PURIFICATION OF TRYPTOPHAN OXYGENASE AND ITS INTERACTION WITH CADMIUM

E.M. Diamond, Anna Jedeikin and J.E. Kench.
South African Medical Research Council Protein Research Unit,
Department of Chemical Pathology, University of Cape Town,
Medical School, Cape Town (South Africa).

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SUMMARY

Purification of tryptophan oxygenase (L-tryptophan oxidoreductase EC 1.13.1.12) from Pseudomonas acidovorans is described. When chromatographed on Sephadex G-200 or on DEAE Sephadex A-50, the enzyme was eluted in two distinct bands. Over the concentration range 10^{-6} - 10^{-4} M, cadmium stimulated the activity of the enzyme but inhibited it non-competitively at higher concentrations. A mechanism is suggested for the behaviour of the enzyme towards cadmium.

INTRODUCTION

One of the effects of cadmium poisoning on mammals is that albumin molecules of relatively low molecular weight appear in the circulation and are excreted in the urine (1). They retain the immunological and electrophoretic characteristics of serum albumin and have been called 'minialbumins'. Minialbumins differ from normal albumin in having a somewhat different amino acid composition, containing less lysine and cystine and no tryptophan (2,3).

It has been shown in rats (4) and in chicks (5), both in vivo and in vitro that tryptophan oxygenase is stimulated by added cadmium. In this study we have demonstrated a similar effect on the bacterial enzyme obtained from Pseudomonas acidovorans. We have also proposed a mechanism which attempts to account for the changes of the activity of tryptophan oxygenase in response to alterations in the concentration of cadmium ions. In addition we have adduced evidence that the enzyme may exist in more than one active form.

MATERIALS AND METHODS

Cultures of Pseudomonas acidovorans (N.C.I.B. 9681, Torry Research Station, Aberdeen, Scotland) were grown at 26° in a medium with the following constituents: NH_4Cl (0.5% w/v), 'Difco' yeast extract (0.05% w/v), $MgSO_4$ (0.02% w/v), K_2HPO_4 (0.15% w/v), KH_2PO_4 (0.05% w/v), L-tryptophan (0.1% w/v). The medium was sterilized by autoclaving at 15 p.s.i. for 15 minutes. All reagents were Analar grade, except for the yeast extract, which was purchased from the Difco Company and L-tryptophan

which was obtained from either the Hopkin and Williams Chemical Company or the Sigma Chemical Company. Sephadex was supplied by Pharmacia, Uppsala, Sweden.

A cell suspension (200 ml) in the above medium was prepared by inoculation with cells from a sloppy agar stock culture and synthesis of tryptophan oxygenase was induced by incubation overnight in the presence of the contained L-tryptophan. The cell suspension was then added aseptically to a flask containing 10 l of medium. Filtered air was bubbled through the solution continuously for 16 - 17 hours at 26°. At the end of this period, the cells were harvested by batchwise centrifugation for 10 mins at 5,000 x g. The bacterial cells (± 10 g) were washed once with 200 ml potassium phosphate buffer (10 mM, pH 7.0) containing L-tryptophan (10 mM) and stored frozen at -15°, usually for not longer than 2 weeks.

ENZYME PURIFICATION

The procedure followed was essentially that described by Poillon et al (6), with certain modifications. In the following account of the purification, "buffer" means a 10 mM potassium phosphate buffer, pH 7.0, containing 10 mM L-tryptophan, unless otherwise stated. All operations were performed at 0 - 4°, except where noted. The cells were suspended in 4 volumes (w/v) of buffer and sonicated for two 3-minute periods at maximum intensity with a "Branson Sonifier" using a 0.5 inch probe. The volume sonicated each time was ± 20 ml and the vessel was maintained at low temperature by cooling in an ice bath. Cell debris was removed by centrifuging at 30,000 x g for 1 hour. Additional quantities of enzyme could be extracted from the debris by resuspension in the buffer and resonicating. This could be repeated three or four times.

An aqueous streptomycin sulphate solution, pH 7.0 (0.25 volumes) was added to the supernatant with stirring which was continued for 20 minutes. The mixture was centrifuged at 12,000 x g for 15 minutes and the precipitate was discarded.

