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

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The six forms of the 17α-hydroxy steroid dehydrogenase purified from rabbit liver cytosol have very similar physical properties. The molecular weights of all the enzymes were within 3% of the average mol.wt. of 39600. Only one of the six enzymes showed a significant difference in amino acid composition. All but one form of the 17α-hydroxy steroid dehydrogenases exhibited greater activities towards the androgen, epitestosterone, than towards oestrogen substrates. With oestrogen substrates one enzyme displayed a high specificity towards the substrate oestradiol-17α 3-glucuronide. This high activity was lost if the glucuronic acid moiety was removed or replaced by glucose or galacturonic acid. The other enzyme forms had approximately equal activity toward oestradiol-17α and its glucuronide or glucoside derivative. However, substitution of galacturonic acid at C-3 of oestradiol-17α substantially decreased the activity of all but one enzyme form.

Both 17α- and 17β-hydroxy steroid dehydrogenase activities are present in rabbit liver (Breuer & Pangels, 1960) and are involved in the metabolism of oestrogens in this animal (Layne *et al.*, 1965; Collins *et al.*, 1967; Williams *et al.*, 1968). We have reported the separation of the soluble 17α- and 17β-hydroxy steroid dehydrogenases of rabbit liver (Hasnain & Williamson, 1974). Moreover, we obtained evidence that the 17α-hydroxy steroid dehydrogenase existed as multiple forms. By the techniques of DEAE-cellulose chromatography and isoelectric focusing we obtained five of the eight demonstrable forms of this enzyme in a homogeneous state (Hasnain & Williamson, 1975). Thaler-Dao *et al.* (1972) have also shown that the 17β-hydroxy steroid dehydrogenase of rabbit liver is present as multiple forms. To determine the biological significance of the multiple forms of the 17-hydroxy steroid dehydrogenases in rabbit liver, we have begun comparative studies on the properties of these enzymes. The present paper describes some of the characteristics of six forms of the 17α-hydroxy steroid dehydrogenase isolated as described by Hasnain & Williamson (1975).

Experimental

Materials

Oestradiol-17α, epitestosterone, $NADP^+$, almond emulsin, bovine serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

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Ketodase was purchased from Warner–Chilcott, Toronto, Ont., Canada, and thermolysin was purchased from Calbiochem, Los Angeles, CA, U.S.A. [1,2-$^3H_2$]Epitestosterone was obtained from New England Nuclear (Canada) Ltd., Montreal, Que., Canada. [1-^{14}C]Acetic anhydride was purchased from Amersham/Searle Corp., Toronto, Ont., Canada. [6,7-$^3H_2$]Oestradiol-17α, [6,7-$^3H_2$]oestradiol-17α 3-glucuronide, [6,7-$^3H_2$]oestradiol-17α 3-glucoside and [6,7-$^3H_2$]oestradiol-17α 3-galacturonide were prepared by methods previously described (Layne *et al.*, 1965; Collins *et al.*, 1967, 1970; Hasnain & Williamson, 1974).

Enzyme preparation

The multiple forms of the soluble 17α-hydroxy steroid dehydrogenase of rabbit liver were separated and purified exactly as described by Hasnain & Williamson (1975). By this method eight distinct forms of the 17α-hydroxy steroid dehydrogenase were resolved and six of these enzymes were homogeneous as judged by polyacrylamide-gel electrophoresis. The six purified enzymes, designated IA, IB, IC, IIC, IID and III (Hasnain & Williamson, 1975), were used for the studies described in this paper.

Subunit-molecular-weight determination

The subunit molecular weight of each purified 17α-hydroxy steroid dehydrogenase was determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate as described by Weber & Osborn (1969). The following protein standards were used: bovine serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome *c*.

Amino acid analysis

Each enzyme sample was dialysed against distilled water and freeze-dried. Approx. 50 μg of each freeze-dried enzyme sample was hydrolysed in 0.5ml of 5.7M-HCl in sealed evacuated tubes at 110°C for 24h. Amino acid analysis was carried out with a Durrum D-500 single-column multi-sample amino acid analyser.

