptophan 2,3-dioxygenases from *Pseudomonas* and rat liver and the indoleamine 2,3-dioxygenase, respectively. The purities of these enzymes were higher than 70% as estimated either by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or from the R_z values. Contamination of other hemoproteins such as catalase and peroxidase was negligible. The ferrous form of the enzymes was obtained by the addition of an appropriate amount of sodium dithionite to the ferric enzyme under anaerobic conditions.

Bovine liver catalase, which was used for the assay of indoleamine 2,3-dioxygenase, was purchased from Sigma Chemical Co. and dialyzed overnight at 4 °C against 5 mM potassium phosphate buffer (pH 7.0) to remove thymol.

Determinations—Activities of *Pseudomonas* and liver L-tryptophan 2,3-dioxygenases were determined by the methods previously reported (22, 24). Assay method of indoleamine 2,3-dioxygenase activity was the same as that reported by Shimizu *et al.* (6) except that the reaction was carried out at 20 °C. Under these conditions, specific activities of the enzymes (micromoles of the product formed/min/mg of protein) were 13 and 2.0 for the *Pseudomonas* and liver enzymes, respectively, and 2.2 for the indoleamine 2,3-dioxygenase. The maximum turnover numbers per heme at 20 °C were 1030, 220, and 210 min^{-1} for the *Pseudomonas* and liver enzymes, and indoleamine 2,3-dioxygenase, respectively. These values for the turnover number were comparable to or higher than those of homogeneous enzyme preparations previously reported (6, 10, 22). Protein and heme were determined as described earlier (22).

Chemicals—L-Tryptophan was purchased from Wako Pure Chemical Co. and methylene blue was from Kanto Chemical Co., Inc., Japan. All other chemicals were of analytical grade from commercial sources.

Spectral Measurements—MCD and CD spectra were recorded with a Jasco J-500C spectropolarimeter equipped with a Jasco electromagnet which produces a longitudinal magnetic field of 11.4 kG. MCD spectra were obtained by subtracting natural CD from apparent MCD with a Jasco data processor DP-500 attached to the J-500C spectropolarimeter (19). CD spectra were obtained by subtracting cell base from apparent CD with the data processor. Experimental conditions for either MCD (300 ~ 700 nm) or CD (300 ~ 500 nm) were as follows; time constant, 0.5 s; scan speed, 100 nm/min; sensitivity, 1 or 2 millidegree/cm; accumulation times, 1 ~ 16 depending on the ratio of signal to noise level of the spectral traces; temperature, 4 °C; slit width was controlled automatically to maintain a spectral band width of 2 nm. Other details are shown in appropriate figures. The intensity of MCD was expressed by the molar ellipticity per G ($[\theta]_M$: degree·cm^2/dmol/G)[3] and that of CD was expressed by the molar ellipticity ($[\theta]$: degree·cm^2/dmol)[3] on the basis of the molar concentration of the heme unit. Optical absorption spectra were recorded with either a Hitachi EPS-3T spectrophotometer or a Union Giken high sensitivity spectrophotometer SM-401. The latter was used for the measurements at both room and cryogenic temperatures. The low temperature attachment designed by Hagihara and Iizuka (25) was used to measure the absorption spectra at cryogenic temperatures as described in detail elsewhere (22).

RESULTS

MCD Spectra of Ferric Enzymes—Figs. 1 and 2 show the MCD, CD, and electronic absorption spectra of ferric *Pseudomonas* L-tryptophan 2,3-dioxygenase and rabbit intestinal indoleamine 2,3-dioxygenase at a neutral pH and 4 °C in the absence of L-tryptophan. The Soret MCD spectra of both enzymes were like the first derivatives of the optical absorption spectra, giving similar MCD parameters to those of high spin ferric myoglobin (metMb) (16). A distinct difference between the two enzymes was, however, noted in the visible region. The visible MCD spectrum of *Pseudomonas* L-tryptophan 2,3-dioxygenase had its main troughs around 546 and 639 nm, while that of indoleamine 2,3-dioxygenase showed the strongest trough at 579 nm in addition to the troughs at 544

