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Steroid 5α-reductase activity of *Treponema denticola*

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Introduction: Previously we have shown that reference and freshly isolated *Treponema denticola* cultures possess 5α-reductase (5α-R) and 3β- and 17β-hydroxysteroid dehydrogenase activity. A gene matching the 3-oxo-5α-steroid 4-dehydrogenase family protein (gene ID: 2739284; locus tag: TDE2697) has been identified in *T. denticola* ATCC 35405. The aim of the work presented here was to optimize assay conditions and determine steroid substrate specificities for the 5α-R activity of *T. denticola* ATCC 33520.
Methods: 5α-R activity of cell-free preparations was assayed with radioactive steroid substrates. 5α-R-reduced products were identified using thin-layer chromatography and a radioisotope scanner. Assay conditions were optimized for co-factor, buffer and pH requirements. Apparent substrate specificities were determined for progesterone, 4-androstenedione, testosterone and corticosterone. The time–course for metabolism of radiolabelled progesterone and cholesterol substrates was investigated with anaerobic cultures.
Results: The optimum pH for 5α-R was 5.5 and the preferred co-factor was NADPH. The order of the steroids with respect to their 5α-R substrate specificities was (in descending order): progesterone, 4-androstenedione, testosterone and corticosterone. There are at least two intermediates in the synthesis of 5α-dihydrocholesterol from cholesterol.
Conclusion: These results suggest that the 3-oxo-5α-steroid 4-dehydrogenase family protein gene of *T. denticola* codes for a functional protein that resembles mammalian 5α-R isoenzyme 2 with regard to co-factor requirement and pH optimum.

Key words: androgen; cholesterol; periodontal disease; progestin; *Treponema denticola*

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Treponema denticola is considered to be a putative periodontal pathogen (10). Previously we have shown that reference and freshly isolated *T. denticola* cultures are capable of metabolizing cholesterol, progesterone, 4-androstenedione, testosterone and corticosterone by means of 5α-reductase (5α-R), 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase activity (4). The genome of *T. denticola* strain American Type Culture Collection (ATCC) 35405 (17) has been sequenced and a gene with a predicted product belonging to the 3-oxo-5α-steroid 4-dehydrogenase family has been reported (gene ID: 2739284; locus tag: TDE2697). The involvement of 3-oxo-4-ene steroid hormones, including certain androgens, progestins and corticosteroid hormones, in periodontal health and disease is well established (14). The level of 5α-R activity is elevated in inflamed gingivae and periodontal tissues and the products of this reaction may differ from the substrates in their hormonal action and susceptibility to further metabolism (18). It is conceivable that the contribution of *T. denticola* to the total 5α-R activity in inflamed gingivae may influence the development of periodontal disease. Variation in serum cholesterol concentrations has been associated with variation in periodontal health (7, 12), thus the 5α-R reduction of cholesterol by *T. denticola* may afford another means of influencing periodontal pathology. Recently we have reported that cholesterol can stimulate the *in vitro* growth of *T. denticola* ATCC 33520 and that certain 3-oxo-4-ene hormonal steroids can inhibit growth (5); this growth modulation may be linked to 5α-R reduction of the substrates.

Steroid 5α-R reduction is a key reaction in both anabolic and catabolic steroid metabolism of host tissues. This is suggestive of a hypothesis that 5α-R reduction may also be a key reaction in the metabolism of steroids by *T. denticola*. As a preliminary to understanding the possible role of treponemal steroid metabolism as a virulence factor, the aims of the work presented in

this paper were to investigate some basic properties of *T. denticola* 5α-R activity, including co-factor requirement, pH optimum, specificity for different 3-oxo-4-ene hormonal steroid substrates and to investigate the 5α-R activity obtained with the 3-ol-5-ene steroid, cholesterol.

Materials and methods

Materials

[4-^{14}C]Testosterone (53 mCi/mmol), [4-^{14}C]cholesterol (53 mCi/mmol) and [1,2,6,7-^{3}H]corticosterone (88 Ci/mmol) were obtained from Amersham International PLC, Amersham, UK. [4-^{14}C]4-Androstene-3,17-dione (54 mCi/mmol) and [4-^{14}C]progesterone (49 mCi/mmol) were obtained from Du Pont de Nemours (Deutschland) GmbH, Dreiech, Germany. Solvents were evaporated in a Gyrovap rotary evaporator (Howe Ltd, Banbury, UK). Stock solutions (50–125 μCi/ml) were prepared in absolute ethanol (Hayman Ltd, Witham, UK) and stored at −20°C. Unlabelled steroids were obtained from Sigma UK Ltd., Poole, UK or Steraloid UK Ltd., Norwich, UK. All other chemicals were obtained at analar or equivalent grade from Merck Ltd., Lutterworth, UK or Sigma Ltd. Buffers were prepared using double-distilled water.

