The Nature and Mechanism of the Tryptophan Pyrrolase (Peroxidase-Oxidase) Reaction of Pseudomonas and of Rat Liver*

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The formation of kynurenine from tryptophan was first observed in liver extracts by Katakai's school and was attributed to a "tryptophan pyrrolase" (1). Partial purification of the system from liver showed it to consist of two separate reactions, an initial oxidation to formylkynurenine followed by hydrolysis (2).

\[ \text{L-Tryptophan} + \text{O}_2 \rightarrow \text{formylkynurenine} \] (1)

\[ \text{Formylkynurenine} + \text{H}_2\text{O} \rightarrow \text{formic acid} + \text{kynurenine} \] (2)

The oxidation reaction was catalyzed by an enzyme that had the following properties: (a) utilization of one molecule of oxygen in a single step, (b) inhibition by catalase, and (c) specific reversal of catalase inhibition by hydrogen peroxide from a donor reaction. Other properties of the enzyme, including cyanide and light reversible CO inhibitions, suggested that it was an iron porphyrin enzyme occurring in both the ferrous and ferric states (3). On the basis of the above properties it was assumed that there was an intermediate formation and utilization of peroxide in the reaction. The reaction was therefore formulated as a coupled oxidation (4), probably catalyzed by a single enzyme, and the enzyme was called the "tryptophan peroxidase-oxidase.''

Further investigation of the reaction mechanism is reported here with both the liver enzyme and a highly purified bacterial enzyme which was first obtained by Hayaishi and Stanier (5) in crude cell extracts from tryptophan-grown Pseudomonas sp. with 100 times the specific activity of liver extracts. The bacterial enzyme shares all of the properties of the animal enzyme. A preliminary report of these results has appeared (6). Peroxide was not directly involved in the oxidation reaction of either enzyme, but was necessary to activate it. The enzyme was identified as an iron-porphyrin protein which was reduced to its active ferrous form by peroxide. In conjunction with the demonstration that gaseous oxygen was incorporated into the product of this reaction (7), the present results indicate that the reaction catalyzed is an oxygenation, a direct oxygen transfer to tryptophan, and it does not involve peroxidation. For this reason we will refer to the enzyme by the original name, tryptophan pyrrolase.

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EXPERIMENTAL

Materials and Methods

Rats—Adult albino rats that had been given a suspension of 200 mg. of DL-tryptophan per 100 gm. of body weight by intraperitoneal injection 5 hours before sacrifice were used for preparation of the liver enzyme. This treatment adaptively increased the amount of enzyme in the livers about 10-fold (8).

Bacteria—Pseudomonas sp. (NTCC 11,980) which metabolized tryptophan via the quinoline pathway (9) was used as the bacterial enzyme source. The medium contained 3.0 gm. of L-tryptophan, 3.0 gm. of KH\(_2\)PO\(_4\), 3.0 gm. of Na\(_2\)HPO\(_4\), 0.2 gm. of MgSO\(_4\), 0.001 gm. of FeSO\(_4\)·7H\(_2\)O and 1.0 gm. of yeast extract (Difco) per l. and was adjusted to pH 7.2. The cells for the inoculum were grown overnight in 1.5 l. of nutrient medium, and then collected by centrifugation, washed with 0.04 M Na phosphate buffer, pH 7.2, and transferred to 15 l. of medium in a 5-gallon Pyrex bottle. The cultures were aerated and stirred vigorously by compressed air passed through two filter candles. The bacteria were harvested in a Sharples Super Centrifuge after 8 to 10 hours at 30°, just before they reached maximal growth. The yield of cells obtained was usually 5 g. wet weight per l. of medium. The cells were washed with cold 0.04 M Na phosphate, pH 7.2, and stored at -9°. About 20 per cent loss in activity of the whole cells was observed during 2 weeks of storage.

Reagents—Kynurenine formamidase (formylase (10)) was prepared catalase-free by a repetition of the heat-treatment step in the procedure described (11). A crystalline commercial preparation of bovine catalase (Worthington) was used. The glucose oxidase was a purified catalase-free preparation of mold origin made from Dee O (Takamine Laboratories) by Dr. V. H. Auerbach. Methylhydroperoxide was kindly provided by Dr. Philip George.