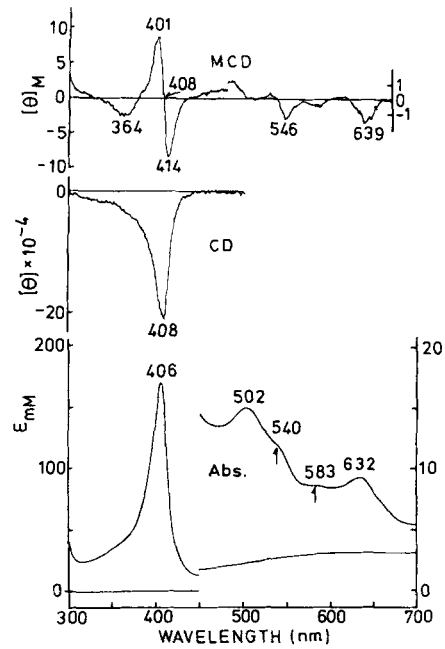

FIG. 1. **MCD, CD, and electronic absorption spectra of ferric *Pseudomonas* L-tryptophan 2,3-dioxygenase in the absence of L-tryptophan.** The enzyme concentrations employed were 7.0 and 10.9 μM in terms of protoheme concentration for the Soret and visible regions, respectively. The measurements were done at 4 °C in 0.1 M potassium phosphate buffer, pH 7.5. *Solid lines* at the bottom of the electronic absorption spectrum represent the base-lines for the Soret or the visible regions. *Abs.* denotes an electronic absorption spectrum.

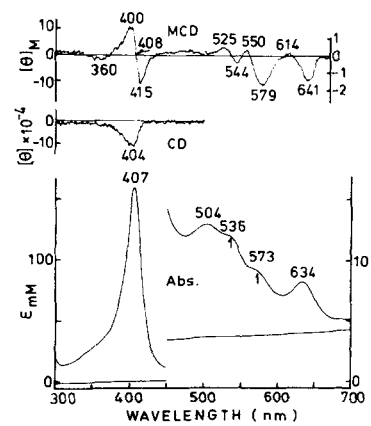

FIG. 2. **MCD, CD, and electronic absorption spectra of ferric indoleamine 2,3-dioxygenase in the absence of L-tryptophan.** The enzyme concentrations employed were 6.8 and 36.4 μM in terms of protoheme concentration for the Soret and visible regions, respectively. The measurements were done at 4 °C in 0.1 M potassium phosphate buffer, pH 7.3. *Solid lines* at the bottom of the electronic absorption spectrum (*Abs.*) represent the base-lines for the Soret or the visible regions.

[3] These units have the following relations to the other unit $\Delta\epsilon/T$ for MCD (the difference in the values for the molar extinction coefficient between that measured by the left and right polarized light per 1*T* = 10,000 G magnetic field), and $\Delta\epsilon$ for CD (the difference in the values for the molar extinction coefficient between that measured by the left and the right polarized light) (20). $[\theta]_M = 1/3 \cdot \Delta\epsilon/T$; $[\theta] = 3{,}300\ \Delta\epsilon$. The CD instrument was calibrated using the natural CD of *d*-10-Camphorsulfonic acid as a standard, with $[\theta]_{290\ nm} = 7{,}260$. The magnetic field was determined with freshly prepared potassium hexacyanoferrate (Fe (III)) with $[\theta]_{M:422\ nm} = 1.0$.

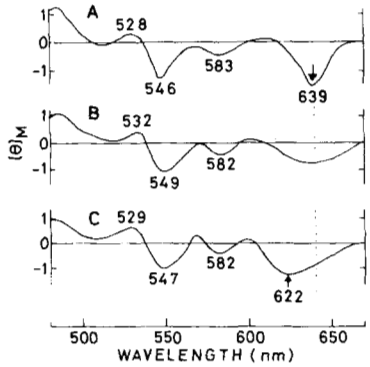

FIG. 3. **Visible MCD spectra of ferric *Pseudomonas* L-tryptophan 2,3-dioxygenase at different pH values with and without L-tryptophan.** *A*, in 0.09 M potassium phosphate buffer, pH 7.5, in the absence of L-tryptophan. *B*, in 0.06 M potassium phosphate buffer, pH 7.5, in the presence of 12 mM L-tryptophan. *C*, in 0.06 M sodium borate-boric acid buffer, pH 8.7, in the presence of 12 mM L-tryptophan. The enzyme concentrations were 10.9, 7.6, and 17.5 μM in terms of protoheme concentration for *A*, *B*, and *C*, respectively. The measurements were done at 4 °C. The *dotted line* indicates 639 nm, the trough position of the ferric enzyme at pH 7.5 in the absence of L-tryptophan.

and 641 nm. The former spectrum is typical of a high spin ferric protohemoprotein, whereas the latter suggests the presence of a low spin species in the indoleamine 2,3-dioxygenase. MCD troughs around 580 nm very similar to those described above have been reported for the low spin complexes of metMb by Vickery *et al.* (16). It should be noted that the described differences between L-tryptophan and indoleamine 2,3-dioxygenases are not clear in the electronic absorption spectra but become evident in the MCD spectra. Rat liver L-tryptophan 2,3-dioxygenase exhibited an MCD spectrum similar to that of the *Pseudomonas* enzyme, although it contained a minute amount of low spin components (see the Miniprint in the accompanying article (21)).

