The Oxygenated Form of L-Tryptophan 2,3-Dioxygenase as Reaction Intermediate*

Yuzuru Ishimura, Mitsuhito Nomura, and Osamu Hayaishi

From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan

Takao Nakamura,† Mamoru Tamura, and Isao Yamazaki

From the Biophysics Division, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan

SUMMARY

In order to clarify the reaction mechanism of L-tryptophan 2,3-dioxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12), a hemoprotein, spectral and kinetic studies were carried out with highly purified enzyme preparations from Pseudomonas fluorescens (ATCC 11299).

A new spectrally distinct species of the enzyme heme (λ_max; 418, 545, and 580 mµ) was observed during the steady state of the catalytic reaction. The formation of the new spectral species was absolutely dependent on the simultaneous presence of both oxygen and L-tryptophan with the ferrous enzyme. In the absence of L-tryptophan, the ferrous heme in the enzyme was oxidized to a ferric state by molecular oxygen. Available evidence indicated that the observed spectrum was due to a ternary complex of oxygen, the enzyme, and L-tryptophan and represented the oxygenated form.

By means of rapid reaction spectrophotometry, the oxygenated form was shown to be an obligatory intermediate of the reaction. The rate of the over-all reaction, as judged by the accumulation of L-formylkynurenine, was always proportional to the amount of the oxygenated form present during the entire course of the reaction. The rate constant for the decomposition of the oxygenated form was estimated to be 10 sec⁻¹, which agreed well with the turnover number of the enzyme (18 sec⁻¹). The rate constant for the binding of oxygen with the ferrous enzyme in the presence of L-tryptophan and that for the reverse reaction were also determined to be 5 X 10⁶ M⁻¹ sec⁻¹ and 230 sec⁻¹, respectively. Thus, the binding of oxygen with the ferrous enzyme in the presence of L-tryptophan is a reversible process, as are those with hemoglobin and myoglobin.

Some properties of the Pseudomonas enzyme also are described.

* This investigation was supported in part by Public Health Service Research Grants CA-04222, from the National Cancer Institute, and AM-05333, from the National Institute of Arthritis and Metabolic Diseases, and by grants from the Squibb Institute for Medical Research and the Scientific Research Fund of the Ministry of Education of Japan. The data were taken from a dissertation submitted by Yuzuru Ishimura in November 1968 to the Graduate School of Kyoto University in partial fulfillment of the requirements for the degree of Doctor of Medical Science.

† Present address, Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka, Japan.

L-Tryptophan 2,3-dioxygenase, commonly known as tryptophan pyrroloase or tryptophan oxygenase, is an enzyme which catalyses the oxidative ring cleavage of L-tryptophan to L-formylkynurenine (1). The enzyme has been isolated both from animal liver and Pseudomonas (2, 3).

Since the enzyme activity was first observed in rat liver extracts by Kotake and Masayama in 1936 (4), the catalytic reaction mechanism as well as its regulatory control mechanism has been the subject of extensive investigations. With the use of the heavy isotope of oxygen (18O), atoms of oxygen incorporated into L-formylkynurenine were found to be derived from molecular oxygen and not from water (5). Tanaka and Knox (6), using partially purified preparations both from Pseudomonas and from rat liver, identified this enzyme as an iron porphyrin protein. Thus, the enzyme was shown to be a unique dioxygenase containing heme as prosthetic group. Later, Feigelson and Greengard (7, 8), using liver enzyme, showed that the apoenzyme could be obtained in an inactive form and that the catalytic activity was restored upon addition of exogenous hematin. Although it has been suggested that the heme iron in the enzyme might be the oxygen-binding site (9), no direct evidence has so far been available either to prove or disprove it. Thus, there has been a considerable discussion in the literature as to the reaction mechanism of the catalytic process, especially the role of heme iron and its valence state during the catalysis (10-14).

The present paper describes the detection and characterization of a new spectral species of L-tryptophan 2,3-dioxygenase which was observed during the steady state of the reaction. Spectral and kinetic analyses of the new species revealed that it is the oxygenated state of heme in the enzyme, and an obligatory intermediate of the reaction. The role of substrate, L-tryptophan, in the oxygenated form is also discussed, together with some other properties of the enzyme. Preliminary accounts of a portion of this work have appeared (15-17).