Enzyme assays

The assay procedure used to determine the 17α-hydroxy steroid dehydrogenase activity towards various substrates was essentially as described previously (Hasnain & Williamson, 1974). Incubation mixtures contained the following: 0.003 μmol of steroid substrate (specific radioactivity 4.5 μCi/μmol) in 30 μl of methanol; 0.5 μmol of $NADP^+$; 5–1000 μl of 17α-hydroxy steroid dehydrogenase; 2ml of 0.1M-glycine/HCl, pH9.5. The volume of each incubation mixture was adjusted to 3ml by the addition of 0.15M-KCl. Samples were incubated at 37°C for 30 min. The extraction of substrate and product from the incubation mixture was modified depending on the substrate used in the assay. Incubations carried out with [6,7-$^3H_2$]oestradiol-17α were extracted with benzene, incubations with [1,2-$^3H_2$]epitestosterone or [6,7-$^3H_2$]oestradiol-17α 3-glucoside were extracted with ethyl acetate and incubations with [6,7-$^3H_2$]-oestradiol-17α 3-glucuronide or [6,7-$^3H_2$]oestradiol 3-galacturonide were extracted with ethyl acetate after adjusting the incubation medium to pH2 with 1.0M-HCl. In the incubations carried out with steroid conjugates, the aglycone was liberated by incubating the extracted conjugates with the appropriate glycosidase. Glucuronic acid and galacturonic acid were removed by incubation with Ketodase, and glucose was removed by incubation with almond emulsin (Hasnain & Williamson, 1974). Oestrogens were analysed by t.l.c. on silica gel H in the solvent system benzene/ethyl acetate (7:3, v/v); the solvent system chloroform/acetone (19:1, v/v) was used for the androgens.

Results and Discussion

Molecular weights

The multiple forms of the soluble 17α-hydroxy steroid dehydrogenase of rabbit liver have very similar properties. The molecular weights of the enzymes, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, are all within 3% of the average mol.wt. of 39600. Since this variation is within the experimental error of the technique, the molecular weights of the different forms of the 17α-hydroxy steroid dehydrogenase can be considered as being essentially the same. The 17α-hydroxy steroid dehydrogenase activity is retained when chromatographed on Sephadex G-75 (Hasnain & Williamson, 1974), indicating a mol.wt. much less than 70000. Since complexes formed from the 40000-mol.wt. subunit determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis would be eluted in the void volume on Sephadex G-75 gel filtration, it can be assumed that the rabbit liver 17α-hydroxy steroid dehydrogenases have no subunit structure. Thus these enzymes differ from the 17β-hydroxy steroid dehydrogenase of human placenta, which has been shown to be a dimer (Burns *et al.*, 1971, 1972; Jarabak & Street, 1971). These results also exclude the possibility that the multiple forms of the 17α-hydroxy steroid dehydrogenase arise from a combination of non-identical subunits. The value of 39600 obtained as the average mol.wt. of the 17α-hydroxy steroid dehydrogenases is similar to the value reported for the 17β-hydroxy steroid dehydrogenase of rabbit liver (Thaler-Dao *et al.*, 1972), the 17β-hydroxy steroid dehydrogenase of guinea-pig kidney (Stevenson & Kochakian, 1974) and the subunit of the 17β-hydroxy steroid dehydrogenase of human placenta (Burns *et al.*, 1971).

Amino acid compositions

The amino acid compositions of the 17α-hydroxy steroid dehydrogenases are shown in Table 1. Although the multiple forms are very similar in amino acid composition, there are two possible differences in the amino acid composition of protein III as compared with the other proteins. The aspartic acid plus asparagine content of protein III is 23 residues/mol, whereas that of the other proteins is from 29 to 33 residues/mol. Similarly, the glutamic acid plus glutamine content of protein III is lower (26 residues/mol) than in the remaining proteins (32–37residues/mol). The amino acid composition of only one other mammalian 17-hydroxy steroid dehydrogenase, human placental 17β-oestradiol dehydrogenase, has been reported (Jarabak & Street, 1971, 1972; Burns *et al.*, 1972; Nicholas *et al.*, 1972). A comparison of the amino acid composition of this enzyme and the rabbit liver 17α-hydroxy steroid dehydrogenases reveals distinct dissimilarities for most of the amino acids. Of particular note is the low $\frac{1}{2}$-cystine content of the 17α-hydroxy steroid dehydrogenase (2 residues/mol, Table 1) when compared with the 17β-hydroxy steroid dehydrogenase (6 residues/mol of subunit).

