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Carbonyl reductase from human testis: purification and comparison with carbonyl reductase from human brain and rat testis

Norihisa Inazu [1], Barbara Ruepp, Hanspeter Wirth [2] and Bendicht Wermuth

University of Berne, Department of Clinical Chemistry, Inselspital, Berne (Switzerland)

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Carbonyl reductase (EC 1.1.1.184) is a cytosolic, monomeric, NADPH-dependent oxidoreductase with broad specificity for carbonyl compounds and a general distribution in human tissues. A carbonyl reductase closely resembling the human enzyme is exclusively expressed in rat reproductive tissues and adrenals (Iwata, N., Inazu, N. and Satoh, T. (1989) J. Biochem. 105, 556–564). In order to investigate the relationship between the human and rat enzyme, carbonyl reductase from human testis was purified to homogeneity. The enzyme was indistinguishable from carbonyl reductase from other human tissues on the basis of physicochemical properties, substrate specificity, inhibitor sensitivity and immunological reactivity. Likewise, the human and rat testis enzymes exhibited greatly overlapping substrate specificities for prostaglandins, steroids as well as many xenobiotic carbonyl compounds, and showed the same susceptibility to inhibition by flavonoids and sulfhydryl-blocking agents. Structural homology between the two enzymes was indicated by the mutual cross-reactivity of antibodies against carbonyl reductase from one species and the enzyme protein from the other species. Unlike the rat enzyme, which is confined to Leydig cells, the human enzyme was detectable in Leydig cells as well as Sertoli and spermatogenic cells.

Introduction

Carbonyl reductase (EC 1.1.1.184), otherwise known as prostaglandin 9-oxoreductase, is a cytosolic, monomeric oxidoreductase that catalyzes the NADPH-dependent reduction of a variety of endogenous and xenobiotic carbonyl compounds. Reflecting the broad substrate specificity, the enzyme has been implicated in the metabolism of prostaglandins [1], anthracycline antibiotics [2] and quinones derived from polycyclic aromatic hydrocarbons [3] and may have other as yet unknown physiological functions.

Carbonyl reductases have been isolated from a number of species and tissues, including human placenta [4], brain [5] and liver [6,7], but appeared to be absent from rat tissues [2,3]. Recently, however, an enzyme was isolated from rat ovary [8] and testis [9], that catalyzes the reduction of oxosteroids and prostaglandins and closely resembles carbonyl reductase from human tissues. In addition to reproductive tissues, the enzyme was also detectable in the adrenals but was absent from other rat tissues, including brain and liver.

To clarify the relationship between carbonyl reductase from human and rat tissues, the enzyme from human testis was purified and its catalytic and immunological properties were compared with those of the rat enzyme. The results indicate that the two enzymes are structurally closely related.

Experimental procedure

Materials

Human tissue was obtained from legal medical autopsies and stored at −20°C. Pyridine nucleotides, prostaglandins and steroids were purchased from Sigma, St. Louis, U.S.A. All other chemicals were obtained from Fluka, Buchs, Switzerland or Merck, Darmstadt, Germany, and were of the highest grade available. Substrates and enzyme inhibitors which are poorly soluble in water were dissolved in ethanol. The final concentration of ethanol in the assay mixture did

Abbreviation: PG, prostaglandin.

Present addresses: [1] Department of Pharmacology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan and [2] Medizinische Poliklinik, Gastroenterologie, Universitätsspital, CH-8091 Zürich Switzerland.

Correspondence: B. Wermuth, Chemisches Zentrallabor, Inselspital, CH-3010 Bern, Switzerland.

not exceed 2%, a concentration which did not affect the activity of the enzyme.

Methods

Enzyme assay. Carbonyl reductase activity was determined spectrophotometrically by recording the change of NADPH absorbance at 340 nm and 30°C. The standard assay mixture consisted of 0.1 M sodium phosphate buffer (pH 6.5), 0.1 mM NADPH, carbonyl substrates as indicated in the text and 10–100 μl enzyme solution in a total volume of 1 ml. Reactions were initiated by the addition of coenzyme, and blanks without substrates or enzyme were routinely included. Dehydrogenase activity was assayed in 0.1 M Tris-HCl (pH 8.5) in the presence of 0.1 mM $NADP^+$ and alcohol substrates. One enzyme unit is defined as the change in absorbance at 340 nm corresponding to the oxidation or reduction of 1 μmol of coenzyme/min.

