Purification and characterization of two forms of microsomal carbonyl reductase in guinea pig liver

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Two forms of microsomal carbonyl reductase, solubilized in Triton X-100, were purified to homogeneity from the liver of male guinea pigs, primarily by affinity, DEAE-Sephacel, gel-filtration and hydroxyapatite chromatography. The major form was a tetrameric glycoprotein of single subunits of M_r 32000 and a pI value of 7.0; another minor form was a monomeric protein with M_r 34000 and a pI value of 7.8. The enzymes were immunologically distinct. Although the enzymes showed similar substrate specificity for exogenous aldehydes and ketones and apparently absolute cofactor specificity for NADPH, their specificity for natural carbonyl compounds differed. The major form irreversibly reduced 5α - and 5β -dihydrotestosterones, menadione and lauryl aldehyde with low K_m values of 10–70 μM , whereas the minor form not only reduced 17-oxosteroids, of which 3α -hydroxy- 5β -androstan-17-one was the best substrate, but also oxidized 17-hydroxysteroids in the presence of NADP⁺. The two forms of carbonyl reductase also exhibited different sensitivity to heavy metal ions, dicoumarol, tetramethyleneglutaric acid, phenobarbitone and corticosteroids.

Mammalian tissues contain some NADPH-dependent aldehyde/ketone reductases which differ from aldehyde reductase (alcohol:NADP⁺ oxidoreductase, EC 1.1.1.2) in their ability to reduce aromatic ketones and quinones and in insensitivity to barbitones (Felsted & Bachur, 1980). The soluble enzymes purified from tissues of man and animals have similar broad substrate specificity for exogenous carbonyl compounds (Culp & McMahon, 1968; Sawada *et al.*, 1979a; Felsted *et al.*, 1980; Daly & Mantle, 1982) and have recently been referred to as carbonyl reductase (Wermuth, 1981), but they differ in specificity for physiological substrates: a role in the metabolism of steroids (Sawada *et al.*, 1979b, 1980; Ikeda *et al.*, 1981), quinones and prostaglandins (Daly & Mantle, 1982; Wermuth, 1981) has been proposed. The enzyme activity is also detected in organelles; the microsomes of rat (Kahl, 1970) and guinea pig (Sawada & Hara, 1978) liver have high enzyme activity. However, the membrane-associated enzymes have not been purified to homogeneity and their physiological roles are not known.

We partially purified a carbonyl reductase from guinea pig liver microsomes and suggested the possibility that this enzyme is 3-oxosteroid reductase, because the enzyme efficiently reduced the 3-oxo group of dihydrotestosterones in addition to exogenous aldehydes and ketones (Sawada *et al.*, 1981). During the course of further purification of the microsomal enzyme to demonstrate this possibility, we found that the enzyme activity was resolved into two forms: one exhibited the dihydrotestosterone reductase activity but the other did not. This report describes the purification of the two forms of the enzyme to homogeneity and their chemical and immunological properties.

Experimental

Chemicals

Steroids, indomethacin, dicoumarol and the standard proteins were obtained from Sigma Chemical Co., 3,3-tetramethyleneglutaric acid from Aldrich Chemical Co., and nicotinamide nucleotides, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase and pI markers from Oriental Yeast Co. (Tokyo, Japan). Chlorpromazine dihydrochloride was supplied by Shionogi Pharmaceutical Co. (Osaka,

Abbreviation used: SDS, sodium dodecyl sulphate.

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Japan), Emulgen 911 by Kao Atlas Co. (Tokyo, Japan), and prostaglandins E_2 and $F_{2\alpha}$ by Ono Central Research Institute (Osaka, Japan). Matrex Red A was obtained from Amicon Co., Ultrogel AcA 34 from LKB Instruments, Bio-Beads SM-2 from Bio-Rad Laboratories, and 2',5'-ADP-Sepharose, DEAE-Sepharcel, Sephadex G-100 and carrier ampholines from Pharmacia Fine Chemicals. Hydroxyapatite was prepared as described by Levin (1962). Human prostatic acid phosphatase was purified by the method of Sawada *et al.* (1978).

