**Indoleamine 2,3-Dioxygenase**

**EQUILIBRIUM STUDIES OF THE Tryptophan BINDING TO THE FERRIC, FERROUS, AND CO-BOUND ENZYMES**

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The equilibrium constants for the tryptophan binding to indoleamine 2,3-dioxygenase, a protohemoprotein, in its ferric (Fe³⁺), ferrous (Fe²⁺), and CO-bound (Fe³⁺-CO) forms were determined by the spectrophotometric titration method. All these enzyme derivatives showed marked spectral changes upon tryptophan binding. Such a spectral change for the ferric enzyme was, however, specific to the l isomer and accompanied the spin state change of the ferric heme from high spin to low spin. The dissociation constants ($K_d$) thus obtained for the complexes of L-tryptophan with the ferric, CO-bound, and ferrous enzymes in 0.1 M potassium phosphate buffer, pH 7.0 at 24°C, were 5.8 mm, 350 μM, and 13 μM, respectively, being in a ratio of 450:27:1. The $K_d$ for the ferric enzyme was about 450 times greater than the $K_m$ and agreed well with the substrate inhibition constant ($K_i$), indicating that l-tryptophan binding to the ferric enzyme is not catalytically significant, but is directly correlated to the substrate inhibition which is stereospecific to the l isomer. The $K_d$ for the ferrous enzyme showed a good agreement with the $K_m$ at various pH values examined. Assuming that the CO-bound enzyme (Fe³⁺-CO) has a tryptophan-binding property similar to the oxygenated enzyme (Fe³⁺-O⁡₂ or Fe²⁺-O⁡₂), it is suggested that the binding of tryptophan to the ferrous enzyme ($K_d = K_m$) rather than that of tryptophan to the oxygenated enzyme ($K_d ≠ K_m$) is involved in a steady state of the catalytic reaction. Available evidence described above provides further support to the following reaction mechanism, in the principal pathway, the ferrous enzyme binds predominantly with tryptophan, followed by molecular oxygen. The ferric enzyme, on the other hand, binds first with the superoxide anion and the resulting oxygenated enzyme reacts with tryptophan either directly or via the ferrous enzyme to form the ternary complex. The ferric enzyme-tryptophan complex appears catalytically inactive such as 5-hydroxy-D- and L-tryptophan, tryptamine, and serotonin (6-8). The tissue distribution in mammals (2, 3) and the molecular properties (3, 8) of the enzyme have already been reported from this laboratory.

Previous studies from this laboratory on the reaction mechanism of indoleamine 2,3-dioxygenase showed that the enzyme was noticeably autoxidizable (1, 7) and, during the catalytic reaction using O₂, it was converted to the ferric enzyme which was catalytically inactive unless O₂⁻ and methylene blue were supplied. Therefore, both O₂ and O₂⁻ could serve as substrate (1, 7, 9), i.e. as oxygen sources, both of which could bind to the ferrous and ferric enzymes, respectively, with the second order rate constants being in the same order of magnitude ($10^8$ M⁻¹ s⁻¹ at pH 8.0 and 24°C) (1).

The other oxygenases such as tryptophan 2,3-dioxygenase (heme-containing dioxygenase) (10), metapyrocatechase (non-heme iron-containing dioxygenase) (11), and cytochrome P-450 (heme-containing monoxygenase) (12-14) all have a common property that the organic substrates bind to the enzyme before O₂. In contrast, tryptophan-free indoleamine 2,3-dioxygenase in a ferrous form could bind to O₂ to form a relatively stable oxygenated enzyme (7). This oxygenated enzyme was catalytically active because the addition of tryptophan to it resulted in the formation of the product, N-formylkynurenine (1, 7). Therefore, it is necessary for elucidating the reaction mechanism to determine whether tryptophan and O₂ bind to the ferrous enzyme in an ordered way or at random in a steady state of the catalytic reaction.

Another novel catalytic property of indoleamine 2,3-dioxygenase is the substrate inhibition which has been reported to be specific to the l isomer of tryptophan (4, 5, 8) and 5-hydroxytryptophan (6, 8). The mechanism of the substrate inhibition has not been understood well.

Tryptophan-binding properties of the enzyme, however, have not been examined yet, except for the earlier observation by Yamamoto and Hayaishi (5) that the addition of L-tryptophan to the enzyme solution at pH 7.5 caused a marked red shift and decrease in absorbance of the Soret peak of the ferric enzyme. This optical change was reported to be specific to the l isomer of tryptophan (5).

In order to understand the organic substrate-binding properties of indoleamine 2,3-dioxygenase, we have performed the spectrophotometric titrations of the enzyme with tryptophan in the present experiment and determined the equilibrium constants for tryptophan binding to the ferric, ferrous, and CO-bound enzymes. The results offer a useful clue to the understanding of the mechanism of the substrate inhibition and of the catalytic reaction sequence.