Identification of reaction products. In addition to following the change in absorbance of NADPH, the reduction of steroids and prostaglandins was monitored by analysis of the alcohol products. Reacations were carried out under standard assay conditions for 2–4 h. Steroids were extracted with a mixture of chloroform/methanol (2:1) and analyzed by thin-layer chromatography on silica gel (Kieselgel 60, Merck, Darmstadt, Germany) using benzene/methanol (19:1) as solvent. Steroids were visualized with H_2SO_4 in methanol (1:1) or iodine vapor. Alternatively, reaction mixtures were dried under a stream of nitrogen, the residues treated with *N*,O-bis(trimethylsilyl)trifluoroacetamide and the trimethylsilyl products analyzed by gas chromatography-mass spectrometry (GC 5890-MSD 5970B, Hewlett-Packard, Palo Alto, CA, U.S.A.) using a HP SE54 capillary column. Reaction mixtures containing prostaglandins were acidified to pH 3 with HCl and prostaglandins were extracted with diethyl ether. Alternatively, aliquots were withdrawn from the reaction mixture and prostaglandins were analyzed directly by reversed-phase HPLC on an aquapore-300 column (Brownlee, Santa Clara, CA, U.S.A.). The solvent was H_20/acetonitrile/acetic acid (65:35:0.1), and prostaglandins were detected spectrophotometrically at 193 nm.

Protein concentration. The concentration of proteins was estimated from the absorbance at 280 and 260 nm [10].

Electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis was carried out on 10% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate according to Laemmli [11]. Protein bands were visualized by silver staining [12]. For immunodetection proteins were blotted onto nitrocellulose sheets using a Bio-Rad (Richmond, CA, U.S.A.) electroblotting apparatus, and the membranes were incubated with antibodies against carbonyl reductase from human liver [13] and rat ovary [8], respectively. Bound antibodies were visualized using horseradish peroxidase coupled to protein A, H_2O_2 and diaminobenzidine.

Enzyme purification. Carbonyl reductase from human testis was purified essentially by following the procedure established for the enzyme from rat ovary [8]. In brief, testes were homogenized with 9 vol. of 10 mM Tris-HCl (pH 7.4), containing 0.154 M KCl, 1 mM dithiothreitol, 0.5 mM EDTA and 0.01% phenylmethylsulfonyl fluoride. The extract was centrifuged at 100 000 $\times g$ for 1 h, and carbonyl reductase was precipitated from the supernatant by ammonium sulfate (30–60% saturation). Precipitated proteins were dissolved in a minimum volume of 10 mM Tris-HCl (pH 7.4), containing 1 mM dithiothreitol, 0.5 mM EDTA and 0.01% phenylmethylsulfonyl fluoride, and fractionated by gel filtration on Sephadex G-100 (4 × 145 cm). Fractions containing carbonyl reductase activity were concentrated by ultrafiltration, dialyzed against 25 mM Tris/acetic acid (pH 8.0), containing 1 mM dithiothreitol, and applied to a column (1 × 40 cm) of PBE 94 Polybuffer exchanger (Pharmacia-LKB, Bromma,

TABLE I

Purification of carbonyl reductase from human testis

	Protein (mg)	Activity (μmol/min)	Specific activity (μmol/min mg)	Recovery (%)
Cytosol	2374	19.37	0.008	100
$(NH_4)_2SO_4$-precipitation/Sephadex G-100	1018	16.96	0.017	88
Chromatofocusing				
CR I	18	3.93	0.22	20
CR II	44	7.41	0.17	38
CR III	30	2.06	0.07	11
AGNADP chromatography [a]				
CR I	3.0	3.10	1.03	16
CR II	3.6	6.78	1.88	35
CR III	3.3	1.58	0.48	8

[a] $NADP^+$ linked to agarose through ribose hydroxyls and hexane spacer.

