ON THE ACTIVATION AND CATALYTIC MECHANISM OF MICROBIAL TRYPTOPHAN PYRROLASE[1]

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Received October 24, 1963

An inconsistency exists between the reports of two laboratories investigating the mechanism of tryptophan pyrrolase (tryptophan 2,3 oxygenase) action. Tanaka and Knox (1959) emphasize that exclusively the Fe^{+2} form of the enzyme is catalytically active. They also propose that the catalytically inactive Fe^{+3} form of the enzyme need be reduced by the concerted action of tryptophan and H_2O_2 or tryptophan and ascorbate and that the substrate tryptophan alone is incapable of reducing the enzyme to a catalytically active ferrous form. On the other hand, Feigelson and Greengard (1961) have reported the preparation from liver of a highly purified apotryptophan pyrrolase which becomes fully active upon addition of ferriprotoporphyrin IX (hematin) to the apoenzyme with no exogenous reductant being necessary (Greengard and Feigelson (1963)). Since it is generally accepted that heme and iron proteins which react with oxygen have their iron in the divalent state, implicit in the hematin catalytic effectiveness in the absence of ascorbate or H_2O_2 is the necessity for the reduction of the hematin iron to the divalent state by the tryptophan during the

[1] This study was supported in part by a travel grant to Dr. Philip Feigelson by the China Medical Board of New York and by grants from the National Institutes of Health, U.S. Public Health Service; the National Science Foundation; The Rockefeller Foundation; the Jane Coffin Childs Memorial Fund for Medical Research; and the Scientific Research Fund of the Ministry of Education of Japan.

The capable technical assistance of Miss Junko Takei is acknowledged with pleasure.

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course of the catalysis. The present report will document three points: a) the ability of tryptophan alone to convert inactivated enzyme to a catalytically active form; b) the activation of bacterial tryptophan pyrrolase preparations by ferriprotoporphyrin IX in the absence of exogenous reductants; and c) the cyclic reduction and reoxidation of the enzyme's metalloporphyrin by tryptophan and oxygen, respectively, during the course of the catalysis.

Tryptophan pyrrolase is an inducible enzyme in Pseudomonas with the enzyme present in the high speed supernatant of the sonicated bacteria (Hayaishi and Stanier (1951)). When assayed for tryptophan pyrrolase activity, in the absence and presence of ascorbate, fresly prepared supernatants show no activation by ascorbate; however, enzyme preparations allowed to age at 4°C manifest a marked ascorbate requirement. Following addition of tryptophan to such ascorbate-requiring enzymes and overnight preincubation at 4°C, then as depicted in Table I the ascorbate-free activity rises indicating the conversion by tryptophan of the inactivated enzyme to a form which is fully active without ascorbate in the assay medium.

TABLE I. THE ACTIVATION OF TRYPTOPHAN PYRROLASE BY TRYPTOPHAN

Preincubation supplement	Tryptophan Pyrrolase Activity -Ascorbate	+ Ascorbate
None	20.4	37.3
Tryptophan	44.6	34.3
Tryptophan + Hematin	42.7	35.1
Prior to Preincubation	19.0	38.0

0.7 ml of a 24-hour-old high speed supernatant of tryptophan adapted Pseudomonas in 0.1 M sodium phosphate, pH 7.0 was incubated, where indicated, with 0.1 ml 0.03 M L-tryptophan and 0.1 ml 5×10^{-6} M hematin in a total volume of 1.0 ml. This was preincubated overnight at 4°C in air. The next morning 0.2 ml aliquots were assayed for tryptophan pyrrolase activity in media containing 1.5 ml 0.1 M sodium phosphate pH 7.0, 0.3 ml 0.03 M L-tryptophan, 0.2 ml 5×10^{-6} M hematin and where indicated 0.2 ml 0.02 M ascorbate, and water to bring the total volume to 2.5 ml. The rates of optical density increase at 321 and 365 mμ were recorded and the tryptophan pyrrolase activity indicated is the sum of the formylkynurenine and kynurenine (formylase being present) formed as mμmoles formed/minute/0.2 ml.

This activation by tryptophan of the Pseudomonas tryptophan pyrrolase can be demonstrated readily with both crude extracts or highly purified prepara-

tions. Furthermore, the tryptophan-mediated activation process was found to proceed with greater efficiency under anaerobic conditions than in the presence of air excluding the possible formation and participation of H_2O_2. It is thus evident that tryptophan acting alone, albeit at a slower rate, is capable of the same activation process as is ascorbate plus tryptophan.