An equal volume of saturated ammonium sulphate, pH 7.0, was added to the supernatant solution dropwise with stirring. When all the ammonium sulphate had been added, stirring was continued for 1 hour. The precipitate was collected by centrifuging at 12,000 x g for 10 minutes and dissolved in a minimal volume of buffer.

The enzyme solution was diluted in 50 mM potassium phosphate, pH 7.0 (5 volumes) containing 60 mM L-tryptophan. Haematin, (6.1 mM), which Poillon et al (6) added at this step of the purification, was omitted in the present work as difficulty was experienced in dissolving this high concentration of haematin at pH 7.0. The solution

was placed in a water bath at 60° and kept at this temperature for 8 minutes with continuous shaking. The solution was then cooled immediately in an ice bath and the precipitated protein was removed by centrifuging at 12,000 x g for 10 minutes. An equal volume of saturated ammonium sulphate was added to the supernatant as described in a previous step and the precipitate was once again collected by centrifugation and dissolved in the minimal volume of buffer.

The enzyme solution was applied to a Sephadex G-200 column (2.8 x 30 cm) which had previously been equilibrated with 10 mM L-tryptophan. The enzyme was eluted overnight with buffer in 5 ml fractions at a flow rate of 30 ml/hour. Aliquots of each fraction were assayed for enzyme activity. The elution pattern is shown in Fig. 1. Fractions 18 - 40 were combined and treated with saturated ammonium sulphate in the usual way. The precipitated protein was isolated by centrifugation and dissolved in potassium phosphate buffer, 50 mM, containing 10 mM L-tryptophan.

This solution was then adsorbed on to a DEAE Sephadex A-50 column (2.8 x 18 cm) previously equilibrated with the same buffer. Gradient elution was carried out between 50 mM and 300 mM potassium phosphate buffers, pH 7.0, containing 10 mM L-tryptophan. Fractions of 5 ml were collected and aliquots were assayed for enzyme activity. The elution profile is shown in Fig. 2. Total protein elution profiles were not determined as tryptophan in the buffer interfered with the photometric assay of the proteins. Fractions 26 - 48 were combined and an equal volume of saturated ammonium sulphate added in the usual way. No precipitate was obtained. The solution was dialysed against 5 changes of buffer for 48 hours. The dialysed solution was freeze-dried. A small quantity of solid remained and was dissolved in a Tris/borate buffer (50 mM, pH 8.5). An aliquot was subjected to polyacrylamide disc gel electrophoresis. When stained for protein with amido black, one prominant band and three or four very light diffuse ones were visible.

TABLE I is a summary of the purification procedure.

ASSAY PROCEDURE

The enzyme (0.35 ml) was incubated with haematin (0.1 ml, 50 mM) and buffer (0.75 ml) for exactly 15 minutes at 37° in a thermostatted Zeiss PMQII spectrophotometer. At the end of this period, 0.1 ml distilled water was added to the blank and the reaction was started by the addition of 0.1 ml L-tryptophan (50 mM) to the test cuvette (final concentration = 3.85 mM). The rate of formation of L-formyl kynurenine was followed by the increase in O.D. at 321 nm over a 15 min period. Activity of tryptophan oxygenase

TABLE 1

STEP	PURIFICATION
Sonic extract	-
Streptomycin Sulphate	1.5 x
Ammonium Sulphate	8 x
Heat treatment	40 x
Sephadex G-200 filtration	50 x
DEAE Sephadex Chromatography	146 x

was calculated from the slope of the plot of optical density against time. The reaction rate was linear for approximately 10 to 15 minutes.

In the routine assay procedure, the buffer used was 10 mM potassium phosphate, pH 7.0. In the investigations involving cadmium, 50 mM Tris, pH 7.0, was employed. In this case the enzyme was incubated with 0.65 ml buffer and 0.1 ml $CdCl_2$ solution for 15 minutes before addition of the substrate.

RESULTS AND DISCUSSION

The enzyme was eluted from the Sephadex G-200 column in two bands (See Fig. 1). This implies that either it exists in two different forms or that it dissociates into two smaller subunits on the column. Also when eluted from the DEAE Sephadex A 50 column, it appeared as two separate entities (See Fig. 2).

The enzyme preparation which was studied in relation to cadmium was that obtained after heat treatment at 60° during the purification procedure. It was considered that heat treatment yielded a preparation which was suitable for our purposes. When the enzyme was purified further, it was found to be progressively less stable, even though the specific activity increased.