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents—L-Tryptophan was purchased from Yoneyama Chemical Industries, Ltd. Tryptophan analogues, including 1-L-Tryptophan 2,3-dioxygenase is preferred because the term L-tryptophan oxygenase may be confused with L-tryptophan 5-monoxygenase and L-tryptophan oxygenase (decarboxylating).
and hence the purified enzyme was usually obtained as the ferric.

The heme in tryptophan 2,3-dioxygenase is autoxidizable under anaerobic conditions (14, 27, 28). Anaerobic conditions were obtained either by gassing the reaction mixture with purified nitrogen for 15 min or by the use of Thunberg type cuvettes.

Preparation of Enzyme

**Growth of Bacteria**—*Pseudomonas fluorescens* (ATCC 11299) was used as the source of enzyme. The organism was subcultured on an agar slant and then grown in a liquid medium containing 0.2% L-tryptophan, 0.13% yeast extract, 0.3% KHPO₄, 0.05% KH₂PO₄, and 0.02% MgSO₄·7H₂O. Inoculations were carried out by transferring the cells from a slant to 5-liter Erlenmeyer flasks containing 1.5 liters of the sterilized liquid medium. Cells were grown at 23°C for 20 to 24 hours with vigorous mechanical shaking and were harvested with a Sharples centrifuge, shortly before they reached maximal growth. The yield of wet packed cells was about 2.5 g per liter of medium. The packed cells could be stored for several weeks with little loss of activity at -15°C.

**Crude Extracts**—All subsequent operations were carried out at about 4°C. Centrifugations were carried out at 10,000 × g for 20 min, and all of the phosphate buffers contained L-tryptophan in a final concentration of 1 mM to stabilize the enzyme, unless otherwise noted.

Wet packed cells, 200 g, were ground mechanically with 400 g of aluminum oxide (Wako W-800) in a chilled porcelain mortar for about 10 min, until the mixture became viscous. Two liters of 0.01 M potassium phosphate buffer, pH 7.0, were added to the mixture and the resulting slurry was centrifuged at 13,000 × g for 30 min to remove cell debris and alumina.

**Streptomycin Treatment**—To the viscous crude extracts (1,800 ml) were added 100 ml of a 10% streptomycin sulfate solution with mechanical shaking. After further stirring for 15 min, the massive precipitate was removed by centrifugation at 13,000 × g for 20 min.

**Ammonium Sulfate Fractionation**—To the streptomycin supernatant solution (1,850 ml) were added 400 g of ammonium sulfate with stirring. After 30 min, the resulting precipitate was collected by centrifugation and dissolved in 0.05 M phosphate buffer, pH 7.0, in a final volume of 200 ml.

**Heat Treatment under Anaerobic Conditions**—To the ammonium sulfate fraction, 20 ml of 0.04 M L-tryptophan were added, and the mixture was kept at room temperature for 10 min under a nitrogen stream. The dissolved oxygen in the medium was almost completely eliminated by this process. Then the solution was heated to 53-55°C for 5 min in a water bath under nitrogen. After cooling to 5°C in an ice bath, denatured protein was removed by centrifugation. To the supernatant solution (195 ml), 28 g of ammonium sulfate were added with stirring, and, after 30 min, the resulting precipitate was collected by centrifugation. The red precipitate was dissolved in a minimum volume of 0.05 M phosphate buffer (approximately 10 ml), pH 7.0. Ammonium sulfate was removed with a Sephadex G-25 column (4 × 15 cm) which had been equilibrated with the same buffer. All of the colored eluate was collected until the presence of ammonium sulfate was detected.

**DEAE-cellulose Chromatography**—The eluate from a Sephadex G-25 column was diluted five times with distilled water and then placed on a DEAE-cellulose column (4.5 × 15 cm), which had previously been equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The column was washed successively with

Preparations of Reduced Form of Enzyme and Anaerobic Conditions—The heme in tryptophan 2,3-dioxygenase is autoxidizable and hence the purified enzyme was usually obtained as the ferric form (native). The ferric enzyme was reduced by either one of the following procedures: (a) quantitative additions of sodium dithionite under anaerobic conditions or (b) illumination of light
with each 200 ml of 0.01 M and 0.05 M phosphate buffer, pH 7.0. The enzyme was then eluted with 200 ml of 0.15 M phosphate buffer, concentrated by the addition of ammonium sulfate (40% saturation), and dissolved in a minimum volume of 0.05 M phosphate buffer, pH 7.0.