In the next series of experiments, effects of L-tryptophan binding on the MCD spectra were examined at various pH values.[4] In the visible region at a neutral pH, the trough intensity around 640 nm of the *Pseudomonas* enzyme decreased upon addition of L-tryptophan accompanied by a considerable broadening of the band width (Fig. 3). The changes were more marked when L-tryptophan was added at an alkaline pH, giving a distinct trough at 622 nm (Fig. 3*C*). No increase in the intensity around 583 nm was observed associated with the changes of 640 nm trough. When effects of pH alone were examined in the absence of L-tryptophan, essentially no change was observed in the spectra by raising the pH from 7.5 to 9.0. The results thus suggested the formation of a different type of high spin form upon binding of L-tryptophan. Changes in other MCD bands were also noticed but were not so remarkable as the above peak shift. In the Soret region, the MCD intensities of both peak and trough of the *Pseudomonas* enzyme decreased slightly upon L-tryptophan binding and/or raising pH. However, these spectral differences between the two high spin forms were too small to analyze the results quantitatively. In addition, the L-tryptophan-bound enzyme thus formed is known to be in multi-equilibria among its acidic and alkaline forms, and the high and low spin species of the latter form (22). Therefore, the apparent dissociation constant of L-tryptophan-ferric enzyme complex was estimated to be 3 ~ 4 mM from a separate series of experiments using electronic absorption spectra under similar conditions. These experiments were all performed at 4 °C to minimize the denaturation of the enzymes during the measurements.

With ferric indoleamine 2,3-dioxygenase in the absence of L-tryptophan, no significant change in the electronic absorption (300 to 700 nm) and MCD (300 to 700 nm) spectra was observable by changing the pH from 6.0 to 8.3. In contrast, the effects of L-tryptophan on the MCD spectra were prominent. As can be seen in Fig. 4, *A* and *B*, a trough around 580 nm, indicative of a low spin component, increased significantly upon addition of L-tryptophan to the enzyme. The effect of L-tryptophan was more remarkable at a higher pH in the sense that the high spin band at 640 nm became no more detectable (Fig. 4*C*). In the Soret MCD spectra, the intensities of the peak and trough of the ferric enzyme increased upon addition of L-tryptophan with slight red shifts of the extrema. The effects were again more pronounced at a higher pH. Such an increase in the intensity of the Soret MCD of ferric indoleamine 2,3-dioxygenase by the addition of L-tryptophan can be explained also as due to the increase in a low spin component (see "Discussion"). Under these conditions, the ratio of the low to the high spin components in the abscence of L-tryptophan at pH 7.3 was calculated to be 0.2, whereas that in the presence of a saturating amount of L-tryptophan was 3.3. Apparent dissociation constants of L-tryptophan-ferric indoleamine 2,3-dioxygenase complex were 0.71, 2.3, and not less than 8 mM at pH 8.3, 7.3, and 6.0, respectively. Methods for the evaluation as well as the significance of these constants will be described under "Discussion." MCD spectra of the ferric indoleamine 2,3-dioxygenase in the presence of a sufficient amount of L-tryptophan was similar to that of the alkaline form of horseradish peroxidase (27, 28) rather than to that of the alkaline form of metMb (16), as judged by both the positions and the intensities of these MCD extrema. Similar changes were induced by other tryptophan analogues

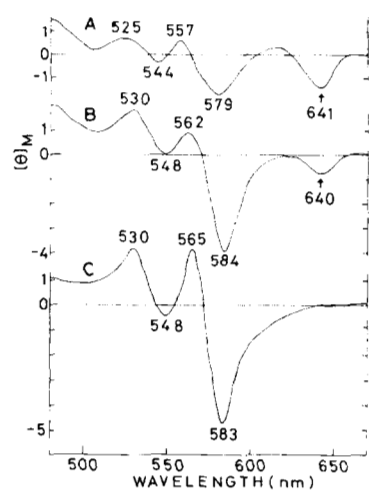

FIG. 4. **Visible MCD spectra of ferric indoleamine 2,3-dioxygenase at different pH values with and without L-tryptophan.** *A*, in 0.08 M potassium phosphate buffer, pH 7.2, in the absence of L-tryptophan. *B*, in 0.07 M potassium phosphate buffer, pH 7.2, in the presence of 3 mM L-tryptophan. *C*, in 0.1 M potassium phosphate buffer, pH 8.3, in the presence of 30 mM L-tryptophan. The enzyme concentrations employed were 36.4, 33.6, and 7.3 μM in terms of the heme for *A*, *B*, and *C*, respectively. The measurements were done at 4 °C.

