Purification and characterization of a novel dimeric 20 α -hydroxysteroid dehydrogenase from *Tetrahymena pyriformis*

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Tetrahymena pyriformis was found to exhibit high NADPH-dependent 20-oxosteroid reductase activity that converted 17 α -hydroxyprogesterone into 17 α ,20 α -dihydroxypregn-4-en-3-one. The enzyme was purified 400-fold from the cytosolic fraction. The purified enzyme with a specific activity of 6.4 μ mol/min per mg of protein had an isoelectric point of 4.9 and M_r of 68000, and was composed of two subunits of equal size. The N-terminal sequence was determined to be LAKTVPLNDGTNFPPIFGG. The enzyme reduced pregnenes and pregnanes possessing a 17 α -hydroxy group to a greater extent than those without the hydroxy group, and oxidized 20 α -hydroxy groups of the steroids in the

presence of NADP⁺. The K_m values for 17 α -hydroxyprogesterone and 17 α -hydroxypregnenolone were 2.9 and 3.4 μ M respectively. Although the enzyme was inactive towards androgens and oestrogens with 3- or 17-oxo groups, it reduced several non-steroidal carbonyl compounds and oxidized *trans*-benzene dihydrodiol. The enzyme activity was inhibited by synthetic oestrogens, barbiturates, aldose reductase inhibitors and quercitrin. Thus, this enzyme is a novel form of 20 α -hydroxysteroid dehydrogenase (EC 1.1.1.149) which structurally and functionally differs from the mammalian and bacterial enzymes.

INTRODUCTION

20 α -Hydroxysteroid dehydrogenase (20 α HSD, EC 1.1.1.149), which catalyses the interconversion of the 20-oxo group of pregnanes and the 20 α -hydroxy group, is distributed amongst various mammalian tissues [1]. From the view of regulation of steroid hormone action in mammals, 20 α HSDs have been purified and characterized from endocrine tissues such as ovary [2,3], testis [4,5] and adrenal gland [6,7]. The enzymes from these tissues are monomeric proteins with M_r values of 35000–40000 and are specific for 20 α -hydroxysteroid substrates. However, the pig adrenal enzyme has been shown to exhibit 3 α -hydroxysteroid oxidoreductase activity for androstanes and pregnanes [8]. There have been several reports on bifunctional or polyfunctional hydroxysteroid dehydrogenases having 20 α HSD activity. Human placental oestradiol 17 β -dehydrogenase (EC 1.1.1.62) exhibits low 20 α HSD activity [9], and 3 α ,3 β ,17 β ,20 α -hydroxysteroid dehydrogenase from rabbit liver [10] and 3 β ,20 α -hydroxysteroid dehydrogenases (EC 1.1.1.210) from bovine and sheep erythrocytes [11,12] have been isolated. In addition, monkey liver indanol dehydrogenase (EC 1.1.1.112) [13] and one form of human liver dihydrodiol dehydrogenase (EC 1.3.1.20) [14] have been shown to have 3 α ,20 α -hydroxysteroid dehydrogenase activity. These enzymes are monomeric proteins with M_r values of 3300–55000, except oestradiol 17 β -dehydrogenase, which is dimeric. Thus, various forms of 20 α HSD are present in mammalian tissues.

20 α HSD activity is also detected in micro-organisms [15], but only one 20 α HSD has been purified from *Clostridium scindens* [16]. The bacterial enzyme is distinct from the mammalian enzymes in its tetrameric structure with an M_r of 162000 and high specificity for 17 α ,21-dihydroxyadrenocorticoids. Although the function of 20 α HSD in micro-organisms remains unknown, steroid-hormone-binding proteins are found in eukaryotic uni-