**Sephadex G-200 Column Chromatography**—The concentrated solution (2 to 3 ml) was passed through a Sephadex G-200 column (2.5 x 50 cm) equilibrated with 0.05 M phosphate buffer, pH 6.5, free of L-tryptophan. Usually, two protein fractions were obtained as judged by absorbance at 280 nm; one appeared at the void volume of the column and the other was eluted a little later. The middle three-fifths of the total volume of the second peak was collected for use.

The purification procedure resulted in an over-all purification of about 180-fold with 5% in yield. The enzyme thus obtained had a specific activity of 5.3 units per mg of protein (13). More recently, Poillon et al. (29) described a method of purification which yielded homogeneous enzyme preparations. However, our preparations after DEAE-cellulose chromatography were essentially free of other hemoproteins as well as other chromoproteins such as flavoprotein and were therefore used for spectral and kinetic studies. Catalase and peroxidase activities were found to be negligible as judged by the spectrophotometric method of Chance (30) and the pyrogallol test (31), respectively. Further attempts to purify the enzyme were usually accompanied by a decrease in activity per hematin molecule. The enzyme can be stored for several days without an appreciable loss of activity if it is kept at 0°C under anaerobic conditions in the presence of 1 mM tryptophan.

**RESULTS**

**Properties of Enzyme**

**Absorption Spectra**—The absorption spectra of both the oxidized (native) and the dithionite-reduced forms of the purified tryptophan 2,3-dioxygenase in the presence and absence of L-tryptophan are shown in Fig. 1. These represent typical absorption spectra of a high spin protohemoprotein having absorption maxima at 404.5, 500, and 635 nm in the ferrie state and at 432, 553, and 588 nm in the ferrous state, respectively. The highest ratio of the absorbance at 404.5 nm to that at 280 nm so far obtained was 1.5 in the ferrie state of enzyme. Almost no change of the spectra was observed between pH 6.0 and 9.0. The ferrie enzyme was instantaneously reduced by the addition of either sodium dithionite or borohydride both in the presence and absence of L-tryptophan. The reduced heme was autooxidizable in the absence of L-tryptophan. The spectral changes in the Soret region caused by substrate were first reported by Maeno and Feigelson (14) and were confirmed by us (15). As shown in the inset of the figure, addition of L-tryptophan to either form of enzyme under anaerobic conditions caused slight but significant changes of their spectra not only in the Soret region but also in the entire visible region. These results suggest that L-tryptophan combines with the enzyme irrespective of the valence state of its heme in the absence of oxygen. No such spectral changes were observed either when other aromatic amino acids such as tyrosine and histidine were added to the enzyme solution or when L-tryptophan was added to other high spin protoporphyrins such as catalase and peroxidase under similar conditions.

In Fig. 2 is shown the absorption spectrum of the pyridine ferrohemochrome of the enzyme as well as that of the authentic hematin. Coincidence of the absorption maxima and troughs indicates that the iron porphyrin in the enzyme is protohematin IX, as reported previously (13). Extinction coefficients of the Soret absorption of the enzyme in the absence of L-tryptophan were estimated to be $175 \times 10^3$ and $145 \times 10^3$ per mole of heme in the ferrie and ferrous state of enzyme, respectively.

**Substrate Specificity and Effects of pH**—In good agreement with the results of previous investigators (6), the enzyme was absolutely specific for L-tryptophan. None of the following tryptophan analogues was active as substrate under the standard conditions.
assay conditions: 2,3-dihydro-DL-tryptophan, 4-methyl-D, L-tryptophan, 5-methyl-D,L-tryptophan, L-tryptophan amide, L- and D-5-hydroxytryptophan, and α-methyl-DL-tryptophan. However, some of these analogues were found to act as inhibitors at higher concentrations. Among those, 5-hydroxy-L-tryptophan was the most potent inhibitor, while 5-hydroxy-D-tryptophan was not. On the other hand, α-methyl-DL tryptophan activated the enzyme at low L-tryptophan concentrations, as has been described already (32, 33). Details of these studies will be described elsewhere.

The pH optimum for enzyme activity was 7.0 to 7.3. The enzyme was most stable at pH 6.5 in 0.05 M potassium phosphate buffer. The isoelectric point of the enzyme protein was determined to be pH 5.2 by the isoelectric focusing method of Vesterberg and Svenson (34).