Preparations of *T. denticola* used for 5α-R assays

Cultures were grown in New Oral Spirochaete (NOS) broth or agar (without the addition of antibiotics) (2) incubated in a Don Whitley Mark 3 anaerobic work station (Don Whitley Scientific Ltd, Shipley, UK) at 37°C, with a gas phase of N_2 (80%), H_2 (10%) and CO_2 (10%). *T. denticola* ATCC 33520 was obtained from the American Type Culture Collection (Rockville, MD). Stock cultures were kept at −70°C in NOS broth supplemented with 10% glycerol. Three-day broth cultures were used as 5% inocula for the preparative cultures. Growth was monitored by measuring OD at 660 nm and cells were harvested at mid-log phase by centrifugation of 200-ml aliquots at 15,000 ***g*** for 20 min at 4°C. The pellets were washed three times in phosphate-buffered saline (PBS) at 4°C and suspended in PBS at 5.0% (wet weight/volume). The cell suspensions were sonicated as 3-ml aliquots in glass tubes in an ice/water bath, using a Soniprep Ultrasonic Disintegrator (Sanyo-Gallenkamp, Loughborough, UK) with an exponential probe set at 28 μm amplitude. The suspensions were sonicated for 30-s intervals followed by 30-s cooling periods, until examination by phase contrast microscopy indicated that more than 99.9% of the cells were lysed. For some assays supernatants and pellets were prepared by centrifugation of sonicates at 15,000 ***g*** for 10 min and Stoscheck's modification of Bradford's Coomassie blue method (20) was used for protein assay. Sonicates, supernatants and pellets were stored at −20°C before use. All incubations of cell-free *T. denticola* preparations (sonicates and supernatants) with steroid substrates were performed aerobically.

Identification and quantification of radiolabelled androgens, progestins, corticosteroids and cholesterol metabolites by thin-layer chromatography (TLC)

Full details of the TLC methodology are given in our earlier paper (4). Briefly, samples were extracted with ethyl acetate, evaporated to dryness, rendered soluble in 50 μl chloroform and separated by TLC on channelled plates (Silica gel 60 Linear-K TLC plate, 20 × 20 × 0.25 Whatman Scientific Ltd., Maidstone, UK). Each sample from the cholesterol experiments was split in two, one portion was brominated while the other was untreated and the two portions were analysed separately. Derivatives were not prepared from any other samples. The developing solvents were benzene : acetone (4 : 1) for androgens and progestins, chloroform : methanol (9 : 1) for corticosteroids and benzene : ethyl acetate (5 : 1) for cholesterol metabolites. Radiolabelled steroid metabolites were identified by comparison of their position with that of unlabelled standard steroids and they were quantified using a radioisotope scanner (Berthold Linear Analyser, Berthold Instruments Ltd., St Albans, UK). Product yields were expressed as percentages of the total quantity of radiolabel. Specific rates of 5α-R reduction were calculated as the yield of 5α-R-reduced products divided by the incubation period of 5.5 min and the quantity of protein in the preparation, in units of pmol/min/mg.

Time–course experiments

Separate experiments were performed with [^{14}C]progesterone or [^{14}C]cholesterol; 15 μl stock radiolabelled steroid solution in ethanol was added to 30 ml of a 4-day culture of *T. denticola* ATCC 33520 in NOS broth. The incubations were performed anaerobically with 1-ml samples being taken at intervals over 10 h and stored at −20°C before assay of steroid metabolites by TLC.

Determination of co-factor requirements and pH optimum

A 0.9-ml aliquot of each reaction mixture was preheated at 37°C before 0.1 ml of 0.5% sonicate in PBS was added to start the reaction. The other constituents were 100 mmol/l buffer (see below for details), 0.89 μmol/l [^{14}C]testosterone (added as an ethanolic solution), 0.1% ethanol and 1 mmol/l co-factor (if present). The reaction was terminated by vortexing with 1 ml ethyl acetate after incubation at 37°C for 5.5 min. The co-factor requirement, pH optimum and preferred buffer were determined as follows: (i) triplicate reaction mixtures, in citrate buffer at pH 5.5 plus NADPH, NADH or no co-factors, were compared for product yields, (ii) product yields were determined in duplicate reaction mixtures using NADPH as the co-factor, in three separate experiments with different buffers and pH ranges: citrate (pH 4.9–5.9), citrate–phosphate (pH 4.3–7.7) and dimethyl glutarate (pH 4.1–7.5), and (iii) Triplicate reaction mixtures with NADPH co-factor and citrate, citrate–phosphate or dimethyl glutarate buffers at pH 5.5, were compared for product yields in a single experiment.

Inhibition by ethanol

Each reaction mixture was prepared in duplicate. One microlitre [^{14}C]testosterone ethanolic stock solution was evaporated to dryness in each reaction tube and rendered soluble by vortexing for 60 s in 0.5 ml of 200 mmol/l citrate buffer at pH 5.5. The final 1 ml reaction mixture incorporated 0.89 μmol/l [^{14}C]testosterone, 1 mmol/l NADPH, 0.1 ml 0.5% sonicate and ethanol at 0.0%, 0.1%, 0.5% or 1.0%. After a 5.5-min incubation the reaction was terminated as described above. The initial velocity (v_0) was calculated as the percentage yield of 5α-R-reduced products divided by 5.5 min. The equation $1/v_0 = K_2/K_1 + [\text{Ethanol}]/K_1$, was fitted to the data by linear regression, where K_1 and K_2 are parameters.

