Cinnabarinate Synthase from Baboon (Papio ursinus) Liver

IDENTITY WITH CATALASE

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The enzyme cinnabarinate 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 Mn²⁺ produces cinnabarinate from 3-hydroxyanthranilic acid.

Doubt is expressed on the existence of a distinct cinnabarinate synthase enzyme.

The enzyme cinnabarinate synthase catalyses the oxidative dimerization of two molecules of 3-hydroxyanthranilic acid to produce cinnabarinate (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 cinnabarinate we wished to isolate and study the enzyme cinnabarinate 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 Mn²⁺ can oxidatively dimerize 3-hydroxyanthranilate to form cinnabarinate, doubt is cast on the existence of a distinct cinnabarinate 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 cinnabarinate 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 cinnabarinate 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 cinnabarinate synthase activity. This was recovered in the supernatant after centrifuging at 10000 g for 30 min.

Ammonium sulphate fractionation. Saturated (NH₄)₂SO₄ solution was added to the supernatant to bring it to 40% saturation. On centrifugation at 35000 g 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 (NH₄)₂SO₄ solution. The small precipitate that formed was collected by centrifugation at 35000 g for 30 min and dissolved in a minimum volume of 66 mM-potassium phosphate buffer, pH 7.4.

Gel filtration on Sephadex G-100. The 60%-satd.- (NH₄)₂SO₄ fraction was placed on a column (3.2 cm × 60 cm) of Sephadex G-100 and eluted with 66 mM-potassium phosphate buffer, pH 7.4. Active fractions were pooled and the protein was recovered by bringing the solution up to 70% saturation with (NH₄)₂SO₄. The precipitate obtained after centri-
fugation was dissolved in 66 mM-potassium phosphate buffer, pH 7.4, and dialysed against 0.01 M-sodium acetate buffer, pH 7.0.

Chromatography on CM-cellulose. The dialysed protein solution was added to a column (1 cm x 10 cm) of CM-cellulose CM-32 equilibrated in 0.01 M-sodium acetate buffer, pH 7.0. Two active fractions were eluted: the first after addition of 0.04 M-KCl to the eluting buffer and the second when the KCl concentration was increased to 0.08 M. 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

Cinnabarinate synthase. The assay incubation contained 3-hydroxyanthranilic acid (5 μmol) in a mixture of 0.05 M-Tris/maleate buffer, pH 7.4 (3.0 ml), and 5.3 mM-MnSO₄ (0.5 ml). The reaction was initiated by the addition of diluted enzyme solution (2–20 μl) 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 A₄₅₀ 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 A₄₅₀ in the spectrophotometer, temperature-controlled at 30°C. The unit of activity is defined as 1 μmol of cinnabarinate produced/min.

Catalase. This was measured spectrophotometrically at 30°C by following the decrease in A₅₄₀. The substrate was prepared by adding 0.1 ml of 30% (w/v) H₂O₂ to 50 ml of 0.05 M-potassium phosphate buffer, pH 7.0. The reaction mixture contained 2.9 ml of the substrate solution and 100 μl of diluted catalase. Under these conditions a decrease in absorbance of 0.05 is equivalent to 3.45 μmol 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 50 mM-Tris/380 mM-glycine, pH 8.4 (running buffer), and 180 mM-Tris/HCl buffer, pH 8.9 (gels), by the method of Davis (1964). After electrophoresis the gels were either stained for protein or used for detecting cinnabarinate synthase activity by immersing the gels in 10 ml of 0.05 M-Tris/maleate buffer, pH 7.4, containing 5 μmol of 3-hydroxyanthranilic acid and 3 μmol 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 0.03 M-potassium phosphate buffer, pH 7.0, 50 mg of 3-hydroxyanthranilic acid and 12.5 ml of 5.4 mM-MnSO₄. Bovine catalase (14 mg) or a purified enzyme fraction (0.1 mg) in 1 ml of 0.03 M-potassium phosphate buffer, pH 7.0, 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-redish-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 cinnabarinate 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 cinnabarinate synthase (Table 2).

