Cinnabarinatase Synthase from Baboon (*Papio ursinus*) Liver

IDENTITY WITH CATALASE

By NERINA SAVAGE and WOLFGANG PRINZ

Department of Physiological Chemistry, Medical School, University of the Witwatersrand,
Johannesburg, Republic of South Africa

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The enzyme cinnabarinatase synthase was purified from the nuclei of baboon liver. Two purified fractions were obtained that exhibited a typical haem protein absorption spectrum; a probable identity with catalase was demonstrated. It was confirmed that catalase in the presence of Mn^{2+} produces cinnabarinatase from 3-hydroxyanthranilate. Doubt is expressed on the existence of a distinct cinnabarinatase synthase enzyme.

The enzyme cinnabarinatase synthase catalyses the oxidative dimerization of two molecules of 3-hydroxyanthranilate to produce cinnabarinatase (2-amino-3-oxo-3H-phenoxazine-1,9-dicarboxylate (Subba Rao & Vaidyanathan, 1966). Both substrate and product induce bladder tumours in experimental animals (Boyland, 1960; Byran *et al.*, 1964). The substrate 3-hydroxyanthranilate is a normal metabolite of dietary tryptophan excreted in the urine in man. 3-Hydroxyanthranilate and other metabolites of tryptophan are increased in the urine of some patients with bladder and breast carcinomas. It has not been unequivocally established whether the abnormal tryptophan metabolites are involved in the aetiology of any of these cancers.

As part of a study on the possible mechanisms of carcinogenesis by 3-hydroxyanthranilate and cinnabarinatase we wished to isolate and study the enzyme cinnabarinatase synthase, a reputed nuclear enzyme (Subba Rao *et al.*, 1965; Subba Rao & Vaidyanathan, 1966).

This present paper concerns the attempted isolation of the enzyme from baboon (*Papio ursinus*) liver. On the basis of experimental observations on this enzyme, and the demonstration that catalase in the presence of Mn^{2+} can oxidatively dimerize 3-hydroxyanthranilate to form cinnabarinatase, doubt is cast on the existence of a distinct cinnabarinatase synthase.

Materials and Methods

Chemicals

3-Hydroxyanthranilic acid and bovine liver catalase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All reagents were of analytical grade. Solutions were prepared with double-distilled water.

Baboon liver catalase

The method described for the preparation of catalase from ox liver by Sarkar & Sumner (1955) was used for the isolation of catalase from baboon liver.

Purification of cinnabarinatase synthase

Unless otherwise stated all procedures were carried out at 4°C. Nuclei were isolated from baboon liver by the procedure described for the preparation of rat liver cinnabarinatase synthase (Subba Rao *et al.*, 1965). Phase-contrast microscopy of the nuclear preparations revealed considerable whole-cell contamination. Addition of Triton X-100 (1%) to the nuclear suspension and overnight storage at 4°C released a considerable amount of cinnabarinatase synthase activity. This was recovered in the supernatant after centrifuging at 10000g for 30 min.

Ammonium sulphate fractionation. Saturated $(NH_4)_2SO_4$ solution was added to the supernatant to bring it to 40% saturation. On centrifugation at 35000g for 30 min the bulk of the Triton X-100 formed a skin on the surface of the fluid and contained the precipitated protein. The clear yellowish-brown solution below the skin was carefully siphoned off and the precipitate discarded. The coloured solution was brought to 60% saturation with further saturated $(NH_4)_2SO_4$ solution. The small precipitate that formed was collected by centrifugation at 35000g for 30 min and dissolved in a minimum volume of 66mM-potassium phosphate buffer, pH 7.4.

Gel filtration on Sephadex G-100. The 60%-satd. $(NH_4)_2SO_4$ fraction was placed on a column (3.2cm × 60cm) of Sephadex G-100 and eluted with 66mM-potassium phosphate buffer, pH 7.4. Active fractions were pooled and the protein was recovered by bringing the solution up to 70% saturation with $(NH_4)_2SO_4$. The precipitate obtained after centri-

fugation was dissolved in 66mM-potassium phosphate buffer, pH7.4, and dialysed against 0.01M-sodium acetate buffer, pH7.0.

Chromatography on CM-cellulose. The dialysed protein solution was added to a column (1 cm $\times$ 10 cm) of CM-cellulose CM-32 equilibrated in 0.01M-sodium acetate buffer, pH7.0. Two active fractions were eluted: the first after addition of 0.04M-KCl to the eluting buffer and the second when the KCl concentration was increased to 0.08M. In both cases the eluate had a greenish-yellow colour.

Spectral determinations were carried out in a Beckman DBG coupled to a 25 cm (10 in) recorder.