Sweden) equilibrated with the dialysis buffer. Elution was carried out using Polybuffer 96 (pH 6.0), containing 1 mM dithiothreitol, and fractions with carbonyl reductase activity were applied directly to AGNADP-type 4 affinity resin (Pharmacia-LKB) equilibrated with 10 mM potassium phosphate buffer (pH 7.0), containing 1 mM dithiothreitol. Elution of proteins was achieved by a linear gradient of 0–600 mM NaCl.

Carbonyl reductase from human brain and rat testis was purified according to Wermuth [5] and Iwata et al. [9], respectively.

Results

Purification and molecular forms

Table I summarizes the results of a typical purification of carbonyl reductase from human testis. The same purification procedure as the one established for rat carbonyl reductase [8,9] was applicable to the human enzyme, with the exception that higher pH values were necessary to bind the human enzyme to the chromatofocusing resin. Elution with a pH gradient yielded three peaks of activity at pH 7.74, 7.63 and 7.28 (Fig. 1). The corresponding molecular forms of the enzyme are further referred to here as CR I, CR II and CR III, respectively, according to the order of their elution from the column. In all preparations the intermediate form was predominant, whereas approximately equal amounts of the two other forms were obtained. The pattern closely resembles the isoelectrofocusing profiles of carbonyl reductase from other human tissues [4–7] as well as from rat testis, although the rat isoenzymes exhibit markedly lower isoelectric points [9].

Electrophoresis on sodium dodecylsulfate-polyacrylamide gels yielded a single band for each of the three enzyme forms (insert Fig. 1). No difference in apparent molecular weight was detectable between the corresponding enzyme forms from testis and brain. On the other hand, slightly lower apparent molecular weights were observed with carbonyl reductase from rat testis.

Immunochemical properties

In order to evaluate the structural relationship between the human and rat enzyme, Western blots of purified carbonyl reductase from human and rat testis as well as human brain were probed with antibodies against the human liver and rat ovary enzyme, respectively. As shown in Fig. 2 the antibodies against the human enzyme recognized all three enzyme forms from rat testis and, vice versa, the antibodies against the rat enzyme cross-reacted with the enzyme from human testis and brain.

Substrate specificity

Aldehydes, ketones and quinones, previously shown to be substrates of carbonyl reductase from human brain [5], placenta [14] and liver [3,7] as well as rat ovary [8] and testis [9], were also substrates of the human testis enzyme. In fact, no significant differences between the corresponding enzyme forms from human testis and brain were detectable (Table II).

In view of the possible role of carbonyl reductase in prostaglandin and/or androgen metabolism in rat re-

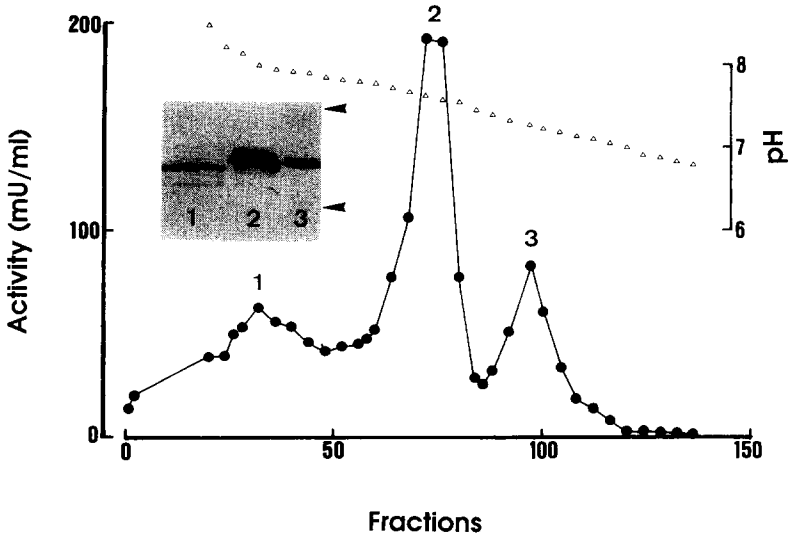

Fig. 1. Resolution of multiple molecular forms of carbonyl reductase from human testis by chromatofocusing. Carbonyl reductase (after elution from the Sephadex G-100 column) was applied on Polybuffer Exchanger 94 and eluted by a pH gradient as described in the experimental procedures. Fractions of 0.5 ml were collected and assayed for pH and enzyme activity using 1 mM benzoylpyridine as substrate. The insert schows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of the three enzyme forms after further purification by affinity chromatography on AGNADP-Sepharose. Numbers 1, 2 and 3 denote the enzyme forms CR I, CR II and CR III, respectively. The arrows in the insert indicate the position of the molecular weight markers ovalbumin (45 000) and bovine carbonic anhydrase (29 000), respectively.