5α-R in particulate and soluble fractions

A 0.5-ml aliquot of 5.0% sonicate was centrifuged at 15,000 ***g*** for 30 min. The supernatant was removed and the pellet

was resuspended in 0.5 ml PBS. The enzyme activity of the supernatant and pellet preparations was determined in duplicate reaction mixtures as described above, using pH 5.5 citrate buffer and NADPH. The enzyme activity was also assayed in triplicate aliquots of boiled sonicate (0.1-ml aliquots in Eppendorf tubes incubated in a boiling water bath for 30 min).

Variation in yield of 5α-R-reduced products with time of incubation

Assays with a range of steroid substrates were conducted as described above using NADPH and citrate buffer at pH 5.5, except that (i) the reactions were started by adding 0.1 ml of a one in five diluted supernatant preparation, and (ii) duplicate reaction mixtures were incubated for 2.5, 5.5, 7.0, 10.0, 15.0 or 20.5 min. The steroid substrates used and their final concentrations were: [^{14}C]progesterone (0.41 μmol/l), [^{14}C]4-androstenedione (0.37 μmol/l), [^{14}C]testosterone (0.85 μmol/l) and [^{3}H]corticosterone (2.84 nmol/l). The coefficient of determination (R^2), for linear regression of the yield of 5α-R-reduced product on incubation time was determined for each substrate.

Variation in yield of 5α-R-reduced products with dilution of 5α-R in the reaction mixture

Reaction mixtures incorporating each of the substrates were prepared as above. The reaction mixtures for [^{3}H]corticosterone were incubated for 20 min while the other substrates were incubated for 5.5 min. Supernatant was prepared from 5.0% sonicate and the following final dilutions of supernatant were used for the different substrates: for [^{14}C]progesterone and [^{14}C]4-androstenedione, 0.002, 0.004, 0.006, 0.008 and 0.01; for [^{14}C]testosterone, 0.01, 0.02, 0.03, 0.04 and 0.05; and for [^{3}H]corticosterone, 0.02, 0.04, 0.06, 0.08 and 0.1. For each substrate R^2 was determined for linear regression of the yield of 5α-R-reduced product on the supernatant dilution.

Substrate specificities for hormonal steroid substrates

Separate stock solutions of [^{14}C]progesterone, [^{14}C]4-androstenedione and [^{14}C]testosterone were prepared by adding 40 μl stock alcoholic steroid solution to 2 ml ethyl acetate in a glass tube, evaporating to dryness on a Gyrovap rotary evaporator and dissolving in 2 ml citrate buffer (pH 5.5, 200 mmol/l) by spinning for 1 min. The steroid concentration of the solution was determined by counting the decays/min in three 25-μl aliquots on a Beckman LS 6000TA scintillation counter (Beckman Ltd, High Wycombe, UK) using 3 ml Ultima Gold Scintillation Cocktail per vial (Canberra Packard Ltd, Reading, UK). The remaining solution was diluted in citrate buffer (pH 5.5, 200 mmol/l) for addition to the reaction mixtures. For each substrate, a series of final concentrations were used, up to a maximum concentration of 4–10 μmol/l. Each reaction mixture was prepared by mixing 0.5 ml steroid substrate solution with a 0.4-ml aliquot of NADPH solution (final concentration 0.5 mmol/l) and incubating the reaction tube at 37°C for 1 min; after which 0.1 ml diluted supernatant (prepared from a sonicate as described above) was added and the reaction mixture was incubated for 5.5 min at 37°C.

In preliminary experiments we found it necessary to make the corticosterone soluble using ethanol, so a modified procedure was used to determine the substrate specificity for corticosterone. (i) A 3-μl aliquot of an ethanolic solution of unlabelled corticosterone (5 mg/ml) was added to 3 ml of 200 mmol/l citrate buffer at pH 5.5. Dilutions were prepared, using a solution of 0.1% ethanol in the same buffer as diluent. A 0.5-ml aliquot of each unlabelled corticosterone dilution was added to a reaction tube. Six final concentrations from 0.4 to 7.2 μmol/l were used in the assay, (ii) A 0.2-ml aliquot of a solution of 7 μl ethanolic stock solution of [^{3}H]corticosterone in 1.5 ml distilled water, was added to each reaction tube. The final ethanol concentration in each reaction mixture was 0.14%, and (iii) A 0.2-ml aliquot of 2.5 mmol/l NADPH (final concentration 0.5 mmol/l) was added and the reaction mixture was incubated at 37°C for 1 min; after which 0.1 ml of a supernatant of 5.0% sonicate in PBS was added and the reaction mixture was incubated for 10.5 min at 37°C.

The initial specific velocities were determined as the total yield of 5α-R-reduced products divided by the incubation period and the quantity of protein in the reaction mixture, data were fitted to the Michaelis–Menten equation, the specific limiting velocity (V) and Michaelis constant (K_m) were calculated by a numerical method based on the direct linear plot method using the enzyme kinetic analysis package LEONORA (6).

Co-factor requirement for 5α-R reduction of cholesterol

Each reaction mixture was incubated in triplicate for 24 h at 37°C and comprised 1 μl [^{14}C]cholesterol in ethanolic solution (final concentrations of cholesterol and ethanol were 1.89 μmol/l and 0.1% respectively), 0.5% sonicate and either 0.5 mmol/l NADPH, 0.5 mmol/l NADH or absence of co-factor, in 1 ml PBS.