Storage of tryptophan pyrrolase at 0-4°C without tryptophan frequently results, in addition to the ascorbate requirement, in a further stimulation in catalytic activity when hematin is added to the assay medium. Such an enzyme preparation provides an opportunity to investigate the relationship between the ascorbate and hematin activation processes. As shown in Figure 1, a purified enzyme preparation with such a dual requirement manifests a 3.2-fold increase in catalytic activity upon saturation with hematin whether ascorbate is present or absent. Likewise, in both the absence of exogenous hematin and at saturation levels of hematin there exists a 2.5-fold stimula-

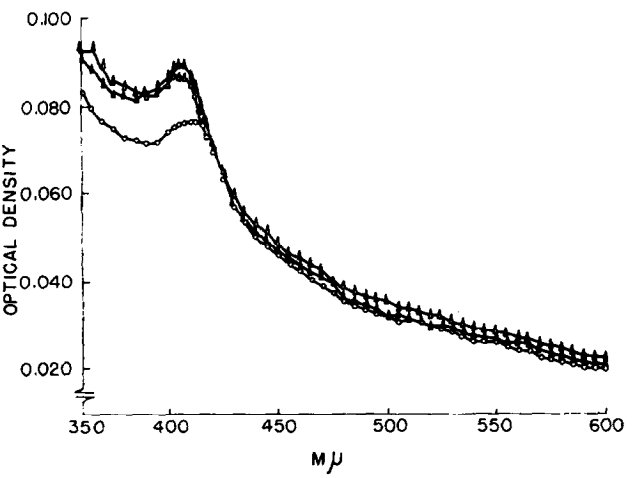

Fig. 1. The activation of tryptophan pyrrolase by hematin and ascorbate. An hematin- and ascorbate-dependent enzyme was obtained by allowing a purified enzyme preparation (specific activity 0.64 μmoles formylkynurenine formed/min./mg protein) to age for 48 hours at 1°C in air; 0.05 ml of this aged enzyme was added to cuvettes containing 0.3 ml 0.03 M L-tryptophan, the indicated amounts of hematin, and 0.1 M sodium phosphate buffer, pH 7.0, to bring the total volume to 2.5 ml (—●—). A parallel series of activity measurements was made in cuvettes which also contained 0.2 ml 0.02 M sodium ascorbate, pH 7.0 (—○—). The rate of formylkynurenine formation was estimated by determination of the linear rate of optical density increase at 321 mμ (Feigelson and Greengard (1961)).

tion by ascorbic acid. Since saturation with respect to one activator does not preclude activation by the other, the ascorbate and hematin stimulatory processes are discrete and independent. It is also evident, even in the absence of ascorbate, that hematin at 10^{-8} molar levels stimulates the tryptophan pyrrolase of Pseudomonas as has been previously found for the comparable liver enzyme (Feigelson and Greengard (1961); Greengard and Feigelson (1963)).

Since tryptophan pyrrolase is an oxygenase catalyzing the insertion of two atoms of molecular oxygen into the pyrrol moiety of tryptophan (Hayaishi et al. (1957)), the stimulatory effects of hematin, in the absence of any exogenous-reducing agent such as ascorbate, poses again the possible mechanisms by which this compound with its trivalent heme iron can become reduced to the divalent state permitting combination with and activation of molecular oxygen. The possible reduction of the iron by the substrate tryptophan was therefore examined spectrophotometrically. In partial agreement with previous reports (Tanaka and Knox (1959)), the purified enzyme was found to have an absorption maximum at 405 mμ, which we find upon reduction with dithionite shifts to 425 mμ with the simultaneous appearance of a new absorption band at 552 mμ (Fig. 2). When the enzyme was incubated anaerobically with the substrate tryptophan, in the absence of any other reducing reagent, a slow reduction of the metalloporphyrin occurs with the appearance of the 552 mμ band and a shift in the Soret absorption maximum to higher wave lengths indicating reduction of the heme iron by the tryptophan. Essentially immediately following admission of air to this substrate-reduced enzyme the absorption band at 552 mμ disappears and the absorption maximum in the Soret region returns to 405 mμ; both changes indicate reoxidation of the enzyme's metalloporphyrin. These and other experiments indicate the ability of tryptophan to reduce the enzyme under anaerobic conditions, and, further, that under steady-state conditions where oxygen is present and the oxygenation of tryptophan to formylkynurenine is proceeding, the state of the iron porphyrin is largely in the oxidized trivalent form.