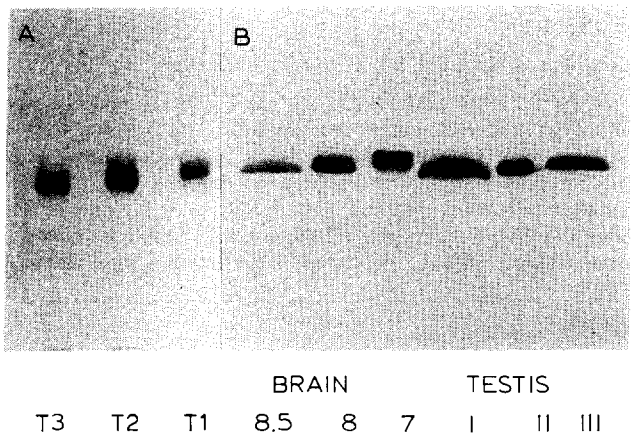

Fig. 2. Immunological cross-reactivity of carbonyl reductase from human and rat tissues. Carbonyl reductase from (A) rat testis and (B) human testis and brain, respectively, was subjected to electrophoresis on sodium dodecylsulfate-polyacrylamide gels, transferred to nitrocellulose and incubated with antibodies against (A) human liver carbonyl reductase and (B) rat ovary carbonyl reductase. Bound antibodies were visualized as described in the experimental procedures.

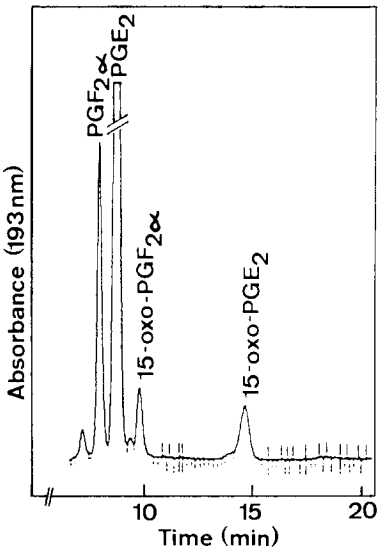

Fig. 3. Reduction of PGE_2 catalyzed by human carbonyl reductase. PGE_2 (1 mM) was incubated with 0.1 mM NADPH in the presence of carbonyl reductase under standard assay conditions for 18 h, and the reaction products were analyzed by reversed phase HPLC as described in the Experimental procedures.

productive tissues [8], the specificity of the human testis enzyme for prostaglandins and androgens was studied in more detail. In accordance with results obtained with carbonyl reductase from human placenta [4], and brain [5] as well as rat tissues [8,9] the human testis enzyme catalyzed the reduction of the 9-oxo as well as the oxidation of the 15-hydroxy group of PGE_2. Time-course studies of the reduction of PGE_2 showed that, in addition to $PGF_{2\alpha}$, significant amounts of 15-oxo-PGE_2 were formed already at the beginning of the reaction when the ratio of NADPH/$NADP^+$ was still high. When the reaction was allowed to proceed to near equilibrium, additionally 15-oxo-$PGF_{2\alpha}$ became detectable (Fig. 3). Similarly, incubation of PGE_2 in the presence of $NADP^+$ and enzyme yielded 15-oxo-PGE_2, 15-oxo-$PGF_{2\alpha}$ as well as $PGF_{2\alpha}$. 13,14-Dihydro-15-oxo-$PGF_{2\alpha}$, which is a better substrate of rat carbonyl reductase than PGE_2, was less efficiently metabolized by the human enzyme. A slight decrease in the absorbance of NADPH was also observed with PGD_2

TABLE II

Substrate specificity of carbonyl reductase from human testis and brain

Carbonyl compounds were incubated at varying concentrations with NADPH or $NADP^+$ and enzyme under standard assay conditions, and kinetic constants (K_m, V) were estimated by graphical extrapolation from double-reciprocal plots.

