Purification of Multiple Forms of the Soluble 17 α -Hydroxy Steroid Dehydrogenase of Rabbit Liver

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Eight distinct forms of the soluble 17 α -hydroxy steroid dehydrogenase of rabbit liver were resolved by DEAE-cellulose chromatography and isoelectric focusing. Five of these enzymes were homogeneous as judged by polyacrylamide-gel electrophoresis. Substrate-specificity studies carried out with oestradiol-17 α and oestradiol-17 α 3-glucuronide revealed a variation in activity toward these substrates among the different purified enzyme forms. Three forms of the 17 α -hydroxy steroid dehydrogenase exhibited equal activity toward both oestrogen substrates, whereas three forms of the enzyme displayed a greater activity toward the glucuronide derivative of oestradiol-17 α . One enzyme in particular is essentially specific for oestradiol-17 α 3-glucuronide, its activity toward oestradiol-17 α being only one-thirtieth that observed with the 3-glucuronide derivative.

The metabolism of oestradiol-17 β in rabbit liver involves the oxidation of this steroid to oestrone, followed by reduction to oestradiol-17 α (Layne *et al.*, 1965; Collins *et al.*, 1967; Williams *et al.*, 1968). These reactions are catalysed by the 17-hydroxy steroid dehydrogenases of rabbit liver (Breuer & Pangels, 1960). Before its excretion in the urine, oestradiol-17 α is conjugated with glucuronic acid at C-3 and with *N*-acetylglucosamine at C-17 (Layne *et al.*, 1964; Layne, 1965). In a previous paper (Hasnain & Williamson, 1974) we described the separation and partial purification of the soluble 17 β - and 17 α -hydroxy steroid dehydrogenases of rabbit liver. Evidence for multiple forms of the 17 α -hydroxy steroid dehydrogenase, some of which exhibited a preference for the 3-glucuronide of oestradiol-17 α as substrate, was also presented. The present paper describes the separation and purification of the multiple forms of the 17 α -hydroxy steroid dehydrogenase of rabbit liver by DEAE-cellulose chromatography and isoelectric focusing. The activities of the different enzyme forms toward oestradiol-17 α and its 3-glucuronide derivative are also compared.

Experimental

Materials

Oestradiol-17 β , oestradiol-17 α and NADP⁺ were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. [6,7-³H₂]Oestradiol-17 β was obtained from Amersham/Searle Corp., Toronto, Ont., Canada. The methods used for the preparation of [6,7-³H₂]oestradiol-17 α , [6,7-³H₂]oestradiol-17 α 3-glucuronide and [6,7-³H₂]oestradiol-17 β 3-glucuronide have

been described elsewhere (Layne *et al.*, 1965; Collins *et al.*, 1967, 1970; Hasnain & Williamson, 1974). DEAE-cellulose (Whatman DE-52) was purchased from Mandel Scientific Co., Montreal, Que., Canada, and carrier ampholytes were from Fisher Scientific Co. Ltd., Ottawa, Ont., Canada.

DEAE-cellulose chromatography

The 17 α -hydroxy steroid dehydrogenase activity of the 105000g supernatant from 100g of rabbit liver was partially purified by calcium phosphate-gel fractionation and gel filtration on Sephadex G-75 as detailed elsewhere (Hasnain & Williamson, 1974). The enzyme fraction obtained by this procedure (280mg of protein) was concentrated to approx. 15ml by ultrafiltration with a PM10 membrane (Diaflo apparatus; Amicon Corp., Lexington, Mass., U.S.A.) and was applied to a DEAE-cellulose column (2.5cm $\times$ 40cm) previously equilibrated with 10mM-Tris-HCl-0.5mM-dithiothreitol-25mM-NaCl, pH8.0. Protein was eluted with a 2.4-litre linear gradient of NaCl from 25mM to 70mM in the equilibrating buffer, at a flow rate of 40ml/h. Fractions (10ml) were collected and assayed for 17 α -hydroxy steroid dehydrogenase activity with the substrates [6,7-³H₂]oestradiol-17 α and [6,7-³H₂]oestradiol-17 α 3-glucuronide as described previously (Hasnain & Williamson, 1974). In this study, a unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of 1 μ mol.

