20α-HYDROXYSTEROID OXIDOREDUCTASE FROM ACTINOMYCES ROSEOCHROMOGENUS ATCC 3347. INDUCTION, PRIMARY PURIFICATION, SUBSTRATE SPECIFICITY.

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We have found that Actinomyces roseochromogenus reduces some 20-oxo-steroids to 20α-alcohols [1, 2] and 3-oxo- to 3β-hydroxysteroids [1], oxidizes 17β-hydroxy- to 17-oxosteroids [3], and is capable of hydrating a Δ^4-double bond [1]. In previous papers we have reported the substrate specificity of the action of a culture of A. roseochromogenus ATCC 3347 [1, 2]. In this paper we give the results of a study of the induction of 20α-hydroxysteroid oxidoreductase (20α-HOR), its primary purification, and the substrate specificity of its action.

It was found that the 20α-HOR of A. roseochromogenus is a constitutive enzyme. We have considered the capacity of a number of steroids for inducing the formation of this enzyme in order to increase its amount in the cell. The capacity for the induction of the 20α-HOR was investigated with respect to the specific activity of a cell-free preparation obtained from a culture grown in the presence of the steroid under investigation. The specific activity of the cell-free preparation was measured from the initial rate of conversion of 17α-hydroxyprogesterone, referred to 1 mg of protein (Table 1).

Table 1

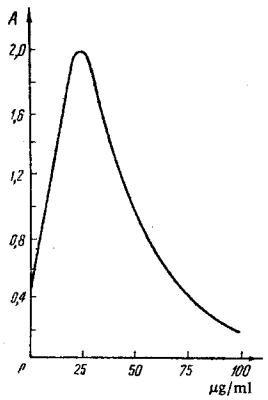
Number	Inducing agent	A*
1	No inducing agent	0.49
2	11α-Hydroxyprogesterone	1.00
3	17α-Hydroxyprogesterone	1.30
4	11-Oxoprogesterone	1.30
5	16β-Hydroxyprogesterone	0.75
6	Corticosterone	0.61
7	11β-Hydroxyprogesterone	0.81
8	Progesterone	0.55
9	Adrenosterone	0.63
10	Androstene-3,17-dione	0.81
11	16α-Hydroxyprogesterone	0.00
12	Deoxycorticosterone	0.01
13	Cortexolone	0.00
14	Hydrocortisone	0.00
15	Prednisone	0.00
16	Cortisone	0.09
17	Prednisolone	0.00
18	Deoxycorticosterone acetate	0.00
19	17α-Hydroxyprogesterone + cortexolone	0.00

*A) relative activity. The activity of a preparation induced by 11α-hydroxyprogesterone (50 μg/ml) was taken as unity.

The steroids studied are divided into two groups according to their influence on the formation of 20α-HOR. The steroids of the first group (2–10) enhance the synthesis of the enzyme. When steroids of the second group (11–18) were added to a growing culture of A. roseochromogenus, the cell-free preparation made from it possessed practically no 20α-HOR activity. This phenomenon can be interpreted in two ways. The steroids of the second group are inhibitors of the biosynthesis of 20α-HOR, or the steroids, possibly passing into the cell-free preparation during its isolation, inhibit the enzymatic reduction reaction even in low concentrations. In order to answer this question, the induction of 20α-HOR was carried out with 17α-hydroxy-progesterone, one of the strongest inducers, in the presence of cortexolone (a steroid of the second group). The cell-free preparation obtained did not possess 20α-HOR activity. At the same time, when 17α-hydroxyprogesterone was incubated in the presence of cortexolone with a cell-free preparation containing 20α-HOR no decrease in the reducing capacity of the cell-free preparation was found. This shows that the steroids of the second group inhibit the biosynthesis of the 20α-HOR.

There were no powerful inducing agents of 20α-HOR among the steroids that we investigated. The strongest of them increased the activity of the preparation by a factor of 2.6 (17α-hydroxyprogesterone and 11-oxoprogesterone); consequently, in the subsequent work as the inducing agent we used 11α-hydroxyprogesterone,

which is more soluble in aqueous media. It was found that the maximum activity of 11α-hydroxyprogesterone is achieved at a concentration of steroid in the culture liquid of 25 μg/ml (figure).



Relative activity (A) of a cell-free preparation as a function of the concentration of inducing agent (11α-hydroxyprogesterone).

A cell-free preparation obtained by breaking down a culture of A. roseochromogenus with ultrasonics and subsequent centrifuging at 1000 × g ("supernatant 1000") possessed not only 20α-HOR activity but also 4,5-dehydrogenase (DH), 3β-hydroxysteroid oxidoreductase (3β-HOR), 17β-hydroxysteroid oxidoreductase (17β-HOR), and 16α-hydroxylase activities. This preparation was subjected to further purification. The activity of the 20α-HOR was checked spectrophotometrically from the initial rate of conversion of 17α-hydroxyprogesterone. Since in the reduction of a 20-oxo group NADP · H_2 takes part as the coenzyme, the initial rate of conversion was measured from the change in absorption at 340 mμ. During the purification of the preparation, the presence of other steroid-converting enzymes was monitored by incubation with the appropriate substrates (progesterone for 16α-hydroxylase, 16α,17-epoxyprogesterone for DH and 3β-HOR, and estradiol for 17β-HOR). When the supernatant 1000 was centrifuged at 13 500 × g, only the 20β-HOR and 17β-HOR activities remained in the supernatant formed (supernatant 13 500). The subsequent purification of the 20α-HOR consisted in the precipitation of the protein from the supernatant 13 500 with ammonium sulfate. The 20α-HOR was contained in the fraction precipitated at a saturation of from 60 to 90%. After the proteins had been centrifuged off, the active fraction was dissolved in a small amount of buffer and was dialyzed on a column of Sephadex G-25. As a result of the partial purification carried out, only the 20α-HOR remained in the preparation; its specific activity was six times higher than the activity of the supernatant 13 500. In the ammonium-sulfate precipitation process, the total activity of the preparation decreased by a factor of 5–6.