cellular organisms such as yeasts [17,18] and *Tetrahymena* [19,20], and the rat glucocorticoid receptor functions in a hormone-dependent manner in the yeast [21]. In addition, *Streptomyces hydrogenans* 3 α ,20 β -hydroxysteroid dehydrogenase has been reported to show sequence identity with 11 β - and 17 β -hydroxysteroid dehydrogenases of mammalian tissues [22]. These findings have suggested that steroid-metabolizing enzymes have a role in the regulation of the concentrations of hydrophobic signal molecules in micro-organisms. In the present study, we investigated the ability of *Tetrahymena pyriformis* to metabolize progesterone and its derivatives, because progesterone is known to markedly inhibit the growth of this protozoan [23,24] and is converted into pregnenolone and unidentified metabolites in cell culture [25]. Furthermore, it was shown that *T. pyriformis* contains a novel dimeric 20 α HSD specific for 17 α -hydroxypregn-4-ene-3,20-dione (17 α -hydroxyprogesterone) and 3 β ,17 α -dihydroxypregn-5-en-20-one (17 α -hydroxypregnenolone).

EXPERIMENTAL

Materials

Steroids and indomethacin were obtained from Sigma Chemical Co.; pyridine nucleotides and pI markers from Oriental Yeast Company (Tokyo, Japan); Sephadex G-100, chromatofocusing exchanger PBE 94, Polybuffer 74 and M_r markers from Pharmacia Fine Chemicals; and gas-phase protein sequencer chemicals from Applied Biosystems. Matrex Red A was purchased from Amicon Co. and HA-Ultrogel was from LKB Produkter. Benzene dihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) was synthesized as described by Platt and Oesch [26]. Aldose reductase inhibitors, tolrestat and AL-1576 (2,7-difluoro-spiro-fluorene-9,5'-imidazolidine-2',4'-dione), were kindly don-

Abbreviations used: 20 α HSD, 20 α -hydroxysteroid dehydrogenase; 17 α -hydroxyprogesterone, 17 α -hydroxypregn-4-ene-3,20-dione; 17 α -hydroxypregnenolone, 3 β ,17 α -dihydroxypregn-5-en-20-one.

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ated by Dr. G.-T. Xu (Alcon Laboratories, Fort West, TX, U.S.A.). Other chemicals were of reagent grade.

Cell growth

Stock cultures of *Tetrahymena pyriformis* W were maintained in a medium of 2% (w/v) proteose-peptone. *Tetrahymena* cells were grown at 28 °C with shaking (80 rev./min) in a 500 ml flask containing 200 ml of a medium composed of 2% (w/v) proteose-peptone (Difco), 0.5% glucose and 0.2% yeast extract (Difco). Cells were grown to early stationary phase, harvested by centrifugation at 2000 *g* and then washed once with 100 ml of 50 mM glycine/NaOH, pH 8.6, and stored frozen at -20 °C.

Enzyme assay

Reductase and dehydrogenase activities were assayed by measuring the absorbance at 340 nm for NAD(P)H. The standard reaction mixture (2 ml) for the reductase assay contained 100 mM potassium phosphate (pH 6.5), 25 μ M 17 α -hydroxyprogesterone, 0.1 mM NADPH and enzyme, and that for the dehydrogenase assay consisted of 100 mM glycine/NaOH (pH 10.0), 25 μ M steroidal alcohol, 0.25 mM NADP⁺ and enzyme. The reaction was initiated by the addition of the enzyme solution. The pH dependency of the enzyme activity and stability was determined with 0.1 M sodium citrate buffer (pH 4.0–5.8) and 0.1 M phosphate buffer (pH 6.0–11.5) which was prepared by mixing solutions of H₃PO₄ and K₃PO₄. Specific activity was expressed as μ mol/min (unit) of NADPH produced or oxidized per mg of protein at 25 °C. Protein concentration was determined by the method of Bradford [27], using BSA as a standard.

