PURIFICATION AND SOME PROPERTIES OF CHICKEN-LIVER AFLATOXIN B₁ REDUCTASE

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(Received 4 March 1980)

Abstract—Aflatoxin B₁ reductase was isolated from the livers of 1- to 2-day-old chickens. The enzyme was purified more than 800-fold with a yield of 29% by the following steps: 100,000-g ultracentrifugation; acid (pH 5.0) precipitation; 40-75% ammonium sulphate fractionation; DEAE-cellulose column chromatography; hydroxyapatite column chromatography; Sephadex G-100 column chromatography; second hydroxyapatite column chromatography. The final enzyme preparation was colourless and contained a trace amount of impurity. The mol wt of the enzyme was estimated to be 46,500 by Sephadex G-100 gel filtration. The 17-ketosteroids androsterone, dehydroisoandrosterone, and oestrone inhibited the described assay conditions. The inhibition by the activity of AFB₁ reductase by 50-70% under the described assay conditions. The inhibition by oestrone appeared to be noncompetitive.

INTRODUCTION

The potent hepatocarcinogenic mycotoxin aflatoxin B₁ (AFB₁) can be converted into various metabolites in animals (Masri, Booth & Hsieh, 1974; Patterson & Allcroft, 1970). It requires metabolic activation to exert its carcinogenic and mutagenic effects (Fahmy, Fahmy & Swenson, 1978; Gurtoo & Bejba, 1977; Gurtoo, Dahms & Paigen, 1978; Martin & Garner, 1977; Patterson & Roberts, 1972a). In the liver, the cyclopentenone ring of AFB₁ can be reduced by cytoplasmic reductase to aflatoxicol (AFL; Patterson & Roberts, 1972b; Salhab, Hsieh, Wong & Ruebner, 1975; Schoenhard, Lee, Howell, Pawlowski, Libbey & Sinnhuber, 1976). AFL has been identified in vivo as the major aflatoxin metabolite in the plasma of rats administered AFB₁ orally or iv (Wong & Hsieh, 1978). The activity of liver AFB₁ reductase was shown to be related to the sensitivity of an animal species to acute aflatoxicosis and to aflatoxin-induced carcinogenesis (Edwards, Rintel & Parker, 1975; Hsieh, Wong, Micas & Ruebner, 1977).

Our preliminary studies established that the liver of a 1-2-day-old chicken possesses much higher AFB₁ reductase activity than the liver of a duckling or a rat (Chen, 1979). The enzyme requires NADPH as co-factor for the reduction and is fairly stable in crude liver preparations. The present report describes the purification of this enzyme from chicken liver and the determination of some of its properties.

EXPERIMENTAL

Animals and chemicals

The chickens (Gallus gallus, Cochins), 1-2 days old, were purchased from the market at Shih-Lin, Taipei, Taiwan. NADP, glucose-6-phosphate (G6P), G6P-dehydrogenase (Torula yeast), tris(hydroxymethyl)aminomethane (Tris) and the reagents for disc gel electrophoresis were products of Sigma Chemical Co. (Saint Louis, MO). Sephadex G-100 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), DEAE-cellulose (Whatman DE 52) from Whatman Inc. (Springfield Mill, England), hydroxyapatite from Bio-Rad Laboratories (Richmond, CA), Liquiflor from New England Nuclear Co. (Boston, MA), crystalline AFB₁ from Calbiochem Co. (San Diego, CA), and ammonium sulphate and silica-gel (Type 60) from E. Merck Co. (Darmstadt, FRG). Ring-labelled [14C]AFB₁ was purified from the chloroform extract of Aspergillus parasiticus incubated in the presence of sodium iodide-[14C]acetate (Hsieh & Mateles, 1971).
Protein determination
Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard.

Purification of AFB₁ reductase
(All of the purification procedures were carried out at 0–4°C.)

Preparation of 100,000-g supernatant. The chickens were killed by decapitation. The livers (30 g) were excised immediately and homogenized in a Waring blender with 3 volumes of chilled 0.15 M-KCl-20 mM-potassium phosphate (KP) buffer (pH 6.8). The homogenate was centrifuged at 100,000 g for 1 hr.