Isoelectric focusing

Isoelectric focusing was performed with an LKB 8102 electrofocusing column of 440ml capacity,

by using the procedure described in the manufacturer's instruction manual. In initial experiments, carrier ampholytes, pH range 5–7 or 5–8, were used at a concentration of 5%. A stabilizing gradient of sucrose (47–5%, w/v) containing 0.5 mM-dithiothreitol was formed stepwise. The lower electrode solution contained 1% (v/v) phosphoric acid in 55% (w/v) sucrose and the upper electrode solution contained 1% (w/v) NaOH. Each of the four 17 α -hydroxy steroid dehydrogenase activities isolated by DEAE-cellulose was concentrated to approx. 10 ml, dialysed against 2 litres of 1 mM-Tris-HCl-0.5 mM-dithiothreitol, pH 8.0, and then concentrated by ultrafiltration to 20 ml. The enzyme was incorporated into the middle portion of the sucrose gradient of the focusing column by substituting the enzyme solution for the less-dense solution when preparing the fractions comprising this portion of the sucrose gradient. Electrofocusing was carried out for 120 h at 4°C, during which time the voltage was adjusted to maintain a total load of 2 W. After electrofocusing was completed, the column contents were drained at a flow rate of 60 ml/h and collected in 2 ml fractions. The measurement of pH was performed at 4°C immediately after emptying the column. A combination pH electrode was used and calibrated at 4°C with a standard buffer solution (Fisher Scientific Co., Ottawa, Ont., Canada) with pH 7.05 at 4°C.

Isoelectric focusing over a narrow pH range was carried out by one of two methods. In one method carrier ampholytes of the desired pH range were prepared by an initial focusing of the commercial ampholytes (pH range 5–7) at a concentration of 5% (w/v) under the conditions described above. These narrow-pH-range ampholytes were then used for focusing the 17 α -hydroxy steroid dehydrogenase. Alternatively, the 17 α -hydroxy steroid dehydrogenase fraction was first focused over the pH range 5–7. Column fractions comprising that portion of the pH gradient having 17 α -hydroxy steroid dehydrogenase activity were combined and refocused. The final ampholyte concentration in both methods was 1.5% (w/v). The sucrose concentration was adjusted to produce a gradient from 47 to 5% (w/v), and dithiothreitol was added to give a final concentration of 0.5 mM. After focusing, the tubes comprising each peak of 17 α -hydroxy steroid dehydrogenase activity were pooled, dialysed against 2 litres of 10 mM-glycine-HCl-0.5 mM-dithiothreitol, pH 9.5, and concentrated by ultrafiltration to a small volume for use in further characterization studies.

Electrophoresis in polyacrylamide gels

Polyacrylamide-disc-gel electrophoresis was carried out as described by Davis (1964). The acrylamide monomer concentration was 10%. Gels were pre-run

in the Tris-glycine buffer system (pH 8.3) before application of the protein sample (2–20 μ g). Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate was performed as described by Weber & Osborn (1969). Gels were fixed with trichloroacetic acid (12.5%, w/v) and stained with Coomassie Brilliant Blue as described in Bulletin AN32 of Ortec Incorporated (1970).

Results

Rabbit liver 17 α -hydroxy steroid dehydrogenase, partially purified by calcium phosphate-gel fractionation and Sephadex-gel filtration (Hasnain & Williamson, 1974), is resolved into four distinct enzyme activities by chromatography on DEAE-cellulose (Fig. 1). Further, one enzyme fraction (peak I) displays greater activity toward the substrate oestradiol-17 α 3-glucuronide than toward oestradiol-17 α , whereas the other enzyme fractions show equal activity toward both substrates. The enzyme activity present in peak IV (Fig. 1) represents a small percentage of the total activity, and further purification of this fraction was not attempted.

Polyacrylamide-gel electrophoresis of the 17 α -hydroxy steroid dehydrogenases isolated by DEAE-cellulose chromatography indicated that only peak III was homogeneous. When the gels were stained for 17 α -hydroxy steroid dehydrogenase activity by the procedure of Karavolas *et al.* (1970) multiple bands were observed with all fractions except peak III.