[4] L-Tryptophan 2,3-dioxygenase is a tetrameric protein which has two distinct binding sites for L-tryptophan, the catalytic and the regulatory sites (26). The binding of L-tryptophan to the regulatory site is considered to facilitate the binding of the second L-tryptophan to the catalytic site which affects the electronic structure of the heme in the enzyme. For this reason, we employed saturating amounts of L-tryptophan with respect to both sites in these experiments.

TABLE I

MCD extrema of ferric L-tryptophan and indoleamine 2,3-dioxygenases and metmyoglobin complexes in the visible region

Hemoproteins (±L-tryptophan[a])	Main MCD troughs		
	nm ([θ]M)		
Pseudomonas dioxygenase			
pH 7.5 (−)	546 (−1.4)	—[b]	639 (−1.6)
pH 8.7 (+)	547 (−1.1)	—	622 (−1.3)
Rat liver dioxygenase			
pH 6.8 (−)	547 (−1.0)	—	641 (−1.8)
pH 8.8 (+)	548 (−0.3)	581 (−2.6)	623 (−1.7)
Intestinal dioxygenase			
pH 7.2 (−)	—	579 (−1.7)	641 (−1.4)
pH 8.3 (+)	—	583 (−4.7)	—
Metmyoglobin complexes[c]			
$-H_2O$ (−)	550 (−0.9)	—	646 (−1.3)
$-OH^-$ (−)	550 (−0.3)	583 (−0.4)	620 (−2.1)
$-N_3^-$ (−)	—	585 (−3.1)	643 (−0.3)
−Imidazole (−)	—	575 (−3.5)	—

[a] A saturated amount of L-tryptophan (3 ~ 30 mM) was employed. For details, see Figs. 3 and 4.

[b] —, negligible or not detectable.

[c] Data were taken from Ref. 16.

such as 5-fluorotryptamine, D-tryptophan, and 5-hydroxy-L-tryptophan.[5] Effects of L-tryptophan binding and pH on the MCD spectrum of the liver enzyme were intermediate between those on the *Pseudomonas* enzyme and indoleamine 2,3-dioxygenase (Fig. 2 in the Miniprint of the accompanying article (21)). These results were summarized and compared with the MCD characteristics of metMb derivatives in Table I.

Thermal Equilibrium between High and Low Spin States in Ferric Indoleamine 2,3-dioxygenase—As described above, results of MCD experiments indicated that the ferric form of indoleamine 2,3-dioxygenase existed as a mixture of high and low spin states irrespective of the pH values of the medium employed. Therefore, low temperature optical studies on the ferric enzyme at a neutral pH were carried out (Fig. 5). As can be seen, absorbance at either 537 or 564 nm due to a low spin component increased as temperature was lowered, with concomitant decrease in a high spin component at 631 nm. The changes were reversible within the temperature range examined. Plots of these changes in absorbance against the reciprocal of absolute temperature are shown in the *inset* of Fig. 5. Similar temperature-dependent changes in absorbance were observed in the presence of L-tryptophan and/or at a higher pH. Thus, the presence of a thermal spin equilibrium in the ferric indoleamine 2,3-dioxygenase was demonstrated. Approximate values for the standard enthalpy and entropy changes and the compensation temperature were estimated to be -1.4 ± 0.2 kcal mol^{-1}, -7.8 ± 1.5 entropy units, and 185 ± 40 *K*, respectively, from these data and the ratio of the high to low spin species at 4 °C described in the previous section.

MCD Spectra of Ferrous Enzymes—Figs. 6 and 7 show the MCD and CD spectra of ferrous *Pseudomonas* enzyme and of ferrous indoleamine 2,3-dioxygenase in the absence of L-tryptophan at a neutral pH and 4 °C. The Soret MCD spectra of the *Pseudomonas* enzyme and the indoleamine 2,3-dioxygenase showed strong positive extrema at 430 and 437 nm, respectively, and weak negative extrema around the positive peaks. The spectra of the liver enzyme were shown in Fig. 3 of the Miniprint in the accompanying article (21). These spectra were very similar to the MCD spectrum of either deoxy-Mb or deoxy-Hb but were entirely different from those

[5] K. Uchida, R. Makino, T. Iizuka, T. Shimizu, Y. Ishimura, T. Nozawa, and M. Hatano, in preparation.