Substrate	Testis						Brain					
	CR I		CR II		CR III		$CR_{8.5}$		CR_8		CR_7	
	K_m (mM)	V (%)	K_m (mM)	V (%)	K_m (mM)	V (%)	K_m (mM)	V (%)	K_m (mM)	V (%)	K_m (mM)	V (%)
4-Benzoylpyridine	0.39	100	0.31	100	0.45	100	0.42	100	0.32	100	0.44	100
4-Nitroacetophenone	1.3	36	2.5	43	1.9	35	2.1	47	1.9	30	2.7	51
4-Nitrobenzaldehyde	1.6	39	1.5	80	1.3	64	1.9	51	2.0	80	2.1	99
4-Carboxybenzaldehyde	0.38	5	0.40	12	0.65	12	0.54	6	1.37	13	0.60	14
Pyridine4-carboxaldehyde	2.0	60	0.49	47	0.71	63	2.8	66	1.2	75	1.3	106
Menadione	0.06	164	0.07	206	0.05	101	0.06	154	0.07	240	0.05	115
5α-Androstane-3,17-dione			0.15	80 [b]					0.08	36 [b]		
5α-Androstan-17β-ol-3-one			0.05	5					0.03	2		
Prostaglandin E_2			0.24	2					0.34	3		
Prostaglandin B_2	0.14	18 [c]	0.39	18	0.21	20	0.16	12	0.33	22	0.51	26

[a] V values are expressed as percentage of the activity with 4-benzoylpyridine.

[b] The reaction is biphasic. Values correspond to initial velocities, whereas final velocities are in the same range as those obtained with 5α-androstan-17β-ol-3-one.

[c] Rate of oxidation.

as substrate. However, when the reaction products were analyzed neither $PGF_{2\alpha}$ nor 11β-$PGF_{2\alpha}$ were detectable. Instead, three less polar compounds had been generated. The same compounds also arose when PGD_2 was incubated with $NADP^+$ in the presence of carbonyl reductase and correspond to 15-oxo-PGD_2 and, probably, the 9-oxo and 9,15-dioxo derivatives of PGD_2. In agreement with the specificity of carbonyl reductase from placenta [4] and platelets [15], PGB_2 was not a substrate of carbonyl reductase from testis and brain in the presence of NADPH, but was efficiently metabolized in the presence of $NADP^+$.

Generally, androgens were metabolized at low rates comparable to the prostaglandins. Exceptionally high initial rates of NADPH disappearance were observed in the presence of 5α-androstane-3,17-dione. Progress curves, however, were biphasic, and after the initial phase the reaction rates were similar to those observed with the other oxosteroids. Purification of the substrate by thin-layer chromatography did not alter the course of the progress curve, indicating that the initial high rate of NADPH oxidation was not due to impurities in the substrate solution. Owing to the non-linear progress curves and low reaction rates, kinetic constants for steroids could only be estimated, and the numbers given in Table II should be considered as approximations. Reduction of androgens was observed only with the 4,5-dihydro derivatives, whereas testosterone was not metabolized at any significant rate. The enzyme did not distinguish, however, between the 5α- and 5β-configuration. Thus, the 3-oxo group of 5α- and 5β-androstanes was converted to the hydroxy group, which exhibited predominantely, though not exclusively, α-configuration (Table III). In addition to the 3-oxo group, carbonyl reductase also catalyzed the reduction of the the 17-oxo group, even though less

TABLE III

Reduction of steroids by human carbonyl reductase

Steroids (0.2 mM), NADPH (0.1 mM) and carbonyl reductase were incubated in 20 mM sodium phosphate (pH 7.0) and 30°C for 4 h. Steroid products were identified as trimethylsilyl derivatives by gas chromatography-mass spectrometry and comparison with authentic compounds. Numbers in parentheses correspond to the ratio of peak areas (total ion) of product and the sum of all products plus substrate and are expressed as percent (mean from two experiments).