Substrate specificities

The relative activities of the different forms of the 17α-hydroxy steroid dehydrogenase towards a series of oestrogen derivatives and towards the androgen epitestosterone are shown in Table 2. The activities of all enzymes, except for enzyme IC, are much higher with the substrate epitestosterone than with any of the oestrogen substrates. In particular, enzyme IA is 30 times more active towards epitestosterone than towards the oestrogens. Enzyme IC is the only form with

Table 1. *Amino acid composition of the multiple forms of the* 17α*-hydroxy steroid dehydrogenase of rabbit liver*
Proteins were hydrolysed in evacuated sealed tubes in 5.7M-HCl at 110°C for 24h, and analyses were performed with a Durrum D-500 amino acid analyser (Spackman *et al.*, 1958). Tryptophan was not determined and no special procedure was used to avoid the destruction of cysteine by acid hydrolysis. Values are not corrected for incomplete recovery and represent a single set of determinations. The residues/mol of protein were estimated on the basis of the molecular weight of each protein determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

	Composition											
	(mol/100mol)						(estimated residues/molecule)					
Enzyme ...	IA	IB	IC	IIC	IID	III	IA	IB	IC	IIC	IID	III
½ Cys	0.5	0.6	0.3	0.2	0.6	0.6	2	2	1	1	2	2
Asx	9.8	9.8	10.3	10.6	10.2	6.9	33	29	31	32	33	23
Thr	3.0	3.2	3.4	3.6	3.7	4.1	10	10	10	11	11	14
Ser	4.5	5.1	5.8	5.5	5.1	5.4	15	15	17	17	16	18
Glx	11.0	10.7	11.5	11.8	11.1	7.9	37	32	35	35	34	26
Pro	5.2	5.3	6.0	5.7	5.2	6.2	18	16	18	17	16	20
Gly	6.7	7.9	7.1	6.8	7.1	7.9	22	24	21	20	22	26
Ala	6.5	7.0	7.4	8.0	7.9	5.9	22	21	22	24	24	19
Val	6.5	7.4	7.2	7.5	7.2	5.9	21	22	22	23	22	19
Met	1.3	1.9	1.6	1.7	1.7	1.4	4	6	5	5	5	5
Ile	5.1	4.9	5.2	5.0	5.7	5.3	17	15	16	15	17	17
Leu	10.2	11.7	11.0	11.0	10.7	10.6	35	35	33	33	32	35
Tyr	3.0	4.2	3.8	4.0	4.1	3.4	10	13	11	12	12	11
Phe	3.9	3.7	3.8	3.6	4.3	3.6	13	11	11	11	13	12
His	2.7	3.7	3.2	2.8	2.6	2.7	9	11	10	8	8	9
Lys	6.8	8.2	8.4	7.5	9.1	7.6	23	25	25	23	27	25
Arg	4.8	5.2	4.6	5.0	4.3	5.4	16	16	14	15	13	18

Table 2. *Substrate specificities of the multiple forms of the* 17α*-hydroxy steroid dehydrogenase of rabbit liver*
Enzyme activities were measured as described in the text. The specific activities of the enzymes for each substrate are reported as the percentages of their activities with oestradiol-17α.

	Specific activity (% of activity with oestradiol-17α)				
Enzyme / Substrate ...	Oestradiol-17α	Oestradiol-17α 3-glucuronide	Oestradiol-17α 3-glucoside	Oestradiol-17α 3-galacturonide	Epitestosterone
IA	100	138	138	50	4100
IB	100	128	128	3	2000
IC	100	3450	100	18	2960
IIC	100	369	134	97	1020
IID	100	89	105	22	957
III	100	187	122	56	1290

higher activity towards oestradiol-17α 3-glucuronide than towards epitestosterone. The 17β-hydroxy steroid dehydrogenase of rabbit liver (Thaler-Dao *et al.*, 1972) and the 17β-hydroxy steroid dehydrogenase of guinea-pig kidney (Liu & Kochakian, 1972) are similar to the 17α-hydroxy steroid dehydrogenases of rabbit liver, having higher activity with androgens than with oestrogens. In contrast, the 17β-hydroxy steroid dehydrogenase of human placenta exhibits a high specificity towards oestrogens (Karavolas & Engel, 1971).

When the oestrogens are compared as substrates (Table 2), the activities of enzymes IA, IB, IID and III are approximately equal towards oestradiol-17α, oestradiol-17α 3-glucuronide and oestradiol-17α 3-glucoside. Thus the presence or absence of a glucuronide or glucoside moiety at C-3 of the oestrogen molecule does not affect the reaction of these substrates with the enzymes. However, enzymes IC and IIC show the highest activity towards oestradiol-17α 3-glucuronide; with enzyme IC the activity towards the glucuronide derivative is 34 times greater than for oestradiol-17α. Enzyme IC does not show this high activity if a glucose moiety is substituted at C-3 of oestradiol-17α, indicating that the carboxylic acid group of the glucuronide substrate is important

Table 3. *K_m and $V_{max.}$ values of the multiple forms of the 17α-hydroxy steroid dehydrogenase for their substrates*
Results were analysed by the double-reciprocal method of Lineweaver & Burk (1934). The assay conditions were as described in the text, except that the amounts of substrates used were as follows: oestradiol-17α, 2.4–24nmol; oestradiol-17α 3-glucuronide, 0.9–9nmol; epitestosterone, 0.5–5nmol.

