INHIBITION OF RABBIT INTESTINAL INDOLEAMINE 2,3-DIOXYGENASE BY COPPER CHELATORS*

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1. Introduction

Indoleamine 2,3-dioxygenase catalyzes a reaction similar to that catalyzed by L-tryptophan 2,3-dioxygenase (EC 1.13.11.11), L-tryptophan + O_2 → *N*-formylkynurenine, except that the former shows little substrate specificity while the latter shows absolute specificity for L-tryptophan. Indoleamine 2,3-dioxygenase was originally purified from rabbit intestine [1–4] and catalyzed the synthesis of D- and L-formylkynurenine from D- and L-tryptophan respectively. More recently, D- and L-5-hydroxytryptophan, tryptamine, and serotonin [5] and melatonin [6] have been shown to be substrates. The enzyme has also been detected in rabbit brain [6] and in rat brain [7,8] and intestine [9]. To elicit catalytic activity in vitro a requirement for methylene blue and ascorbate has been demonstrated [1,2], catalysis being inhibitable under these conditions by superoxide dismutase [10].

My previous work with L-tryptophan 2,3-dioxygenases from *Pseudomonas acidovorans* and rat liver (See Ref. 11, 12 for review) demonstrated that these enzymes contain two g-atoms copper and two moles heme per mole protein as functioning cofactors [13]. Copper must be present as Cu(I) for catalysis to proceed, while the heme may be ferro or ferri [14,15]. Of particular usefulness in those studies in the establishment of copper as being essential for catalysis was the use of the copper chelators, bathocuproinesulfonate [Cu(I) specific] and bathophenanthrolinesulfonate [Cu(I) and Fe(II) specific] [16], as inhibitors of catalysis [15]. Subsequent analysis of homogeneous preparations of pseudomonad and rat hepatic L-tryptophan 2,3-dioxygenases confirmed the presence of copper [15], as did the recent demonstration of the presence of radiocopper in the pseudomonad enzyme prepared from bacteria grown in a medium containing ^{64}Cu(II) [17].

Reported herein is the inhibition by bathocuproinesulfonate and by bathophenanthrolinesulfonate of catalysis by rabbit intestinal indoleamine 2,3-dioxygenase, suggesting that this enzyme is also a copper-containing enzyme in which copper must by present as Cu(I) for catalysis to proceed. The relative lack of inhibition by diethyldithiocarbamate [Cu(II) specific [18]] of catalysis by indoleamine 2,3-dioxygenase is supportive of this suggestion.

2. Materials and methods

Rabbit small intestines, Type I mature, were purchased fresh-frozen from Pel-Freez, Rogers, Arkansas. Methylene blue, sodium ascorbate, D-tryptophan, catalase, bathocuproinesulfonate, bathophenanthrolinesulfonate, and diethyldithiocarbamate were purchased from Sigma Chemical Company. All other chemicals were reagent grade quality or better.

Indoleamine 2,3-dioxygenase was prepared by a modification of the method of Yamamoto and Hayaishi [2]. Details will be published elsewhere. The purification involved homogenization, high speed centrifugation, streptomycin sulfate treatment, ammonium sulfate fractionation and precipitation, and DEAE-cellulose column chromatography. Enzyme

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preparations had a specific activity of 0.16–0.60 μmoles D- formylkynurenine synthesized min^{-1} mg $protein^{-1}$ at pH 7.0 and 37°C.

Assays contained 0.10 M potassium phosphate, pH 7.0, 3.0 mM D-tryptophan, 1.0 mM sodium ascorbate, 3.0 μM methylene blue and enzyme in a total volume of 1.0 ml [1,2]. *N*-formylkynurenine production was monitored at 321 nm using a Gilford spectrophotometer equipped with a recorder, automatic sample changer, and a thermostatted circulating water bath. Protein concentrations were determined turbidometrically [19].

3. Results

As shown in fig.1, both of the Cu(I) complexing agents, bathocuproinesulfonate and bathophenanthrolinesulfonate, progressively inhibit indoleamine 2,3-dioxygenase as their concentrations are increased. 50% inhibition occurs at concentrations of inhibitor near 50 μM under the conditions of this assay; 100% inhibition occurs near 0.75–1.0 mM concentration of inhibitor. Inhibition of catalysis by these two inhibitors occurs to the same extent whether they are present in the assay mixture initially or are added during the course of a catalytic reaction.

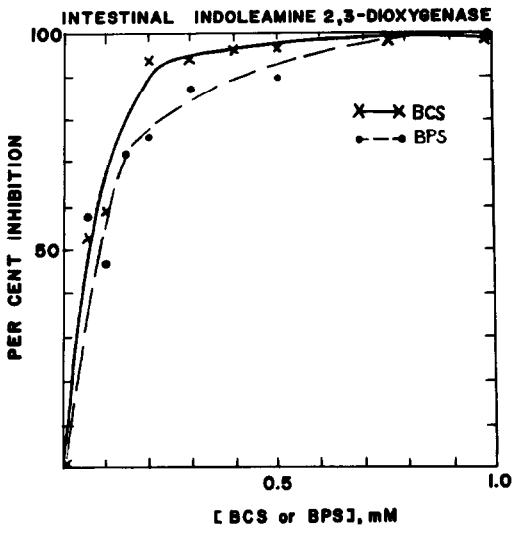

Fig.1. Per cent inhibition by bathocuproinesulfonate (BCS) and bathophenanthrolinesulfonate (BPS) of catalysis by rabbit intestinal indoleamine 2,3-dioxygenase. Assays were as described in Materials and methods, monitoring D-*N*-formylkynurenine formation at 321 nm, with the indicated concentrations of inhibitor added. For each determination the rate of a blank assay containing all reagents except enzyme was subtracted from that of an assay containing enzyme. 245 μg enzyme, specific activity 0.16, was used in each assay. Assays were initiated by the addition of enzyme last.