**EXPERIMENTAL PROCEDURES**

Materials—L-Tryptophan, d-tryptophan, 5-hydroxy-L-tryptophan, 5-hydroxy-d-tryptophan, tryptamine HCl, serotonin creatinine sulfate α-methyl-DL-tryptophan, indole, and indoleacetic acid were purchased from Sigma. To remove possible contamination by...
Tryptophan Binding to Indoleamine 2,3-Dioxygenase

the L isomer in the D-tryptophan sample, the commercial product of D-tryptophan was recrystallized twice from methanol. The biological assay showed that the contamination by L-tryptophan in the D isomer (about 1% in commercial products) was less than 0.1% after recrystallization.

Preparation of Indoleamine 2,3-Dioxygenase—Indoleamine 2,3-dioxygenase was prepared from rabbit small intestine by the method of Shimizu et al. (8) through the Step 6 (Sephadex G-100). The ratio of the absorbance at 406 nm to that at 280 nm at pH 6.0 was between 1.5 and 1.8 and the turnover number for L-tryptophan at pH 8.0 and 24°C was around 1.8 s⁻¹ on the basis of the enzyme concentration experiments with its hemin content. The molar extinction coefficients (εₘ) of the absorbance peaks of the enzyme in various forms were determined spectrophotometrically using the εₘ value of 33.4 m⁻¹ cm⁻¹ (16) at 557 nm for the α-peak of the reduced pyridine hemochromogen.

Assay of Indoleamine 2,3-Dioxygenase—The Kₑₐ for tryptophan and the substrate inhibition constant (Kₛ) were determined under normal atmospheric oxygen partial pressure (approximately 220 μM O₂ in buffer media at 24°C) using the following assay method essentially the same as that described by Shimizu et al. (8). The assay mixture contained in a total volume of 1.0 ml, 100 mM potassium phosphate buffer, 0.025 mM methylene blue, 10 mM ascorbic acid, 250 μg of catalase, various concentrations of L- or D-tryptophan, and enzyme. The increase in absorbance at 321 nm of the assay mixture due to the formation of N-formylkynurenine was continuously followed at 24°C. Since L-tryptophan showed marked substrate inhibition at concentrations over 0.2 mM, Kₛ was determined from the Lineweaver-Burk plot in the low L-tryptophan concentration range where no substrate inhibition was observed.

Titrations of Indoleamine 2,3-Dioxygenase in Various Forms with Tryptophan—The ferrous enzyme sample was prepared by diluting the enzyme stock solution (100 to 200 μM) in a buffer medium. For the preparations of the ferrous and CO-bound enzyme samples, the enzyme stock solution was diluted in buffer media previously bubbled with nitrogen and carbon monoxide, respectively, followed by the addition of a small amount of dithionite to reduce the ferric enzyme. The titration of the enzyme with tryptophan was made in 600 μl of a medium by measuring the absorbance changes of the enzyme upon successive additions of tryptophan. For the titrations of the ferrous and CO-bound enzymes, the cuvette was sealed tightly with parafilm in order to prevent the entering of air into the sample solution and a gas-tight microsyringe was used for the additions of the tryptophan stock solution, and Kₑₐ was determined from the Lineweaver-Burk plot in the low L-tryptophan concentration range where no substrate inhibition was observed.

Equation 2 indicates that a plot of log AA against log [Trp] should yield a straight line with the slope of Kₑₐ/ΔAₐ and the intercept of 1/ΔAₑₐ can be then calculated as (slope)/(intercept). The Hill plots of the tryptophan binding to the various enzyme derivatives are also presented (see below) according to the Equation 3 derived from Equation 1.

\[ \log \frac{\Delta A}{\Delta A_e} = \log[Trp] - \log K_e \]  

Equation 3 indicates that a plot of log ΔA/ΔAₑₐ against log[Trp] should yield a straight line with a slope of unity. Kₑₐ is then equal to the concentration of free tryptophan where ΔA = ΔAₑₐ/2, i.e. 50% of the enzyme is bound by tryptophan. RESULTS

Absorption Spectral Change of Enzyme upon Tryptophan Binding

As shown in Fig. 1, marked spectral changes were observed upon additions of L-tryptophan to the ferric (Fig. 1A), ferrous and CO-bound (Fig. 1B) enzymes. The absorption maxima and their extinction coefficients of the above three enzyme derivatives in the tryptophan-free and-tryptophan-bound forms are summarized in Table I.