Acid precipitation. The 100,000-g supernatant was diluted with an equal volume of 20 mM-KP buffer (pH 6.8), and the pH was slowly adjusted to 5.0 with 1 N-acetic acid. The suspension was spun at 20,000 g for 30 min to remove the protein precipitate. The pH of the supernatant was then brought back to neutral with 1 N-NH₄OH.

Ammonium sulphate fractionation. To the enzyme-containing supernatant solid ammonium sulphate was added slowly to 40% saturation under continuous stirring. The mixture was spun at 20,000 g for 30 min. The supernatant was brought to 75% saturation with ammonium sulphate. The protein precipitate was collected by centrifugation, dissolved in a small volume of buffer A (5 mM-Tris-HCl pH 8.0, 1 mM-EDTA) and used for DEAE-cellulose column chromatography.

DEAE-cellulose column chromatography. A DEAE-cellulose column (2.6 x 40 cm) was pre-equilibrated with two bed volumes of buffer A. The enzyme solution was applied to the column and washed with 400 ml buffer A. It was followed by a linear continuous gradient of 0–170 mM-KCl in buffer A (total volume 1200 ml). The flow rate was 0.6 ml/min. Fractions of 14 ml were collected and assayed for enzyme activity. Only fractions with high enzyme activity were combined and concentrated by precipitation with ammonium sulphate to 80% saturation. The concentrated protein was dissolved in a minimum volume of buffer B (10 mM-KP, pH 6.8, 2 mM-EDTA, 5 mM-2-mercaptoethanol) and dialysed overnight against the same buffer.

First hydroxyapatite column chromatography. The dialysed enzyme solution was applied to an equilibrated hydroxyapatite column (1.5 x 12 cm) and washed with 100 ml buffer B. The column was then eluted with a linear gradient formed from 200 ml buffer B and 200 ml of a mixture containing KP (pH 6.8), EDTA and 2-mercaptoethanol at concentrations of 200 mM, 2 mM and 5 mM respectively. The flow rate was 0.45 ml/min. Fractions of 12 ml were collected and assayed for AFB₁ reductase activity. The fractions with high enzyme activity were combined and concentrated by ammonium sulphate precipitation to 80% saturation.

Sephadex G-100 column chromatography. The concentrated enzyme solution was chromatographed on an equilibrated Sephadex G-100 column (2.6 x 65 cm) in buffer C (40 mM-KP, pH 6.8, 2 mM-EDTA, 5 mM-2-mercaptoethanol). The flow rate was 20 ml/hr at a constant hydrostatic pressure of 35 cm H₂O. Fractions of 10 ml were collected and assayed for AFB₁ reductase activity.

Second hydroxyapatite column chromatography. The active fractions from the Sephadex G-100 column were combined, diluted with an equal volume of de-

Fig. 1. Separation by DEAE-cellulose column chromatography of the 40–75% ammonium sulphate fraction: enzyme activity (—); protein concentration, estimated by the optical density of eluate at 280 nm (——); KCl (eluent) gradient (••••). The arrow shows the start of the KCl gradient. One enzyme unit is defined as the amount required to reduce 1 nmol AFB₁ to aflatoxicol in 40 min. The volume of each fraction was 14 ml. Only the fractions with high enzyme activity (55–75) were combined for further purification. For details, see experimental section.
ionized water, and then rechromatographed on the hydroxyapatite column. The sample was eluted with a linear gradient formed from 200 ml 30 mM-KP (pH 6.8) and 200 ml 200 mM-KP (pH 6.8). The flow rate was 10 ml/hr. Fractions of 7 ml were collected.