Isoelectric focusing of the pooled fractions from peak I of the DEAE-cellulose chromatography (Fig. 1) over the pH range 5–7 produced the profile of enzyme activity shown in Fig. 2. Three 17 α -hydroxy steroid dehydrogenase activities are separated. Each peak has activity toward both oestradiol-17 α and oestradiol-17 α 3-glucuronide, but peak 1C exhibits a marked preference for the glucuronide derivative. When these combined enzyme fractions (Fig. 2) are refocused over a narrower pH range, 5.6–6.6, the same enzyme profile is obtained and a more complete separation of the activities is achieved. The pooled fractions from peak II of the DEAE-cellulose chromatography are resolved into two enzyme activities by isoelectric focusing over the pH range 5–8. When the focusing is carried out over a narrower pH range (5.6–6.7), four enzyme activities are observed (Fig. 3). Peaks IIA and IIC display a greater activity toward oestradiol-17 α 3-glucuronide than toward oestradiol-17 α . The isoelectric points of the enzymes comprising peaks IIA and IIB are very similar and these activities are not clearly separated. Further attempts to resolve these enzymes by refocusing have been unsuccessful.

The purity of the isolated 17 α -hydroxy steroid dehydrogenases has been established by poly-

Fig. 1. DEAE-cellulose chromatography of the partially purified 17 α -hydroxy steroid dehydrogenase of rabbit liver

The 17 α -hydroxy steroid dehydrogenase was first partially purified by calcium phosphate-gel fractionation and Sephadex-gel filtration. Enzyme (280mg of protein) was applied to the DEAE-cellulose column (2.5cm $\times$ 40cm). Enzyme activities were measured as described in the text. The amount of each column fraction (10ml) assayed was as follows: for oestradiol-17 α , 0.05ml; for oestradiol-17 α 3-glucuronide, 0.01ml. ●, E_{280} ; ▲, activity with oestradiol-17 α ; □, activity with oestradiol-17 α glucuronide; ····, NaCl gradient.

Fig. 2. Isoelectric focusing of the peak-I 17 α -hydroxy steroid dehydrogenase fraction isolated by DEAE-cellulose chromatography

Fractions comprising peak I of the DEAE-cellulose column (fractions 59–70) were pooled and dialysed as described in the text. Enzyme (34 mg of protein) was focused over the pH range 5–7. The ampholyte concentration was 5% (w/v). The amount of each column fraction (2ml) assayed for enzyme activity was 0.1ml for oestradiol-17 α and 0.01ml for oestradiol-17 α 3-glucuronide. ▲, Activity with oestradiol-17 α ; □, activity with oestradiol-17 α 3-glucuronide; ····, pH gradient.

Fig. 3. Isoelectric focusing of the peak-II 17α -hydroxy steroid dehydrogenase fraction isolated by DEAE-cellulose chromatography

Fractions comprising peak II of the DEAE-cellulose column (fractions 83–102) were pooled and dialysed as described in the text. Enzyme (52mg of protein) was focused over the pH range 5.6–6.7. The ampholyte concentration was 1.5% (w/v). The amount of each column fraction (2ml) assayed for enzyme activity was 0.1ml for oestradiol- 17α and 0.05ml for oestradiol- 17α 3-glucuronide. ▲, Activity with 17α -oestradiol; □, activity with oestradiol- 17α 3-glucuronide; ·····, pH gradient.

acrylamide-gel electrophoresis by the method of Davis (1964) (Plate 1a) and by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Plate 1b). The enzyme of peak 1A is the only fraction that is not homogeneous, two protein bands being evident in the gel (Plate 1a). Whether both of these proteins are 17α -hydroxy steroid dehydrogenases, or whether one of these proteins is a contaminant, remains to be determined. It is possible that a protein is associated with the 17α -hydroxy steroid dehydrogenase of peak 1A having an isoelectric point identical with that of the enzyme, but a different electrical charge at the pH (8.3) used in the polyacrylamide-gel electrophoresis. The two protein bands could also result from a chemical modification or conformational change of the enzyme before or during polyacrylamide-gel electrophoresis (Kaplan, 1968; Epstein & Schechter, 1968).