![Fig. 1. Optical spectral change of (A) ferric (Fe⁴⁺), (B) ferrous (Fe²⁺), and CO-bound (Fe⁴⁺CO) enzymes upon binding of L-tryptophan. All measurements were made in 0.1 M potassium phosphate, pH 8.0, at 24°C. The enzyme concentration was 4.5 μM for all cases. L-Tryptophan was added to the final concentrations of 3.6, 0.2, and 3.0 mM for the ferric, ferrous, and CO-bound enzymes, respectively, to form the corresponding complexes with L-tryptophan. (A) tryptophan-free (---) and tryptophan-bound (-----) ferric enzymes. (B) tryptophan-free (-----) and tryptophan-bound (-----) ferrous enzymes and tryptophan-free (-----) and tryptophan-bound (-----) CO-bound enzymes. See "Experimental Procedures" and Footnote f in Table I for sample preparations.](http://www.jbc.org/content/early/1966/109/1340/F1.large.jpg)
TABLE I

<table>
<thead>
<tr>
<th>Derivative</th>
<th>pH</th>
<th>Soret (nm)</th>
<th>Visible (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe³⁺</td>
<td>8.0</td>
<td>568(2.9)</td>
<td>539(8.97)</td>
</tr>
<tr>
<td>Trp-Fe³⁺</td>
<td>8.0</td>
<td>540(10.4)</td>
<td>530(9.62)</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>6.0-8.0</td>
<td>530(8.97)</td>
<td>558(13.7)</td>
</tr>
<tr>
<td>Trp-Fe²⁺</td>
<td>8.0</td>
<td>520(8.97)</td>
<td>558(12.6)</td>
</tr>
<tr>
<td>Fe³⁺CO</td>
<td>6.0-8.0</td>
<td>520(8.97)</td>
<td>539(14.7)</td>
</tr>
<tr>
<td>Trp-Fe³⁺CO</td>
<td>6.0-8.0</td>
<td>530(14.7)</td>
<td>570(15.6)</td>
</tr>
</tbody>
</table>

* Wavelength (λ) is given in nanometers: the millimolar extinction coefficients (ε) are given in mm⁻¹ cm⁻¹ and are indicated in parentheses. All values were obtained at 24°C in 0.1 M potassium phosphate.

** Spectrum was pH-dependent (see text).

*** L-Tryptophan-enzyme complex.

** Shoulder.

* The ε value was slightly greater at lower pH.

When CO was bubbled through the ferrous enzyme solution, slightly smaller ε values for the CO-bound enzyme (ε₅₃₈ ~ 190 at pH 6.0) were obtained especially at higher pH values (ε₅₃₈ ~ 170 at pH 8.0). Because of this reason, the CO-enzyme complex was prepared by bubbling CO through the ferrous enzyme solution, followed by the addition of a small amount of sodium dithionite. Identical and the greatest ε values were obtained by this method.

**Ferrous Enzyme**

Absorption spectrum of the ferrous enzyme was dependent on pH and this was attributed to the acid-alkaline transition spectral change (pK' = 8.10 in the absence of tryptophan at 24°C), just as observed for the ferric hemoglobin or myoglobin. Details of such spectral analysis will be published elsewhere. The high spin type absorption spectrum (solid line in Fig. 1A) of the tryptophan-free enzyme shifted to the almost complete low spin type spectrum (broken line in Fig. 1A) upon L-tryptophan binding to the ferrous enzyme (see also Fig. 2). Among the other indoleamine derivatives examined, only 5-hydroxy-L-tryptophan induced such a marked spectral change. D-Tryptophan showed a very small effect, less than 1% of that of the L isomer in the same concentration. Other indole compounds which serve as substrates such as 5-hydroxy- and a-methyl-DL-tryptophan caused no significant spectral changes. D-Tryptophan showed a very small effect, less than 1% of that of the L isomer in the same concentration. Other indole compounds which serve as substrates such as 5-hydroxy- and a-methyl-DL-tryptophan caused no significant spectral changes.

**Ferric Enzyme**

Absorption spectrum of the ferric enzyme was dependent on pH and this was attributed to the acid-alkaline transition spectral change (pK' = 8.10 in the absence of tryptophan at 24°C), just as observed for the ferric hemoglobin or myoglobin. Details of such spectral analysis will be published elsewhere. The high spin type absorption spectrum (solid line in Fig. 1A) of the tryptophan-free enzyme shifted to the almost complete low spin type spectrum (broken line in Fig. 1A) upon L-tryptophan binding to the ferrous enzyme (see also Fig. 2). Among the other indoleamine derivatives examined, only 5-hydroxy-L-tryptophan induced such a marked spectral change. D-Tryptophan showed a very small effect, less than 1% of that of the L isomer in the same concentration. Other indole compounds which serve as substrates such as 5-hydroxy- and a-methyl-DL-tryptophan caused no significant spectral changes. D-Tryptophan showed a very small effect, less than 1% of that of the L isomer in the same concentration. Other indole compounds which serve as substrates such as 5-hydroxy- and a-methyl-DL-tryptophan caused no significant spectral changes.

**Ferrous Enzyme**

Substrate-specific Spectral Change—The ferrous enzyme exhibited typical high spin absorption spectrum in both tryptophan-free (solid line in Fig. 1B) and tryptophan-bound (broken line in Fig. 1B) forms. This result indicates that the sixth coordination position of the ferrous heme of the enzyme is still vacant in the tryptophan-bound form, i.e., the added tryptophan or other certain base ligand is not bound to the ferrous heme even in the absence of another substrate, O₂ which should occupy the above vacant position of the heme in a catalytic reaction. On addition of L-tryptophan (0.2 mM) to the ferrous enzyme, a slight decrease in absorbance of the peaks at both the Soret (429 nm) and the visible (558 nm) regions of the tryptophan-free enzyme occurred without significant changes in its spectral form. Similar spectral changes were also observed by d-tryptophan, 5-hydroxy-D- and L-tryptophan, tryptamine, and serotonin which are all known to be the substrates of the enzyme. However, as described below, a secondary spectral change was also caused on further additions of tryptophan.