*Kₘ Values for Oxygen and L-Tryptophan and Turnover Number of Enzyme—Reciprocal plots of the over-all reaction rate against oxygen concentrations at fixed tryptophan concentrations were linear within analytical limits and the *Kₘ* value for oxygen with a saturated concentration of L-tryptophan (5 mM) was found to be 60 μM. In contrast, plots of reciprocal velocity against reciprocal tryptophan concentrations were nonlinear at all levels of oxygen and the apparent *Kₘ* value for L-tryptophan determined in a concentration range between 50 μM and 10 mM under an air atmosphere was 240 μM. Mutual effects of oxygen and L-tryptophan concentrations were observed on each *Kₘ* value and the apparent dissociation constants for each substrate were increased by decreasing the concentration of the other substrate. These results are in agreement with those reported previously (6, 33).

The maximal turnover number of the enzyme per hematin molecule was estimated to be 1100 min⁻¹ under the standard assay conditions.

**Absorption Spectra of Enzyme during Reaction**

Detection of Oxygenated Form of Enzyme at 5°—We have previously reported that, when the valence state of heme in the enzyme was examined during the steady state of the reaction, a new spectrum appeared in the Soret region, which was indicative of neither ferric heme nor ferrous heme, nor a mixture of these, suggesting the possible presence of a transient intermediate (27, 28). This finding was further confirmed and extended in the following experiments. Incubations were carried out at 5° to slow down the reaction rate in a wide cuvette with 1-cm light path into which a continuous supply of gas can be added through bubbling without interference with optical measurements (35). When oxygen was introduced to a solution containing both the ferrous enzyme and tryptophan, a new absorption spectrum appeared immediately, having its absorption maxima at 418, 545, and 580 μM (Fig. 3, solid line). These peak positions are very close to those of known oxygenated heme proteins such as oxyhemoglobin, oxymyoglobin, and Compound III of horseradish peroxidase (15).

The ferric enzyme was reduced by the addition of 30 mmoles of sodium dithionite under anaerobic conditions. Excess dithionite was removed by bubbling commercial nitrogen which contained a trace amount of oxygen. The reaction was then initiated by the addition of 0.15 ml of 0.1 M potassium phosphate buffer, pH 7.0, which had previously been equilibrated with pure oxygen. The spectra were recorded at 1 min intervals.

Fig. 3. Spectrum of L-tryptophan 2,3-dioxygenase obtained at 5° by continuous bubbling of oxygen. The reaction mixture contained 22.7 mmoles of the reduced form of L-tryptophan 2,3-dioxygenase (specific activity, 3.7), 500 mmoles of potassium phosphate buffer (pH 7.0), and 50 mmoles of L-tryptophan in a final volume of 5.0 ml. The reaction was initiated by introducing gaseous oxygen into a cuvette by bubbling (35). ---, recorded during the continuous bubbling of oxygen; -- , recorded when the supply of oxygen was stopped; ---, ferrous enzyme before and after the reaction.

Fig. 4. Spectral changes during the autoxidation of ferrous L-tryptophan 2,3-dioxygenase in 5% oxygen. The reaction mixture contained 17.1 mmoles of ferric L-tryptophan 2,3-dioxygenase (specific activity, 2.3) and 300 mmoles of potassium phosphate buffer, pH 7.0, in a final volume of 3.0 ml under anaerobic conditions. The ferric enzyme was reduced by the addition of 30 mmoles of sodium dithionite under anaerobic conditions. Excess dithionite was removed by bubbling commercial nitrogen which contained a trace amount of oxygen. The reaction was then initiated by the addition of 0.15 ml of 0.1 M potassium phosphate buffer, pH 7.0, which had previously been equilibrated with pure oxygen. The spectra were recorded at 1 min intervals.
Fig. 5. Spectra of the oxygenated form of L-tryptophan 2,3-dioxygenase obtained by the use of rapid scan spectrophotometry at 24°C. The incubation mixture contained 14.5 mmoles of reduced L-tryptophan 2,3-dioxygenase (specific activity, 3.7), 500 μmoles of potassium phosphate buffer (pH 7.0), and 30 μmoles of L-tryptophan in a final volume of 2.4 ml under anaerobic conditions. The reaction was initiated by the injection of 0.5 ml of 0.1 M potassium L-tryptophan. These results, together with the kinetic evidence described below, indicate that the observed spectrum is due to a ternary complex of oxygen, the enzyme, and tryptophan, and represents the oxygenated form.