Enzyme Assay—Tryptophan oxidation was assayed at 25° by continuous spectrophotometric estimation of the kynurenine formed in the presence of an excess of kynurenine formamidase (12) to catalyze the reaction of Equation 2. The standard assay system contained in a 1-cm. cell, 0.1 ml. of the enzyme solution (0.2 to 0.6 unit), 0.1 ml. of formamidase (2 units (11)), 0.1 ml. of 0.02 M ascorbic acid solution (freshly dissolved and adjusted to pH 7.2), 0.4 ml. of 0.02 M L-tryptophan solution, 2.5 ml. of Na phosphate buffer (0.1 M, pH 7.2) and water in a total volume 3.5 ml. The reaction was started by the addition of the substrate. The readings were made at 365 m\(\mu\) in a Beckman spec-
trophotometer, continuously (model DK) or at 1-minute intervals (model DU), against a blank which contained the same components minus the substrate. The reaction rate was linear with time at optical densities less than 0.8, and was proportional to the enzyme concentration. The assays with atmospheres other than air were run in Thunberg type cells kindly lent by Dr. Eric Ball.

The enzyme activity was expressed as μmoles of kynurenine formed in 10 minutes per ml. of the enzyme solution (1 μmole = 1 unit). Under the above assay conditions 0.128 optical density corresponded to 0.1 μmole of kynurenine (the molecular extinction coefficient of kynurenine at 365 μm in 4.5 × 10⁻⁴ (9)). Specific activity was expressed as units per mg. of protein. The protein was measured in the crude extract by the biuret method (13), and after the first ammonium sulfate fractionation by a spectrophotometric method (14). The values determined by these two methods agreed within 10 per cent. Absorption spectra were measured in a Beckman model DK recording spectrophotometer. The points given on the figures were used in redrawing these continuously recorded curves. The wave length scales of the spectrophotometers used were calibrated with sodium dichromate solution and with a mercury lamp.

### RESULTS

#### Purification of Enzyme

Because of the lability of the purified enzyme from bacteria and liver, it was studied immediately after preparation. All manipulations were carried out at 5° or below.

**Bacterial Enzyme—Step I.** Wet bacteria, 25 gm., were vigorously ground with 50.0 gm. of levigated alumina (Norton Company) in a chilled porcelain mortar for 5 minutes (15). Grinding was discontinued when the mixture became viscous. Crystalline DNase, 50 μg., was then added and 80 ml. of 0.1 m Na phosphate buffer, pH 7.2, were mixed in slowly. The precipitate obtained after centrifugation at 10,000 × g for 40 minutes was again extracted with 80 ml. and once with 40 ml. of the buffer, and the three supernatant fractions were combined.

Step II. One mg. of crystalline RNase was added to the combined extracts and the mixture was allowed to stand for 30 minutes in an ice bath. Two-thirds volume of saturated ammonium sulfate solution adjusted to pH 7.2 with concentrated ammonia solution was added. The mixture was centrifuged 30 minutes later at 6,000 × g for 30 minutes at 0°. The supernatant was discarded, and the precipitate was dissolved in ⅔ volume of the 0.1 m phosphate buffer and dialyzed overnight against cold 0.01 m phosphate, pH 7.2, which contained 0.0001 m sodium Versenate. The precipitate formed during dialysis was removed by centrifugation at 10,000 × g for 30 minutes and discarded.

Step III. The dialyzed supernatant fraction was adjusted to pH 6.0 with dropwise addition of acetic acid, and any precipitate formed during this treatment was removed by centrifugation. Calcium phosphate gel suspension (20 mg. dry weight per ml.) (16) was added and the mixture was allowed to stand for 15 minutes. Usually 80 to 90 per cent of the enzyme was adsorbed by ⅔ volume of the gel suspension. The gel was collected by centrifugation at 6,000 × g for 10 minutes and was washed once with cold water. The enzyme was eluted three times with ⅔ volume of 0.2 m Na phosphate buffer, pH 6.5, and the eluates combined.

Step IV. The eluate was adjusted to pH 7.2 with 1 N sodium hydroxide, then ⅔ volume of saturated ammonium sulfate, pH 7.2, was added slowly. After 30 minutes the mixture was centrifuged at 10,000 × g for 30 minutes. The precipitate was dissolved in 7 ml. of the 0.1 m Na phosphate buffer pH 7.2.