	Oestradiol-17α		Oestradiol-17α 3-glucuronide		Epitestosterone	
Enzyme	K_m (μM)	$V_{max.}$ (nmol/min per mg)	K_m (μM)	$V_{max.}$ (nmol/min per mg)	K_m (μM)	$V_{max.}$ (nmol/min per mg)
IA	8.8	8	4.7	5	0.74	74
IB	2.7	15	1.0	3	0.63	110
IC	8.6	13	5.1	180	5.2	200
IIC	2.4	9	3.6	47	0.43	41
IID	3.1	11	2.7	9	0.20	72
III	2.8	7	1.0	6	0.44	48

for the increased reaction with this enzyme. If galacturonic acid is substituted at C-3 of the oestrogen molecule, the activity with all forms of the 17α-hydroxy steroid dehydrogenase, except for enzyme IIC, is decreased considerably, indicating that the change in configuration at C-4 of the sugar moiety of the substrate affects its interaction with the enzyme.

The K_m and $V_{max.}$ of each 17α-hydroxy steroid dehydrogenase was determined for the substrates oestradiol-17α, oestradiol-17α 3-glucuronide and epitestosterone, and the data are shown in Table 3. All enzymes except enzyme IC have a significantly lower K_m with epitestosterone than with either oestrogen substrate. Enzymes IA, IB, IID and III also have higher $V_{max.}$ for the androgen substrate than for the oestrogens. These results are in agreement with the relative activities of these enzymes towards the androgen and oestrogen substrates (Table 2). The relatively higher activity of enzyme IC towards oestradiol-17α 3-glucuronide when compared with oestradiol-17α is primarily due to the high $V_{max.}$ of this enzyme for the glucuronide derivative and not to any significant difference in the K_m of the enzyme for each oestrogen substrate. The greater activity of enzyme IIC towards epitestosterone results from the lower K_m of this enzyme for the androgen than for oestradiol-17α 3-glucuronide. All of these enzymes showed a characteristic substrate inhibition with epitestosterone at steroid concentrations higher than 3μM. Inhibition was not observed with oestradiol-17α or its glucuronide derivative at steroid concentrations as high as 8μM. Thaler-Dao *et al.* (1972) did not observe a similar substrate inhibition of rabbit liver 17β-hydroxy steroid dehydrogenase when incubated with testosterone. However, the 3(17)β-hydroxy steroid dehydrogenase from *Pseudomonas testosteroni* (Talalay, 1963) and the 17β-hydroxy steroid dehydrogenase from mature male rat testes (Oshima & Ochiai, 1973) both have been shown to exhibit substrate inhibition. In both cases inhibition was observed with testosterone, but not with oestradiol-17β.

The variation in substrate specificity among the different forms of the 17α-hydroxy steroid dehydrogenase supports the previous evidence (Hasnain & Williamson, 1975) that the multiple forms of this enzyme are present *in vivo* and are not artifacts generated during purification of the enzymes. The presence of a 17α-hydroxy steroid dehydrogenase in rabbit liver that shows a specificity with oestrogen substrates for the glucuronide derivative suggests a close relationship between conjugation with glucuronic acid at C-3 of the steroid and oxidation–reduction at C-17 in this tissue. With unconjugated oestrogens the predominant interconversion is between oestrone and oestradiol-17β (Hasnain & Williamson, 1974). However, after formation of the 3-glucuronide derivative conversion into the 17α-epimer predominates. Thus conjugation of the oestrogen molecule at C-3 with glucuronic acid directs oestrogen metabolism towards elimination of this molecule owing to the presence in rabbit tissues of a 17α-hydroxy steroid dehydrogenase specific for the glucuronide derivative. Oestradiol-17α 3-glucuronide is the specific substrate for the enzymic transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to the 17α-position of the steroid (Collins *et al.*, 1968). The double conjugate formed is then excreted in the urine (Layne *et al.*, 1964; Layne, 1965; Collins *et al.*, 1967).

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