**Substrate-nonspecific Spectral Change—**A secondary spectral change (Spectra I and II in Fig. 3A) was induced when L-tryptophan concentration exceeded about 0.5 mM. Similar spectral changes were also observed not only by some other substrates such as d-tryptophan, tryptamine but also by indole and indoleacetic acid which do not serve as substrates. On the other hand, the other substrates such as 5-hydroxy-D- and L-tryptophan and serotonin, having a hydroxyl group at position 5 of the indole ring in common, did not cause such a secondary spectral change. The rate of the secondary spectral change was stimulated by stirring of the titration mixture. When more L-tryptophan was added to the solution containing 0.2 mM L-tryptophan, a CO-bound enzyme could hardly be formed whereas an immediate spectral change was observed due to the formation of the CO-bound enzyme (peak at 418 nm) upon CO bubbling through the "normally" tryptophan-bound enzyme solution containing 0.2 mM L-tryptophan (peak at 427 nm). This result strongly indicates that the sixth coordination position of the heme of the ferrous enzyme, where CO could bind, is occupied by a certain ligand. (b) The spectral change from high spin to low type spectrum observed for the ferrous enzyme in the visible region, i.e., appearance of the α (558 nm) and β (533 nm) peaks due to the secondary spectral change to the ferrous enzyme, also indicates that a certain ligand such as nitrogen base is bound to the heme.

(c) The secondary spectral change exhibited the isosbestic points (416 and 435 nm, the crossing points of Spectrum I and II in Fig. 3A) with normally tryptophan-bound enzyme, indicating that the initially bound tryptophan does not dissociate from the tryptophan-enzyme complex during the secondary tryptophan binding.

The secondary tryptophan binding to the ferrous enzyme may not occur in a steady state of the catalytic reaction and may be an artificial tryptophan binding under a completely anaerobic condition in the absence of any ligand which binds to the heme because CO-bound enzyme did not show such secondary tryptophan binding (see below). However, as a practical problem, the secondary spectral change on additional indoleamine binding to the ferrous enzyme interfered the titrations of the ferrous enzyme with d-tryptophan and tryptamine (see below for titrations).

**CO-bound Enzyme**

On L-tryptophan binding to the CO-bound enzyme, decrease in absorbance at the α (570 nm), β (539 nm), and Soret (420 nm) peaks and small optical blue shifts (about 2 nm) were observed as indicated by the chain (tryptophan-free) and
dotted (tryptophan-bound) lines in Fig. 1B. Such spectral changes were also detected for other indoleamine derivatives including D-tryptophan, 5-hydroxy-D and L-tryptophan, serotonin, and tryptamine. The effects of these substrates were, however, smaller than that of L-tryptophan. No secondary spectral change was observed in the case of CO-bound enzyme even when L-tryptophan concentration was increased to 10 mM.

Spectrophotometric Titrations of Enzyme in Ferric, Ferrous, and CO-bound Forms with Tryptophan

A spectral change with one set of isosbestic points was observed for the titration of each enzyme derivative in both the Soret and the visible regions upon additions of tryptophan, indicating that there is only one form of tryptophan-enzyme complex for each derivative. As an exception, the secondary tryptophan binding was observed in the case of the ferrous enzyme as described above. Identical results (Kd values) were obtained from the titrations in both the Soret and the visible regions.

Ferric Enzyme

Fig. 2 shows the optical absorption changes of the ferric enzyme on successive additions of L-tryptophan. One set of isosbestic points is observed for the spectra in both the visible and Soret regions. Due to the reasons described under “Experimental Procedures,” the final spectrum for the complete formation of the tryptophan-enzyme complex could not be obtained. In order to know the maximum absorbance change (ΔA1) at 406 nm, a peak position of the tryptophan-free ferric enzyme, a double reciprocal plot is made in Fig. 2, inset, according to “Data Analysis.” A straight line with a slope of 1.28 mm and an intercept of 2.50 were obtained, indicating that L-tryptophan binds to the ferric enzyme in a molar ratio of 1:1 with the dissociation constant of 1.28/2.5 = 0.51 (mm). Similar results were obtained for 5-hydroxy-L-tryptophan binding to the ferric enzyme. The dissociation constants of the ferric enzyme for L-tryptophan and 5-hydroxy-L-tryptophan at pH 8.0 and 24°C were thus determined to be 0.51 mm and 4.3 mm, respectively. The dissociation constant for D-tryptophan under the same conditions was more than 30 mm.