Rapid Scan Spectrophotometry of Oxygenated Form—A spectral species similar to that described above was also shown to exist under the standard assay conditions (20% oxygen at 24°C) by the following experiments with the use of a Hitachi rapid scan spectrophotometer. This instrument permits full spectral scans of the entire visible region three times per sec with a scanning time of 0.15 sec. In Fig. 5 are shown results of these experiments in which the reaction was initiated by the addition of oxygen to a solution containing ferrous enzyme and tryptophan. It can be seen that the main portion of enzyme had been converted to the new species after 1 sec but it reverted to the original ferrous enzyme after 5 sec. A lowering of the peak height in Fig. 5B was due to a dilution caused by the addition of the buffer solution containing oxygen. Fig. 5C shows a record only at 1 set after the initiation of the reaction. In addition to the changes in the Soret region, the formation of two new peaks at 545 and 580 μm is evident. These processes could be observed repeatedly by the addition of oxygen, as shown in Fig. 5D.

Stopped Flow Experiments

Spectrum of Tryptophan 2,3-Dioxygenase in Steady State of Reaction—A typical record of a stopped flow experiment is shown in Fig. 6, in which a solution containing both the ferrous enzyme and L-tryptophan was allowed to react with molecular oxygen and changes in absorbance were followed at 415 μm. An almost instantaneous increase in optical density was observed followed by its gradual disappearance. The maximum change in absorbance as represented by the peak height is denoted by \( p_{\text{max}} \) and the time for changes in optical density to fall from \( p_{\text{max}} \) to \( p_{\text{max}/2} \) is designated as \( t_{\text{off}} \) according to the Chance's
**Oxygenated Form of L-Tryptophan 2,3-Dioxygenase**

Vol. 245, No. 14

FIG. 7. Difference spectra of the steady state and ferrous enzyme in the Soret region. Concentrations of ferrous L-tryptophan 2,3-dioxygenase, L-tryptophan, and potassium phosphate buffer, pH 7.0, were 1.4 μM, 5 mM, and 0.1 M, respectively. The initial concentrations of oxygen were 600 μM (O—O) and 60 μM (X—X), respectively.

FIG. 8. Absolute spectra of L-tryptophan 2,3-dioxygenase during the steady state of the reaction reconstructed from the data in Fig. 7. -, the ferrous form of enzyme; O—O and X—X, the same as in Fig. 7.

Fig. 7. Difference spectra of the steady state and ferrous enzyme in the Soret region. Concentrations of ferrous L-tryptophan 2,3-dioxygenase, L-tryptophan, and potassium phosphate buffer, pH 7.0, were 1.4 μM, 5 mM, and 0.1 M, respectively. The initial concentrations of oxygen were 600 μM (O—O) and 60 μM (X—X), respectively.

FIG. 9. Time course of the accumulation of the reaction product and the amount of the oxygenated enzyme. Concentrations of ferrous L-tryptophan 2,3-dioxygenase (specific activity, 3.7), L-tryptophan, and potassium phosphate buffer, pH 7.0, were 1.4 μM, 5 mM, and 0.1 M, respectively. Initial concentration of oxygen was 120 μM. Accumulation of the reaction product, L-formylkynurenine (lower figure), and the amount of the oxygenated form (upper figure) were recorded in parallel experiments as described in the text under identical experimental conditions.

FIG. 10 (left). Linear relationship between the rate of over-all reaction and the amount of the oxygenated form replotted from the data in Fig. 9.

FIG. 11 (right). Effects of oxygen concentrations on the kinetics of the oxygenated form of L-tryptophan 2,3-dioxygenase. Concentrations of ferrous L-tryptophan 2,3-dioxygenase (specific activity, 3.7), L-tryptophan, and potassium phosphate buffer, pH 7.0, were 1.4 μM, 5 mM, and 0.1 M, respectively. Numerals in the figure represent the initial micromolar concentrations of oxygen.