**Disc gel electrophoresis**

Using the final enzyme preparation, disc gel electrophoresis was carried out on 7% acrylamide gel at pH 9.5 according to the methods of Ornstein (1964) and Davis (1964). A current of 5 mA/gel was applied for approximately 1 hr. The gel was stained with 0.25% (w/v) aqueous Coomassie Brilliant Blue R-250 in 45% (v/v) aqueous methanol and 9% (v/v) aqueous acetic acid and destained in a solution of 75% (v/v) aqueous acetic acid and 5% (v/v) aqueous methanol.

**Determination of molecular weight**

The molecular weight of the enzyme was estimated from the data obtained from Sephadex G-100 gel filtration. Aliquots (20 mg) of the standard proteins bovine serum albumin (mol wt 67,000), chymotrypsinogen A (mol wt 25,000) and cytochrome c (mol wt 12,000) were used for calibration. The values of $K_v = v_e - v_0/v_e - v_0$ ($v_e$ = elution volume; $v_t$ = total volume of the column; $v_0$ = void volume) were plotted against the logarithms of the known molecular weights.

**Effect of 17-ketosteroids on enzyme activity**

To investigate the previous report that AFB$_1$ reductase activity in crude liver preparations is inhibited by 17-ketosteroids, the activity of purified AFB$_1$ reductase was assayed in the presence of androsterone, dehydroisoandrosterone or oestrone. To study the type of inhibition, the enzyme was assayed at various concentrations of AFB$_1$ in the presence of 20 $\mu$m- and 40 $\mu$m-oestrone. The data were presented as Lineweaver-Burk plots.

**RESULTS AND DISCUSSION**

Figure 1 shows the results of separation of the 40–75% ammonium sulphate fraction on the DEAE-cellulose column. The enzyme activity was distributed over several peaks. Only fractions with high enzyme activity (55–75) were combined for further purification. The apparent activity exhibited by the discarded trailing peaks may be attributed to some less specific liver reductases, since various cytoplasmic NADPH-dependent aldo-keto reductases with broad specificities have been reported (Bachur, 1976). Figure 2 shows the distribution of protein and of enzyme activity after separation on the first hydroxyapatite chromatographic column. The active fractions (16–27) were combined and subjected to Sephadex G-100 filtration. The pooled active fractions from this filtration (those eluting between 175 and 205 ml; Fig. 3) were applied to a second hydroxyapatite column, from which a broad single peak (fractions 28–36) containing the enzyme activity was eluted (Fig. 4). The molecular weight of the purified AFB$_1$ reductase was estimated to be 46,500, by Sephadex G-100 gel filtration (Fig. 5).

Table 1 shows that the specific activity of AFB$_1$ reductase in the fractions collected from the final stage of purification was more than 800 times greater than that of the chicken-liver homogenate. About 29% of the enzyme could be recovered. The final enzyme preparation was colourless and was shown by
Fig. 3. Sephadex G-100 gel filtration of the enzyme preparation obtained from the hydroxyapatite column: enzyme activity (---); protein concentration (----), estimated by the optical density of the eluate at 280 nm. One enzyme unit is defined as the amount required to reduce 1 nmol AFB₁ to aflatoxicol in 40 min. Only the fractions with high enzyme activity (those eluting between 175 and 205 ml) were combined for further purification.

Fig. 4. Separation on the second hydroxyapatite chromatographic column of the enzyme preparation obtained from the Sephadex G-100 filtration: enzyme activity (---); protein concentration (----) estimated by the optical density of the eluate at 280 nm; potassium phosphate (eluent) gradient (-----). The arrow indicates the start of the eluent gradient. One enzyme unit is defined as the amount required to reduce 1 nmol AFB₁ to aflatoxicol in 40 min. The volume of each fraction was 7 ml. A broad single peak (fractions 28-36) containing the enzyme activity was eluted. For details, see experimental section.

