THE PARTICIPATION OF COPPER IN TRYPTOPHAN PYRROLASE ACTION[+]

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The mechanism by which tryptophan pyrrolase, a hemoprotein, catalyzes the oxygenation of L-tryptophan to formylkynurenine has been under active investigation. Three alternative hypotheses concerning the valence state of the heme coenzyme during the catalytic process, have been proposed: (a) only the divalent form is active (Tanaka and Knox, 1959; Tokuyama and Knox, 1964); (b) the active heme form is trivalent, but this hematin coenzyme may undergo oscillation in valence state during the catalytic process (Feigelson et al. 1964, 1965); (c) only the divalent form is active but the heme coenzyme undergoes cyclic oxidation and reduction during the steady state catalytic reaction (Hayaishi, 1964). A new finding, which may ultimately clarify the catalytic process and the role of the hematin cofactor therein is herein reported, in which the participation of enzyme bound copper in the microbial tryptophan pyrrolase catalytic reaction is strongly suggested by virtue of the presence of copper in the purified enzyme and the sensitivity of the catalytic process to specific chelating agents.

Pseudomonas ATCC 11299 was cultivated in the presence of 0.1 %

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L-tryptophan. The enzyme purification was carried out essentially as previously described (Feigelson et al. 1965) with a further purification by zone electrophoresis. All the solutions used in the enzyme purification contained 10^{-3}M L-tryptophan to stabilize the enzyme (Greengard and Feigelson, 1962). The enzyme was extracted from induced bacteria, fractionated with streptomycin and ammonium sulfate, and then further purified by DEAE column chromatography using gradient elution at pH 7.0. After elution, the enzyme solution was concentrated by adding ammonium sulfate, dialyzed against veronal buffer (μ = 0.1, pH 8.3) and finally subjected to zone electrophoresis for 16 hours at 7.5 volts/cm., using starch grains as supporting medium in the same veronal buffer. Under these conditions, the holoenzyme moves towards the anode as a single brown band. The enzyme was eluted from the excised starch band with 10^{-2}M potassium phosphate buffer, pH 7.0. Tryptophan pyrrolase activity and protein concentrations were measured as previously reported (Feigelson et al. 1965). One enzyme unit is defined as catalytic formation of one μmole of formylkynurenine per minute at 25°C. Total copper content was determined using cuproine according to the method of Poillon and Dawson (1963).

The summary of a typical enzyme purification starting with 10 grams of wet bacteria is presented in Table I. Twenty-nine percent of the enzyme activity was recovered with an approximately seventy-fold increase in purity. The copper content of the purified enzyme increased as the purification proceeded. Enrichment of the tryptophan pyrrolase fraction with respect to copper is particularly evident following DEAE treatment wherein much contaminating protein was removed. It may be noted that when the enzyme was further purified 2.6 fold by zone

Table I.

Purification and Copper Content of Pseudomonas Tryptophan Pyrrolase

Preparation	Volume ml.	Protein mg./ml.	Activity			Copper	
			E.U./ml.	E.U./mg. protein	Recovery	mμmole/ml.	mμmole/mg. protein
Extract	45.5	50.0	1.67	0.033	100	38	0.76
Streptomycin	54.0	29.0	1.36	0.047	97	30	1.03
Ammonium sulfate	4.5	77.0	12.6	0.164	75	114	1.48
DEAE	1.3	35.0	30.0	0.857	51	144	4.11
Electrophoresis	1.0	10.0	22.0	2.20	29	108	10.8

electrophoresis, the copper content was also increased 2.6 fold, indicating a constant ratio of copper to enzyme activity. The copper content of such purified tryptophan pyrrolase preparations could not be decreased by treatment of the enzyme with chelex-100 (Bio-Rad Co., Richmond, Calif.) which is capable of removing extraneous copper which may be loosely bound to a protein. These findings suggest that the copper is tightly bound to the tryptophan pyrrolase molecule and should be considered an integral part of its structure.

To determine the functional role of the enzyme bound copper, inhibitor studies were initiated employing specific copper chelating agents. As shown in Table II, diethyldithiocarbamate (10^{-2}M) (McFarlane, 1932), salicylaldoxime (4×10^{-3}M)(Simonsen and Burnett, 1955), cuprizone (4×10^{-3}M) (Peterson and Bollier, 1955) which all specifically chelate Cu^{++}, and 10^{-4}M levels of bathocuproine disulfonated disodium salt which is specific to Cu^{+} (Diehl and Smith, 1958), all markedly inhibited the enzyme. Other chelating agents, such as 8-hydroxyquinoline, o-phenanthroline, α , α' - dipyridyl, and EDTA, did not show any inhibitory effect at comparable concentrations.

It has been reported that, in addition to heme iron, mammalian cytochrome oxidase contains copper and is inhibited by 10^{-2} M salicylaldoxime (Okunuki, 1962). However, tryptophan pyrrolase is the only oxygenase, catalyzing the insertion of oxygen into an organic molecule, in which both a dissociable hematin coenzyme (Greengard and Feigelson, 1962) and copper have been shown to partake in the catalytic process. It is evident that existing hypotheses concerning the catalytic mechanism of this enzyme will require revision and expansion to include a role for its copper component. At this stage in our knowledge one may suspect

Table II.

Inhibition of Tryptophan Pyrrolase Activity By Chelating Agents

Inhibitors	Concentrations	Inhibition (%)
8-Hydroxyquinoline	1×10^{-2}	0
α, α'-dipyridyl	1×10^{-2}	0
EDTA	1×10^{-2}	11
Sodium Azide	1×10^{-3}	11
KCN	1×10^{-3}	100
	1×10^{-4}	80
Diethyldithiocarbamate	1×10^{-2}	68
	1×10^{-3}	12
Salicylaldoxime	8×10^{-3}	86
	4×10^{-3}	62
	1×10^{-3}	27
Cuprizone	4×10^{-3}	52
Bathocuproinedisulfonate	9×10^{-4}	100
	3×10^{-4}	39
	9×10^{-5}	22

The enzyme was preincubated with chelating agents for 10 minutes and then L-tryptophan, hematin and ascorbate were added. The rate of formylkynurenine formation was then measured spectrophotometrically as previously described (Feigelson et al., 1965). Cuprizone, being water insoluble, was dissolved into 50% ethanol; in this control experiment, the same volume of 50% ethanol was added to the incubation mixture.

that the substrates of tryptophan pyrrolase, oxygen and tryptophan, are bound respectively to the cuprous and hematin components of the enzyme. The specific catalytic roles of, and the possible interaction between, the enzyme bound copper and hematin moities in tryptophan pyrrolase are under further investigation.

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