Comparison of pH 5.5 and pH 7.2 for 5α-R reduction of cholesterol

Triplicate reaction mixtures at pH 5.5 (100 mmol/l citrate buffer) and pH 7.2 (PBS) were incubated for 24 h at 37°C. The sonicate, [^{14}C]cholesterol, ethanol and NADPH were at the same concentrations as above.

Comparison of product yields

The mean values of product yields obtained by different experimental treatments were compared by analysis of variance.

Results

Time–course experiments

5α-Dihydroprogesterone was the initial product and 5α-pregnan-3β-ol-20-ene was the final product from progesterone. 5α-Dihydrocholesterol was the final product from cholesterol. There were at least two intermediate products (referred to as unidentified product 1 and unidentified product 2) from cholesterol with different chromatographic mobilities that we were unable to identify. The maximum yields of the unidentified products were low compared to that of the final product. Details are presented in Fig. 1.

Determination of co-factor requirements

There was a significantly greater yield ($P = 0.0002$) of 5α-R-reduced products in the presence of NADPH (6.81%) compared to the presence of NADH (0.87%) or the absence of co-factor (0.80%).

Determination of pH optimum and comparison of buffers

The optimum yield of 5α-R-reduced product was at pH 5.6 for citric–citrate buffer, at pH 5.4 for citrate–phosphate buffer and at pH 6.0 for dimethyl glutarate buffer. Data are presented in Fig. 2. At pH 5.5, significantly greater yields of 5α-R-reduced products ($P = 0.02$) were obtained

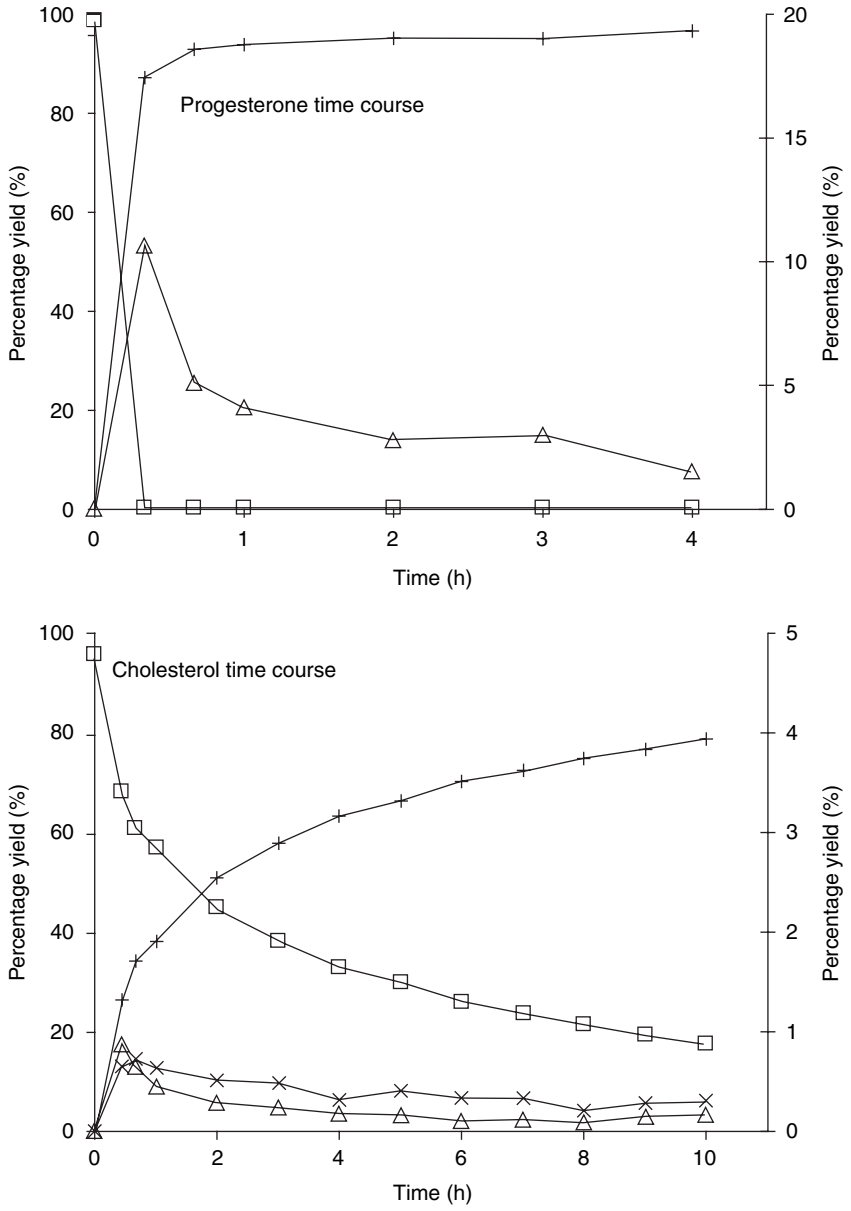

Fig. 1. Time courses for metabolism of progesterone and cholesterol. The Progesterone Time Course shows the percentage yield of progesterone (□, left x-axis), 5α-dihydroprogesterone (△, right x-axis) and 5α-pregnan-3β-ol-20-one (+, left x-axis) plotted against time of incubation (H). The Cholesterol Time Course shows the percentage yield of cholesterol (□, left x-axis), unidentified product #1 (△, right x-axis), unidentified product #2 (×, right axis) and 5α-dihydrocholesterol (+, left x-axis) plotted against time of incubation (H).

with citric–citrate buffer (7.31%) and citric–phosphate buffer (7.35%) compared to dimethyl glutarate buffer (5.77%). Since there was no significant difference between the yield in the citrate-based buffers the optimum pH was taken to be 5.5, the mean of the two experiments.