At this final stage the enzyme was usually purified about 35-fold with a yield of 25 per cent (see Table I). The highest specific activity obtained was 17.6 and the spectrum of this preparation is illustrated in Fig. 1 to show its porphyrin nature.

Most of the kynurenine formamidase activity was removed by the first ammonium sulfate fractionation (Step II) and the ratio of the rates of formation of formylkynurenine to its hydrolysis was about 300 to 1 after the second ammonium sulfate fractionation (Step IV). The Step IV preparation contained a negligible amount of catalase (usually less than 2 μg. per ml.), measured by the spectrophotometric assay of hydrogen peroxide decompo-

### Table I

| Purification of tryptophan pyrrolase from tryptophan-adapted Pseudomonas |
|------------------|---|---|---|---|---|
|                  | Volume | Activity | Protein concentration | Specific activity | Yield % |
| Step I           | 162.0  | 4.3      | 15.3                  | 0.28             | 100.0   |
| Step II          | 32.0   | 18.1     | 6.6                   | 2.70             | 83.5    |
| Step III         | 46.0   | 5.3      | 1.3                   | 4.10             | 35.2    |
| Step IV          | 8.0    | 21.3     | 2.2                   | 9.70             | 24.5    |

**Fig. 1.** Spectra of highly purified bacterial tryptophan pyrrolase (■) and of its dipyridine hemochrome (○). The enzyme (from Step IV of purification) contained 3.5 mg. protein and 61.5 units per ml. Its specific activity was 17.6. The hemochrome was made from another preparation (specific activity 5.9) by HCl-acetone and reduction with sodium dithionite. It was measured in 0.2 N NaOH.
sition (17). It did not show any peroxidase activity with pyrogallol in the presence of hydrogen peroxide (18). The purified enzyme was very labile and about 50 per cent loss in activity occurred on over-night storage. The enzyme was almost completely inactivated by ethanol, methanol, and acetone fractiona-
tion procedures.

**Rat Liver Enzyme**—Freshly removed livers of six tryptophan-
adapted rats were homogenized, centrifuged, and the supernatant fraction precipitated and washed at pH 5.4 as previously de-
scribed (2). The solution of this precipitate in 0.1 M Na phos-
phate buffer, pH 7.2, was reprecipitated by addition of 3 volume of saturated ammonium sulfate adjusted with ammonia to pH 7.2. The precipitate was then taken up in a small volume of buffer and recentrifuged to clarify it for the optical assays. The specific activity usually was about 0.25. The preparations were catalase-free, but they were not sufficiently concentrated to iden-
tify a porphyrin spectrum.

### Table II

**Reaction of purified Pseudomonas tryptophan pyrrolase**

<table>
<thead>
<tr>
<th>L-Tryptophan</th>
<th>Oxygen uptake</th>
<th>Formylkynurenine formed</th>
<th>Kynurenine formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Experiment I</td>
<td>4.0</td>
<td>0.0</td>
<td>4.45</td>
</tr>
<tr>
<td>Experiment II</td>
<td>8.0</td>
<td>0.0</td>
<td>8.65</td>
</tr>
</tbody>
</table>

* Formylkynurenine was determined at 320 mµ (ε = 3750) (19) then kynurenine formamidase was added and kynurenine deter-
mined at 365 mµ.

**Table III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>KCN</td>
<td>4 × 10⁻⁴</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁻³</td>
<td>84</td>
</tr>
<tr>
<td>NH₂OH</td>
<td>4 × 10⁻⁴</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁻³</td>
<td>75</td>
</tr>
<tr>
<td>NaN₃</td>
<td>4 × 10⁻⁴</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁻³</td>
<td>70</td>
</tr>
<tr>
<td>NaF</td>
<td>2 × 10⁻³</td>
<td>9</td>
</tr>
<tr>
<td>CO (in dark)</td>
<td>80% (20% O₂)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20% (20% O₂, 60% N₂)</td>
<td>77</td>
</tr>
<tr>
<td>Na dithionite</td>
<td>5.7 × 10⁻⁴</td>
<td>100</td>
</tr>
<tr>
<td>K ferricyanide</td>
<td>5.7 × 10⁻⁴</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine</td>
<td>5.7 × 10⁻⁴</td>
<td>88</td>
</tr>
<tr>
<td>Glutathione</td>
<td>5.7 × 10⁻⁴</td>
<td>70</td>
</tr>
<tr>
<td>Thiomalic acid</td>
<td>5.7 × 10⁻⁴</td>
<td>25</td>
</tr>
<tr>
<td>Na Versenate</td>
<td>5.7 × 10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>5.7 × 10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>Na diethyldithiocarbamate</td>
<td>5.7 × 10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>5.7 × 10⁻⁴</td>
<td>100*</td>
</tr>
</tbody>
</table>