Preparation of microsomes and solubilization by Triton X-100

The microsomes and cytosols from liver and other tissues of male albino guinea pig (approx. 400 g body wt.) of the Hartley strain were prepared as previously described (Sawada & Hara, 1978). The tissues were homogenized in 4 vol. of 0.25 M-sucrose/1 mM-EDTA. The homogenates were centrifuged at 10000g for 15 min and the microsomal fraction was obtained by centrifugation of the resulting supernatant at 105000g for 1 h and then was washed three times with the same medium. The washed liver microsomes contained 6, 1 and 1% of glucose-6-phosphate dehydrogenase, alcohol dehydrogenase and succinate dehydrogenase activities, respectively, of the total activity of these enzymes in the homogenate. When the microsomal fraction was washed with 0.15 M-KCl instead of 0.25 M-sucrose/1 mM-EDTA, a significant loss of carbonyl reductase activity in microsomes was not observed, and when 1.0 M-KCl was used to wash the microsomes, the reductase activity was 5% less than that in microsomes before washing. The microsomes were suspended to 20 mg of protein/ml in 0.5 mM-EDTA/1 mM-2-mercaptoethanol/20 mM-Tris/HCl buffer, pH 7.0 (Buffer A). Triton X-100 was added to the suspension to a final concentration of 1% (v/v). The mixture was gently stirred for 1 h and was centrifuged at 105000g for 1 h to give a clear solubilized supernatant. All procedures were carried out at 0–4°C.

Enzyme assay

Carbonyl reductase activity was assayed at 37°C by monitoring NADPH absorbance at 340 nm. The standard assay mixture consisted of 5 mM-pyridine-4-aldehyde/80 μ M-NADPH/0.1% Triton X-100/80 mM-sodium citrate buffer, pH 5.2, or 80 mM-potassium phosphate buffer, pH 6.3, and enzyme in a total volume of 2.5 ml. Although most of the other aldehydes and ketones were made up in aqueous solution when used as substrate, steroids and prostaglandins were dissolved in methanol, and vitamin K_1 and lauryl aldehyde were suspended in 1.6% (w/v) Emulgen 911, and 50 μ l portions were added to the reaction mixture.

The concentrations of methanol (1%) and Emulgen (0.04%) had no effect on the rates of reduction with the two carbonyl reductases. The reaction was initiated by the addition of NADPH. A molar absorption coefficient of $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for NADPH. One unit of enzyme was defined as the amount of enzyme oxidizing 1 μ mol of NADPH/min at 37°C.

The reverse reaction was measured by recording NADPH formation in 2.5 ml of 160 μ M-NADP⁺/0.1% Triton X-100/80 mM-glycine/NaOH buffer, pH 10.0, various concentrations of alcohols and enzyme at 37°C.

The activities of catalase, acid phosphatase, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase and succinate dehydrogenase were assayed by the methods of Beers & Sizer (1952), Sawada *et al.* (1978), Chung & Langdon (1963), Racker (1950) and Ackrell *et al.* (1978), respectively.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) and by the modified Lowry procedure (Mather & Tamplin, 1979) with bovine serum albumin as a standard. For routine column monitoring, protein concentration was estimated by measurement of A_{280} .

Carbohydrate determination

Concentration of hexose in the purified preparation was determined by the phenol/ H_2SO_4 colorimetric method (Hodge & Hofreiter, 1962) with D-glucose as a standard, and that of sialic acid by the method of Warren (1959) with N-acetylneuraminic acid as a standard.

Electrophoresis

Disc gel electrophoresis in 7.5%-polyacrylamide gels containing 0.1% Triton X-100 was performed at 4°C according to the method described by Davis (1964). SDS/polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969). The gels were stained for protein with Coomassie Brilliant Blue R-250 and for carbohydrate with HIO_4 /Schiff's reagent (Zacharius *et al.*, 1969). Isoelectric focusing of the purified reductases was performed in thin-layer polyacrylamide gels containing 0.1% Triton X-100 and 2% (v/v) Pharmalyte, pH 3–10.5, as described by the manufacturer. The pH gradient in the gel was assessed by focusing pI markers simultaneously.