5α-R activity in particulate and soluble fractions of sonicated cells

The protein in the sonicate suspension (1.76 mg/ml) was approximately equally divided between supernatant (52%) and pellet (48%) while the 5α-R activity in the pellet was approximately 7.8 times that in the supernatant. No activity was detected in boiled sonicate.

Variation in yield of 5α-R-reduced products with time of incubation and dilution of reaction mixture

For all the steroid substrates, R^2 for the linear regression of yield on times of incubation was > 0.97 and R^2 for the linear regression of yield on dilution of 5α-R in reaction mixtures was 0.94.

Inhibition by ethanol

At ethanol concentrations of 0.0%, 0.1%, 0.5% and 1.0%, the respective mean percentage yields of 5α-R-reduced products were 5.97%, 5.72%, 5.12% and 4.48%. For the equation $1/v_0 = K_2/K_1 + [\text{Ethanol}]/K_1$, the fitted statistics are $K_1 = 3.30$ (percentage yield of product per min), $K_2 = 3.05$ (percentage concentration of ethanol)$^{-1}$ and $R^2 = 0.99$.

Substrate specificities for 3-oxo-4-ene substrates

The substrate specificity (V/K_m) of the 5α-R activity was greatest for progesterone, followed by 4-androstenedione and testosterone with the lowest specificity being for corticosterone. The estimates of apparent enzyme kinetic parameters are presented in Table 1. The estimate of V for progesterone exceeds that of 4-androstenedione by a factor of only 1.035; however the other estimated parameters, particularly K_m and V/K_m, clearly distinguish between the substrates. The Michaelis–Menten curves fitted to the data are presented in Fig. 3.

Co-factor requirement for 5α-R reduction of cholesterol

A significantly greater yield ($P < 0.0001$) of 5α-dihydrocholesterol occurred in the presence of added NADPH (34.6%) compared to the presence of added NADH (2.9%) or the absence of added co-factor (3.2%). The presence of added NADPH compared to the presence of added NADH or the absence of added co-factor significantly reduced ($P < 0.0001$) the respective yield of unidentified product 1 (28.2%, 74.0%, 74.6%) and increased ($P < 0.0001$) the respective yield of unidentified product 2 (22.5%, 4.5%, 2.2%).

Comparison of pH 5.5 and pH 7.2 5α-R reduction of cholesterol

Significantly greater yields ($P < 0.003$) of 5α-dihydrocholesterol, unidentified product 1 and unidentified product 2 occurred in the reaction mixtures that were buffered at pH 7.2 (respective yields; 14.5%, 53.6%, 8.8%) with PBS compared to those buffered at pH 5.5 with citrate buffer (respective yields; 5.0%, 29.8%, 6.3%).

Discussion

Previously we have demonstrated by TLC and gas chromatography–mass spectroscopy, the presence of 5α-R, 3β-hydroxy