Ferrous Enzyme

L-Tryptophan titration spectra of the ferrous enzyme in the Soret band at pH 7.0 and 24°C are shown in Fig. 3A. The corresponding absorption change was also seen in the visible region (not indicated). Under the experimental conditions employed for the result of Fig. 3A, the addition of 0.2 mM L-tryptophan induced almost complete spectral change for the formation of the tryptophan-enzyme complex. L-Tryptophan also binds to the ferrous enzyme in a molar ratio of 1:1 with the dissociation constant of 13 μM at 7.0 and 24°C. The dissociation constant for 5-hydroxy-L-tryptophan under the same conditions was 63 μM.

CO-bound Enzyme

A typical titration curve for L-tryptophan binding to the CO-bound enzyme is shown in Fig. 3B. One mole of L-tryptophan binds to 1 mol of the CO-bound enzyme as judged from the straight line in Fig. 3B, inset. The dissociation constant at pH 7.0 and 24°C for L-tryptophan was 350 μM. This value is 1 order of magnitude greater than that for L-tryptophan binding to the ferric enzyme (Kd = 13 μM) under the same experimental conditions. The dissociation constants for the other indoleamine derivatives were between 1 and 10 mM for D-tryptophan, 5-hydroxy-D- and L-tryptophan, and that for serotonin was especially greater (more than 10 mM).

Hill Plots and pH Profile of Tryptophan Binding to the Ferric, Ferrous, and CO-bound Enzyme

According to Equation 3 under “Data Analysis,” the Hill plots of the formation of the L-tryptophan-enzyme complexes for the (A) ferric, (B) ferrous, and (C) CO-bound enzymes at various pH values are shown in Fig. 4. In all cases, straight lines with a slope of unity were obtained, indicating the absence of cooperativity in tryptophan binding to the enzyme in various forms. Fig. 5 shows the pH profile of the dissociation constants for the tryptophan-enzyme complexes. From Figs. 4 and 5, the following conclusions may emerge. (a) The affinities of the ferric and ferrous enzymes for L-tryptophan are sensitive to the pH change and increase with the increase in pH, suggesting possible involvement of a certain ionizable amino acid residue of the enzyme protein. The sensitivity of the constants to the pH change is much greater for the ferric enzyme than the ferrous enzyme. For instance, the ratios of the constants at pH 8.0 to those at pH 6.0 are about 60 and 13 for the ferric and the ferrous enzymes, respectively (see also

**Fig. 2.** Spectral change of the ferric enzyme on titration with L-tryptophan. Titration was performed in 0.1 M potassium phosphate, pH 8.0, at 24°C. The concentration of the enzyme was 9.9 μM. The arrows indicate the decrease (↓) or increase (↑) in absorbance of each absorption peak on successive additions of L-tryptophan (0 to 1.28 mM). A double reciprocal plot, 1/ΔA versus 1/[L-Trp], is shown in inset. Detailed procedures are described in the text.

**Fig. 3.** Spectral changes of the (A) ferrous and (B) CO-bound enzymes on titrations with L-tryptophan. The measurements were carried out in 0.1 M potassium phosphate buffer, pH 7.0, at 24°C. The concentrations of the ferrous and CO-bound enzymes were 3.5 and 4.5 μM, respectively. Double reciprocal plots for each titration, 1/ΔA versus 1/[L-Trp], are shown in the insets of A and B. In both titrations for the ferrous and CO-bound enzymes, decrease in absorbance of the Soret peaks (429 and 420 nm, respectively) was observed on L-tryptophan binding. Note the absence wavelength scale for the CO-bound enzyme (B) is expanded twice as that for the ferrous enzyme (A) for a clearer presentation. Spectra I and II for the ferrous enzyme in A are due to the secondary additional binding of L-tryptophan to the enzyme. See the text for detail.
bound enzymes.

enzyme derivatives
zyme
pH 6.0
being in a ratio of 446:27:1. This result indicates that the L-tryptophanoenzyme
ferric
Table
phosphate, at 24°C. The ordinate represents the logarithm of the
affinity of the enzyme for L-tryptophan is distinctly altered
ferrous enzyme. At pH
phan increases in the order of the ferric, CO-bound, and
tryptophan is independent of pH and the dissociation constant
stays at 350 p~. (c) The affinities of the enzyme for L-trypto-
molar dissociation constants.

dependning on the valence state and liganded form of the heme,
complexes of L-tryptophan and these enzyme derivatives are
FIG. 4.
The Hill plots of the formation of t-tryptophan-enzyme complexes for the (A) ferric, (B) ferrous, and (C) CO-bound enzymes. All data are obtained in 0.1 M potassium phosphate, pH 6.0 (C), 7.0 (A), and 8.0 (O), at 24°C. The abscissa for each of all enzyme derivatives is logarithmic presentation of the enzyme-free L-tryptophan concentration.

FIG. 5. The pH profile of the dissociation constants (Kd) of the t-tryptophan-enzyme (E) complexes. The results are for the ferric (O), CO-bound (L), and ferrous (A) enzymes in 0.1 M potassium phosphate, at 24°C. The ordinate represents the logarithm of the molar dissociation constants.

