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Transformations of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895. III. Metabolism of Progesterone during Esterase Inhibition with Clomiphene Citrate

KJELL CARLSTRÖM

Department on Pure and Applied Biochemistry, Royal Institute of Technology, and the Department of Obstetrics and Gynaecology, Sabbatsberg Hospital, Karolinska Institutet, S-113 82 Stockholm, Sweden*

The steroid esterase activity in cell-free preparations from *P. lilacinum* was completely inhibited by 3×10^{-3} M clomiphene citrate. During esterase inhibition with this compound progesterone was directly transformed into testosterone acetate, thus indicating the "biochemical Baeyer-Villiger oxidation" as the C_{17-20}-lyase mechanism in this microbe. Clomiphene citrate partially inhibited the lyase and the 20-reductase activities, the latter mainly by suppression of the formation of the 20α-epimer.

Testosterone acetate or its 1-dehydro analog is the first intermediate formed in the side chain degradation of progesterone in a number of microorganisms.[1–7] The isolation and identification of this important metabolite is often complicated by a rapid hydrolysis catalyzed by esterases. Thus, it was necessary to separate the C_{17-20}-lyase and the esterase activities by chromatography on DEAE-cellulose before the formation of testosterone acetate from progesterone could be demonstrated in cell free extracts from *Cylindrocarpon radicicola*.[2] In progesterone fermentations with *Aspergillus flavus* and *Septomyxa affinis*, significant amounts of the 17β-acetate could be detected only after selective esterase inhibition with 2.15×10^{-3} M DFP.[4,7] In similar experiments with *Penicillium lilacinum*, formation of testosterone acetate could not be demonstrated in the presence of 2.15×10^{-3} M DFP[4] and this was later shown to be due to the insensitivity of the esterase activity of this fungus to DFP.[8] During the continued studies of the mechanism of the side chain degradation of progesterone by *P. lilacinum*, clomiphene citrate was found to be an efficient in-

* Present address.

hibitor of the esterase activity. The present communication describes the identification of testosterone acetate isolated after incubation of progesterone with cell-free preparations of *P. lilacinum* in the presence of clomiphene citrate.

MATERIALS AND METHODS

Abbreviations and trivial names. DFP: diisopropyl-fluorophosphate: GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; dimethyl-POPOP: 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]benzene; PPO: 2,5-diphenyloxazole; TLC: thin layer chromatography; UV: ultraviolet. Clomiphene citrate: 1-(*p*-2-diethylaminoethoxyphenyl)-1,2-diphenyl-2-chloroethylene dihydrogen citrate; progesterone: 4-pregnene-3,20-dione; testosterone: 17β-hydroxy-4-androsten-3-one; testololactone: 17a-oxa-4-androstene-3,17-dione; testolic acid: 13-hydroxy-3-oxo-13,17-*seco*-4-androsten-17-oic acid.

Radioactive steroids. [7-^{3}H]Progesterone (specific activity 16 Ci/mmol), [21-^{14}C]progesterone (specific activity 0.0556 Ci/mmol) and [7-^{3}H]testosterone (specific activity 25 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. They were purified by TLC before use. In the substrate solutions they were diluted with non radioactive steroids to give a final radioactivity corresponding to 18 000 – 100 000 cpm per 10 μl solution.

Non radioactive steroids. Progesterone, 20α-hydroxy-4-pregnene-3-one and 20β-hydroxy-4-pregnene-3-one were purchased from Ikapharm Ltd., Ramat – Gan, Israel, and 5α-cholestane, testosterone, testosterone acetate, and 4-androstene-3,17-dione from Sigma Chemical Co., St. Louis, Mo. All steroids were tested for purity by TLC and GLC.

Clomiphene citrate. Two batches of clomiphene dihydrogen citrate were used, kindly donated by Star OY, Tampere, Finland, and by Draco AB, Lund, Sweden, respectively. The preparations contained 60 % of the *cis* and 40 % of the *trans* epimer. Both batches had the same effect on the steroid transformations studied.

Other chemicals. DFP was obtained from the Research Laboratory of National Defence (FOA), Ursvik, Sweden. Other reagents were from commercial sources and were of analytical grade. Solvents were redistilled.[9]

Chromatographic systems. TLC on Silica gel GF_{254} (system I), GLC on OV-17 and GC – MS were carried out as previously described.[9]

Growth of organism and preparation of cell-free extracts. The following media and inducers were used:

Experiment	Medium	Inducer
1	Czapek-Dox with the addition of 5 mg $ZnSO_4.7H_2O$, 5 mg $MnSO_4.3H_2O$ and 0.5 mg $CuSO_4.5H_2O$ per litre.[10]	Progesterone or 4-androstene-3,17-dione (50 mg in 1.5 ml dimethylformamide per 200 ml culture).
2.3.5	Czapek-Dox (50 g glucose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, and 0.01 g $FeSO_4.7H_2O$ per litre).	Progesterone (70 mg in 2 ml dimethylformamide per 300 ml culture.
4	Czapek-Dox with the addition of 5 mg $ZnSO_4.7H_2O$ per litre.	As in 2, 3, 5.