Fig. 5. Determination of molecular weight of AFB₁ reductase by Sephadex G-100 gel filtration. The enzyme had a $K_{av}$ value of 0.279, corresponding to a mol wt of 46,500. $K_{av} = V_e - V_0/V_i - V_0$ where $V_e = $ elution volume, $V_0 = $ void volume, and $V_i = $ total volume of the column.
Table 1. Purification of aflatoxin B₁ (AFB₁) reductase from chicken liver

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Protein (mg)</th>
<th>Specific activity (enzyme units*/mg protein)</th>
<th>Recovery (%)</th>
<th>Purification factor†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>5400</td>
<td>1.91</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Supernatant, 100,000 g</td>
<td>2320</td>
<td>4.05</td>
<td>91.1</td>
<td>2.12</td>
</tr>
<tr>
<td>Acid precipitation</td>
<td>1512</td>
<td>6.2</td>
<td>90.9</td>
<td>3.25</td>
</tr>
<tr>
<td>Ammonium sulphate fractionation (40–75%)</td>
<td>901</td>
<td>10.6</td>
<td>87.4</td>
<td>5.55</td>
</tr>
<tr>
<td>DEAE-cellulose column</td>
<td>150</td>
<td>56.3</td>
<td>81.8</td>
<td>29.48</td>
</tr>
<tr>
<td>Hydroxyapatite column</td>
<td>27</td>
<td>192.9</td>
<td>50.5</td>
<td>101.00</td>
</tr>
<tr>
<td>Sephadex G-100 column</td>
<td>6.22</td>
<td>535.1</td>
<td>32.3</td>
<td>280.15</td>
</tr>
<tr>
<td>Second hydroxyapatite column</td>
<td>1.95</td>
<td>1534.5</td>
<td>29.0</td>
<td>803.40</td>
</tr>
</tbody>
</table>

*1 enzyme unit is defined as the amount required to reduce 1 nmol AFB₁ to aflatoxicol in 40 min.
†The purification factor is the specific activity of a purified sample as a fraction of the specific activity of the homogenate.

Table 2. Effect of 17-ketosteroids on AFB₁ reductase activity

<table>
<thead>
<tr>
<th>Addition to enzyme assay mix*</th>
<th>Relative AFB₁ reductase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Androsterone, 100 µM</td>
<td>36.2</td>
</tr>
<tr>
<td>Dehydroisoandrosterone, 100 µM</td>
<td>28.5</td>
</tr>
<tr>
<td>Oestrone, 20 µM</td>
<td>49.0</td>
</tr>
<tr>
<td>40 µM</td>
<td>28.0</td>
</tr>
</tbody>
</table>

*Androsterone and dehydroisoandrosterone were predissolved in dimethylsulphoxide and oestrone was predissolved in methanol before addition to the incubation medium.

analytical disc gel electrophoresis to contain a trace amount of impurity. The purified enzyme was unstable and storage at 0°C for 1 wk resulted in 50% less activity. Compared with other cytoplasmic aldoketo reductases (Bachur, 1976; Chen, 1979; Culp & McMahon, 1968; Felsted, Gee & Bachur, 1974), the purified AFB₁ reductase showed similar physicochemical characteristics such as sulphydryl sensitivity, pH optimum (pH 6–8), and low molecular weight. Patterson & Roberts (1972) reported that 17-ketosteroids were able to inhibit AFB₁ reductase activity in the high-speed supernatant of avian livers and that
these livers also possessed NADPH-linked 17-ketosteroid reductase activity. The three 17-ketosteroids tested in the present study inhibited the activity of purified chicken-liver AFB₁ reductase by 50-70% (Table 2). The inhibition by oestrone was noncompetitive (Fig. 6), which implies that AFB₁ and oestrone bind at different sites on the enzyme. Whether the inhibition by the other 17-ketosteroids is also noncompetitive requires further studies.

The substrate of the purified enzyme in vitro is not clear. Patterson & Roberts (1972) suggested 17-ketosteroids as the natural substrates. A direct assay of the enzyme with these steroids and a comparison of the Km values are necessary to answer this question.

Acknowledgements—This work was supported by the National Science Council Grant No. NSC-66B-0412-19(10), Republic of China, and was performed while one of us (R. D. Wei) received a Research Chair Award from the Tjing-Ling Medical Foundation. We thank Ms. Bruni Kobbe for her editorial assistance.

REFERENCES