Substrate	Product	
5α-Androstan-17β-ol-3-one	5α-androstane-3α,17β-diol	(11)
	5α-androstane-3β,17β-diol	(trace)
5β-Androstan-17β-ol-3-one	5β-androstane-3α,17β-diol	(27)
	5β-androstane-3β,17β-diol	(2)
5α-Androstan-3α-ol-17-one	no product detectable	
5α-Androstan-3β-ol-17-one	5α-androstane-3β,17β-diol	(4)
5β-Androstan-3α-ol-17-one	5β-androstane-3α,17α-diol [a]	(9)
5β-Androstan-3β-ol-17-one	no product detectable	
5α-Androstane-3,17-dione	5α-Androstan-3α-ol-17-one	(32)
	5α-Androstan-3β-ol-17-one	(5)
	5α-Androstan-17β-ol-3-one	(9)
	5α-Androstane-3α,17β-diol	(trace)
5β-Androstane-3,17-dione	5β-Androstan-3α-ol-17-one	(37)
	5β-Androstan-3β-ol-17-one	(1)

[a] The 17α-configuration was tentatively assigned on the basis of the mass spectrum and elution time.

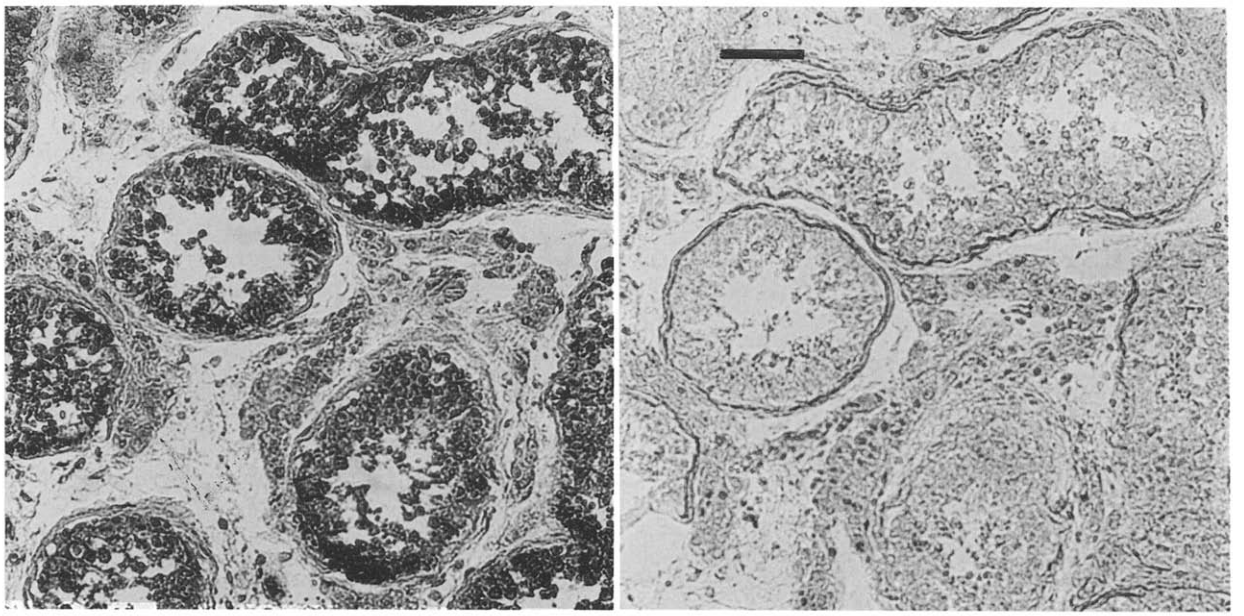

Fig. 4. Immunohistochemical localization of carbonyl reductase in human testis. Sections of paraformaldehyde-fixed human testis were partially digested with trypsin and incubated with antibodies against human liver carbonyl reductase. Bound antibodies were visualized using horse radish peroxidase coupled to protein A and diaminobenzidine as chromogen. Controls (right panel) were incubated with serum from non-immunized rabbits. The black bar corresponds to 0.1 mm

17-hydroxy than 3-hydroxy product was formed from 5α- and 5β-androstane-3-17-dione (Table III). No NADPH production was detectable when the enzyme was incubated with $NADP^+$ and 100 μM 5α-androstan-17β-ol-3-one, 5β-androstan-17β-ol-3-one 5α-androstane-3α,17β-diol and testosterone respectively.