Table II). (b) The affinity of the CO-bound enzyme for L-tryptophan is independent of pH and the dissociation constant stays at 350 m~. (c) The affinities of the enzyme for L-tryptophan increases in the order of the ferric, CO-bound, and ferrous enzyme. At pH 7.0, the dissociation constants for the complexes of L-tryptophan and these enzyme derivatives are 5.8 m~, 350 m~, and 13 m~ (see also Table II), respectively, being in a ratio of 446:27:1. This result indicates that the affinity of the enzyme for L-tryptophan is distinctly altered depending on the valence state and liganded form of the heme,
suggesting marked conformational differences in the ferric, ferrous, and CO-bound enzymes, especially in the vicinity of the tryptophan binding site.

Substrate Inhibition by L-Tryptophan

The effects of tryptophan concentration on the catalytic activity of indoleamine 2,3-dioxygenase were examined in detail at various pH values in the present experiment. The result for L-tryptophan is shown in Fig. 6. The degree of the substrate inhibition was pH-dependent and increased as the pH is raised. Defining the tryptophan concentration which inhibits the half-maximum enzyme activity as the "substrate inhibition constant, Ks," the Ks values of 30, 4.2, and 0.40 mM at pH values 6.0, 7.0, and 8.0, respectively, were obtained for L-tryptophan (Fig. 6). As will be mentioned under "Discussion," these Ks values were quite close to the dissociation constants for the complexes of L-tryptophan and the ferric enzyme at respective pH values. Small substrate inhibition, less than 1% as compared with L-tryptophan, was observed for D-tryptophan, indicating that the substrate inhibition is quite stereospecific to the L isomer.

DISCUSSION

Previous studies from this laboratory on the reaction mechanism of indoleamine 2,3-dioxygenase (1, 5, 7, 17) have suggested that all of the ferric (Fe3+), ferrous (Fe2+), and oxygenated (Fe3+O2 or Fe2+O2-) enzymes were involved in a steady state of the catalytic reaction. This was based on the following observations that (a) both CO and KCN which are ligands tightly binding to the ferrous and ferric hemes of some hemoproteins, respectively, inhibited the reaction (5, 17) when added at a steady state, and (b) the oxygenated enzyme produced the corresponding products on additions of various substrates (7).

The present study of the spectrophotometric measurements of tryptophan binding to the enzyme in various valence states and liganded forms shows that all of the ferric, ferrous, and CO-bound enzymes undergo spectral changes upon L-tryptophan binding. This is the first analysis of the effects of tryptophan binding on the spectral property of the enzyme except for such a spectral change of the ferric enzyme observed earlier by Yamamoto and Hayaishi (5). Since the oxygenated enzyme which is a possible intermediate in a catalytic reaction, is unstable, especially in the presence of tryptophan (7), and is rapidly converted to the ferric enzyme by autoxidation (7), the precise determination of its absorption spectra in the tryptophan-free and tryptophan-bound forms and of the equilibrium constant for the tryptophan binding to the oxygenated enzyme was difficult by the ordinary titration technique em-
Tryptophan Binding to Indoleamine 2,3-Dioxygenase

employed in the present work. Therefore as a substitution of the oxygenated enzyme, a stable CO-bound enzyme was used. The CO-bound enzyme is considered to be a proper substitution of the oxygenated enzyme because CO is a ligand very similar to O₂ in size of the molecule and both ligands bind to the ferrous heme of various hemoproteins. The CO-bound hemoglobin (or myoglobin) was also demonstrated to have a similar structure to that of the oxygenated derivative by the x-ray analyses of the difference in their crystalline structure (18) or by the measurements of the optical rotatory dispersion (19).

Affinities of Various Enzyme Derivatives for Tryptophan—
Taking advantage of the spectral changes of the enzyme observed upon additions of tryptophan, we made the titrations of various enzyme derivatives with L-tryptophan and were able to determine the precise dissociation constants (Kₐ) for their complexes with L-tryptophan. The number of the tryptophan binding site was assumed to be one for each enzyme derivative except for the secondary binding site (heme moiety) of the ferrous enzyme. This assumption is based on the following fact that no co-operativity was observed for the tryptophan binding to any of the three enzyme derivatives as judged from the slope of the Hill plots (n = 1.0). The dissociation constants thus obtained for the ferrous, CO-bound, and ferric enzymes at pH values 6.0, 7.0, and 8.0 at 24°C are summarized in Table II together with the Kₐ values for the catalytic reaction and the Kᵦ values for the substrate inhibition. First, a good agreement of the Kᵦ for the ferrous enzyme with the Kᵦ, both being in the order of micromolar is noticeable. This agreement of both values may not be accidental because such agreement is seen at various pH values examined. Second, the Kᵦ for the CO-bound enzyme (350 μM) was pH-independent and was one to 2 orders of magnitude greater than that for the ferrous enzyme. This result indicates that the ligand (such as CO) binding to the ferrous heme of the enzyme markedly weakens the affinity of the enzyme for L-tryptophan. This may be either due to a possible conformational change of the ferrous enzyme upon ligand binding to the heme just as seen in the distinct conformational difference between the ferrous (deoxy) and oxygenated (or CO-bound) hemoglobin (20, 21) or myoglobin (29) or due to a possible interaction between the enzyme-bound tryptophan and the heme-bound ligand (CO). Third, the dissociation constants for the ferric enzyme, being in the order of millimolar, is markedly greater than that for the ferrous enzyme, being in the order of micromolar. In other words, the affinity of the ferric enzyme for L-tryptophan is 2 to 3 orders of magnitude lower than that for the ferrous enzyme. The ratios of the Kᵦ for the ferric enzyme to that for the ferrous enzyme at pH values 6.0, 7.0, and 8.0 are about 670, 450, and 90, respectively.