* The inhibition by Cu²⁺ was specific in that similar concentra-
tions of Mn²⁺, Mg²⁺, Zn²⁺, Co²⁺, Ni²⁺, Fe²⁺, and Al³⁺ did not inhibit. Fe³⁺ caused slight inhibitions (20 per cent at 6 × 10⁻⁴ M).

**Reaction**—L-Tryptophan oxidation by *Pseudomonas* sp. ex-
tracts has yielded kynurenine and formic acid, which have been isolated (7, 9). Although formylkynurenine was not accumu-
lated, the extracts contained an active formylkynurenine form-
amidase (5). Table II shows that the reaction catalyzed by the purified *Pseudomonas* enzyme is identical with that cata-
lized by the animal enzyme (Equations 1 and 2). One molecule each of L-tryptophan and of O₂ disappeared to form 1 molecule of formylkynurenine. The latter compound was accumulated quan-
titatively by the purified bacterial enzyme in the absence of added formamidase, and identified by its absorption curve (10) and by its conversion to kynurenine on addition of purified formamidase. The same kind of identification was made with the liver enzyme. There was no evidence throughout the puri-
ification of either enzyme for the separation of the oxidation reac-
tion into two steps.

No other substrate than L-tryptophan has been found for the liver enzyme. D-Tryptophan was neither oxidized by the puri-
ified bacterial enzyme nor did it inhibit the oxidation of L-trypt-
ophan. dl-α-Methyltryptophan, contrary to the report of Sourkos and Townsend (20), was not oxidized by the liver en-
zyme or by the bacterial enzyme (Dr. Morton Civen, unpub-
lished results). dl-α-Methyltryptophan did inhibit tryptophan oxidation slightly. The affinity of the bacterial enzyme for L-
tryptophan (Kₘ, 3.5 × 10⁻⁴ M) was almost identical with that observed for the animal enzyme (Kₘ, 4 × 10⁻⁴ M). The pH
optimas for the two reactions were substantially the same (pH 7.0 to 7.2).

The affinity of the bacterial enzyme for oxygen, determined by its per cent maximal activity in different concentrations of oxygen, is shown in Fig. 2. About 50 per cent of the full activity occurred in the presence of 13 volume per cent O₂. The af-

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**Figure 2.** Activity of bacterial tryptophan pyrrolase as a function of oxygen concentration. Reactions were run in Thunberg-type cuvettes equilibrated with various N₂-O₂ mixtures.
Inhibitors of Tryptophan Pyrrolase—The effects of a number of inhibitors on the reaction of the bacterial enzyme is given in Table III. Of particular interest was the sensitivity of the reaction to cyanide, hydroxylamine, and azide, common inhibitors of ferric-porphyrin enzymes, and the light-reversible CO inhibition and inhibition by Cu++. The liver enzyme was inhibited by these same substances.

The light reversible CO inhibition, characteristic of ferrous porphyrins, is shown in Fig. 3. As with the animal enzyme, complete CO inhibition was not reversed by light, but partial inhibition was partially reversed. The bacterial enzyme when completely inhibited by CO was fully reactivated by aeration.

The bacterial and liver enzymes therefore had nearly identical properties in addition to the effects of catalase, peroxide and cyanide described below. The hemoprotein nature of the tryptophan pyrrolase, suggested by the properties of the liver enzyme and reaffirmed by the properties of the bacterial enzyme, was supported by the spectrum of the purified bacterial enzyme given in Fig. 1.