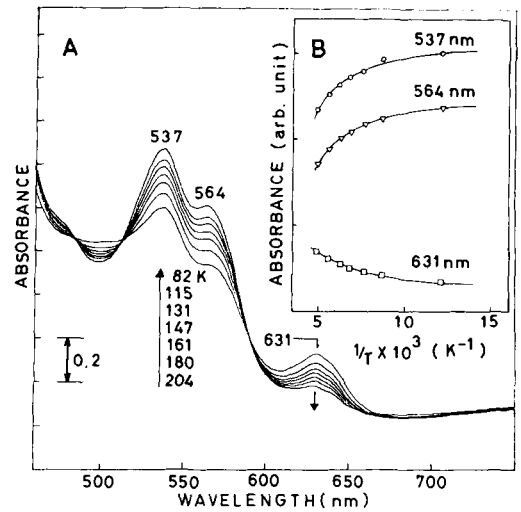

FIG. 5. **Low temperature electronic absorption spectra of ferric indoleamine 2,3-dioxygenase in the absence of L-tryptophan at various temperatures (*A*) and plots of absorbance at the peaks against reciprocal absolute temperature (*B*).** The enzyme concentration employed was 30 μM in terms of protoheme concentration, and the apparent optical path length was 2 mm. The medium used was 0.16 M Tris-HCl buffer, pH 7.2. The temperature was monitored by a thermocouple of gold-cobalt alloy *versus* copper. *arb.*, arbitrary.

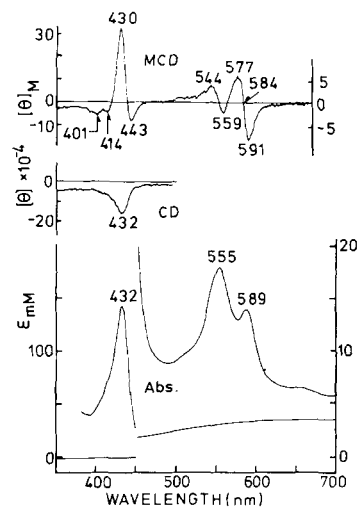

FIG. 6. **MCD, CD, and electronic absorption spectra of ferrous *Pseudomonas* L-tryptophan 2,3-dioxygenase in the absence of L-tryptophan.** The enzyme concentration employed was 5.1 μM in terms of protoheme concentration. The measurements were done at 4 °C in 0.1 M potassium phosphate buffer, pH 7.5. *Solid lines* at the bottom of the electronic absorption spectrum (*Abs.*) represent the base-lines for the Soret and the visible regions, respectively.

of low spin ferrous hemoproteins such as CO- and oxy-Mb and cytochrome b_5 (16, 29). It is suggested therefore that the heme of these enzymes was in a typical ferrous high spin state. However, some differences among the three dioxygenases were found in the visible MCD spectra. For example, a strong peak at 577 nm was found for the *Pseudomonas* enzyme, while no such a peak was seen for indoleamine 2,3-dioxygenase. The visible MCD spectrum of the ferrous *Pseudomonas* enzyme was very similar to those of deoxy-Hb (30) and ferrous horseradish peroxidase (27) rather than to that of deoxy-Mb (16). On the other hand, the spectra of both indoleamine 2,3-dioxygenase and liver enzyme were rather similar to that of deoxy-Mb.

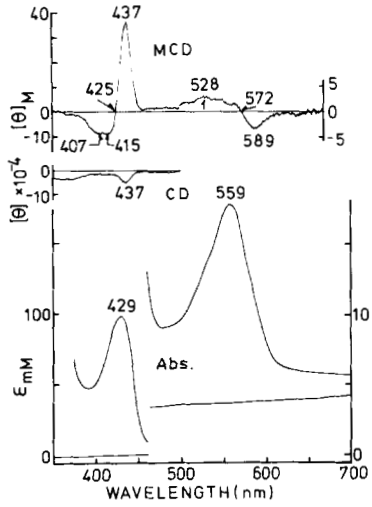

FIG. 7. **MCD, CD, and electronic absorption spectra of ferrous indoleamine 2,3-dioxygenase in the absence of L-tryptophan.** The enzyme concentration employed was 5.7 μM in terms of protoheme concentration. The measurements were done at 4 °C in 0.1 M potassium phosphate buffer, pH 7.3. *Solid lines* at the bottom of the electronic absorption spectrum (*Abs.*) represent the base-lines for the Soret and the visible regions, respectively.