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme assays

Cinnabarinatase synthase. The assay incubation contained 3-hydroxyanthranilic acid (5 μ mol) in a mixture of 0.05M-Tris/maleate buffer, pH7.4 (3.0 ml), and 5.3mM-MnSO₄ (0.5 ml). The reaction was initiated by the addition of diluted enzyme solution (2–20 μ l) and allowed to proceed at 30°C for 30 min. The reaction was stopped by the addition of 1.0 ml of 20% (w/v) orthophosphoric acid and the A_{450} was read. Blanks were prepared by adding all the reagents to a boiled enzyme solution. Enzymic activity could also be followed by monitoring directly the change in A_{450} in the spectrophotometer, temperature-controlled at 30°C. The unit of activity is defined as 1 μ mol of cinnabarinatase produced/min.

Catalase. This was measured spectrophotometrically at 30°C by following the decrease in A_{240} . The substrate was prepared by adding 0.1 ml of 30% (w/v) H₂O₂ to 50 ml of 0.05M-potassium phosphate buffer, pH7.0. The reaction mixture contained 2.9 ml of the substrate solution and 100 μ l of diluted catalase. Under these conditions a decrease in absorbance of 0.05 is equivalent to 3.45 μ mol of H₂O₂ decomposed (Sigma Chemical Co. catalogue).

Gel electrophoresis

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (0.1%) was carried out by the method of Weber & Osborn (1969). Electrophoresis of purified fractions was also carried out in 50mM-Tris/380mM-glycine, pH8.4 (running buffer), and 180mM-Tris/HCl buffer, pH8.9 (gels), by the method of Davis (1964). After electrophoresis the gels were either stained for protein or used for detecting cinnabarinatase synthase activity by immersing the gels in 10 ml of 0.05M-Tris/maleate buffer, pH7.4, containing 5 μ mol of 3-hydroxyanthranilic acid and 3 μ mol of MnSO₄. The development of an orange-yellow band indicated the position of the enzyme. Gels were scanned in the linear attachment to the Gilford spectrophotometer.

Isolation of the enzymic reaction product of 3-hydroxyanthranilate

The reaction mixture contained 100 ml of 0.03M-potassium phosphate buffer, pH7.0, 50 mg of 3-hydroxyanthranilic acid and 12.5 ml of 5.4mM-MnSO₄. Bovine catalase (14 mg) or a purified enzyme fraction (0.1 mg) in 1 ml of 0.03M-potassium phosphate buffer, pH7.0, was added to the reaction mixture. Boiled enzyme preparations added to the reaction mixtures served as controls. After 7 h incubation at 30°C the reaction was terminated by adding 40 ml of 20% (w/v) orthophosphoric acid solution. Extraction with ethyl acetate, drying over anhydrous Na₂SO₄, and evaporation to dryness on a rotary evaporator at 30°C yielded a reddish-brown residue. This was dissolved in acetone and crystallized, to obtain dark-reddish-brown crystals. The product was compared with authentic cinnabarinic acid by t.l.c. on silica-gel plates (Merck 5553).

Results

A summary of the purification of cinnabarinatase synthase is given in Table 1. For comparison, the various stages of the purification of baboon catalase is also shown, indicating the co-purification with cinnabarinatase synthase (Table 2).

The optimum pH for the synthase was found to be 7.4 from assays in Tris/maleate buffer (0.05M) in the pH range 6.07–8.05.

Comparison of purified enzyme with catalase

Cinnabarinatase synthase activity was proportional to protein concentration with baboon catalase (tested up to 300 ng/ml) and with bovine catalase (tested up to 800 ng/ml). With the purified synthase, however, proportionality was not sustained at protein concentrations greater than 100 ng/ml, conceivably owing to the presence of a protein inhibitor.

Enzymic activity is dependent on the presence of Mn²⁺ and is very low in its absence (Table 3). Azide inhibited the cinnabarinatase synthase activity with the purified enzyme fractions and with catalase.

The enzyme protein isolated from impure nuclei of baboon liver exhibited a typical visible spectrum of a haem protein and resembled that of catalase. Further, catalase purified from baboon liver showed cinnabarinatase synthase activity (Table 2). Significantly, the ratio of catalase/cinnabarinatase synthase activity remained constant for successive stages of purification, with one exception: it is possible that the abnormally high ratio observed for the eluate from the tricalcium phosphate gel is attributable to inhibition of cinnabarinatase synthase activity by Ca²⁺.

After incubation of 3-hydroxyanthranilic acid with baboon or bovine catalase, or with either of the purified enzyme fractions, the reaction product was isolated and compared with authentic cinnabarinic acid.