Fig. 3 shows that as the concentration of cadmium was raised from 1.0×10^{-7} M, the percentage stimulation increased and reached a maximum in the region of 7.0×10^{-6} M-

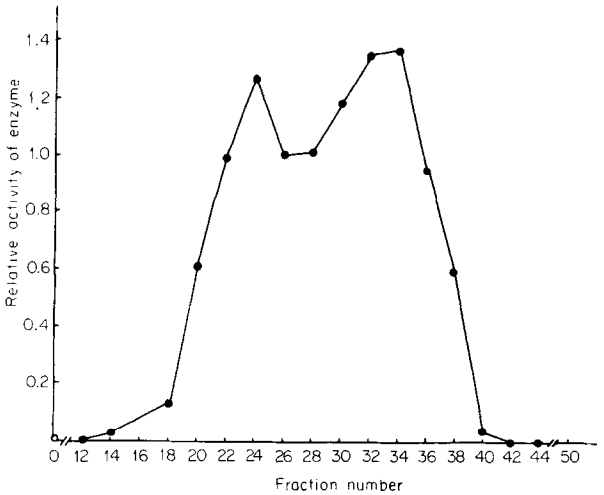

Fig. 1 COLUMN CHROMATOGRAPHIC PATTERN OF TRYPTOPHAN OXYGENASE ON SEPHADEX G-200

See text for experimental details. Aliquots of each fraction were assayed as described in the text. The enzyme activity is expressed as ΔO.D. at 321 nm/ 10 minutes.

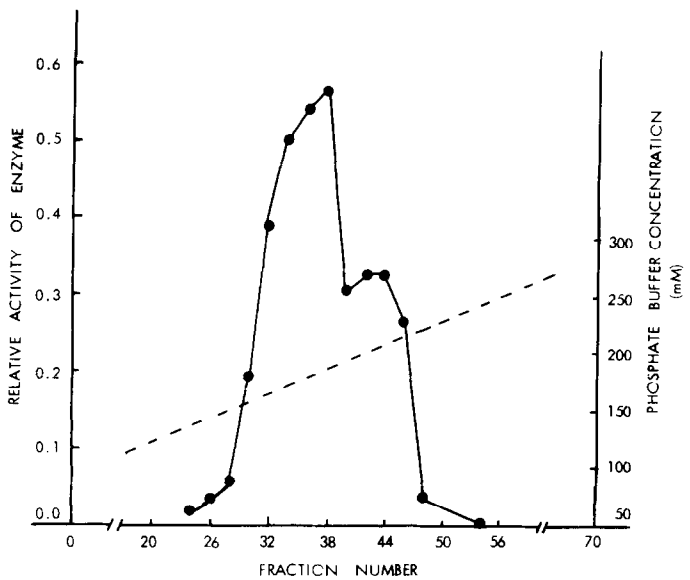

Fig. 2 COLUMN CHROMATOGRAPHIC PATTERN OF TRYPTOPHAN OXYGENASE ON DEAE SEPHADEX A50

Experimental details appear in the text. The enzyme activity is expressed as ΔO.D. at 321 nm/10 minutes.

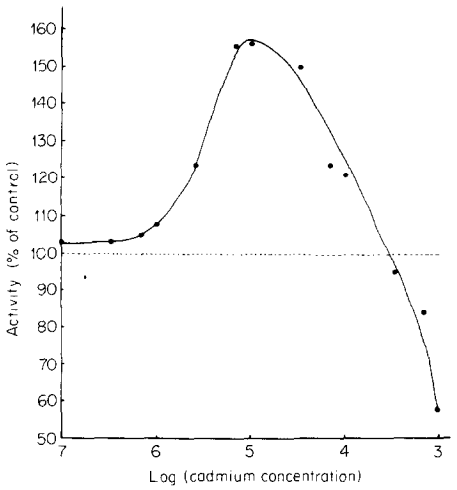

Fig. 3 EFFECT OF VARYING THE CONCENTRATION OF CADMIUM ON TRYPTOPHAN OXYGENASE

The enzyme activity was determined as described in the text, $CdCl_2$ solutions being added to the incubation mixture.