TABLE II

Soret CD extrema of the dioxygenases

Hemoproteins	CD extrema (ellipticity)	
	Ferric	Ferrous
	nm ([θ] × 10⁻⁴)	
Pseudomonas dioxygenase		
pH 7.5	408 (−21)	432 (−15)
pH 9.0 + Trp[a]	408 (−13)	—[b]
Rat liver dioxygenase		
pH 7.5	409 (−12)	436 (−9)
pH 9.0 + Trp[a]	413 (−11)	—
Intestinal dioxygenase		
pH 6.0	404 (−12)	437 (−5)
pH 8.3 + Trp[a]	418 (−11)	—
Myoglobin		
Sperm whale[c]	409 (10.7)	435 (10.6)
Sperm whale[d]	408 (11.7)	437 (17.6)
Horse heart[e]	405 (9)	434 (11)
Hemoglobin (human)[e]	410 (8)	432 (13)

[a] A saturated amount of L-tryptophan (3 ~ 30 mM) was employed.
[b] —, not measurable.
[c] From Ref. 39.
[d] From Ref. 40.
[e] From Ref. 41.

Upon addition of 0.3 to 10 mM L-tryptophan, MCD spectra of these ferrous enzymes showed gradual changes in their shapes and intensities. The process was very slow and, when completed after several hours, the enzymes showed spectra attributable to a hemochrome-type low spin complex, which was no longer catalytically active. An example of such changes was shown in Fig. 4 of the Miniprint in the accompanying article.

CD Spectra of Ferric and Ferrous Enzymes—All of the three dioxygenases in both their ferric and ferrous states showed negative Cotton effects in the Soret region as shown in the corresponding figures. Among them, the intensity value of −21 ($[\theta] \times 10^{-4}$) at 408 nm for the ferric *Pseudomonas* enzyme in the absence of L-tryptophan was considerably greater than those for the other dioxygenases as well as for those of Mb and Hb (Table II). However, the addition of L-tryptophan and/or high pH caused a marked decrease in the intensity. The value for the *Pseudomonas* enzyme was −13 at pH 9.0 in the presence of a saturating amount of L-tryptophan. No change in the position of the CD trough was observed associated with the decrease in the intensity. On the other hand, the trough position of indoleamine 2,3-dioxygenase shifted to a longer wavelength either by the addition of L-tryptophan (0.1 to 10 mM) or by raising the pH from 6.0 to 8.3 in the presence of a small amount of L-tryptophan (0.05 mM). Essentially no change in the intensity was observed associated with the peak shift. From these results, it appears that the shift of CD peak position is somehow related to the spin state conversion induced by the substrate binding, whereas the changes in CD intensity reflect another kind of change in the structure such as changes in the interaction between the heme prosthetic group and nearby aromatic amino acid residue(s) from the protein moiety (31). The interaction between the heme and the added substrate, L-tryptophan, is another possible cause for the changes in the CD intensity.

Ferrous L-tryptophan 2,3-dioxygenases and indoleamine 2,3-dioxygenase showed negative CD extrema around 435 nm corresponding to their Soret optical absorption spectra. The CD intensities ($[\theta] \times 10^{-4}$) decreased in the following order; *Pseudomonas* enzyme (−15), liver enzyme (−9), and indoleamine 2,3-dioxygenase (−5). These differences in the intensity may be correlated with the differences in interaction between the heme and nearby aromatic amino acid as has been suggested above for the ferric enzymes. The effects of L-tryptophan binding to the ferrous enzymes were not precisely determined because denaturation of the enzymes occurred rather rapidly in the presence of L-tryptophan under the experimental conditions. However, a small increase in the CD intensity of ferrous indoleamine 2,3-dioxygenase was consistently observed upon addition of L-tryptophan, suggesting again the changes in the interaction between the heme and nearby aromatic amino acid residue(s).

DISCUSSION

Ferric and ferrous forms of L-tryptophan 2,3-dioxygenases and indoleamine 2,3-dioxygenase showed that MCD spectra closely resembled those of corresponding forms of myoglobin. They were clearly different from MCD spectra of the cytochrome P-450 family, a group of protoheme-containing monooxygenases (18–20, 32). The results are not surprising because the heme (protoheme IX) and its proximal (fifth) ligand (nitrogen) of the dioxygenases studied in this paper were the same as those of myoglobin (6, 33), whereas the proximal ligand of cytochrome P-450 is considered to be a thiolate anion (34). However, some delicate differences in the MCD spectra have been found among the three dioxygenases and led us to propose the following differences in their distal (sixth) ligands.