**Porphyrin Nature of Enzyme**

It was not possible to show parallel increases in specific activity of tryptophan pyrrolase with increases in the concentration of the porphyrin by absolute measurement at 403 mp because large amounts of other substances absorbing nonspecifically near 400 mp were removed during purification. The evidence that the porphyrin obtained was associated with the enzyme activity consisted of (a) its virtual absence from crude and purified extracts of bacteria which were not grown in tryptophan and which had almost no tryptophan pyrrolase activity, (b) its presence in all active preparations of the enzyme, (c) the absence of other porphyrin protein activities (peroxidase, catalase, cytochrome) in these active enzyme fractions, and (d) the specific alterations in the spectrum of this porphyrin by the substrate and by inhibitors during the tryptophan pyrrolase reaction. These alterations will be described.

The spectrum of the undiluted enzyme as isolated (ferric form) with its Soret band at 403 mp (Figs. 1 and 4) was changed to one with the Soret band at 433 mp (Fig. 4) by ascorbic acid plus L-tryptophan (but not n-tryptophan), very slowly (80 minutes) if incubated anaerobically, but immediately if incubated aerobically. No change occurred with ascorbic acid in the absence of L-tryptophan. The new spectrum was identified as that of the ferrous form of the enzyme, since the same spectrum was produced in the absence of L-tryptophan by reduction of the enzyme with sodium dithionite or with H₂ in the presence of Pd. In the latter instance the enzyme was reoxidized by air to the ferric form and was still active in the assay system. Further evidence that the 433 mp Soret band formed in each of the above reductions represented the ferrous form of the enzyme was its conversion by CO to a new spectrum with a 420 mp Soret band (Fig. 4). The locations of the Soret bands produced by various...
reagents are summarized in Table IV. The location of the α- and β-bands could not be precisely fixed with the concentrations of enzyme available. The ferrie enzyme showed peaks near 625 μm and near 545 μm and 500 μm. These were changed in the ferrous enzyme and its CO derivative to double peaks above 550 μm. Also given in Fig. 1 is the spectrum of the dipyridine hemochrome prepared from the prosthetic group which was cleaved with HCl-acetone by standard procedures. The bands at 417, 520, and 557 μm were not identical with any described.

**Action of Catalase**

The basis for the postulated role of peroxide in the tryptophan oxidation reaction with the animal enzyme was its inhibition by catalase. When the bacterial enzyme was obtained catalase free (Step IV), catalase added before the start of the reaction was readily demonstrated to inhibit the reaction about 70 per cent. Generation of small amounts of peroxide during the reaction, either with glucose oxidase and glucose (Fig. 5) or with ascorbic acid, prevented this inhibition. An amount of catalase too small to inhibit significantly the enzyme reaction became an effective inhibitor in the presence of ethanol as a result of the higher affinity for peroxide which catalase shows in its peroxidatic reaction with ethanol than when functioning catalatically (22). With very dilute solutions of hydrogen peroxide itself, it was also possible to activate the catalase-inhibited reaction (Table V). Peroxide in the absence of tryptophan pyrrolase had no measurable effect on L-tryptophan.

The action of peroxide on the enzyme was clarified by determining the time when catalase exerted its effect when added to the continuously assayed reactions of the bacterial and liver enzymes. As shown in Fig. 6, catalase did not inhibit the reactions of the bacterial or liver enzymes when added after the reaction had begun. The requirement for peroxide was therefore limited to a period at the start of the reaction during which the enzyme became activated, and peroxide was apparently not needed in the tryptophan oxidation reaction itself.

**Action of Cyanide**

By the same type of experiment used to determine when catalase acted, the inhibition by cyanide was found to be much diminished and slow in developing if added to the reaction after it had begun (Figs. 7 and 8). Since the ferrie forms of the iron porphyrin enzymes react with cyanide, this form apparently existed only before the reaction started, at the same period when peroxide was required to activate the enzyme. The disappearance of the enzyme's sensitivity to cyanide may be referred to a reduction of inactive Fe+++ enzyme to Fe++ enzyme by peroxide. Thereafter, peroxide was unnecessary and the reaction was no longer inhibited by catalase and only slowly by cyanide.

**Table IV**

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Peak (Fe++)</th>
<th>+CN</th>
<th>+CN</th>
<th>+CN</th>
<th>Reduced (Fe++)</th>
<th>Fe++</th>
<th>CO</th>
<th>Dipyridine hemochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Band (μm)</td>
<td>403</td>
<td>415</td>
<td>418</td>
<td>418</td>
<td>433</td>
<td>420</td>
<td>417 (γ = 557, β = 520)</td>
<td></td>
</tr>
</tbody>
</table>

* Reduced enzyme was prepared by incubation with L-tryptophan, with H2 and Pd, or with Na dithionite.