In fig.2 are shown the results of a kinetic analysis of the type of inhibition of indoleamine 2,3-dioxygenase caused by bathocuproinesulfonate and bathophenanthrolinesulfonate. As can be seen, both of these compounds are uncompetitive inhibitors of catalysis with respect to D-tryptophan. K_m for D-tryptophan (pH 7.0, 37°C) was calculated to be 8.9 μM, K_i for bathocuproinesulfonate was 220 μM, and K_i for bathophenanthrolinesulfonate was 54 μM.

In table 1 are shown the results of an experiment in which the Cu(II) complexing agent, diethyldithiocarbamate, was tested as an inhibitor of catalysis by indoleamine 2,3-dioxygenase. As can be seen, a very high concentration of diethyldithiocarbamate (10 mM) inhibited D-formylkynurenine synthesis only slightly (12%). Catalase was included in these assays to eliminate a large blank rate due to the nonenzymic reaction of diethyldithiocarbamate with H_2O_2 generated by methylene blue and ascorbate [20]. Diethyldithiocarbamate at lower concentrations did not inhibit significantly.

4. Discussion

The data presented in this report strongly implicate the involvement of copper in catalysis by rabbit intestinal indoleamine 2,3-dioxygenase. Inhibition by bathocuproinesulfonate and bathophenanthrolinesulfonate suggests Cu(I) in the enzyme as being essential for catalysis. The refractoriness of the enzyme to inhibition by diethyldithiocarbamate, which binds Cu(II) but not Cu(I), is consistent with this suggestion.

The uncompetitive nature of the inhibition by these chelators with respect to D-tryptophan implies that only in the D-tryptophan enzyme complex does Cu(I) become accessible to the chelators. Either a conformational change in the protein occurs or a valence change of the copper from Cu(II) to Cu(I) occurs in the complex.

Confirmation of these proposals awaits analysis

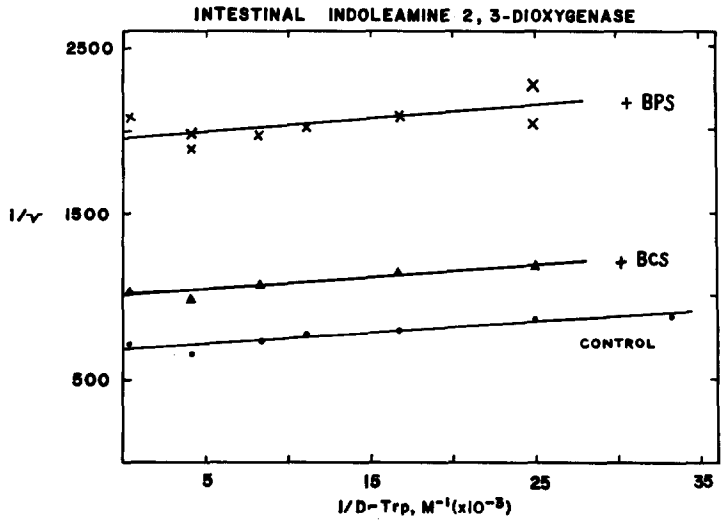

Fig.2. Lineweaver–Burk plots of D-*N*-formylkynurenine synthesis by indoleamine 2,3-dioxygenase as a function of D-tryptophan concentration in the absence and presence of bathocuproinesulfonate and bathophenanthrolinesulfonate. Assays were as described in Materials and methods with the concentration of D-tryptophan being varied as indicated. Control, no further addition; + BCS, including 0.1 mM bathocuproinesulfonate; + BPS, including 0.1 mM bathophenanthrolinesulfonate. Appropriate blanks lacking enzyme were subtracted from the measured rates in each case. Lines were drawn as a best fit for the data using a linear regression computor program. 190 μg enzyme, specific activity 0.60, was used in each assay. Assays were initiated by the addition of enzyme last. (v) is nmoles D-*N*-formylkynurenine synthesized min^{-1}.

of homogeneous preparations of rabbit intestinal indoleamine 2,3-dioxygenase for copper. Such studies are currently underway, but will take time due to the amounts of protein necessary for adequate analysis of stoichiometric amounts of copper and to the relatively low amounts of enzyme present in rabbit small intestine. It will be of great interest to determine if indoleamine 2,3-dioxygenase contains the same cofactors as do the bacterial and hepatic L-tryptophan 2,3-dioxygenases. The similarity of their catalyzed reactions would suggest this, but their different idiosyncrasies, such as the methylene blue–ascorbate requirement and

Table 1
Effect of diethyldithiocarbamate on catalysis by indoleamine 2,3-dioxygenase[a]

Addition			ΔA_{321} min^{-1}		D-*N*-Formylkynurenine Synthesized	
Enzyme[b]	Catalase[c]	Diethyldithiocarbamate[d]	Observed	Net	nmoles min^{-1}	(%) Inhibition
–	+	–	0.0008			
+	+	–	0.0215	0.0207	5.52	0
–	+	+	0.0018			
+	+	+	0.0200	0.0182	4.85	12

[a] Assays were as described in Materials and methods.
[b] 245 μg indoleamine 2,3-dioxygenase, specific activity 0.16.
[c] 100 μg catalase, specific activity 5.1×10^4.
[d] 10 mM diethyldithiocarbamate.

inhibition of catalysis by superoxide dismutase of the former, imply that there are dissimilarities between the two types of enzymes which deserve more detailed study. The importance of indoleamine 2,3-dioxygenase in regulating the cellular levels of tryptophan metabolites is only beginning to be explored. The properties of this enzyme deserve further study, especially if copper is indeed found to be a cofactor in addition to the previously discovered heme cofactor.

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