M_r determination

The M_r values of native microsomal carbonyl reductases were determined by gel filtration on Sephadex G-100 or Ultrogel AcA 34 column (1.9 cm $\times$ 97 cm) with bovine liver catalase (M_r

232000), rabbit muscle aldolase (M_r 158000), prostatic acid phosphatase (M_r 100000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and myoglobin (M_r 17800) as standards. The Sephadex G-100 column was equilibrated with 0.05% Triton X-100/0.3M-NaCl/20mM-Tris/HCl buffer, pH 8.0, and the Ultrogel column with 0.1% Triton X-100/1M-NaCl/20mM-Tris/HCl, pH 8.0. For M_r determination of denatured enzymes, SDS/polyacrylamide-gel electrophoresis was employed in the presence of the following standards; bovine serum albumin, ovalbumin, trypsin (M_r 23000) and myoglobin.

Sucrose density-gradient centrifugation

The sedimentation coefficient ($s_{20,w}$) of the purified reductase was determined by density-gradient centrifugation in a 5–20% (w/v) sucrose density-gradient containing 0.1% Triton X-100/1M-NaCl/20mM-Tris/HCl buffer, pH 7.5, as described by Clarke (1975). The sample, containing the following reference proteins: catalase ($s_{20,w}$ 11.3S), yeast alcohol dehydrogenase ($s_{20,w}$ 7.2S), haemoglobin ($s_{20,w}$ 4.6S) and cytochrome *c* ($s_{20,w}$ 1.7S) in 0.2ml, was layered on a 4.5ml gradient and centrifuged at 4°C for 20 h at 38000 rev./min in a Hitachi SW-40 rotor (r_{av} 7.3cm). Portions (0.2ml) were collected from the gradient, enzyme activities were assayed as described above and the concentrations of haemoproteins were determined at 415nm.

Immunochemical experiments

Antibody against the purified carbonyl reductase was raised in a young female rabbit. Before the immunization, Triton X-100 in the enzyme solution was first removed at 4°C with a 2',5'-ADP-Sepharose column (1cm × 5cm). The enzyme (3mg) was dialysed at 4°C against 20 vol. of Buffer A and absorbed onto the column. The column was washed with Buffer A until the A_{280} became zero. The enzyme was then eluted with 1M-NaCl in Buffer A, and dialysed against 50 vol. of 0.9% NaCl/10mM-sodium phosphate buffer, pH 7.2, for 12 h at 4°C. The enzyme preparation was emulsified with an equal volume of Freund's complete adjuvant. The rabbit was immunized initially with 1 mg of the enzyme by subcutaneous injection, and similarly injected with 0.5mg of the enzyme at 1 week intervals thereafter. Blood was withdrawn from an ear vein 10 days after the fourth injection had been given, and the serum was separated.

The antiserum was tested by the Ouchterlony (1949) double diffusion technique. The effect of the antiserum on carbonyl reductase activity was examined as follows: a mixture containing 10mM-sodium phosphate buffer, pH 7.2, 25 μ g of the enzyme solution and various amounts of the anti-

serum (0–100 μ l) in a final volume of 0.2ml was preincubated for 30min at 30°C, and then the remaining activity was measured in the standard assay mixture.

Identification of reaction product

For identifying the reduced products, 0.12mM-3 α -hydroxy-5 β -androstane-17-one or 0.12mM-5 β -dihydrotestosterone as a substrate was incubated at 37°C for 60min with the purified reductase (0.1mg of protein), 80mM-potassium phosphate buffer, pH 6.3, and 80 μ M-NADPH in a total volume of 5.0ml. The reaction products were extracted with 10ml of ethyl acetate. The organic phases were separated and concentrated by evaporating the solvent. Aliquots of organic extracts were applied to silica t.l.c. plates (Merck; silica gel 60 F-254) and chromatographed in solvent A [benzene/acetone (4:1, v/v)] or solvent B [benzene/methanol (19:1, v/v)]. After the products were visualized by spraying with H₂SO₄/methanol (1:1, v/v), R_F values of the products were compared with those of the authentic steroids 5 β -androstane-3 α ,17 β -diol and 5 β -androstane-3 β ,17 β -diol.