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

Comparison of purified enzyme with catalase

Cinnabarinate 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 cinnabarinate 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 cinnabarinate synthase activity (Table 2). Significantly, the ratio of catalase/cinnabarinate 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 cinnabarinate 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 cinn-
IDENTITY OF CINNABARINATE SYNTHASE WITH CATALASE

Table 1. Purification of cinnabarinate synthase from baboon liver

Enzyme activity is expressed as μmol of cinnabarinate produced/min. Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100 extract</td>
<td>230</td>
<td>9660</td>
<td>1104</td>
<td>0.11</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Precipitate 40–60% satd. (NH₄)₂SO₄</td>
<td>9</td>
<td>630</td>
<td>655</td>
<td>1.0</td>
<td>9.5</td>
<td>59.3</td>
</tr>
<tr>
<td>Chromatography on Sephadex G-100</td>
<td>98</td>
<td>136</td>
<td>170</td>
<td>1.7</td>
<td>15.8</td>
<td>15</td>
</tr>
<tr>
<td>Chromatography on CM-cellulose</td>
<td>0.08M-KCl</td>
<td>2.7</td>
<td>2.65</td>
<td>21.6</td>
<td>8.0</td>
<td>72.7</td>
</tr>
<tr>
<td></td>
<td>0.04M-KCl</td>
<td>1.2</td>
<td>0.55</td>
<td>10.7</td>
<td>8.9</td>
<td>80.9</td>
</tr>
</tbody>
</table>

Table 2. Purification of catalase from baboon liver

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

<table>
<thead>
<tr>
<th>State</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol/min per mg)</th>
<th>Activity ratio catalase/cinnabarinate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate after chloroform/ethanol treatment</td>
<td>1100</td>
<td>5863</td>
<td>1810</td>
<td>3600</td>
</tr>
<tr>
<td>Supernatant after acidification</td>
<td>1000</td>
<td>5750</td>
<td>840</td>
<td>3800</td>
</tr>
<tr>
<td>Eluate from calcium phosphate gel</td>
<td>120</td>
<td>690</td>
<td>3300</td>
<td>10000</td>
</tr>
<tr>
<td>Dialysis precipitate</td>
<td>—</td>
<td>9.4</td>
<td>22300</td>
<td>3700</td>
</tr>
</tbody>
</table>

Table 3. Effect of Mn²⁺ concentration on the formation of cinnabarinate

(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.

<table>
<thead>
<tr>
<th>Conc. of MnSO₄ (mm)</th>
<th>Cinnabarinate formed in 30min (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>0.076</td>
<td>8.3</td>
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<tr>
<td>0.15</td>
<td>23.9</td>
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<td>0.38</td>
<td>46.8</td>
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<td>0.76</td>
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<td>1.5</td>
<td>80.7</td>
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<tr>
<td>(b)</td>
<td></td>
</tr>
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<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.076</td>
<td>20.0</td>
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<td>0.37</td>
<td>55</td>
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<tr>
<td>0.75</td>
<td>64</td>
</tr>
</tbody>
</table>

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 Rᵥ 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 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 cinnabarinate synthase activity and the major protein band when stained with Coomassie Blue.

**Discussion**

The chromophore formed in the incubation system that contained Mn²⁺, catalase and 3-hydroxyanthranilic acid has characteristics similar to that produced by cinnabarinate synthase (Subba Rao & Vaidyanathan, 1966). Ishiguro et al. (1971) have observed a similar reaction with 3-hydroxyanthranilic acid, Mn²⁺ and haemoglobin. It must be
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 cinnabarinate synthase have been unsuccessful. Our evidence so far suggests that cinnabarinate 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-hydroxyanthranilic acid is similarly ascribed to catalase. In this instance the enzyme did not require Mn$^{2+}$ for activity.

Since it has been shown that cinnabarinate is carcinogenic when implanted into bladders of mice (Boyland, 1960) and further that 3-hydroxyanthranilic acid, the precursor of cinnabarinate, has an affinity for the bladder epithelium of rats and mice, it would be worth while to investigate the synthesis of cinnabarinate in vitro and in vivo. The observation that cinnabarinate inhibits mitochondrial respiration and the suggestion that this interaction of cinnabarinate 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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References