Enzyme purification

In a typical purification of 20 α HSD, *Tetrahymena* cells (12 g wet weight) were lysed by homogenization in a Teflon-glass homogenizer with 80 ml of 50 mM Tris/phosphate, pH 7.4, containing 0.15 M KCl, 5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A). The lysate was centrifuged at 105 000 *g* for 1 h. The supernatant was fractionated by adding (NH₄)₂SO₄ with constant stirring. The 45–75% (NH₄)₂SO₄ precipitate was collected by centrifugation at 12 000 *g* for 15 min and dissolved in 10 ml of buffer A. The solution was applied to a 4.6 cm $\times$ 95 cm Sephadex G-100 column in 10 mM Tris/HCl buffer, pH 7.5, containing 0.5 mM EDTA, 1 mM 2-mercaptoethanol and 0.15 M KCl. The enzyme fractions were concentrated to 20 ml by ultrafiltration using an Amicon YM-10 membrane, dialysed overnight against 10 mM Tris/HCl, pH 7.5, containing 0.5 mM EDTA, 1 mM 2-mercaptoethanol and 20% (v/v) glycerol (buffer B), and then applied to a 1.2 cm $\times$ 10 cm Matrex Red A column that had been pre-equilibrated with buffer B. The column was washed with 50 ml of buffer B plus 0.5 mM NAD⁺ and eluted with buffer B plus 0.5 mM NADP⁺. The enzyme fractions were concentrated to 10 ml by ultrafiltration, dialysed against buffer B and then applied at a flow rate of 12 ml/h to a 0.7 cm $\times$ 15 cm PBE 94 column that had been previously equilibrated with 25 mM histidine/HCl, pH 6.2, containing 20% (v/v) glycerol. When the column was eluted at a flow rate of 12 ml/h with 120 ml of 1:8 diluted Polybuffer 74, pH 4.0, containing 20% (v/v) glycerol, the enzyme activity emerged as a single peak at pH 5.1. The enzyme fractions were pooled and directly applied to a 1.2 cm $\times$ 8 cm HA-Ultrogel column equilibrated with buffer B without EDTA.

Figure 1 Product identification of 17 α -hydroxyprogesterone reduction by h.p.l.c.

The substrate (25 μ M) was incubated for 7 min at 25 °C with the 105 000 *g* supernatant of *T. pyriformis* in the presence of 0.1 mM NADPH, and the reaction products were extracted with CH₂Cl₂. After the solvent was evaporated, the residue was dissolved in 0.2 ml of methanol and then a portion (10 μ l) was analysed by h.p.l.c. (solid curve). The separation of the authentic 17 α ,20 α -dihydroxypregn-4-en-3-one (1), 17 α ,20 β -dihydroxypregn-4-en-3-one (2) and 17 α -hydroxyprogesterone (3) are shown as a broken curve. The steroids adsorbed on a reverse-phase μ Bondasphere 5 μ C18 column were developed with a linear gradient of 25–75% (v/v) acetonitrile (—) at a flow rate of 1 ml/min.

The enzyme was eluted with a linear 0–50 mM potassium phosphate gradient in the buffer. The enzyme fractions were concentrated to 2 ml by ultrafiltration.

Product analysis

Identification of products by the enzymic reduction and oxidation of steroid substrates was performed by t.l.c. using silica-gel plates in a solvent system of benzene/acetone (8:2, v/v) as described previously [7,13]. The products of the reduction of 17 α -hydroxyprogesterone by the enzyme was further analysed according to the h.p.l.c. method [3]. The retention times of 17 α ,20 α -dihydroxypregn-4-en-3-one, 17 α ,20 β -dihydroxypregn-4-en-3-one and 17 α -hydroxyprogesterone were 15.6, 17.0 and 18.7 min respectively (Figure 1). The mass spectrum of the isolated product was measured on a JEOL TMS-D300 spectrometer to estimate the configuration of the C-20 hydroxy group of the product [28]. The mass spectrometer was operated in the electron-impact mode at an ionization energy of 70 eV. The stoichiometry of the enzymic reduction of 17 α -hydroxyprogesterone was also analysed by determining the levels of substrate and product, 17 α ,20 α -dihydroxypregn-4-en-3-one, according to the h.p.l.c. method [3]. The rate of oxidation of NADPH by the enzyme was first recorded at 25 °C in the standard reaction mixture for the 17 α -hydroxyprogesterone reductase assay. At different times, aliquots (0.1 ml) were mixed with 0.1 ml of acetonitrile. After the mixture was centrifuged at 12 000 *g* for 15 min, 50 μ l of the supernatant was subjected to h.p.l.c. The concentrations of the substrate and product were determined by their peak area at 240 nm.