In Table I are summarized our visible MCD parameters for the ferric forms of the three dioxygenases together with the values for metMb derivatives described by Vickery *et al.* (16). As can be seen, both *Pseudomonas* and rat liver enzymes at a neutral pH showed similar MCD parameters to that of metMb-H_2O which is predominantly in a high spin ferric state at an acidic pH. Upon addition of L-tryptophan at an alkaline pH, the spectrum turned into that having a negative trough at around 620 nm which is almost the same as the spectrum of metMb-OH^-. On the other hand, the MCD spectrum of the substrate-free ferric indoleamine 2,3-dioxygenase at a neutral pH was that of a mixture of high and low spin states as judged from the simultaneous presence of two bands at 641 and 579 nm. This interpretation was in accord with the findings that the Soret MCD intensity of this enzyme ($[\theta]_{M\ \text{peak-trough}} = 22.1$) was greater than that of *Pseudomonas* (17.5) or liver (15.6) enzyme (see the figures). On the basis of magnetic suscepti-

bility and MCD measurements, Vickery *et al.* (16) have demonstrated a direct correlation between the intensity of the Soret MCD spectrum and the low spin content of a hemoprotein; the larger the amount of a low spin component, the stronger the intensities of the Soret MCD band of the ferric hemoprotein.

Several possibilities can be considered for the coexistence of low and high spin components observed in the MCD spectra of indoleamine 2,3-dioxygenase: (*a*) an equilibrium ligand exchange between a strong field ligand and a weak field ligand; (*b*) an acid-base transition; and (*c*) a thermal spin equilibrium between the high and low spin states. Among them, the ligand exchange reaction is unlikely because such reaction may not occur under the frozen conditions (Fig. 5). For the second possibility, we could not detect any transition in MCD, CD, and optical spectra by changing the pH between 6.0 and 8.3. Then, the last possibility is most likely and was actually shown to be the case by examining the temperature-dependent spectral changes in Fig. 5. Similar temperature-dependent spin equilibria have been demonstrated with both *Pseudomonas* and rat liver enzymes and their significance has been discussed in detail (Ref. 22).

Upon addition of L-tryptophan, the MCD trough around 580 nm of indoleamine 2,3-dioxygenase increased, while the trough around 640 nm decreased, indicating that the spin equilibrium shifted upon binding of L-tryptophan. The effects of L-tryptophan was more remarkable at a higher pH. To explain such multi-equilibria among the high and low spin states of both L-tryptophan-bound and -free forms of indoleamine 2,3-dioxygenase, the following scheme is postulated;

$$\begin{array}{ccc} HT & \overset{K'}{\rightleftharpoons} & LT \\ \uparrow\downarrow K_1 & & \uparrow\downarrow K_2 \\ H+T & \overset{K}{\rightleftharpoons} & L+T \end{array}$$

where H and L denote high and low spin forms of the ferric enzyme, T denotes L-tryptophan, K and K' are the spin equilibrium constants, and K_1 and K_2 are the dissociation constants as specified in the scheme. According to this scheme, the following relationships among these equilibrium constants are obtained.

$$K = \frac{[L]}{[H]} \tag{1}$$

$$K' = \frac{[LT]}{[HT]} = \frac{K \cdot K_1}{K_2} \tag{2}$$

$$K_{app} = \frac{K+1}{\frac{1}{K_1} + \frac{K}{K_2}} \tag{3}$$

where K_{app} is the apparent dissociation constant of the L-tryptophan-enzyme complex, which was 2.3 and 0.71 mM at pH 7.3 and 8.3, respectively (see "Results"). Among them, the values for K and K' are calculated from the Soret intensities of the MCD spectra by taking those of ferric L-tryptophan 2,3-dioxygenase at a neutral pH and of cyanide complex of metMb as the standards for the purely high and purely low spin forms, respectively. Then, the remaining equilibrium constants, K_1 and K_2, are easily calculated from Equations 2 and 3 with the given values for K_{app}, K, and K'.

The values for the four equilibrium constants at pH 7.3 and 4 °C were calculated to be as follows: K, 0.2; K', 3.3; K_1, 8.4 mM and K_2, 0.52 mM.[6] Both K and K' were independent of pH between 6.0 and 8.3, while the values of K_1 and K_2 decreased to 2.6 and 0.16 mM respectively, by raising the pH to 8.3. Thus, the observed effects of both L-tryptophan and pH on the spin equilibrium are well explained on the basis of these constants as due to (*a*) the shift of spin equilibrium from a high to a low spin state upon binding of L-tryptophan ($K' > K$); (*b*) a higher affinity of the low spin form for L-tryptophan than that of the high spin form ($K_1 > K_2$); and (*c*) the effects of pH on K_1 and K_2 values. These interpretations are in accordance with the previous observation of Sono *et al.* (35) who showed by spectrophotometric titration that the apparent affinity of indoleamine 2,3-dioxygenase for L-tryptophan greatly increased upon raising the pH from 6 to 8.