Results

Enzyme purification

The solubilized supernatant of liver microsomes, containing both carbonyl reductase and 5 β -dihydrotestosterone reductase activities, was directly applied to a Matrex Red A column (2.4cm × 22cm) equilibrated with Buffer A containing 0.05% Triton X-100. Both enzyme activities were absorbed on the resin and were then eluted with 0.05% Triton X-100/0.5mM-EDTA/1mM-2-mercaptoethanol/20mM-Tris/HCl buffer, pH 8.0 (Buffer B), containing 1M-NaCl. The enzyme fractions were pooled, dialysed against 4 litres of Buffer B, and applied to a DEAE-Sepharose column (2.4cm × 23cm) equilibrated with Buffer B. The column was washed with Buffer B, then eluted with a linear 0–0.3M-NaCl gradient in Buffer B. This resulted in the resolution of two distinct peaks of carbonyl reductase activity which were designated CR1 and CR2; 5 β -dihydrotestosterone reductase activity was eluted coincidentally with CR2 but was not detected in CR1 (Fig. 1). The two enzyme fractions were separately concentrated through an Amicon YM-10 membrane and were each dialysed overnight against 2 litres of Buffer A. CR1 was further purified according to the following scheme. The dialysed enzyme solution was applied to a 2',5'-ADP-Sepharose column (1.6cm × 5cm) equilibrated with Buffer A containing 0.05% Triton X-100. After the column was washed with the same buffer and Buffer B, the enzyme was

Fig. 1. DEAE-Sephacel chromatography of carbonyl reductase from guinea pig liver microsomes. Fractions (10 ml) were collected and analysed for protein (A_{280}). Carbonyl reductase (O) and 5β -dihydrotestosterone reductase (●) were assayed with pyridine-4-aldehyde and 5β -dihydrotestosterone as substrates, respectively, in 80 mM-potassium phosphate buffer, pH 6.3, as described in the text.

Table 1. Co-purification of carbonyl reductase and 5β -dihydrotestosterone reductase from guinea pig liver microsomes. All the procedures were performed at 4°C. In the purification of CR2, 1 M-NaCl/0.1% Triton X-100/0.5 mM-EDTA/1 mM-2-mercaptoethanol/20 mM-Tris/HCl buffer, pH 8.0, was used for the elution of the 2',5'-ADP-Sepharose column and for the pre-equilibration and elution of Ultrogel AcA 34 and hydroxyapatite columns. The reductase activities were assayed in a reaction mixture consisting of 3.4 mM-pyridine-4-aldehyde or 60 μ M- 5β -dihydrotestosterone/80 μ M-NADPH/80 mM-potassium phosphate buffer, pH 6.3, as described in the text. Units are μ mol of NADPH/min. Abbreviation used: n.d., no activity detected.

Step	Volume (ml)	Total protein (mg)	Carbonyl reductase			5β -Dihydrotestosterone reductase			Specific activity ratio of carbonyl reductase to 5β -dihydrotestosterone reductase
			Specific activity (units/mg)	Yield (%)	Purification (fold)	Specific activity (units/mg)	Yield (%)	Purification (fold)	
Microsomes	240	4840	0.0146	100	1	0.0102	100	1	1.43
Solubilized supernatant	216	2130	0.0320	96	2.2	0.0149	83	1.5	1.65
Matrex Red A	118	677	0.0924	89	6.3	0.0539	74	5.3	1.72
CR1									
DEAE-Sephacel	21.5	120	0.158	27	11	n.d.	—	—	—
2',5'-ADP-Sepharose	2.6	3.20	2.23	10	153	n.d.	—	—	—
Sephadex G-100	3.3	1.61	3.31	8	227	n.d.	—	—	—
Hydroxyapatite	1.2	1.05	4.16	6	285	n.d.	—	—	—
CR2									
DEAE-Sephacel	23.5	187	0.184	49	13	0.147	56	14	1.25
2',5'-ADP-Sepharose	6.5	49.1	0.476	33	33	0.479	47	47	1.00
Ultrogel AcA 34	5.5	30.1	0.664	28	45	0.654	40	64	1.01
Hydroxyapatite	4.2	19.3	0.891	24	61	0.881	34	86	1.01

eluted with Buffer B containing 0.3 M-NaCl. Following concentration by ultrafiltration, the sample was chromatographed on a Sephadex G-100 column (2.6 cm $\times$ 95 cm) in Buffer B containing 0.3 M-NaCl. The enzyme fractions were pooled, concentrated by ultrafiltration and applied to

a hydroxyapatite column (1.2 cm $\times$ 7 cm) equilibrated with Buffer B. The enzyme was eluted with a linear 0–0.15 M-potassium phosphate, pH 8.0, gradient in Buffer B. CR2 was purified by the same series of procedures as those for CR1 following their initial separation by DEAE-Sephacel

chromatography, except that Ultrogel AcA 34 was used in place of Sephadex G-100 in the gel filtration (Table 1). Subsequent to the DEAE-Sephacel fractionation, no additional heterogeneity of carbonyl reductase was detected and the ratios of carbonyl reductase activity and 5β -dihydrotestosterone reductase activity were constant during the chromatographic steps of purification of CR2. CR1 and CR2 as eluted from the hydroxyapatite columns each consisted of a single band when examined by polyacrylamide disc gel electrophoresis with or without SDS (Fig. 2).