Other methods

Electrophoresis on a 7.5% polyacrylamide disc gel [29] or 12.5% polyacrylamide slab gel containing SDS [30] was carried out as described. Isoelectric focusing on polyacrylamide gel with a pH range of 4–10.5 was performed as described [31]. The gels were stained for protein with Coomassie Brilliant Blue R-250. The *M_r* value of the native enzyme was estimated by gel filtration on a 1.5 cm $\times$ 90 cm Sephadex G-100 column in 10 mM Tris/HCl,

pH 7.5, containing 0.15 M KCl, and that of the denatured enzyme was estimated by SDS/PAGE standardized by the use of M_r markers. The amino acid sequence of the protein (0.2 nmol) was determined by automated Edman degradation using an Applied Biosystems 473A gas-phase sequencer with an Applied Biosystems 120A phenylthiohydantoin analyser for the on-line identification of amino acid derivatives.

RESULTS

20 α HSD activity in the cytosolic fraction

When the NADPH-linked reductase activity in the 105000 g supernatants of *T. pyriformis* lysate was assayed spectrophotometrically at 340 nm with several steroids as substrates, significant

NADPH oxidation rates (14–20 m-units/mg of protein) were observed only with 17 α -hydroxyprogesterone as the substrate. The reductase activities for 5 β -androstane-3,17-dione, 17 β -hydroxy-5 β -androstane-3-one, progesterone, 5 β -pregnane-3,20-dione, cortisone and oestrone were less than 3 m-units/mg or could not be detected. Product analyses by t.l.c. and h.p.l.c. (Figure 1) indicated that only 17 α ,20 α -dihydroxypregn-4-en-3-one was produced by the enzymic reduction of 17 α -hydroxyprogesterone. The electron-impact mass spectrum of the isolated product showed a molecular ion (M^+) at m/z 332, and fragment ion peaks at m/z 314 ($M^+ - H_2O$) and 287 ($M^+ - CH_3CHOH$). The ratios of peak heights, ($M^+ - H_2O$)/(M^+) and ($M^+ - CH_3CHOH$)/(M^+), were 1.0 and 15 respectively, which were also coincident with those measured with the authentic 17 α ,20 α -dihydroxypregn-4-en-3-one. In contrast, the spectrum of the authentic 17 α ,20 β -dihydroxypregn-4-en-3-one exhibited low values of 0.72 and 4.5 for ($M^+ - H_2O$)/(M^+) and ($M^+ - CH_3CHOH$)/(M^+) respectively. The results indicate that *T. pyriformis* has a soluble 20 α HSD.

Table 1 Purification of 20 α HSD from *T. pyriformis*

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	5.70	354	0.016	100	1.0
40–75% (NH ₄) ₂ SO ₄	5.70	191	0.030	100	1.9
Sephadex G-100	3.01	37	0.081	53	5.1
Matrex Red A	2.12	1.0	2.12	37	132
Chromatofocusing	1.71	0.58	2.96	30	185
HA-Ultrogel	1.61	0.25	6.44	28	402

Purification and purity

20 α HSD activity in the fraction from the Sephadex G-100 chromatography rapidly decreased during storage at 4 °C; about 50 % of the activity was lost in 2 days. The enzyme was partially stabilized by the addition of 20 % (v/v) glycerol, with 80 % of the original activity remaining after storage for 2 days. Because of the instability of the enzyme, we performed repeated small-scale purification of the enzyme from *T. pyriformis* to shorten the overall purification time, and added 20 % (v/v) glycerol to the buffers used. Representative results are shown in Table 1. Although the specific activity varied from 6.2 to 6.9 units/mg of protein, depending on the preparations, PAGE of the native and denatured enzymes gave single protein bands (Figure 2).

M_r , isoelectric point and N-terminal-sequence analysis

The M_r value of the denatured 20 α HSD estimated by SDS/PAGE was 33000, whereas M_r 68000 was obtained by analytical gel filtration of the native enzyme. As the enzyme was eluted at pH 5.1 at the chromatofocusing step, the enzyme was sharply focused at pH 4.9 on gel isoelectric focusing. The results indicate that the enzyme is a dimeric protein with a pI value of 4.9.