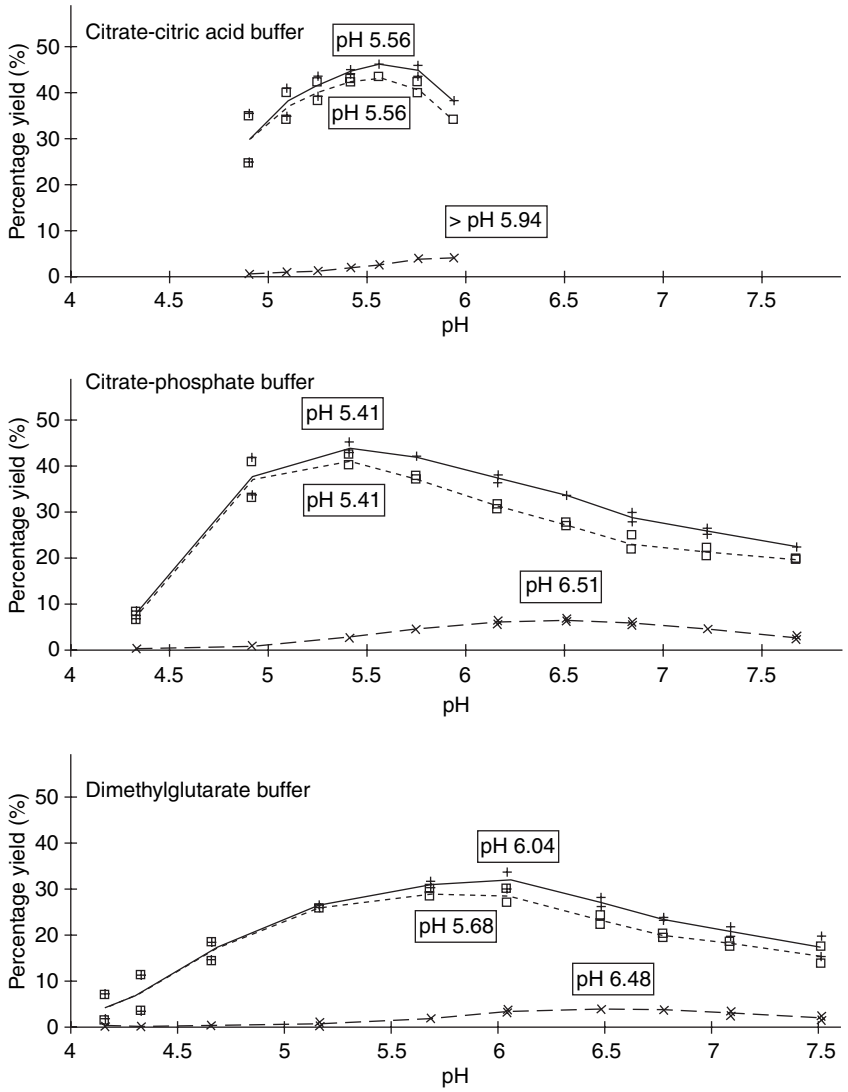

Fig. 2. Optimum pH for yield of 5α-reduced product from testosterone. The charts shown the variation of percentage yields of 5α-dihydrotestosterone (□), 5α-androstan-3β, 17β-diol (×) and total 5α-reduced product (+) with the pH of the three buffer systems used in reaction mixtures; citrate, citrate-phosphate and dimethyl glutarate. The icons shown individual data points and the lines show mean values.

steroid dehydrogenase and 17β-hydroxysteroid dehydrogenase activities in *T. denticola* preparations (4). The time–course experiments confirm our previous findings that 5α-R reduction precedes 3β-hydrogenation for a 3-oxo-4-ene-steroid substrate; however, with cholesterol (a 3β-ol-5-ene-steroid) there are apparently at least two intermediate products that we were unable to identify. As we reported, the mobility of unidentified product 1 on the thin-layer chromatogram corresponded to that of 4-cholesten-3-one while the mobility of unidentified product 2 corresponded to that of 5-cholesten-3-one and 5α-cholestan-3-one. Both 5-cholesten-3-one and 5α-dihydrocholesterol could be detected in preparations of sonicated *T. denticola* by gas chromatography–mass spectroscopy (4). The data suggest a pathway involving 3β-hydroxydehydrogenation of cholesterol to 5-cholesten-3-one, isomeration to 4-cholesten-3-one, 5α-reduction to 5α-cholestan-3-one followed by 3-keto reduction to 5α-dihydrocholesterol. The accumulation of unidentified product 1 in the absence of added NADPH and the influence of pH on product yield are consistent with this interpretation. Further investigations are required to determine the pathway of cholesterol metabolism used by *T. denticola*, in particular to identify the substrate of the 5α-R, for this reason we were only able to compare the substrate specificities of the hormonal steroids in this paper. Stationary-phase cultures at close to maximum optical density were used in the time–course experiments, with the aim of maximizing biomass, enzyme activity and product yield. In future work it would be of interest to investigate the variation of enzyme activity with growth phase.

The linear relationship of product yield to both incubation time and dilution of enzyme validated the use of 5α-R-reduced product yield divided by the 5.5-min incubation period as an approximation for initial velocity. During this period, total 5α-R-reduced products were < 10% and 3β-R-reduced products were < 1% of the initial substrate. The linear relationship between product yield and incubation time is evidence against a progressive inactivation of 5α-R activity by exposure to aerobic conditions during incubations. Further work would be required to determine if properties of the 5α-R were modified by exposure to aerobic conditions during preparation of the cell-free supernatants.

In our substrate specificity determinations, we found it necessary to render the corticosterone soluble with ethanol at a final concentration of 0.14%. An experiment with testosterone as substrate demonstrated that 5α-R activity was weakly inhibited by ethanol, a concentration of 0.15% ethanol only reduced 5α-R activity by approximately 5%. Thus it is unlikely that the much lower substrate specificity of corticosterone, compared to the other

Table 1. Apparent kinetic parameters of *Treponema denticola* 5α-reductase activity for four steroid substrates determined at initial NADPH concentration of 1 mmol/l

Substrate	Michaelis constant (K_m) (μmol/l)	Specific limiting velocity (V) (pmol/min/mg)	Substrate specificity (V/K_m)	Relative substrate specificity (Relative V/K_m)
Progesterone	0.28	1.29	4.67	190
4-Androstenedione	1.15	1.25	1.08	44
Testosterone	5.85	0.85	0.15	6
Corticosterone	16.59	0.41	0.02	1