Tryptophan Binding to the Ferric Enzyme and Substrate Inhibition—It should be stressed from the result of Table II that the Kᵦ for the ferric enzyme is quite close to the Kᵦ for the substrate inhibition at all pH values examined. This result strongly suggests that tryptophan binding to the ferric enzyme is not essential and rather inhibitory for the catalytic reaction. For instance, while 0.2 mM L-tryptophan is enough to gain the maximum enzyme activity at pH 7.0 (100% in Fig. 6), almost all of the ferric enzyme present (more than 97%) is considered to be in the tryptophan-free form. This suggestion is also supported by the fact that D-tryptophan can serve as substrate, exhibiting the maximum enzyme activity to similar extent as L-tryptophan (8), without causing any spectral change due to the binding to the ferric enzyme. The above discussion for the ferric enzyme is, however, made on the basis of an assumption that no tryptophan binding occurs without inducing any spectral change of the enzyme. Such a possibility of tryptophan binding to the ferric enzyme without spectral change can not be completely ruled out at the present time. Our preliminary measurements of the circular dichroism (CD) spectra of indoleamine 2,3-dioxygenase exhibited no CD spectral change in 250 to 700 nm for the ferric enzyme on addition of 0.2 mM L-tryptophan at pH 6.6. In contrast, a marked CD spectral change was observed for the ferrous enzyme by the same treatment. These CD spectral observations again support the speculation made based on the spectrophotometric observations.

Affinities of Various Enzyme Derivatives for Tryptophan—Taking advantage of the spectral changes of the enzyme observed upon additions of tryptophan, we made the titrations of various enzyme derivatives with L-tryptophan and were able to determine the precise dissociation constants (Kₐ) for their complexes with L-tryptophan. The number of the tryptophan binding site was assumed to be one for each enzyme derivative except for the secondary binding site (heme moiety) of the ferrous enzyme. This assumption is based on the following fact that no co-operativity was observed for the tryptophan binding to any of the three enzyme derivatives as judged from the slope of the Hill plots (n = 1.0). The dissociation constants thus obtained for the ferrous, CO-bound, and ferric enzymes at pH values 6.0, 7.0, and 8.0 at 24°C are summarized in Table II together with the Kₐ values for the catalytic reaction and the Kᵦ values for the substrate inhibition. First, a good agreement of the Kᵦ for the ferrous enzyme with the Kᵦ, both being in the order of micromolar is noticeable. This agreement of both values may not be accidental because such agreement is seen at various pH values examined. Second, the Kᵦ for the CO-bound enzyme (350 μM) was pH-independent and was one to 2 orders of magnitude greater than that for the ferrous enzyme. This result indicates that the ligand (such as CO) binding to the ferrous heme of the enzyme markedly weakens the affinity of the enzyme for L-tryptophan. This may be either due to a possible conformational change of the ferrous enzyme upon ligand binding to the heme just as seen in the distinct conformational difference between the ferrous (deoxy) and oxygenated (or CO-bound) hemoglobin (20, 21) or myoglobin (29) or due to a possible interaction between the enzyme-bound tryptophan and the heme-bound ligand (CO). Third, the dissociation constants for the ferric enzyme, being in the order of millimolar, is markedly greater than that for the ferrous enzyme, being in the order of micromolar. In other words, the affinity of the ferric enzyme for L-tryptophan is 2 to 3 orders of magnitude lower than that for the ferrous enzyme. The ratios of the Kᵦ for the ferric enzyme to that for the ferrous enzyme at pH values 6.0, 7.0, and 8.0 are about 670, 450, and 90, respectively.