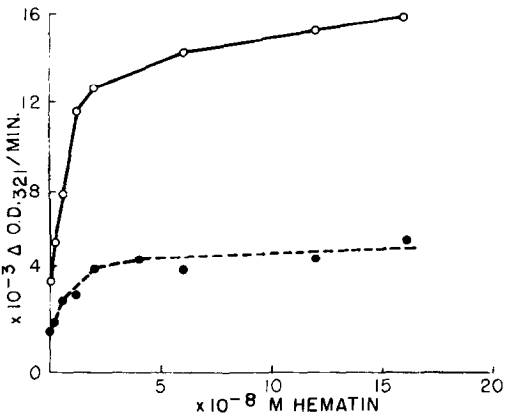

Fig. 2. The reduction by tryptophan and reoxidation by oxygen of tryptophan pyrrolase.

To Thunberg-type quartz cuvettes were added: 0.2 ml of purified tryptophan pyrrolase (5.4 mg protein/ml; specific activity 0.43 μmoles formylkynurenine formed/min./mg protein), 2.3 ml 0.1 M sodium phosphate, pH 7.0, and to the side arm 0.3 ml 0.03 M L-tryptophan. The tube was evacuated, then equilibrated with oxygen-free "Q" gas and evacuated again. This process was repeated three times to insure anaerobiasis. The absorption spectrum was then taken using a Cary automatic spectrophotometer model 15 with 0.1 M sodium phosphate as the optical blank (—▲—). Tryptophan was then added to the enzyme from the side arm and the spectrum taken 46 minutes thereafter (—o—). Air was then admitted into the cuvette and the spectrum recorded one minute later (—△—).

The reduced form of the enzyme augments when the system becomes anaerobic due to consumption of the dissolved oxygen. These facts indicate a cyclic reduction and oxidation of the pyrrol iron by tryptophan and oxygen respectively during the course of the catalysis. The following reaction mechanism is compatible with the experimental evidence:

$$1)\quad Fe^{+3} + T \longrightarrow \overset{T}{Fe^{+3}} \longrightarrow \overset{T\bullet}{Fe^{+2}} \longrightarrow Fe^{+2} + T\bullet$$

$$2)\quad Fe^{+2} + O_2 \longrightarrow \overset{O_2}{Fe^{+2}} \longrightarrow \overset{O_2^-}{Fe^{+3}} \longrightarrow Fe^{+3} + O_2^-$$

$$3)\quad T\bullet + O_2^- \longrightarrow FK$$

$$T + O_2 \longrightarrow FK$$

The above scheme, while it certainly will require expansion and revision, constitutes the simplest probable model which fits all the known facts.

Tryptophan reduces the heme Fe^{+3} to Fe^{+2}, the tryptophan becoming a free radical. The E-Fe^{+2} and/or E-T-Fe^{+2} reacts with oxygen to form a transient intermediary in which the oxygen reoxidizes the heme iron to the original trivalent state, the oxygen necessarily becoming reduced in the process. Then, presumably still on the enzyme surface, the tryptophan radical and superoxyl ion combine yielding the reaction product formylkynurenine (FK). Reaction 1) is analogous to the reported reduction of hematin a_2 by pyridine, yielding a hemochromogen in the absence of exogenous-reducing reagents (Yamanaka and Okunuki (1963)). Oxidation of the ferrous heme by oxygen to the trivalent state yielding O_2^-, Reaction 2), is identical with that proposed for cytochrome oxidase (King and Lee (1960)). The resultant electrophilic tryptophan and nucleophilic oxygen moieties may interact, presumably on the enzyme surface, yielding the reaction product formylkynurenine with the enzyme being regenerated to its original, catalytically active, ferric state. This constitutes the first evidence for substrate and oxygen activation by cyclic reduction and reoxidation of a metallo-organic cofactor during oxygenase activity and may preshadow a similar functioning of inorganic iron in other oxygenases (Oxygenases, Hayaishi, ed. (1962)).

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