The N-terminal sequence (residues 1 to 18) was determined by gas-phase sequencing to be LAKTVPLNDGTNFPFPGG.

Stability

The purified 20 α HSD was relatively stable; it could be stored in buffer B at –20 °C for at least 2 weeks with little loss of activity. The effects of pH and temperature on the stability of the enzyme was investigated. The enzyme was stable during incubation at 25 °C for 10 min in the pH range from 6.0 to 10.0 but was gradually inactivated below pH 5.8 or above pH 10.5 and lost almost all the activity at pH 4.5 and 11.5. When the enzyme was incubated in buffer B at various temperatures for 5 min, it retained about 95 % of its initial activity up to 40 °C, but rapidly became inactivated above 45 °C.

Catalytic properties

The substrate specificity of *T. pyriformis* 20 α HSD is summarized in Table 2. The enzyme reduced 20-oxosteroids, of which 17 α -hydroxyprogesterone was the best substrate, exhibiting the highest V_{max}/K_m value. The enzyme also reduced 17 α -

Figure 2 PAGE of purified *T. pyriformis* 20 α HSD

(a) SDS/PAGE of the denatured enzyme and M_r markers and (b) PAGE of the native enzyme on a disc gel without SDS. The direction of migration was from top to bottom.

Table 2 Substrate specificity of *T. pyriformis* 20 α HSD

The NADPH-linked reductase and NADP⁺-linked dehydrogenase activities were assayed at pH 6.5 and 10.0 respectively. Kinetic constants were determined from Lineweaver–Burk analyses of the assays. Values in parentheses are the specific activities with the indicated concentrations of the substrate.

Substrate	K_m (μ M)	V_{max} (units/mg)	V_{max}/K_m (units/mg per μ M)
Reductase activity			
17 α -Hydroxyprogesterone	2.9	6.99	2.41
17 α -Hydroxypregnenolone	3.4	3.50	1.01
17 α ,21-Dihydroxy-5 β -pregnane-3,20-dione	22	2.02	0.09
17 α ,21-Dihydroxy-5 β -pregnane-3,11,20-trione	20	1.29	0.06
5 β -Pregnane-3,20-dione (50 μ M)		(0.36)	
Pregnenolone (10 μ M)		(0.18)	
Cortisone (10 μ M)		(0.15)	
Corticosterone (10 μ M)		(0.15)	
Progesterone (20 μ M)		(0.05)	
3 α -Hydroxy-5 β -pregnan-20-one (20 μ M)		(0.05)	
Ethyl acetoacetate	67	22.8	0.34
Isatin	64	16.9	0.26
Pyridine-3-aldehyde	81	20.0	0.25
Camphorquinone	78	9.01	0.11
2,4-Pentanedione	210	20.4	0.09
1,2-Cyclohexanedione	125	9.94	0.08
Phenylglyoxal	430	22.6	0.05
D-Glyceraldehyde	670	17.8	0.03
4-Nitrobenzaldehyde	340	4.60	0.01
Dehydrogenase activity			
17 α ,20 α -Dihydroxypregn-4-en-3-one	13	6.99	0.54
20 α -Hydroxy-5 β -pregnan-3-one	73	8.65	0.12
5 α -Pregnane-3 α ,20 α -diol (25 μ M)		(0.37)	
5 β -Pregnane-3 α ,20 α -diol (25 μ M)		(0.18)	
Benzene dihydrodiol	2100	20.6	0.01

Table 3 Stoichiometry of the 17 α -hydroxyprogesterone reductase reaction by *T. pyriformis* 20 α HSD

Reaction time (min)	Substrate (nmol)		Product (nmol)
	NADPH oxidized	17 α -Hydroxyprogesterone reduced	17 α ,20 α -Dihydroxypregn-4-en-3-one formed
2.5	14.8	15.0	14.5
5.0	27.6	27.4	28.0
10.0	36.7	36.6	36.6

hydroxypregnenolone efficiently, but its reactivity to 20-oxopregnenes without a 17 α -hydroxy group, 20-oxopregnenes and corticosteroids was low. It should be noted that the enzyme did not reduce 3-oxosteroids, such as 17 α ,20 α -dihydroxypregn-4-en-3-one, 20 α -hydroxy-5 β -pregnan-3-one and 17 β -hydroxy-5 β -androstane-3-one, and 17-oxosteroids such as 5 β -androstane-3,17-dione and oestrone 3-sulphate. When the stoichiometry of 17 α -hydroxyprogesterone reduction by the enzyme was determined, NADPH oxidation, 17 α -hydroxyprogesterone reduction and 17 α ,20 α -dihydroxypregn-4-en-3-one production were almost in a ratio of 1:1:1 (Table 3).