**Fig. 5.** Catalase inhibition of bacterial tryptophan pyrrolase (C) and its prevention by peroxide generated from glucose oxidase plus glucose (D). Oxygen deficiency caused by the donor reaction eventually slowed Reaction B. Standard assay without catalase (A).

**Table V**

<table>
<thead>
<tr>
<th>Catalase</th>
<th>H2O2</th>
<th>Observed activity</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>μ mole</td>
<td>μ mole/10 min.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.934</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0.205</td>
<td>22</td>
</tr>
<tr>
<td>1.0</td>
<td>2.85 X 10⁻³</td>
<td>0.452</td>
<td>40</td>
</tr>
<tr>
<td>1.0</td>
<td>5.70 X 10⁻³</td>
<td>0.710</td>
<td>76</td>
</tr>
<tr>
<td>1.0</td>
<td>8.55 X 10⁻³</td>
<td>0.823</td>
<td>88</td>
</tr>
</tbody>
</table>

Hydrogen peroxide was added last to standard assay reactions of purified Pseudomonas enzyme not containing ascorbic acid or other sources of peroxide.
Activation of Tryptophan Pyrrolase by Peroxide

A lag period of at least several minutes often occurred at the start of the reaction with preparations of both the bacterial and the animal enzymes, especially if they were aged for several days.

In the experiments of Fig. 7, and especially in Fig. 8, it can be seen that ascorbic acid or methylhydroperoxide, added as a source of peroxide, shortened the lag period. In Fig. 5 the lag period is lengthened by catalase and shortened by the generation of peroxide.

**Fig. 6.** Effects of catalase on bacterial (Fig. 6A) and liver (Fig. 6B) tryptophan pyrrolase reactions. Standard reaction assay (Curve A). Inhibition by 1 mg. of catalase added before the reaction was started (Curve C). No inhibition when catalase was added 4 minutes after reaction had begun (Curve B).

**Fig. 7.** Effects of cyanide on bacterial tryptophan pyrrolase reaction. Standard assay (B), plus ascorbic acid (A). Inhibition by KCN (2 x 10^-5 M) added before the reaction was started (C). Small and slowly developing inhibition when KCN was added at arrows after the reaction started (A and B). The presence of ascorbic acid (5.7 x 10^-4 M) in the system (A) minimized the KCN inhibition, presumably by accelerating the reactivation of any enzyme which became oxidized to the ferrie form. Note that ascorbic acid also shortened the initial lag period (dotted lines in A cf. B).

**Fig. 8.** Effects of cyanide on liver tryptophan pyrrolase reaction. Inhibition by KCN (1.2 x 10^-4 M) added before reaction was started (Curve E). Small and slowly developing inhibition when KCN was added after reaction started (at second arrow) in Curves B and C. The presence of ascorbic acid (2.8 x 10^-4 M) in Reaction B minimized development of KCN inhibition. Curve D shows the long lag period of this one week old liver enzyme preparation (cf. Curve A, with CH3OOH added). To all other reactions CH3OOH (8.7 x 10^-4 M) was added at the first arrow to abolish this lag.
Experiment 1

Addition of hydrogen peroxide and methylhydroperoxide significantly shortened the lag period. The spectra in Fig. 9 of the undiluted Pseudomonas enzyme recorded immediately after addition of L-tryptophan plus 1.0 x 10^{-2} M H_2O_2 to B. The evidence thus far described indicated that the tryptophan pyrrolase was isolated in an inactive ferric form which was reduced by peroxide in the presence of L-tryptophan to the active ferrous form. Peroxide was then no longer necessary, except to reactivate small amounts of enzyme which might be oxidized in side reactions, since the formation of the CO-complex of the active enzyme and the enzyme's insensitivity to cyanide indicated that it remained in the ferrous form during the reaction. Additional evidence for this view was obtained by the use of ferricyanide, already described as an effective inhibitor of the enzyme reaction. Ferricyanide would be expected to oxidize the active ferrous enzyme to the inactive ferric form. The prompt inhibition of the reaction after addition of ferricyanide is shown in Fig. 10. The inhibited enzyme was reactivated by ascorbic acid, which served as a source of peroxide for the reduction. Less complete reactivation occurred if cyanide was added to react with the ferric form of the enzyme. It was concluded that ferricyanide inhibited the reaction by forming the inactive ferric enzyme.