**Table II**

<table>
<thead>
<tr>
<th>pH</th>
<th>Kᵦ</th>
<th>Kᵦ</th>
<th>Kᵦ</th>
<th>Kᵦ</th>
</tr>
</thead>
<tbody>
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<td>μM</td>
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<td>μM</td>
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<td>30</td>
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<td>8.0</td>
<td>6.5</td>
<td>5.6</td>
<td>350</td>
<td>0.51</td>
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</table>

**Dissociation constant for L-tryptophan-enzyme complex.**

**Substrate inhibition constant (see text for definition).**

3 M. Sono, W. Watanabe, and O. Hayashi, unpublished observation.
following three cycles (A, B, and C) seem to be most probable candidates,

$$\text{Cycle A: } \text{Fe}^{2+} \xrightarrow{K_1} \text{Fe}^{3+} \xrightarrow{O_2} \text{Trp. Fe}^{2+} \xrightarrow{K_2} \text{Product}$$

$$\text{Cycle B: } \text{Fe}^{3+} \xrightarrow{K_1} \text{Fe}^{2+} \xrightarrow{O_2} \text{Trp. Fe}^{3+} \xrightarrow{K_2} \text{Product}$$

$$\text{Cycle C: } \text{Fe}^{2+} \xrightarrow{K_1} \text{Fe}^{3+} \xrightarrow{O_2} \text{Trp. Fe}^{2+} \xrightarrow{K_2} \text{Product}$$

Tryptophan binds to the oxygenated enzyme (Kd = K) in both Cycle B and Cycle C, while tryptophan reacts with the ferrous enzyme (Kd = Kd) in Cycle A. Assuming that the CO-bound enzyme has a tryptophan-binding property similar to the oxygenated enzyme and that the dissociation constant for the tryptophan-enzyme complex directly corresponds to the Kd for tryptophan, it is then suggested that the Cycle A is a main pathway (Kd = Kd) rather than Cycles B and C (Kd ≠ Kd) during a steady state of the catalytic reaction and the latter two pathways (B and C) are minor ones. This indicates that tryptophan may bind to the ferrous enzyme before oxygen.

On the basis of the results obtained in the present experiments and of the above discussion, we propose a new possible reaction sequence of indoleamine 2,3-dioxygenase in Fig. 7. In the principal pathway in this sequence, the ferrous enzyme binds predominantly with tryptophan, followed by molecular oxygen. The tryptophan binding to the ferric enzyme is of no catalytic significance, i.e. the tryptophan-ferric enzyme complex is not involved in a catalytic reaction cycle. The ferric enzyme binds with superoxide anion in the tryptophan-free form to yield the oxygenated enzyme (Fe<sup>3+</sup>O<sub>2</sub>) in Fig. 7 and the oxygenated enzyme thus formed or produced by the binding of molecular oxygen to the tryptophan-free ferrous enzyme (Fe<sup>2+</sup>) is presented as a possible final intermediate. This oxygenated enzyme may react with tryptophan either directly or via the ferrous enzyme to form the ternary complex (Trp-Fe<sup>3+</sup>O<sub>2</sub>). For a final conclusion, not only equilibrium but also transient and steady state kinetic studies of the enzyme reaction may be necessary. Kinetic studies of tryptophan binding to the enzyme are under investigation at the present time in our laboratory.

Acknowledgments—We wish to thank Doctors H. Hori and H. Morimoto of Laboratories of Biophysics, Faculty of Engineering Science, Osaka University, for the performance of the measurements of the electron paramagnetic spectra and Mr. J. Ukon of Union Giken Co. for the measurements of the circular dichroism spectra.

REFERENCES
Additions and Corrections

Vol. 255 (1980) 2465-2471

The mechanism of sugar binding to the periplasmic receptor for galactose chemotaxis and transport in Escherichia coli.

David M. Miller, III, John S. Olson, and Florante A. Quiocho

Page 2468, Line 6 of legend to Fig. 5 should read:
Panel A is 0 to 2.0 s (0.2 s per division); the time scale for Panel B is

Page 2469, Line 12 in Column 1 should read:

The first entry under “Component” should read:
4-Hydroxyproline

Page 2470, Line 3 in Column 2 should read:
steady state rate of galactose uptake on the order of 10^{-3} M


Studies on the carbohydrate of collagens. Characterization of a glucuronic acid-mannose disaccharide unit from Nereis cuticle collagen

Robert G. Spiro and Vishnu D. Bhoyroo

Page 5348, right column, Table I

The first entry under “Component” should read:
4-Hydroxyproline

Vol. 255 (1980) 4073-4080

Purification and structural analysis of the soluble sn-glycerol-3-phosphate dehydrogenase isozymes in Drosophila melanogaster

David W. Niesel, Glenn C. Bewley, Steven G. Miller, Frank B. Armstrong, and Chi-Yu Lee

Page 4079, Note Added in Proof

The data cited in this note was conducted in collaboration with Dr. S. S.-L. Li and the authors failed to acknowledge this fact. The acknowledgment appears below:

In collaboration with Drs. S. S.-L. Li and Y. C. Pan, Laboratory of Animal Genetics, National Institute of Environmental Health Science, NIH, Research Triangle Park, N.C., the amino acid composition of preparative soluble tryptic peptides of both isozymic forms has been analyzed and demonstrated to be identical except for a single peptide which differs electrophoretically between the maps. This difference peptide has been determined to be the COOH-terminal peptide.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Indoleamine 2,3-dioxygenase. Equilibrium studies of the tryptophan binding to the ferric, ferrous, and CO-bound enzymes.
M Sono, T Taniguchi, Y Watanabe and O Hayaishi


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