The reverse reaction of the enzyme was explored by testing its ability to oxidize a variety of steroidal alcohols. The enzyme oxidized 20 α -hydroxysteroids (Table 2). The oxidized products

Figure 3 pH dependency of oxidoreductase activity by *T. pyriformis* 20 α HSD

Reductase (○) and dehydrogenase (●) activities were assayed with 25 μ M 17 α -hydroxyprogesterone and 17 α ,20 α -dihydroxypregn-4-en-3-one respectively, as substrates.

of 17 α ,20 α -dihydroxypregn-4-en-3-one and 20 α -hydroxy-5 β -pregnan-3-one were identified as 17 α -hydroxyprogesterone and 5 β -pregnane-3,20-dione respectively, by t.l.c. No NADPH formation was observed with 20 β -hydroxysteroids (such as 17 α ,20 β -dihydroxypregn-4-en-3-one and 5 β -pregnane-3 α ,20 β -diol), 3-hydroxysteroids (such as 3 α -hydroxy-5 β -pregnan-20-one, lithocholic acid, 5 β -androstane-3 α ,17 β -diol and 3 β -hydroxy-5 α -androstane-17-one), or 17-hydroxysteroids (such as 17 β -hydroxy-5 β -androstane-3-one, 17 β -oestradiol and 17 α -oestradiol) as substrate.

While the maximal NADPH-linked reduction rate of the enzyme with 17 α -hydroxyprogesterone as the substrate was observed at pH 6.0–6.5, the optimal pH of the reverse reaction was about 10.5 (Figure 3). The enzyme showed a strict specificity for NADPH and NADP⁺; no activity in either direction was observed with 0.1 mM NADH or 0.5 mM NAD⁺ as a cofactor under any pH conditions. The apparent K_m values for NADPH and NADP⁺, determined at pH 7.0 with the 20-oxo- and 20 α -hydroxy-steroid substrates (25 μ M), were 0.2 μ M and 8 μ M respectively.

When non-steroidal compounds were tested as substrates for *T. pyriformis* 20 α HSD, the enzyme rapidly reduced various carbonyl compounds and oxidized benzene dihydrodiol, although the K_m values for the non-steroidal substrates were much higher than those for the 20-oxosteroid substrates (Table 2). The enzyme also exhibited reductase activities of 111, 82, 48 and 11 % (relative to that for 17 α -hydroxyprogesterone) for 1 mM pyridine-4-aldehyde, 1 mM pyridine-3-aldehyde, 0.1 mM acenaphthenequinone and 1 mM 4-nitroacetophenone respectively. However, the enzyme was almost inactive towards other aromatic aldehydes and ketones, such as 4-chlorobenzaldehyde, 4-carboxybenzaldehyde, benzaldehyde, menadione and 9,10-phenanthrenequinone, and aldoses such as D-glucose, D-glucuronate and D-xylose. It should be noted that the enzyme did not oxidize alcohols such as ethanol, 1-propanol, glycerol, cyclohexanol and benzyl alcohol.

Inhibitors

The 17 α -hydroxyprogesterone reductase activity of *T. pyriformis* 20 α HSD was inhibited by synthetic oestrogens; the IC₅₀ (concentration required for 50% inhibition) values were 29, 24 and 23 μ M for hexestrol, stilbestrol and dienestrol respectively. However, natural oestrogens such as oestrone and 17 β -oestradiol at a concentration of 25 μ M caused less than 15% inhibition of the enzyme activity. Although 0.1 mM Cu²⁺ inhibited the enzyme activity by 40%, no significant inhibition was observed with 1 mM thiol reagents such as 4-chloromercuribenzoate and 4-chloromercuribenzenesulphonate, 1 mM reducing agents such as glutathione and 2-mercaptoethanol, 1 mM chelating agents such as EDTA and 1,10-phenanthroline, or 1 mM alcohol dehydrogenase inhibitors such as pyrazole and isobutyramide.