**DISCUSSION**

The properties of the *Pseudomonas* tryptophan pyrrolase were found to be comparable in every respect to those of the liver enzyme, so that a postulated coupled oxidation mechanism was equally appropriate for both enzymes. However, the bacterial enzyme was available in a form sufficiently purified to identify it as a hemoprotein and to permit direct testing of the two fundamental assumptions of the earlier postulated reaction mechanism: that peroxide was formed and used up in the reaction itself, and that the iron changed valence in the course of the reaction. The evidence given indicated that the reaction of the bacterial enzyme needed only very small and not stoichiometric amounts of peroxide, amounts such as might be generated by side reactions in the assay system. The peroxide acted only at the beginning of the reaction in connection with the reduction of the bacterial enzyme to the active ferrous form. Though the mechanism of this activation is still not clear, it would be analogous to the reduction of methemoglobin and of catalase-azide complex to their oxygenated ferrous forms by peroxide under anaerobic conditions (25, 26).

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The evidence indicated that there was no

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TABLE VI

**Shortening of lag period in tryptophan pyrrolase reaction by hydrogen peroxide or methylhydroperoxide**

<table>
<thead>
<tr>
<th>Peroxide concentration</th>
<th>Lag period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.06</td>
</tr>
<tr>
<td>CH_3OOH: 9.3 x 10^{-5}</td>
<td>0.42</td>
</tr>
<tr>
<td>CH_3OOH: 2.8 x 10^{-5}</td>
<td>0.82</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.55</td>
</tr>
<tr>
<td>HOOC: 2.85 x 10^{-6}</td>
<td>0.32</td>
</tr>
<tr>
<td>HOOC: 9.3 x 10^{-4}</td>
<td>0.66</td>
</tr>
<tr>
<td>HOOC: 2.85 x 10^{-4}</td>
<td>0.82</td>
</tr>
</tbody>
</table>

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Fig. 9. Reduction of bacterial tryptophan pyrrolase by hydrogen peroxide in presence of L-tryptophan. Spectra determined immediately after addition of L-tryptophan to A, and L-tryptophan plus 1.0 x 10^{-4} M H_2O_2 to B.

Peroxide with glucose oxidase and glucose. These observations suggested that the reaction lagged during the period of obligatory reduction by peroxide of inactive ferric enzyme to the active ferrous form. Direct evidence of this role for peroxide was obtained by the use of dilute solutions of hydrogen peroxide and methylhydroperoxide. The lag periods of reactions without catalase or ascorbic acid, determined by extrapolation of the linear portion of the curve to the time axis as in Fig. 7, are given in Table VI. Addition of hydrogen peroxide and methylhydroperoxide significantly shortened the lag period. The spectra in Fig. 9 of the undiluted *Pseudomonas* enzyme recorded immediately after addition of L-tryptophan without and with dilute hydrogen peroxide shows that the enzyme was reduced to the active ferrous form only in the presence of hydrogen peroxide. This did not occur in the absence of L-tryptophan. The reduced enzyme formed the typical CO derivative of the ferrous enzyme. Similar results were obtained with methylhydroperoxide. In the absence of L-tryptophan the enzyme was very sensitive to peroxide; the same dilute peroxide solutions decreased the height of the Soret band, shifted the maximum to about 408 m\(\nu\), and irreversibly inactivated the enzyme. The experiments in Fig. 8 were done with an aged animal enzyme which showed a prolonged lag period which was eliminated by methylhydroperoxide.

No activation or reduction of the enzyme occurred with potassium chloridrate or potassium molybdocyanide (23), samples of which were kindly given by Dr. P. George, nor did these reagents or peroxide produce transient spectral changes in the tryptophan pyrrolase resembling the FeIV or FeV forms they produce with horseradish peroxidase, methemoglobin, and catalase (24). There is no evidence, therefore, that the reduction of tryptophan pyrrolase from the ferric to the ferrous form is other than a direct one occurring in the presence of L-tryptophan and peroxide, although the mechanism of this remains to be clarified.