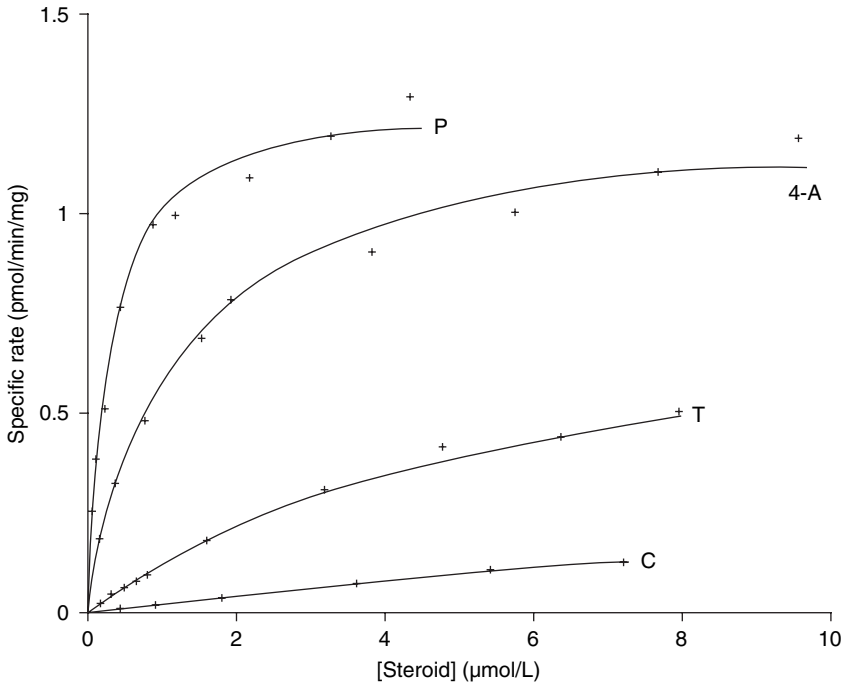

Fig. 3. The specific 5α-reduction rate of different hormonal steroid substrates. The chart shows the specific 5α-reduction rate plotted against steroid concentration for progesterone (P), 4-androstenedione (4-A), testosterone (T) and corticosterone (C). The Michaelis-Menten curves (——) have been fitted to the data points (+).

steroids, was the result of the incorporation of ethanol in the reaction mixture.

The *T. denticola* 5α-R activity has the same co-factor requirement as the mammalian 5α-R enzymes. The acidic pH optimum reported here resembles that presented by rat and human 5α-R type 2 isoenzymes under certain experimental conditions but not that of isoenzyme 1, which has a neutral optimum. However, one study of human isoenzyme 2 reported an optimum of pH 5.0 for the limiting velocity parameter while the substrate specificity parameter had an optimum of pH 7.0; in addition the yield from lysed cells had an acidic optimum while a neutral optimum was shown using whole cells (21). Further investigation of *T. denticola* 5α-R activity is required to determine if such factors influence the pH, giving an optimum product yield. Previously it has been shown that the 5α-R activity does not appear to be released into the culture broth *in vitro* (3). This suggests that *in vivo* (i) the 5α-R operates in an intracellular compartment with a low pH, (ii) pH 5.5 is not the optimum pH within the cell, or (iii) the enzyme operates at a pH that is not its optimum. The 5α-R activity of the particulate fraction exceeds that of the soluble fraction, this supports the hypothesis that the *T. denticola* 5α-R is membrane-associated. Interestingly, the requirement for NADPH, but not the acidic yield optimum, is shared with other bacterial 5α-R enzymes; e.g. *Rhodococcus corallina* (8), *Eubacterium* sp. 144 (9) and *Mycobacterium smegmatis* (11, 13).

The ability of progesterone, 4-androstenedione and testosterone to inhibit the *in vitro* growth of *T. denticola* ATCC 33520 (5) is in the same order as the 5α-R specificity for these substrates, suggesting that 5α-R reduction is important in this growth inhibition. In the same paper we also reported that cholesterol could stimulate the *in vitro* growth of this culture. Cholesterol has been shown to stimulate the *in vitro* growth of *Brachyspira* (formerly *Serpulina, Serpula* and *Treponema*) *hyodysenteriae*. Dependent upon the conditions of incubation, this spirochaete incorporates 5α-dihydrocholesterol in its cell membranes (16, 19). It would be of interest to determine whether this product is also incorporated in the cell membranes of *T. denticola*. 5α-R reduction of cholesterol by *T. denticola* may also have a deleterious influence on host cell function by altering the distribution of cholesterol metabolites in host cell membranes.

Sex steroids are involved in the regulation of immune and inflammatory responses and the formation and destruction of bone and matrix tissue components; all these regulatory functions may have implications in periodontal disease (14). Like rat and human isoenzymes, the apparent substrate specificities of *T. denticola* 5α-R activity for androgens and progesterone exceed that for corticosterone. The apparent Michaelis constants of the treponemal enzyme for these sex steroids are in the micromolar range, as are those reported for rat and human isoenzyme 1 (1, 15). This suggests that *in vivo* the treponemal enzyme could compete effectively with human isoenzyme 1 for substrates and thus disrupt regulatory functions of the host sex steroid metabolism. Some specific instances where the metabolism of androgens and progestins by *T. denticola* might modulate the inflammatory response, including hormonal control of prostaglandin, interleukin-1α, interleukin-1β, interleukin-6 and metalloproteinase production, are discussed in our earlier paper (4).