As the enzyme exhibited reductase activity towards non-steroidal carbonyl compounds, we further examined its sensitivity to several inhibitors for mammalian aldo-keto reductases [32]. The enzyme activity was inhibited by barbital (IC₅₀ = 370 μ M), phenobarbital (IC₅₀ = 28 μ M), tolrestat (IC₅₀ = 56 μ M), AL-1576 (IC₅₀ = 85 μ M) and quercitrin (IC₅₀ = 29 μ M), but less than 17% inhibition of the enzyme activity was observed with 1 mM diphenic acid, 1 mM valproic acid and 0.1 mM indomethacin.

DISCUSSION

This study has, for the first time, demonstrated the reductase activity of 17 α -hydroxyprogesterone to 17 α ,20 α -dihydroxypregn-4-en-3-one in *T. pyriformis*, from which a 20 α HSD was isolated. The specific activity of *T. pyriformis* 20 α HSD is comparable with that of rat ovary 20 α HSD [2,3], and is much higher than those of the enzymes from other mammalian tissues [4–7] and *Clostridium scindens* [16].

T. pyriformis 20 α HSD has an *M_r* of 68 000 and is composed of two subunits of equal size. Such a dimeric 20 α HSD has not been reported yet. Most 20 α HSDs [2–7] and polyfunctional dehydrogenases with 20 α HSD activity [10–14] in mammalian tissues are monomers, while high-*M_r* 20 α HSD from *C. scindens* is tetrameric [16]. The dimeric structure and *M_r* of *T. pyriformis* 20 α HSD are similar to those of bifunctional oestradiol 17 β -dehydrogenase from human placenta [9], but the *T. pyriformis* enzyme did not exhibit oxidoreductase activity for oestradiol and oestrone.

The *T. pyriformis* enzyme did not show 3 α -, 3 β -, 17 β - or 20 β -hydroxysteroid dehydrogenase activity, indicating that it differs from bifunctional or polyfunctional hydroxysteroid dehydrogen-

ases from mammalian tissues [8,10–12]. The specificity of the *T. pyriformis* enzyme from 17 α -hydroxypregnenes is similar to those of mammalian 20 α HSDs from testis [4,5] and adrenal gland [7], but differs from that of the rat ovary enzyme which reduces progesterone more rapidly than 17 α -hydroxyprogesterone [3]. On the other hand, the strict cofactor requirement of the *T. pyriformis* enzyme for NADP(H) is the same as that of the rat ovary enzyme [2,3], but is different from dual cofactor specificity for NADP(H) and NAD(H) of the testicular and adrenal enzymes. The substrate- and cofactor-specificity of the *T. pyriformis* enzyme is also distinct from that of the *C. scindens* enzyme [16]. In addition, the inhibitor sensitivity of the *T. pyriformis* enzyme is different from 20 α HSDs from mammalian tissues. The activity of the *T. pyriformis* enzyme was not affected by reducing agents and thiol reagents, inhibitors of the testicular enzymes [4,5], and was inhibited by synthetic oestrogens which do not affect the activity of pig adrenal 20 α HSD [8]. Thus, *T. pyriformis* 20 α HSD is enzymically distinct from the mammalian and bacterial enzymes.