1.0×10^{-5}M. When the concentration was further elevated, the extent of stimulation declined. The enzyme was progressively inhibited by cadmium in the range 5.0×10^{-4}M-1.0×10^{-3}M. When the cadmium concentration reached 1.0×10^{-3}M, activity of tryptophan oxygenase was only approximately 60% of the control, to which no cadmium had been added. The control preparations contained less than 1.0×10^{-8}M cadmium. At concentrations above 1.0×10^{-3}M, cadmium was precipitated in the reaction mixture.

Fig. 4 shows that the inhibition of the enzyme by cadmium was non-competitive. Km for the enzyme with respect to L-tryptophan was found to be 1.42×10^{-2}M. As can also be seen from the figure, when an activating cadmium concentration (3.3×10^{-5}M) was present, Km was lowered. In this case it was found to be 7.7×10^{-3}M.

It would appear that there are two sites on the enzyme molecule which are receptive to cadmium. The first may be designated "stimulatory" and the second "inhibitory".

The results in Table II indicate that in the presence of a stimulatory concentration of cadmium (3.3×10^{-5}M), the activity of the enzyme was independent of the period of incubation with the metal ion. It is also evident that the attachment of the cadmium to these stimulatory sites must be an extremely fast reaction.

When the enzyme was incubated with an inhibitory concentration of cadmium ($7.0 \times$

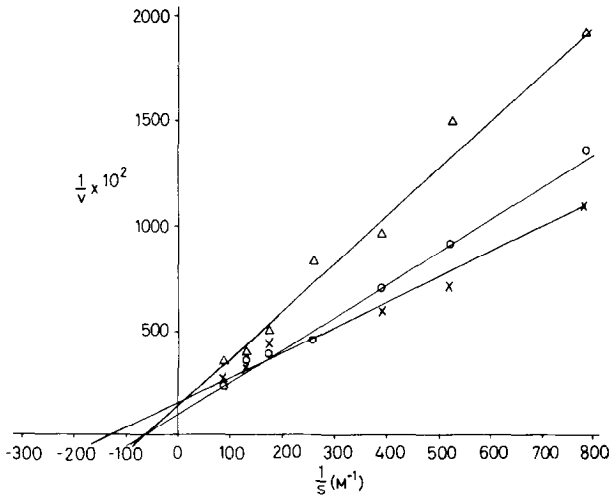

Fig. 4 LINEWEAVER AND BURKE PLOTS

The enzyme was assayed as described in the text. The concentration of tryptophan was varied, whilst the enzyme concentration was kept constant. Δ, 7.0×10^{-4}M $CdCl_2$; X, 3.3×10^{-5}M $CdCl_2$; 0, ABSENCE OF CADMIUM.[2]

10^{-4}M), there was a fall in the activity of the enzyme on prolonging the period of incubation. The observed changes could be accounted for in the following way : Cadmium saturated the stimulatory sites prior to attachment to the inhibitory ones. Possibly certain inhibitory sites exist which have almost, but not quite, the same affinity for cadmium as the stimulatory ones, and a correspondingly rapid response to metal binding. This would explain the somewhat diminished activity (89.6%) of the enzyme when T=0. The subsequent gradual fall in the activity of the enzyme on prolonging the time of incubation with 7.0×10^{-4}M cadmium is indicative of further, albeit delayed, binding to the remaining inhibitory sites on the enzyme molecule. These sites appear to have a lesser affinity for cadmium than those which are saturated initially.

The number of atoms of cadmium involved in the interaction with receptor sites on tryptophan oxygenase remains to be determined on a pure, homogeneous, preparation of the enzyme. The bacterial enzyme appears to offer the best opportunity in this regard. In our experience, the isoenzymes of the liver of rabbit and chicken are much less stable than the bacterial enzyme.

TABLE II

EFFECT OF INCUBATING TRYPTOPHAN OXYGENASE WITH CADMIUM FOR DIFFERENT LENGTHS OF TIME

The enzyme was incubated with the $CdCl_2$ solutions for the indicated periods of time. The assay was commenced in the usual way by the addition of tryptophan. In the case where T=O, $CdCl_2$ and tryptophan were added simultaneously to the reaction cuvette.

Incubation Time (T)	Cadmium Concentration	Activity of Enzyme
(Minutes)	(M)	(% of control)
15	0 (Control)	100
0	7.0×10^{-4}	89.6
2	7.0×10^{-4}	79.8
15	7.0×10^{-4}	67.8
0	3.3×10^{-5}	145
2	3.3×10^{-5}	147
15	3.3×10^{-5}	144

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