In conclusion, we have demonstrated that *T. denticola* ATCC 35520 displays steroid 5α-R activity with a specificity for certain hormonal steroids in the order progesterone, 4-androstenedione, testosterone and corticosterone. This supports the hypothesis that the 3-oxo-5α-steroid 4-dehydrogenase family protein gene identified in the genome of *T. denticola* ATCC 35405 codes for a functional protein. Further work is required to determine the role of 5α-R in the pathogenicity of *T. denticola*. This should include the determination of the identity and substrate specificity of the substrate that is 5α-R-reduced during synthesis of 5α-dihydrocholesterol and a survey of 5α-R activity with different substrates in isolates from patients and sites with different clinical presentations.

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References

1. Andersson S, Russell DW. Structural and biological properties of cloned and expressed human and rat steroid 5α-reductases. Proc Natl Acad Sci U S A 1990: **87**: 3640–3644.
2. Cheng SL, Chan ECS. The routine isolation, growth, and maintenance of the intermediate-size anaerobic oral spirochetes from periodontal pockets. J Periodontal Res 1983: **18**: 362–368.
3. Clark DT. Investigations of the metabolism of testosterone, 4-androstenedione, progesterone, corticosterone and cholesterol by

Treponema denticola. In: An investigation of the metabolism of testosterone, 4-androstenedione, progesterone, corticosterone, cortisol and cholesterol by *Treponema denticola*. Thesis, UK: London University, 2001: 161–192.
4. Clark DT, Soory M. The metabolism of cholesterol and certain hormonal steroids by *Treponema denticola*. Steroids 2006: **71**: 352–363.
5. Clark DT, Soory M. The influence of cholesterol, progesterone, 4-androstenedione and testosterone on the growth of *Treponema denticola* ATCC 33520 in batch cultures. Anaerobe 2006: **12**: 267–273.
6. Cornish-Bowden A, *Leonora* Version 1.0. 25 March 1994. Analysis of enzyme kinetic data. Oxford: Oxford University Press, 1995.
7. D'Aiuto F, Nibali L, Parkar M, Suvan J, Tonetti MS. Short-term effects of intensive periodontal therapy on serum inflammatory markers and cholesterol. J Dent Res 2005: **84**: 269–273.
8. Germaine P, Lefebvre G, Bena B, Gay R. Etude *in vitro* des characteristiques de la $5\Delta^{-4}$ stéroïd de *Nocardia corallina*. C R Seances Soc Biol Fil 1973: **166**: 1123–1128.
9. Glass TL, Saxerud MH, Casper HH. Properties of a 4-ene-3-ketosteroid-5α-reductase in cell extracts of *Eubacterium* sp. 144. J Steroid Biochem Mol Biol 1991: **39**: 367–374.
10. Haffajee AD, Teles RP, Socransky SS. Association of *Eubacterium nodatum* and *Treponema denticola* with human periodontitis lesions. Oral Microbiol Immunol 2006: **21**: 269–339.
11. Hörhold C, Groh H, Dänhardt S, Lestrovaja NN, Schubert K. Steroid transforming organisms from microorganisms. III: properties of 4-ene-3-oxosteroid 5α-reductase from *Mycobacterium smegmatis*. J Steroid Biochem 1977: **8**: 701–707.
12. Katz J, Flugelman MY, Goldberg A, Heft M. Association between periodontal pockets and elevated cholesterol and low density lipoprotein cholesterol levels. J Periodontol 2002: **73**: 494–500.
13. Lestrovaja NN, Groh H, Höhold C, Dähardt S, Scgubert K. Steroid transforming enzymes from microorganisms. IV: purification and cofactor requirement of the 4-ene-3-oxosteroid-5α-reductase from *Mycobacterium smegmatis*. J Steroid Biochem 1977: **8**: 313–317.
14. Mascarenhas P, Gapski R, Al-Shammari K, Wang H-L. Influence of sex hormones on the periodontium. J Clin Periodontol 2003: **30**: 671–681.
15. Normington K, Russell DW. Tissue distribution and kinetic characteristics of rat steroid 5α-reductase isoenzymes. J Biol Chem 1992: **267**: 19548–19554.
16. Ochiai S, Adachi Y, Mori K. Unification of the genera *Serpulina* and *Brachyspira*, and proposals of *Brachyspira hyodysenteriae* Comb. Nov., *Brachyspira innocens* Comb. Nov. and *Brachyspira pilosicoli* Comb. Nov.. Microbiol Immunol 1997: **41**: 445–452.
17. Seshadri R, Myers GS, Tettelin H et al. Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. Proc Natl Acad Sci U S A 2004: **101**: 5646–5651.
18. Soory M. Hormone mediation of immune responses in the progression of diabetes, rheumatoid arthritis and periodontal diseases. Curr Drug Targets Immune, Endocr and Metabol Disord 2002: **2**: 13–25.
19. Stanton TB. Cholesterol metabolism of *Treponema hyodysenteriae*. Infect Immun 1987: **55**: 309–313.
20. Stoscheck CM. Increased uniformity in the response of the Coomassie Blue G protein assay to different proteins. Anal Biochem 1984: **184**: 111–116.
21. Thigpen AE, Cala KM, Russell DW. Characterization of Chinese hamster ovary cell lines expressing human steroid 5α-isozymes. J Biol Chem 1993: **268**: 17404–17412.