Table 1. *Purification of cinnabarinatase from baboon liver*Enzyme activity is expressed as μmol of cinnabarinatase produced/min. Experimental details are given in the text.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification	Yield (%)
Triton X-100 extract	230	9660	1104	0.11	1	100
Precipitate 40–60%–satd. $(\text{NH}_4)_2\text{SO}_4$	9	630	655	1.0	9.5	59.3
Chromatography on Sephadex G-100	98	136	170	1.7	15.8	15
Chromatography on CM-cellulose						
0.08M-KCl	2.7	2.65	21.6	8.0	72.7	2.9
0.04M-KCl	1.2	0.55	10.7	8.9	80.9	

Table 2. *Purification of catalase from baboon liver*

Experimental details are given in the text. The method used was that of Sarkar & Sumner (1955).

State	Volume (ml)	Total protein (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)		Activity ratio catalase/cinnabarinatase
			Catalase	Cinnabarinatase	
Filtrate after chloroform/ethanol treatment	1100	5863	1810	0.50	3600
Supernatant after acidification	1000	5750	840	0.22	3800
Elate from calcium phosphate gel	120	690	3300	0.33	10000
Dialysis precipitate	—	9.4	22300	6.1	3700

Table 3. *Effect of Mn^{2+} concentration on the formation of cinnabarinatase*

(a) 0.04M-KCl fraction from baboon liver; (b) catalase purified from baboon liver. The reaction medium was that described in the text except that in (a) 4.4 μmol of substrate and 0.08 μg of enzyme and in (b) 5.6 μmol of substrate and 0.03 μg of enzyme protein were added.

	Concn. of MnSO_4 (mM)	Cinnabarinatase formed in 30 min (nmol)
(a)	0	5.2
	0.076	8.3
	0.15	23.9
	0.38	46.8
	0.76	56.8
	1.1	65.1
	1.5	80.7
(b)	0	1.0
	0.076	20.0
	0.15	29.2
	0.37	55
	0.75	64

those of cinnabarinic acid. In ethanol the absorption maxima were at 450, 430 and 235 nm and appeared to be identical with those reported for cinnabarinic acid by Butenandt *et al.* (1957).

Gel electrophoresis

After polyacrylamide-gel electrophoresis in sodium dodecyl sulphate/phosphate buffer, the purified enzyme showed one major band and a minor band. The apparent molecular weight of the protein of the major band, by comparison with standard markers [serum albumin (68000), bovine catalase (60000), ovalbumin (44000), lactate dehydrogenase (36000) and cytochrome *c* (12500)] ranged between 65000 and 68000. Bovine catalase and baboon catalase gave values of 60000 and 65000 respectively for the subunits of these two enzymes.

Electrophoresis in Tris/glycine buffer, pH 8.9, showed close correspondence between the cinnabarinatase activity and the major protein band when stained with Coomassie Blue.

Discussion

The chromophore formed in the incubation system that contained Mn^{2+} , catalase and 3-hydroxy-anthranilic acid has characteristics similar to that produced by cinnabarinatase (Subba Rao & Vaidyanathan, 1966). Ishiguro *et al.* (1971) have observed a similar reaction with 3-hydroxy-anthranilic acid, Mn^{2+} and haemoglobin. It must be

barinic acid. The products were examined by t.l.c. with butanol/acetic acid/water (4:2:1, by vol.), ethanol and ethyl acetate/methanol (99:1, v/v). The R_f values (0.54, 0.24 and 0.0 respectively) were identical with those of authentic cinnabarinic acid for all enzyme incubations. The visible and u.v. spectra of the reaction products were in good agreement with

presumed that oxidation of 3-hydroxyanthranilic acid occurs by action of a metal-bound haem protein in the presence of oxygen.

Attempts to isolate a specific cinnabarinatase synthase have been unsuccessful. Our evidence so far suggests that cinnabarinatase synthase and catalase activities are properties of the same protein. It is unclear whether the enzyme described by Morgan *et al.* (1965) to be present in a soluble liver fraction from poikilothermic vertebrates that also catalyses the oxidation of 3-hydroxyanthranilate to cinnabarinatase can be similarly ascribed to catalase. In this instance the enzyme did not require Mn^{2+} for activity.

Since it has been shown that cinnabarinatase is carcinogenic when implanted into bladders of mice (Boyland, 1960) and further that 3-hydroxyanthranilate, the precursor of cinnabarinatase, has an affinity for the bladder epithelium of rats and mice, it would be worth while to investigate the synthesis of cinnabarinatase *in vitro* and *in vivo*. The observation that cinnabarinatase inhibits mitochondrial respiration and the suggestion that this interaction of cinnabarinatase with the oxidative processes in mitochondria may be involved in the induction of bladder tumours in man (Zollner, 1976) would support this suggestion.

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