The procedures for cultivation of the organism and preparation of cell-free extracts (in 0.06 M Tris-HCl, pH 7.2) have been described previously.[9]

Incubation of steroids with cell-free extracts. The procedure was basically the same as that described previously, using 1.0 ml aliquots of the 100 000 *g* supernatant.[9] DFP was added as a freshly prepared 10 % (v/v) solution in ethanol and the samples were preincubated with that inhibitor for 30 min. Clomiphene citrate was added in Tris buffer. The addition of clomiphene citrate caused the formation of a light, white-greyish pre-

cipitate which was not removed. After the addition of the inhibitors, NADPH was added in 0.1 ml of Tris buffer. Steroids were added in 10 µl of ethanol and the final volume was adjusted with Tris buffer to 1.4 ml. The final concentrations of inhibitors, cofactor, and steroids are given in Tables 1 and 2.

Table 1. Effect of inducers and of DFP on the transformation of progesterone by cell-free homogenates of *P. lilacinum*.

Experiment [a]	DFP added	Steroid composition of the reaction mixture (mol %) Progesterone	20(α + β)-Hydroxy-4-pregnen-3-one	Testosterone	4-androstene-3,17-dione	Testololactone [b]
1 P	None	49.6	5.8	19.9	24.7	N. d.
1 P	1×10^{-3} M	65.9	8.1	8.1	17.9	N. d.
1 P	1×10^{-2} M	72.0	17.9	4.5	5.6	N. d.
1 A	None	71.9	5.1	6.5	10.9	5.6
1 A	1×10^{-3} M	89.1	5.0	0.9	5.0	N. d.
1 A	1×10^{-2} M	74.7	19.5	1.6	4.2	N. d.

[a] [21-^{14}C] Progesterone was incubated with homogenates from cells induced with progesterone (1 P) and with 4-androstene-3,17-dione (1 A). The protein concentration of the homogenates were 1.37 mg/ml (1 P) and 1.62 mg/ml (1 A). The initial concentration of progesterone was 2.28×10^{-4} M and of NADPH 9.7×10^{-4} M. Quantitative steroid analysis was made by GLC. [b] Tentatively identified from TLC behaviour and mass spectrum.[9]

After incubation for 60 min at 26° the reaction was terminated by addition of 0.1 ml 1 M HCl and 1.0 ml 30 % (w/v) $(NH_4)_2SO_4$. The steroids were extracted with two 4 ml portions of chloroform. The chloroform phase was dried over Na_2SO_4 and was evaporated to dryness. The residue was dissolved in 100 µl of acetone.

Steroid analysis. From each sample 10 – 30 µl were subjected to TLC in system I. The chromatoplates were examined in UV light. When the samples contained radioactive steroids the steroid zones were scraped off and were eluted with two 1.0 ml portions of ethanol. To the eluates 15 ml of a solution containing 50 mg of dimethyl-POPOP and 4 g of PPO in 1 litre of toluene was added. Radioactivity was measured in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. The percentage distribution of radioactivity (^{3}H) in the different metabolites was calculated. No significant radioactivity was found in UV-positive zones not corresponding to the steroids (from fungal pigments and from clomiphene citrate) and in the UV-negative zones.

When a mixture of progesterone labelled with ^{3}H at C-7 and with ^{14}C at C-21 is used as substrate, the ^{3}H/^{14}C ratio will reflect the relative amount of steroids without the side chain. This facilitates the identification of the metabolites and therefore [7-^{3}H, 21-^{14}C]-progesterone was used as substrate in experiments 2 – 5. The ^{3}H/^{14}C ratio was calculated for all metabolites in these experiments.

Quantitative GLC was carried out using OV-17 as stationary phase.[9] The samples from experiment 1 were acetylated prior to GLC, other samples were not derivatized.