Physicochemical properties

CR2 stained with HIO_4 /Schiff's reagent on the polyacrylamide gel (Fig. 2), but CR1 did not. The phenol/ H_2SO_4 colorimetric assay for neutral sugar yielded a colour response equivalent to 16 mol of glucose/mol of enzyme in the preparation of CR2, but sialic acid was not detectable by the Warren (1959) procedure.

The M_r values estimated by gel filtration and SDS/polyacrylamide-gel electrophoresis were 37 500 and 34 000, and 32 000 and 115 000 for CR1

and CR2, respectively (Fig. 3). The sedimentation coefficient ($s_{20,w}$) of CR2, which was determined by sucrose density-gradient centrifugation, was about 5.5S. These results suggested that CR1 exists as a monomer whereas CR2 exists as a tetramer in the native state.

Samples of CR1 and CR2 were subjected to isoelectric focusing and stained with Coomassie Blue. Both carbonyl reductases produced a single band and the corresponding isoelectric points for CR1 and CR2 were 7.8 and 7.0, respectively.

Catalytic properties

The dependence of reductase activity on pH was examined in 80 mM-sodium citrate and 80 mM-potassium phosphate buffers. CR1 exhibited maximal activity with either 4-nitroacetophenone or pyridine-4-aldehyde as a substrate between pH 5.8 and 6.2. The optimum for CR2 with the above two compounds and 5β -dihydrotestosterone as substrate occurred at about pH 4.8–5.3.

Table 2 compares substrate specificity of the carbonyl reductases for various carbonyl compounds. CR1 and CR2 reduced aromatic aldehydes efficiently. Although aliphatic aldehydes such as DL-glyceraldehyde, acetaldehyde and crotonaldehyde were poor substrates for the two enzymes, CR2 showed relatively high affinity and a moderate $V_{\max}$ value for lauryl aldehyde. Of the ketone substrates, CR1 showed high reactivity

Fig. 2. Polyacrylamide-gel electrophoresis of two purified carbonyl reductases

About 10 μg of purified CR1 and CR2 were applied to gels (a) and (d) and (b) and (e), respectively, and 50 μg of CR2 to gel (c). (a)–(c), polyacrylamide-gel electrophoresis at pH 8.3; (d) and (e), SDS/polyacrylamide-gel electrophoresis. Gels (a), (b), (d) and (e) were stained for protein, and gel (c) for carbohydrate.

Fig. 3. M_r values of CR1 and CR2

Curve (a), gel filtration on Ultrogel AcA 34; curve (b), that on Sephadex G-100; curve (c), SDS/polyacrylamide-gel electrophoresis. The standard proteins were: 1, catalase; 2, aldolase; 3, prostatic acid phosphatase; 4, bovine serum albumin; 5, ovalbumin; 6, trypsin; 7, myoglobin.

Table 2. *Substrate specificities of microsomal carbonyl reductases*

The activity was assayed with 80 mM-potassium phosphate buffer, pH 6.3, and 80 mM-sodium citrate buffer, pH 5.2, for CR1 and CR2, respectively. Relative $V_{\max}$ values are percentages of those obtained with pyridine-4-aldehyde. Actual $V_{\max}$ values were 8.9 and 3.8 $\mu\text{mol}/\text{min}$ per mg for CR1 and CR2, respectively. Abbreviation used: n.d., not determined.