**Relationship between Rate of Over-all Reaction and Amounts of Oxygenated Form of Enzyme**—Since 415 μM is the most prominent peak of the difference spectra, all of the following stopped flow experiments were carried out at this wave length unless otherwise noted. In Fig. 9 are shown the relative amounts of the oxygenated form during the entire course of the reaction (upper curve) as well as the accumulation of the reaction product, L-formylkynurenine, as determined by the increase in optical density at 321 μM (lower curve). There were recorded in parallel experiments under identical conditions in which the initial concentration of oxygen was 120 μM. When the rates of over-all reaction at different time intervals calculated from the tangents of the lower curve were plotted against the relative amounts of

...
The rate constants for the decomposition of the oxygenated form (product formation) calculated by Chance's equation are shown in Table I. The values (19.2 - 19.6 sec⁻¹) agree quite well with the turnover number of the enzyme determined by the conventional method (18 sec⁻¹) and the rate constant for the decomposition obtained from the data in Fig. 9 by dividing the rate of formylkynurenine formation by the amount of oxygenated form (18 sec⁻¹).

Effect of Tryptophan Concentrations—The next experiments were carried out in order to learn the effect of tryptophan concentration on the formation and decomposition of the oxygenated form. The results of typical experiments in which an anaerobic solution of ferrous enzyme was mixed with solutions containing varying amounts of L-tryptophan and 60 μM of oxygen are shown in Fig. 13.

The ferrous enzyme solution contained 50 μM L-tryptophan to eliminate a trace amount of dissolved oxygen and to prevent the oxidation of enzyme heme. When the solutions in both syringes of the flow apparatus were allowed to react, the tryptophan was diluted to below 25 μM, which was approximately one-tenth of its Kₐ value under these conditions and was therefore disregarded for the calculations. It is seen from the figure that the rate of formation of the oxygenated form as well as pₘₐₓ decreased as L-tryptophan concentration decreased. For experimental details, see the legend for Fig. 13. Kₐ is the rate constant for the decomposition of the oxygenated form of L-tryptophan 2,3-dioxygenase.

<table>
<thead>
<tr>
<th>L-Tryptophan concentration</th>
<th>pₘₐₓ</th>
<th>t½ off</th>
<th>pₘₐₓ/t½ off</th>
<th>Kₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.13</td>
<td>20</td>
<td>2.6</td>
<td>23</td>
</tr>
<tr>
<td>0.15</td>
<td>0.23</td>
<td>11</td>
<td>2.5</td>
<td>24</td>
</tr>
<tr>
<td>0.20</td>
<td>0.29</td>
<td>8.5</td>
<td>2.46</td>
<td>24.4</td>
</tr>
<tr>
<td>0.40</td>
<td>0.61</td>
<td>4.5</td>
<td>2.75</td>
<td>21.8</td>
</tr>
<tr>
<td>1.0</td>
<td>0.82</td>
<td>4.0</td>
<td>3.29</td>
<td>18.3</td>
</tr>
<tr>
<td>2.0</td>
<td>0.83</td>
<td>3.8</td>
<td>3.15</td>
<td>19.0</td>
</tr>
<tr>
<td>5.0</td>
<td>0.86</td>
<td>3.6</td>
<td>3.09</td>
<td>19.4</td>
</tr>
</tbody>
</table>

The time required for reaching pₘₐₓ in Fig. 13 is probably due to the conformational change of the enzyme induced by L-tryptophan since the formation of the oxygenated form was accelerated by the preliminary incubation of enzyme with high concentrations of L-tryptophan.
Our interest in the general mechanism of dioxygenase reaction led us to undertake a study of these discrepancies. As cited above (39, 40), we once reported in collaboration with Feigelson that both ferric and ferrous enzyme could be regarded as the active form of L-tryptophan 2,3-dioxygenase. Later, however, it was noticed that the enzyme preparations used at that time contained some endogenous reductants which could reduce the ferric enzyme to active ferrous state in the presence of L-tryptophan (13). With the use of more purified preparations, we further demonstrated that, when the ferric enzyme was gradually reduced by illumination under anaerobic conditions, the process resulted in a proportional increase in the activity (27, 28, 41). Likewise, a gradual oxidation of the ferrous enzyme by air resulted in a parallel decrease in the activity. These results, together with the fact that the ferrous enzyme was fully active in the absence of the activator whereas the ferric form was almost inactive (41), indicated that the enzyme must be in the ferrous state to initiate the reaction. Thus, the original view of Tanaka and Knox (6) was revitalized. However, we also found that a new spectrum of the enzyme, which was due to neither the ferric nor the ferrous state of the heme, appeared in the Soret region during the steady state of the reaction (27, 28). The new spectral species was observed only in the presence of both L-tryptophan and oxygen, and therefore was interpreted to be due to a ternary complex of oxygen, L-tryptophan, and the enzyme. These findings were now confirmed and extended in this paper.