The above findings together with the results of low temperature spectroscopy also indicated that the ground state of both L-tryptophan-bound and free forms of indoleamine 2,3-dioxygenase is the low spin state. Further, the extremum position of the trough around 580 nm due to the low spin form was not affected either by pH or by the addition of L-tryptophan. Such findings together with the MCD characteristics shown in Table I suggest that the sixth ligand of indoleamine 2,3-dioxygenase is a strong field axial ligand such as an imidazole nitrogen in both L-tryptophan-bound and free forms. OH^- is unlikely because the low spin trough around 580 nm was pH insensitive. Thermal spin equilibrium between high and low spin states of imidazole complex of metMb has been well established (36). It is interesting to note that the binding of L-tryptophan causes the shift in spin equilibrium without exchanging the sixth ligand. On the other hand, the sixth ligand of the heme iron in L-tryptophan 2,3-dioxygenase is considered to be H_2O which is in equilibrium with OH^- on the basis of their MCD characteristics and pH sensitivity. Our previous study (22) on the acid-base transition and substrate-binding equilibria of ferric L-tryptophan 2,3-dioxygenases also supported this conclusion.

Sharonov *et al.* (30) have proposed that the visible MCD spectrum of a protohemoprotein in a ferrous high spin state is influenced by the "constraints on heme group imposed by quaternary and/or tertiary protein structure." According to them, hemoproteins could be classified into two groups: the proteins with MCD intensity ratio of the Q_{00}-transition (α-band) to Q_V-transition (β-band) greater than 2 and those with the value below 1.8. The former corresponds to a "tensed state" with a low ligand affinity as exemplified by deoxy-Hb and the latter to a "relaxed state" with a high ligand affinity such as in deoxy-Mb. In this context, the visible MCD patterns of ferrous L-tryptophan 2,3-dioxygenases in the L-tryptophan-free states (2.2 ~ 2.6) were those of the former class, *i.e.* the tensed state, and that of ferrous indoleamine 2,3-dioxygenase (no two discernible transitions) could be one of the latter class which represents the relaxed state. Such an interpretation may be consistent with the facts that both L-tryptophan 2,3-dioxygenases are tetrameric whereas indoleamine 2,3-dioxygenase is a monomeric protein (1, 2, 6) and that *Pseudomonas* L-tryptophan 2,3-dioxygenase in the absence of L-tryptophan has a lower ligand affinity than that bound with L-tryptophan (33, 37). Significance of the ratio of intensities of the α- to β-bands on the electronic structure of a hemoprotein was also pointed out by Wang and Brinigar (38).

Although the heme itself is optically inactive, CD spectra of the heme in proteins have been extensively studied in the past (17). Hsu and Woody (31) have demonstrated that the

[6] When the cyanide form of indoleamine 2,3-dioxygenase in the absence of L-tryptophan (21) was taken as the standard for the pure low spin state, the following values were obtained for the equilibrium constants: K, 0.13; K', 1.13; K_1, 2.6 mM; K_2, 0.30 mM. Although the values were somewhat smaller than those shown in the text, the relationships among them ($K < K'$, $K_1 > K_2$) were found to be unchanged.

origin of CD spectra in myoglobin and hemoglobin is "a coupled oscillator interaction between the heme transitions and allowed π-π^* transitions in nearby aromatic side chains." CD spectra of hemoproteins thus reflect the steric relationship between the heme and aromatic residues in the heme pocket. Therefore, the observed differences and the changes in CD spectra of these dioxygenases could be related to the differences or changes in the interaction between the heme and nearby aromatic residue(s) of these hemoproteins. It was not possible, however, to identify the aromatic residues involved in the interaction because no information is available on the protein structures of these enzymes at present. MCD and CD studies on the ferric and ferrous low spin complexes of these enzymes are described in the accompanying paper (21).

Acknowledgments—We are greatly indebted to Dr. Kazuo Kuratsuka and his associates, National Institute of Health, Japan, for their kind supply of fresh rabbit intestine. Skillful technical assistances of Y. Tanizaki and R. Kobayashi are also acknowledged.

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