Protein assays were made by the biuret method.[11]

Identification of steroids. The testosterone acetate formed from progesterone was isolated by TLC from the samples containing 3×10^{-3} M clomiphene citrate in experiments 2 and 3. It was subjected to GLC on OV-17 and to GC-MS analysis. One half of the testosterone acetate fraction was heated with 1 M KOH in methanol at 60° for 60 min and the resulting testosterone was isolated by TLC, converted into its trimethyl silyl ether and subjected to GLC on OV-17 and to GC-MS analysis.

The identification of other progesterone metabolites has been described previously.[9] As an additional criterion of identity, the ^{3}H/^{14}C ratios of the metabolites of [7-^{3}H, 21-^{14}C] progesterone was used.

Table 2. Effect of clomiphene citrate on the transformation of progesterone and the hydrolysis of testosterone acetate by cell-free homogenates of *P. lilacinum*.

Experiment [a]	Protein mg/ml	Substrate	Clomiphene citrate added	Steroid composition of the reaction mixture, (mol %) Progesterone	20(α+β)-Hydroxy-4-pregnen-3-one	Testosterone acetate	Testosterone	4-Androstene-3,17-dione	Testololactone [b]
2	0.910	[7-^{3}H,21-^{14}C] Progesterone	None	58.3	10.6	0.4[c]	23.9	5.6	1.2
2	0.910	»	3×10^{-3} M	84.3	5.5	7.1	1.4[d]	1.5[d]	0.2[d]
3	0.665	»	None	78.3	7.4	0.3[c]	10.6	2.5	0.9
3	0.665	»	1×10^{-3} M	86.8	4.5	0.3[c]	6.8	0.9	0.7
3	0.665	»	2×10^{-3} M	87.9	4.7	0.3[c]	5.5	0.9[d]	0.7
3	0.665	»	3×10^{-3} M	90.0	5.0	3.3	0.3[d]	1.2[d]	0.2[d]
3	0.665	Testosterone acetate	None	—	—	3.6	96.4	N.d.	N.d.
3	0.665	»	1×10^{-3} M	—	—	3.6	96.4	N.d.	N.d.
3	0.665	»	2×10^{-3} M	—	—	12.1	87.9	N.d.	N.d.
3	0.665	»	3×10^{-3} M	—	—	> 99	Trace	N.d.	N.d.
4	1.165	[7-^{3}H,21-^{14}C]-Progesterone	None	62.5	22.0	0.2c	5.3	8.1	1.9
4	1.165	»	3×10^{-3} M	85.9	7.8	2.7	1.5	1.2[d]	0.9
4	1.165	Testosterone acetate	None	—	—	Trace	> 99	N.d.	N.d.
4	1.165	»	3×10^{-3} M	—	—	> 99	Trace	N.d.	N.d.
5	0.729	[7-^{3}H,21-^{14}C]-Progesterone	3×10^{-3} M	90.0	4.3	3.7	0.6[d]	1.1[d]	0.3[d]

[a] The initial concentration of progesterone was 2.28×10^{-4} M, of testosterone acetate 2.16×10^{-4} M, and of NADPH 9.7×10^{-4} M. With [7-^{3}H, 21-^{14}C] progesterone as substrate the quantitative analysis was made by TLC and liquid scintillation counting, in other cases by GLC. In experiment 5, parallel incubations were also made with unlabelled progesterone+a tracer amount of [7-^{3}H]testosterone (see text). [b] Tentatively identified from TLC behaviour and mass spectrum.[9] [c] Probably insignificant, not detected by TLC. [d] Insignificant, ^{3}H/^{14}C ratio not exceeding that of the C_{21} steroids.

RESULTS

Effect of inducers and of DFP on the enzyme activity. As seen in Table 1 progesterone was the best inducer of the C_{17-20}-lyase activity. In separate experiments this was found to be true also for the esterase activity. Extracts from non induced cells had none of these activities. However, independently of the inducer used, the extracts contained very strong esterase activity. It was not possible to detect any formation of testosterone acetate even after treatment with 1×10^{-2} M DFP. The C_{17-20}-lyase was sensitive to DFP and a concentration of 1×10^{-3} M caused an inhibition of 42 % in the extract from progesterone induced cells. Thus, the attempts to bring about selective suppression of the esterase activity by selective induction and by treatment with DFP proved unsuccessful.

Effect of clomiphene citrate on the enzyme activity. Addition of 3×10^{-3} M clomiphene citrate completely inhibited the esterase activity whereas 30 – 40 % of the C_{17-20}-lyase activity was still present (Table 2). Thus, formation of testosterone acetate could be demonstrated in four independent experiments. Incubation of [7-^{3}H, 21-^{14}C] progesterone with boiled extracts in the presence of 3×10^{-3} M clomiphene citrate did not yield significant amounts of progesterone metabolites.