Table 1. Comparison of the specific activities of the purified multiple forms of the 17α -hydroxy steroid dehydrogenase of rabbit liver

The enzyme fractions are those described in Figs. 1, 2 and 3. Enzyme activity was measured as described in the text. Protein was measured by the method of Warburg & Christian (1942).

Enzyme	Specific activity (nmol of product/min per mg of protein)	
	Substrate ... Oestradiol- 17α	Oestradiol- 17α 3-glucuronide
IA	0.8	1.1
IB	3.5	4.4
IC	1.1	38
IIC	2.9	11
IID	3.3	3.7
III	2.3	4.3

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of the 17 α -hydroxy steroid dehydrogenases purified by DEAE-cellulose chromatography and isoelectric focusing

The enzyme fractions are those denoted in Figs. 1, 2 and 3. (a) Polyacrylamide-gel electrophoresis at pH 8.3 by the method of Davis (1964). (b) Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis.

The specific activities of the purified 17 α -hydroxy steroid dehydrogenases are summarized in Table 1. The specific activity of enzyme 1C is 34 times greater for oestradiol-17 α 3-glucuronide than for oestradiol-17 α . Enzyme IIC has a threefold greater activity toward the 3-glucuronide derivative, whereas the remaining enzymes display similar activities with both oestrogen substrates.

Discussion

In a previous paper (Hasnain & Williamson, 1974) we obtained evidence for multiple forms of the soluble 17 α -hydroxy steroid dehydrogenase of rabbit liver by DEAE-cellulose chromatography and isoelectric focusing. However, these procedures did not provide a complete separation of the enzymes, since some forms had very similar isoelectric points. The present paper describes an improved procedure for the purification of the 17 α -hydroxy steroid dehydrogenase. By modifying the NaCl gradient used in eluting the 17 α -hydroxy steroid dehydrogenase from the DEAE-cellulose column, four enzyme activities were obtained. Subsequent isoelectric focusing of these enzyme activities resulted in the separation of a total of eight forms of the 17 α -hydroxy steroid dehydrogenase. Polyacrylamide-gel electrophoresis indicated that five of these enzymes were homogeneous.

Recent evidence suggests that heterogeneity may be a common feature of the 17-hydroxy steroid dehydrogenases of different species. Thaler-Dao *et al.* (1972) have reported that the 17 β -hydroxy steroid dehydrogenases of rabbit liver can be separated into several enzyme activities by DEAE-cellulose chromatography and polyacrylamide-gel electrophoresis. Multiple forms of the 17 β -hydroxy steroid dehydrogenase of guinea pig kidney have been observed by Liu & Kochakian (1972a,b), and Stevenson & Kochakian (1974) have purified one of these forms. Engel & Groman (1974) have reported that multiple forms of the 17 β -hydroxy steroid dehydrogenase of human placenta are obtained by isoelectric focusing of the native enzyme on polyacrylamide gels.

Conclusions as to the origin of the multiple forms of the 17 α -hydroxy steroid dehydrogenase must await a detailed study of the structural features of these enzymes. The molecular heterogeneity could result from genetic variants of the enzyme, chemical modification, conformation changes, or the binding of small molecules (Kaplan, 1968; Epstein & Schechter, 1968). It is unlikely that the multiple 17 α -hydroxy steroid dehydrogenases are artifacts produced during

the purification of the enzymes. The same relative amounts of the different forms are obtained from different rabbit liver preparations. Storage of the liver supernatant at 4°C for several days does not alter the enzyme pattern, excluding the possibility that proteinases present in rabbit liver supernatant generate the different enzyme forms. Illingworth (1972) has reported that isoelectric focusing can produce multiple enzyme forms. He has attributed this effect to artifacts formed by interaction of the protein with Ampholine. However, in our studies the heterogeneity of the 17 α -hydroxy steroid dehydrogenase is evident after DEAE-cellulose chromatography, before isoelectric focusing. Moreover, refocusing of the isolated enzymes does not alter the observed enzyme profile.

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