Inhibition studies

In agreement with the known sensitivity of human brain [5] and rat testis [9] carbonyl reductase, sulfhydryl modifying reagents, e.g., 4–OH-mercuribenzoate, and flavonoids (quercitrin) were potent inhibitors of the human testis enzyme, whereas phenobarbital, valproic acid and 3,3′-tetramethylene-glutarate, potent inhibitors of aldehyde and aldose reductase, were markedly less effective. No significant differences between the various human brain and testis enzyme forms were detectable.

Immunohistochemical localization of carbonyl reductase

In human testis, carbonyl reductase was detectable in all three main cell types, i.e., Leydig, Sertoli and spermatogenic cells (Fig. 4). In the seminal vesicle and vas deferens the enzyme was localized primarily in the muscularis with very little or no staining of the epithelium. This is in contrast to the distribution of the enzyme in rat testis and accessory tissues, where immunostaining was limited to the Leydig cells in testis and the epithelium in vas deferens [9].

Discussion

We have purified three molecular forms of carbonyl reductase from human testis and compared their properties with those of carbonyl reductase from rat testis. The observed immunological cross-reactivity together with the many other similarities, including physicochemical properties, substrate specificity and sensitivity to inhibitors, strongly suggest a close relationship between the human and rat enzyme. Nevertheless, within the frame of general similarity marked differences exist between the enzymes from the two species. In the rat, carbonyl reductase is expressed specifically in the gonads and accessory tissues and the adrenals [8,9], whereas in man the enzyme also occurs in other tissues, including liver, kidney, brain and placenta [16]. Moreover, the enzymes expressed by rat testis, vas deferens and ovary, respectively, exhibit slightly but distinctly different specificities for steroids and prostaglandins. In man, no tissue-specific isoenzymes have so far been observed. On the contrary, in this study we showed that within experimental error the human testis and brain enzymes do not differ in their substrate specificities, and analogous results were obtained from the comparison of the brain enzyme with the enzymes from placenta [5,14] and liver [3,6,7], respectively.

Little is known about the physiological role of carbonyl reductase. Originally, prostaglandins were considered to be the physiological substrates [1]. Later, however, when a number of other carbonyl compounds, including aldehydes, ketones and quinones, were found to be metabolized much more efficiently than the prostaglandins, the role of the enzyme in the metabolism of prostaglandins was called into question and a more general role in the detoxication of reactive, potentially toxic carbonyl compounds was suggested [3,5,14,17]. Nevertheless, the significant 15-hydroxyprostaglandin dehydrogenase activity which was observed in the present study with both 9-oxo- and 11-oxoprostaglandins suggests that in tissues with little NAD-dependent 15-OH-prostaglandin dehydrogenase activity, e.g., the brain [18], carbonyl reductase may be responsible for the inactivation of prostaglandins.

The finding of the specific expression of carbonyl reductase in rat reproductive tissues and adrenals, tissues involved in the synthesis of steroid hormones, suggested a possible 'new' role of the enzyme in steroid metabolism. In keeping with such a function carbonyl reductase from rat tissues efficiently catalyzes the reduction of dihydrotestosterone and 3,17-androstanedione, and discriminates between the 5α- and 5β-configuration [8]. In addition, the activity of the ovarian enzyme changes during the estrous cycle [19] and in pregnancy [20] and is influenced by corticosteroid hormones and gonadotropins [21]. In human tissues, the evidence for a physiological role of carbonyl reductase in steroid metabolism is less convincing. All the steroids tested in this study were poor substrates and little discrimination was detectable between androstanones and etiocholanones. Moreover, the recent finding of a dihydrodiol dehydrogenase (3α-hydroxysteroid dehydrogenase) in human liver exhibiting K_m values for androgens 30–300-times lower than carbonyl reductase [22] suggests that in tissues where both enzymes are present, androgens will be metabolized by 3α-hydroxysteroid dehydrogenase rather than carbonyl reductase. Studies on the localization of the two enzymes will be needed to further evaluate the role of carbonyl reductase in steroid metabolism.

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