Valence State of Iron in Tryptophan Pyrrolase Reaction

The evidence thus far described indicated that the tryptophan pyrrolase was isolated in an inactive ferric form which was reduced by peroxide in the presence of L-tryptophan to the active ferrous form. Peroxide was then no longer necessary, except to reactivate small amounts of enzyme which might be oxidized in side reactions, since the formation of the CO-complex of the active enzyme and the enzyme's insensitivity to cyanide indicated that it remained in the ferrous form during the reaction. Additional evidence for this view was obtained by the use of ferricyanide, already described as an effective inhibitor of the enzyme reaction. Ferricyanide would be expected to oxidize the active ferrous enzyme to the inactive ferric form. The prompt inhibition of the reaction after addition of ferricyanide is shown in Fig. 10. The inhibited enzyme was reactivated by ascorbic acid, which served as a source of peroxide for the reduction. Less complete reactivation occurred if cyanide was added to react with the ferric form of the enzyme. It was concluded that ferricyanide inhibited the reaction by forming the inactive ferric enzyme.
Inhibition of bacterial tryptophan pyrrolase reaction by ferricyanide (1.4 \times 10^{-4} M) added at first arrows. Reactivation by ascorbic acid (5.7 \times 10^{-4} M) was nearly complete (Fig. 10A), unless KCN (1.1 \times 10^{-3} M) was added first to react with the inactive ferric enzyme (Fig. 10B).

Further change in the valence of the active enzyme in the course of the reaction itself, although the occurrence of a continuing slow conversion of the enzyme to the ferric form followed by reactivation was indicated by the slowly developing inhibition when cyanide was added to the going reaction. The crucial points of this evidence were the lack of inhibition by catalase and cyanide after the reaction had started. These points were also demonstrated with the liver enzyme. The several changes going on in the reaction with tryptophan pyrrolase from either source can therefore be formulated as follows:

\[
\begin{align*}
\text{(CN and catalase inhibition)} \\
\text{Inactive} & \quad \text{Fe}^{3+}.\text{enzyme} \\
\quad & \quad \text{H}_2\text{O}_2 + \text{L-tryptophan} \\
\text{Active} & \quad \text{Fe}^{2+}.\text{O}_2.\text{enzyme} \quad \text{L-tryptophan} \\
\quad & \quad \text{Fe}^{2+}.\text{enzyme} \quad \text{formylkynurenine} \\
\quad & \quad \text{(CO inhibition)}
\end{align*}
\]

This scheme offers a satisfactory explanation for the major properties of the enzyme which have been described. The lag period frequently seen at the start of the reaction with aged enzyme (in which sources of peroxide formation are diminished), probably represents the time needed for the conversion of the inactive ferric to the active ferrous enzyme by substrate and slowly accumulated hydrogen peroxide. Without an adequate source of peroxide, a prolonged lag period could occur, as in Fig. 8, and this could possibly be misinterpreted as the time needed to induce the enzyme in cell-free preparations. Since quantitative measurements of tryptophan pyrrolase are always made in the presence of a source of peroxide, the new knowledge of the reaction suggested no way in which a given amount of enzyme could become more active under the usual assay conditions. The new knowledge of the reaction mechanism therefore strengthens the belief that increases in enzyme activity which have been obtained by adaptation of animals with the substrate or with adrenal corticoids represent increased amounts of enzyme (27).

**SUMMARY**

The enzyme of *Pseudomonas* catalyzing the oxidation of L-tryptophan to formylkynurenine was partially purified and identified as an iron porphyrin protein. Its properties were nearly identical to those of the liver enzyme catalyzing the same reaction.

The reaction mechanism, previously formulated as a coupled oxidation, is a direct oxygenation of the substrate with molecular oxygen by the ferrous enzyme. The role of peroxide in the reaction was demonstrated to be its conversion of the inactive ferric enzyme in the presence of substrate into the active ferrous form of the enzyme. Tryptophan pyrrolase is suggested as a more appropriate name than the familiar but incorrect tryptophan peroxidase-oxidase.

**REFERENCES**

The Nature and Mechanism of the Tryptophan Pyrrolase (Peroxidase-Oxidase) Reaction of Pseudomonas and of Rat Liver
Takehiko Tanaka and W. Eugene Knox


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