	Concentration (mM)	CR1		CR2	
		K_m (mM)	Relative $V_{\max}$	K_m (mM)	Relative $V_{\max}$
Pyridine-4-aldehyde		3.8	100	2.3	100
Benzaldehyde		0.43	23	0.87	61
4-Nitrobenzaldehyde		0.18	81	0.11	126
3-Nitrobenzaldehyde		0.092	204	0.063	256
2-Nitrobenzaldehyde		0.11	125	0.098	203
Phenylglyoxal		2.6	111	0.62	89
Lauryl aldehyde*	0.2	n.d.	(10)	0.070	55
4-Benzoylpyridine*	3.4	n.d.	(4)	0.009	84
Acetophenone*	10.0	n.d.	(8)	1.6	96
4-Nitroacetophenone		0.23	78	0.082	91
3-Nitroacetophenone		0.86	36	0.037	168
Propiophenone*	5.0	n.d.	(2)	1.4	103
Benzalacetone*	3.4	n.d.	(6)	3.7	84
Diacetyl		8.6	214	7.2	182
5 α -Androstane-3,17-dione*	0.06	n.d.	(2)	n.d.	(0)
5 β -Androstane-3,17-dione*	0.06	n.d.	(13)	n.d.	(1)
3 α -Hydroxy-5 β -androstane-17-one*	0.06	0.050	82	n.d.	(0)
3 β -Hydroxy-5 β -androstane-17-one*	0.06	n.d.	(20)	n.d.	(0)
5 α -Dihydrotestosterone*	0.06	n.d.	(0)	0.013	22
5 β -Dihydrotestosterone*	0.06	n.d.	(0)	0.010	129
Cortisone*	0.06	n.d.	(0)	n.d.	(8)
Prednisone*	0.06	n.d.	(0)	n.d.	(12)
Menadione*	0.2	n.d.	(1)	0.036	127
Vitamin K ₁ *	0.08	n.d.	(0)	n.d.	(2)
Potassium ferricyanide†	1.0	n.d.	(1)	n.d.	(177)
NADPH		0.004		0.006	

* With these substrates, the velocities with the indicated concentrations relative to that with 3.4 mM-pyridine-4-aldehyde are expressed in parentheses.

† Activity of this substrate was determined at 410 nm by using an absorption coefficient of $10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

only to nitroacetophenones, and diacetyl, whereas CR2 reduced various ketones. A clearer difference in substrate specificity between CR1 and CR2 was observed with steroids, quinones and potassium ferricyanide. CR1 essentially reduced 17-oxosteroids, whereas CR2 reduced 3-oxosteroids. The K_m value of CR1 for 3 α -hydroxy-5 β -androstane-17-one was lower than those for the other substrates, and the K_m values of CR2 for dihydrotestosterones were as low as that for the best ketone substrate, 4-benzoylpyridine. The reduced product of 3 α -hydroxy-5 β -androstane-17-one by CR1 was identified as 5 β -androstane-3 α ,17 β -diol and that of 5 β -dihydrotestosterone by CR2 as 5 β -androstane-3 α ,17 β -diol on t.l.c. Only CR2 had the ability to reduce quinones and potassium ferricyanide. It should be noted that 0.04 mM-2,6-dichlorophenol-indophenol, 0.1 mM-cytochrome *c*, 1.0 mM-prostaglandin E₂ and 60 μM -testosterone, androsterone,

epiandrosterone, progesterone, oestrone and 5 β -cholestan-3-one were not reduced by the two enzymes.

Both CR1 and CR2 preferred NADH to NAD⁺ as a cofactor, the relative velocities with 80 μM -NADH to those with 80 μM -NAD⁺ were 0.25% and 0.1% for CR1 and CR2, respectively.

The reverse reactions of the carbonyl reductases were explored by testing their ability to oxidize a variety of alcohols. CR1 oxidized 60 μM -5 β -androstane-3 α ,17 β -diol, 60 μM -5 β -dihydrotestosterone, 60 μM -testosterone and 50 mM-cyclohexanol at rates of 24, 13, 4 and 6%, respectively, of reductase activity obtained with pyridine-4-aldehyde under its optimal pH conditions of 6.3. The optimal pH of 5 β -androstane-3 α ,17 β -diol dehydrogenase activity was 10.0 when assayed in 80 mM-glycine/NaOH buffers. However, no NADPH formation was observed with 17 α -epitestosterone, 5 α -andro-

Table 3. *Effects of inhibitors and steroids on microsomal carbonyl reductases*

Aliquots of the inhibitors at the final concentration indicated were added into the reaction mixtures containing 1.0mM-4-nitroacetophenone, 80mM-potassium phosphate buffer, pH6.3, for CR1 or 80mM-sodium citrate buffer, pH5.2, for CR2 and the reductase at 37°C, and then the activity was assayed immediately by the addition of 80 μ M-NADPH. When steroids were added, 60 μ M-3 α -hydroxy-5 β -androstan-17-one and 5 β -dihydrotestosterone were as substrates for CR1 and CR2, respectively. The values are expressed as means $\pm$ S.E.M. obtained from three separate preparations.