The spectrum of the new spectral species over the entire visible region was obtained and found to be almost identical with those of known oxygenated hemoeproteins (15). These spectral characteristics together with the kinetic evidence that the binding of oxygen to the ferrous heme is a reversible process strongly support our view that the new spectral species is due to an “oxygenated” form of heme in L-tryptophan 2,3-dioxygenase. These interpretations are in accord with the previous observation of Maeno and Feigelson (9) that the photochemical action spectrum, with a maximum at 421 mp, is identical with the Soret absorption spectrum of the tryptophan-carboxyferroprotoporphyrin-enzyme complex.

The oxygenated form of enzyme was observed whenever the reaction proceeded. It was detected under various experimental conditions with several different techniques, i.e. at different temperatures, varying concentrations of oxygen, L-tryptophan, and the enzyme by an ordinary spectrophotometric method, rapid scan spectrophotometry, and the stopped flow method. Furthermore, we were able to demonstrate that the rate constant for the decomposition of the oxygenated form was in good agreement with the maximal turnover number of the enzyme. Thus, the oxygenated form was proved to be an obligatory intermediate of the reaction.

Recently, Feigelson and Maeno (12) and Maeno and Feigelson (14) proposed a reaction mechanism which postulates the participation of ferric enzyme in the catalytic process. Their postulate is based mainly on the observation that, when the reaction was initiated with a ferric enzyme in the presence of ascorbate, no increase in optical densities at 432 mp due to the formation of ferrous enzyme was observed during the steady state of catalysis. However, this does not necessarily mean that the enzyme is in the ferric state during the process. The present data as well as those reported previously (27, 28) clearly indicate that the heme iron in the enzyme oscillates in charge, being sequentially reduced by L-tryptophan and reoxidized by oxygen.

Dr. K. Hiromi of Osaka Prefectural University, it was possible to observe reaction rates from 0.6 msec after mixing. A typical trace of a stopped flow experiment with Dr. Hiromi’s instrument, in which final concentrations of oxygen and L-tryptophan were 120 μm and 5 mM, respectively, and that of the enzyme as heme was 6 mM, is shown in Fig. 14. Experiments were also carried out at several different oxygen concentrations (30 to 240 mM) and the rate of reaction was directly proportional to the oxygen concentration, indicating that the reaction was first order with respect to oxygen. The average rate constant calculated from all of our experiments is 5.0 x 10^6 sec^-1. Based on this value as well as other kinetic constants described earlier, the reverse rate constant is determined to be 231 sec^-1.

**DISCUSSION**

There has been considerable discussion in the literature as to the reaction mechanism of L-tryptophan 2,3-dioxygenase. Tanaka and Knox (6) and Tokuyama and Knox (11) presented evidence that the enzyme with its heme in the ferric state was catalytically inactive but could be reduced to an active ferrous form by the addition of either ascorbate or hydrogen peroxide in the presence of L-tryptophan. In their hypothetical reaction mechanism, Tanaka and Knox assumed that oxygen binds first with ferrous heme and then reacts with L-tryptophan, giving L-formylkynurenine as product. On the other hand, Feigelson, Ishimura, and Hayashi (39, 40) and subsequently Feigelson and Maeno (12) and Maeno and Feigelson (14) reported that the ferric as well as the ferrous enzyme was catalytically active. A reaction mechanism was proposed by these authors in which the formation of the oxygenated form; a, level of the ferrous enzyme at 415 nm; b, changes in optical transmission at 415 nm due to the formation of the oxidized form; c, changes in optical transmission at 415 nm due to the formation of the oxygenated form; c, level of the ferrous enzyme at 415 nm; d, changes in optical transmission at 415 nm due to the formation of the oxygenated form; e, level of the ferrous enzyme at 415 nm; f, changes in optical transmission at 415 nm due to the formation of the oxygenated form; g, level of the ferrous enzyme at 415 nm; h, changes in optical transmission at 415 nm due to the formation of the oxygenated form; i, level of the ferrous enzyme at 415 nm; j, changes in optical transmission at 415 nm due to the formation of the oxygenated form; k, level of the ferrous enzyme at 415 nm; l, changes in optical transmission at 415 nm due to the formation of the oxygenated form; m, level of the ferrous enzyme at 415 nm; n, changes in optical transmission at 415 nm due to the formation of the oxygenated form; o, level of the ferrous enzyme at 415 nm; p, changes in optical transmission at 415 nm due to the formation of the oxygenated form; q, level of the ferrous enzyme at 415 nm; r, changes in optical transmission at 415 nm due to the formation of the oxygenated form; s, level of the ferrous enzyme at 415 nm; t, changes in optical transmission at 415 nm due to the formation of the oxygenated form; u, level of the ferrous enzyme at 415 nm; v, changes in optical transmission at 415 nm due to the formation of the oxygenated form; w, level of the ferrous enzyme at 415 nm; x, changes in optical transmission at 415 nm due to the formation of the oxygenated form; y, level of the ferrous enzyme at 415 nm; z, changes in optical transmission at 415 nm due to the formation of the oxygenated form.
enzyme is mainly in an oxygenated state and, therefore, almost no free ferrous enzyme can be detected during the steady state of the reaction.