The homogeneous 20 α HSD from *T. pyriformis* rapidly reduced non-steroidal aldehydes and ketones, and oxidized benzene dihydrodiol. While the oxidoreductase activity for non-steroidal substrates had not been investigated with mammalian and bacterial 20 α HSDs, it has very recently been reported that monomeric 20 α HSD of bovine testis is structurally and functionally identical with aldose reductase (EC 1.1.1.21) [33]. Although the *T. pyriformis* enzyme was inhibited by the aldose reductase inhibitors, it was inactive towards benzaldehyde and glucose, the substrates of the bovine testicular enzyme. With respect to the substrate specificity, *T. pyriformis* 20 α HSD resembles carbonyl reductase (EC 1.1.1.184) and dihydrodiol dehydrogenase rather than aldose reductase. As 3 α -hydroxysteroid dehydrogenases [34,35], 17 β -hydroxysteroid dehydrogenases [36,37] and 20 β -hydroxysteroid dehydrogenase [38] from mammalian tissues have been shown to exhibit such carbonyl reductase and/or dihydrodiol dehydrogenase activities, further studies will be necessary to elucidate whether the carbonyl reductase and dihydrodiol dehydrogenase activities are associated with only the purified 20 α HSD in this protozoan. In addition, further kinetic and affinity labelling experiments are required to prove the bifunctional nature of the same active site on the dimeric enzyme.

The amino acid sequence deduced from a cDNA for bovine testicular 20 α HSD [33] and N-terminal sequence of *C. scindens* 20 α HSD [16] have been reported. In addition, the sequences of cDNAs encoding pig testicular 20 β -hydroxysteroid dehydrogenase [38] and rat liver 3 α -hydroxysteroid dehydrogenase [39] with the oxidoreductase activities for xenobiotic compounds have been determined. When the N-terminal sequence of *T. pyriformis* 20 α HSD was aligned with those of the hydroxysteroid dehydrogenases of other species, it showed no homology with those of *C. scindens* 20 α HSD and pig testicular 20 β -hydroxysteroid dehydrogenase, but exhibited a low degree of similarity to those of bovine testicular 20 α HSD and rat liver 3 α -hydroxysteroid dehydrogenase (Figure 4). As the latter two enzymes have been shown to belong to the superfamily of aldo-keto reductases, the N-terminal sequence of *T. pyriformis* 20 α HSD also shows low similarity to those of other members of the aldo-keto reductase superfamily such as human placental aldose reductase [40], human liver aldehyde reductase [40] and bovine lung prostaglandin F synthase [41]. To test whether the sequence similarity among the *T. pyriformis* enzyme and the aldo-keto reductase superfamily proteins is evolutionarily significant, molecular cloning of *T. pyriformis* 20 α HSD is now in progress.

20 α HSDt:	L A K T V P L N D G T N F P I F G G
20 α HSDb:	G A K M P I L G L
3 α HSDr:	M D S I S L R V A L N D G N F I P V L G F
ARh:	M A S R I L L N N G A K M P I L G L
ALRh:	M A A S C V L L H T G Q K M P L I G L
PGFb:	M D P K S Q R V K L N D G H F I P V L G F

Figure 4 Comparison of the N-terminal sequence of *T. pyriformis* 20 α HSD with those of aldo-keto reductase superfamily proteins

The following abbreviations are used: 20 α HSDt, *T. pyriformis* 20 α HSD (this paper); 20 α HSDb, bovine testicular 20 α HSD [33]; 3 α HSDr, rat liver 3 α -hydroxysteroid dehydrogenase [39]; ARh, human placental aldose reductase [40]; ALRh, human liver aldehyde reductase [40]; and PGFb, bovine lung prostaglandin F synthase [41]. Boxed residues indicate exact matches between the sequence of 20 α HSDt and that of a given enzyme.

The identification of the novel 20 α HSD in *T. pyriformis* supports the previous idea that steroid-metabolizing enzymes may be capable of regulating the concentrations of hydrophobic signal molecules in unicellular organisms [22], although the hydrophobic signal molecules are unknown. Since the *Tetrahymena* enzyme reduced various non-steroidal compounds and oxidized benzene dihydrodiol, further studies on substrate specificity of the enzyme might lead to the elucidation of the signal molecule(s) in this protozoan. Alternatively, the broad substrate specificity of the enzyme for non-steroidal compounds suggests that it is one of the enzymes incorporated into the protozoa responsible for detoxifying xenobiotic chemicals, in the same way as carbonyl reductase and dihydrodiol dehydrogenase in mammalian tissues have been thought to play a role in drug metabolism [42].

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