Inhibitor and steroid	Concentration (mM)	Inhibition	
		CR1	CR2
HgCl ₂	0.001	45 $\pm$ 3	99 $\pm$ 1
AgNO ₃	0.001	77 $\pm$ 2	1 $\pm$ 1
<i>p</i> -Chloromercuribenzoic acid	0.01	71 $\pm$ 2	99 $\pm$ 1
Dicoumarol	0.01	2 $\pm$ 1	35 $\pm$ 1
3,3-Tetramethyleneglutaric acid	0.1	18 $\pm$ 1	76 $\pm$ 1
Ammonium molybdate	0.1	56 $\pm$ 2	8 $\pm$ 1
CuSO ₄	0.1	53 $\pm$ 1	1 $\pm$ 1
Quercitrin	0.1	53 $\pm$ 1	34 $\pm$ 1
Indomethacin	0.1	32 $\pm$ 1	35 $\pm$ 1
Sodium phenobarbitone	1.0	16 $\pm$ 2	43 $\pm$ 2
Chlorpromazine	1.0	46 $\pm$ 1	38 $\pm$ 1
Progesterone	0.06	76 $\pm$ 1	63 $\pm$ 1
5 β -Pregnane-3,20-dione	0.06	42 $\pm$ 1	49 $\pm$ 1
5 α -Pregnane-3,20-dione	0.06	20 $\pm$ 1	56 $\pm$ 2
21-Hydroxy-5 α -pregnane-3,20-dione	0.06	49 $\pm$ 1	80 $\pm$ 1
Deoxycorticosterone	0.06	43 $\pm$ 1	60 $\pm$ 1
Androsterone	0.06	1 $\pm$ 1	31 $\pm$ 1
Testosterone	0.06	20 $\pm$ 1	20 $\pm$ 1
5 β -Androstane-3 α ,17 β -diol	0.06	20 $\pm$ 2	23 $\pm$ 1
Oestrone	0.06	2 $\pm$ 1	9 $\pm$ 1

stane-3 β ,17 β -diol and 5 α -dihydrotestosterone as substrates. CR2 did not show the reverse reaction with the above alcohols.

Inhibition studies

CR1 and CR2 were similarly inhibited by Hg²⁺, *p*-chloromercuribenzoic acid, quercitrin, indomethacin and chlorpromazine, but differed from each other in sensitivity to the other inhibitors (Table 3). Effects of steroids which were not substrates for the two enzymes were examined, since the two enzymes efficiently reduced 17- or 3-oxosteroids. The two enzyme activities with the natural substrates were inhibited more highly by gestagens and deoxycorticosterone than by androgens and oestrone. CR2 was non-competitively inhibited by progesterone and 21-hydroxy-5 α -pregnan-3,20-dione, for which *K_i* values were both 10 μ M, while the reduced product, 5 β -androstan-3 α ,17 β -diol, gave competitive inhibition at a *K_i* value of 87 μ M.

Immunochemical properties

The antiserum against CR2 formed a single and fused precipitin line with both CR2 and the microsomes treated with 1.0% Triton X-100, but did not react with CR1 and the liver cytosol fraction (Fig. 4a). When the effect of the antiserum on carbonyl reductase activity in the purified

Fig. 4. Double immunodiffusion of carbonyl reductases and microsomes from guinea pig tissues against anti-CR2 serum

Anti-CR2 serum (A) was placed in the centre wells on plates (a) and (b). The microsomes (20mg of protein/ml) were incubated with 1% Triton X-100 at 4°C for 30min and their 10 μ l portions were analysed. Peripheral wells on plate (a) contain CR2 (5 μ g, 1 and 4), CR1 (5 μ g, 2), liver microsomes (3 and 6) and liver cytosol (1 mg, 5) and those on plate (b) contain CR2 (5 μ g, 1 and 2) and microsomes from heart (3), testis (4), spleen (5), liver (6), kidney (7), muscle (8), lung (9) and brain (10).

enzymes was examined, CR2 was maximally inhibited by about 50%, but the activity of CR1 was not affected at all. An enzyme immunologically similar to CR2 was also found in the microsomes from guinea pig kidney (Fig. 4b).