Since microbial acetylation of testosterone has been reported,[12,13] it was necessary to establish that the testosterone acetate was directly formed from progesterone and not by acetylation of testosterone, formed from progesterone *via* an alternative pathway. The fact that the ^{3}H/^{14}C ratio of the testosterone acetate never exceeded that of the substrate progesterone speaks against the the latter possibility. Furthermore, when unlabelled progesterone was incubated in the presence of a trace amount of [7-^{3}H] testosterone, only 0.5 % of the radioactivity could be found in the testosterone acetate which represented 2 – 4 % of the total steroids in the reaction mixture (experiment 5). More than 95 % of the radioactivity remained in the TLC fraction corresponding to testosterone, but no testosterone was formed from progesterone.

The identity of the testosterone acetate was established by its chromatographic behaviour and mass spectrometric properties before and after hydrolysis. Mass spectra of authentic testosterone acetate and of testosterone acetate isolated from the reaction mixture were identical. This was also true for the spectra of the trimethyl silyl ethers of authentic testosterone and of the product formed by hydrolysis of the testosterone acetate from the reaction mixture.

The reduction of the 20-oxo group of progesterone was partially inhibited by clomiphene citrate at all concentrations used (Table 2). TLC and GC-MS analyses revealed that the formation of the 20α-alcohol was greatly suppressed by clomiphene citrate. Thus, the 20α-hydroxysteroid represented only about 15 % of the mixture of epimers compared to about 50 % in the mixtures isolated from the control incubations.

Effect of Zn^{2+} on the enzyme activity. In experiments 1 and 4, the Czapek-Dox substrate was fortified with Zn^{2+}.[10] This resulted in an increased yield of biomass and a deeper lilaceous colour of the cells. When extracts from such cells were incubated with progesterone, 4-androstene-3,17-dione was the main C_{19} metabolite, whereas extracts from cells grown on a zinc-deficient medium yielded testosterone as the major product (Tables 1 and 2).

DISCUSSION

The formation of testosterone acetate from progesterone under the conditions described in this paper strongly supports the "biochemical Baeyer-Villiger oxidation" as the mechanism of the pregnane side chain cleavage by *P. lilacinum*.[14] The sequence of the degradation of progesterone by this fungus may therefore be outlined as follows: Progesterone ⟶ testosterone acetate ⇌ testosterone ⇌ 4-androstene-3,17-dione ⟶ testololactone ⇌ testolic acid.[10] Hitherto the "biochemical Baeyer-Villiger oxidation" seems to be the only clearly established pathway for the microbial degradation of the pregnane side chain.[1–7] The lactonization of ring D in 17-ketosteroids proceeds *via* an analogous mechanism and is carried out in several fungi, including *P. lilacinum*.[1] The lactonizing enzyme in this fungus is induced by its substrate, 4-androstene-3,17-dione.[10] As shown in this paper the latter steroid also induced the C_{17-20}-lyase, although less efficiently than progesterone. Similar observations have been made by Miller concerning the induction of the lactonizing enzyme in *Septomyxa affinis*.[7] The progesterone metabolism of this fungus parallels that of *P. lilacinum*, except that 1-dehydro derivatives are formed. Miller found progesterone to be a better inducer than 1,4-androstadiene-3,17-dione and suggested that the two "biochemical Baeyer-Villiger oxidations" are carried out by the same enzyme. The fact that 4-androsten-3,17-dione induces the side chain splitting enzyme activity in *P. lilacinum* may also be taken as a support for this suggestion.

Previous studies have shown 4-androstene-3,17-dione to be the first and major metabolite appearing in progesterone fermentations with whole cells of *P. lilacinum*.[4,7,15] On this basis it was suggested that 4-androstene-3,17-dione might be the primary C_{19} steroid metabolite of progesterone.[4,15] The cells used in those studies were grown on beer wort and on a medium fortified with Zn^{2+} to a final concentration of 2.1×10^{-6} M. In beer wort, Zn^{2+} levels of $0.8 - 8.3 \times 10^{-6}$ M have been reported.[16] The present paper shows that when the cells are grown on media fortified with Zn^{2+}, the 17-ketone/17β-alcohol ratio increases drastically. This might be due to increased levels of 17β-hydroxysteroid dehydrogenase in the cells since it is well known that many NAD-linked dehydrogenases contain zinc.[17] This might also explain the high levels of 4-androstene-3,17-dione found in the whole cell experiments. A possible role of 4-androstene-3,17-dione as the primary C_{19} steroid metabolite of progesterone is ruled out by the results given in the present communication.

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