It is to be noted that the oxygenation spectrum of enzyme heme was observed only in the presence of L-tryptophan. On the other hand, additions of L-tryptophan either to the ferrous or the ferric enzyme in the absence of oxygen caused modifications in their spectra over the entire visible region. Furthermore, the rate of decomposition of the oxygenated form was independent of L-tryptophan concentrations. Therefore, it seems reasonable to assume that first L-tryptophan and then oxygen binds to the ferrous enzyme to form a tryptophan-enzyme-oxygen complex, the oxygenated intermediate. If the observed intermediate does not contain the L-tryptophan that reacts with oxygen, the rate constant for its degradation should be affected by the L-tryptophan concentrations, but the data in Table II show that this is not the case.

The only enzymatically active species of oxygenated heme so far known is the Compound III of peroxidase, in which case divalent iron in the heme is oxygenated in the absence of its organic substrate (42). In contrast, in the case of L-tryptophan 2,3-dioxygenase, the organic substrate is necessary in order to form the oxygenated intermediate in a detectable quantity. In the absence of L-tryptophan, the ferrous form of enzyme is oxidized to the ferric form very slowly. Presumably tryptophan, by binding with the enzyme, evokes a conformational change in the enzyme protein and an increase in reactivity of the heme moiety towards oxygen. This interpretation is in accord with the findings that the dissociation constants of heme-binding substances such as cyanide and carbon monoxide with the enzyme protein and an increase in reactivity of the heme moiety towards oxygen. This interpretation is in accord with the findings that the dissociation constants of heme-binding substances such as cyanide and carbon monoxide with the enzyme are greatly altered by L-tryptophan (9, 15, 41, 43, 44).

The following reaction mechanism is therefore compatible with the above data. Tryptophan first combines with the ferrous enzyme and activates the heme in the enzyme. The activated enzyme then reacts with oxygen to form an intermediary ternary complex. Both substrates, tryptophan and oxygen, are activated in the complex and interact, yielding formylkynurenine as the product.

The oxygenated iron compound reported here requires much more detailed characterization. Nevertheless, our findings are quite consistent with the view that dioxygenase reactions in general involve a ternary complex of enzyme, oxygen, and organic substrate (16, 17, 27, 45). The role of tryptophan(s) in the oxygenation of the enzyme heme is especially noteworthy in the oxygenation of the enzyme heme is especially noteworthy.

Acknowledgments—The authors are grateful to Dr. K. Hiromi for his kind collaboration in the determination of the rate constant for the formation of the oxygenated form. We are also indebted to Dr. C. A. Tyson for his aid in the preparation of this paper, to Mr. M. Fujita for his assistance in the use of the rapid scan spectrophotometer, and to Mrs. T. Kizu for her excellent technical assistance.

REFERENCES


43. Ishimura, Y., Nozaki, M., Hayashi, O., Tamura, M., and Yamazaki, I., in K. Okunuki, M. D. Kamen, and I. Suzuki (Editors), Structure and function of cytochromes, University of Tokyo Press, Tokyo, 1968, p. 188.
The Oxygenated Form of L-Tryptophan 2,3-Dioxygenase as Reaction Intermediate
Yuzuru Ishimura, Mitsuhiro Nozaki, Osamu Hayaishi, Takao Nakamura, Mamoru Tamura and Isao Yamazaki


Access the most updated version of this article at [http://www.jbc.org/content/245/14/3593](http://www.jbc.org/content/245/14/3593)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/245/14/3593.full.html#ref-list-1](http://www.jbc.org/content/245/14/3593.full.html#ref-list-1)