Discussion

The main objective of this study was the purification and elucidation of the biochemical properties of microsomal carbonyl reductase from guinea pig liver. The enzyme activity solubilized from the microsomes was resolved into two peaks on DEAE-Sephacel chromatography and both enzymes were subsequently purified to homogeneity. The physicochemical and immunochemical properties of the two enzymes were clearly distinctive. Judging from the amount contained in the microsomes, M_r and substrate specificity for CR2, the enzyme is the carbonyl reductase which was partially purified by Sawada *et al.* (1981). The enzyme was here further characterized as a tetrameric glycoprotein composed of a subunit of M_r 32000 and with a pI value of 7.0. CR1 is a new enzyme, which was first found in this study, and does not share most physical and enzymic properties with CR2, although the two enzymes similarly reduced several carbonyl compounds. Thus, the multiplicity is due to the presence of two distinct enzymes in guinea pig liver microsomes. This contrasts with the multiplicity of cytosolic carbonyl reductases from tissues of man (Wermuth, 1981) and animals (Sawada *et al.*, 1979a; Hara *et al.*, 1982; Ikeda *et al.*, 1981; Felsted *et al.*, 1980; Daly & Mantle, 1982), in which the multiple forms of the enzyme are similar in physical, enzymic or immunochemical properties.

CR1 differs from cytosolic carbonyl reductases in human brain (Wermuth, 1981) and chicken kidney (Hara *et al.*, 1982), with respect to its inability to reduce quinones, which are good substrates for the cytosolic enzymes, but resembles cytosolic carbonyl reductases from guinea pig liver in the ability to reduce 17-oxosteroids and to oxidize 17-hydroxysteroids (Sawada *et al.*, 1979a,b). However, the sensitivity of CR1 to SH-reagents and heavy metal ions was higher than that of the cytosolic enzymes of guinea pig liver. The enzyme also differs from NAD⁺-dependent 17 β -hydroxysteroid dehydrogenase from the same source in its cofactor specificity, M_r and substrate specificity (Blomquist *et al.*, 1977). While the NAD⁺-dependent dehydrogenase efficiently oxidized both 5 α - and 5 β -androstanes, CR1 showed specificity for 5 β -androstanes and its reduction rates for 17-oxosteroids were higher than oxidation rates for 17-hydroxysteroids. The present enzyme

may have a physiological role in the metabolism of 5 β -androstanes.

CR2 is a glycoprotein with M_r 115000. No cytosolic carbonyl reductase containing carbohydrates have so far been reported, and the highest M_r of the cytosolic enzymes is 86000 for guinea pig lung enzyme (Nakayama *et al.*, 1982). The results of co-purification of CR2 and 5 β -dihydrotestosterone reductase support our previous suggestion that the enzyme is 3-oxosteroid reductase (Sawada *et al.*, 1981). Since the enzyme showed high specificity and affinity for 5 α - and 5 β -dihydrotestosterones as steroid substrates, it may be involved in testosterone metabolism as dihydrotestosterone 3-oxoreductase. 3-Oxosteroid reductase has been partially purified from rat liver microsomes (Billheimer *et al.*, 1981). CR2 produced a 3 α -hydroxysteroid from 5 β -dihydrotestosterone, whereas the rat enzyme reduces 4 α -methyl-5 α -cholest-7-en-3-one to its 3 β -hydroxy derivative. CR2 showed a more broad substrate specificity than did CR1 and reduced other hydrophobic carbonyl compounds as vitamin K₁ and lauryl aldehyde. The enzyme may have alternative roles in vitamin K₁-dependent carboxylation (Wallin & Hutson, 1982) and in formation of long-chain fatty alcohols (Kawalek & Gilbertson, 1973).

Thus, the two enzymes purified as carbonyl reductase from guinea pig liver microsomes must be steroid-metabolizing enzymes and also must have a role as drug-metabolizing enzyme in detoxification of biologically active carbonyl compounds to more hydrophilic and less active alcohols.

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