It was shown previously that the substrates of the 20α-HOR from a culture of A. roseochromogenus are pregnane steroids having a 17-hydroxy or a 16α,17-epoxy group [1,2]. We have checked the capacity of the cell-free preparation obtained from reducing a number of pregnane derivatives in order to elucidate whether substituents other than a 17α- oxygen group promote the reduction of the 20-oxo group. In addition to this, it appeared of interest to compare the substrate specificity of the action of the cell-free preparation and of the intact cells, since it was found previously that a cell-free preparation of the 20α-HOR from a culture of A. albus has a broader substrate specificity than the intact culture [4]. To study the substrate specificity of 20α-HOR we took supernatant 13 500. As can be seen from Table 2, the introduction of a hydroxyl group into one of positions 7β, 11α, 11β, 12α, 15α, 15β, 16α, and 21 and of a keto group into position 11 of the pregnane molecule (in the absence of a 17-oxygen function) did not lead to an active substrate since the corresponding progesterone derivatives were not reduced by the cell-free preparation. The substrate specificity of the cell-free preparation of 20α-HOR coincided with the substrate specificity of a growing culture of A. roseochromogenus. It may be concluded from this that the role of the 17- oxygen function (determining the possibility of reduction) is connected neither with the induction of the formation of 20α-HOR (since the enzyme is certainly present in the cell-free preparation) nor with a difference in the permeability of the cells for 17-hydroxy- and 17-deoxysteroids. This characteristic property of the 20α-HOR from A. roseochromogenus distinguishes it from the 20β-HOR from A. albus. The latter enzyme does not require the presence of any one definite function in the molecule of the substrate for the exhibition of its activity.

Table 2

No.	Substance	System of solvents *	Reduction of the 20-oxo group	
			by a cell-free preparation	by the intact cell
1	17α-Hydroxyprogesterone	1	+	+
2	17β-Hydroxyisoprogesterone	2	+	+
3	11α,17α-Dihydroxyprogesterone	3	+	+
4	16α,17-Epoxyprogesterone	2	+	+
5	Cortexolone	4	+	+
6	17α-Hydroxyprogesterone acetate	1	—	—
7	Cortexolone 21-acetate	3	—	—
8	7β-Hydroxyprogesterone	3	—	—
9	11α-Hydroxyprogesterone	5	—	—
10	11β-Hydroxyprogesterone	5	—	—
11	12α-Hydroxyprogesterone	3	—	—
12	15α-Hydroxyprogesterone	3	—	—
13	15β-Hydroxyprogesterone	5	—	—
14	16α-Hydroxyprogesterone	1	—	—
15	11-Deoxycorticosterone	3	—	—
16	11-Oxoprogesterone	3	—	—
17	Cortisone	3	—	—
18	Prednisone	3	—	—
19	Progesterone	3	—	—
20	16β-Methylprogesterone	1	—	—
21	17α-Methylprogesterone	1	—	—

*1) Ether, 2) Benzene-acetone (4 : 1), 3) Benzene-acetone (2 : 1), 4) Ethyl acetate, 5) Benzene-acetone (3 : 1).

20α-HORs are very widely distributed and are found in the tissues of various organs of animals [5–9]. It is known that 20α-HORs take part in some cases in the regulation of the biosynthesis of C-19 hormones, the 20α-HORs acting on that part of the biosynthetic route where the splitting off of the C-11 side chain of steroids takes place. When 17α-hydroxyprogesterone is used as substrate, the 20α-HOR competes with 17,20-lyase and, moreover, the product of the reduction of 17α,20α-dihydroxypregn-4-en-3-one inhibits the splitting off of the side chain. It is an interesting fact that the 20α-HOR isolated from rat testes, which is also an oxidoreductase of this type, it similar to 20α-HOR from A. roseochromogenus, according to preliminary information. Thus, in both cases the best substrate is 17α-hydroxyprogesterone, both enzymes require NADP · H_2 as the cofactor, and the reverse reaction scarcely takes place. This gives grounds for hoping that the readily accessible 20α-HOR from A. roseochromogenus can be used for investigating the mechanism of the action of oxidoreductases of this type.

EXPERIMENTAL

The steroid substrates were purified by crystallization, and their purity was checked by their melting points and chromatographically. Chromatographic checking was carried out on micro plates with a fixed layer of silica gel [10]. The chromatographic spots were revealed by spraying with H_2SO_4, followed by heating, or by means of Lugol's solution. Before use, the ammonium sulfate was crystallized from a solution of the tetrasodium salt of EDTA (1 g of EDTA to 1 *l* of double-distilled water).

Growth of a culture of A. roseochromogenus. A flask containing 100 ml of nutrient medium [2] was inoculated with 10 ml of seed material [2]. The microorganism was grown for 6 hr, after which 2.5 mg of 11α-hydroxyprogesterone in 0.5 ml of ethanol was added to the growing mycelium, and the cultivation of the mycelium was continued for another 18 hr.

Preparation of the homogenate and fractional centrifuging. The mycelium produced was filtered from the culture liquid, carefully suspended on 0.5% NaCl solution (10 ml per gram of mycelium), and again filtered off. The mycelium was washed with NaCl solution four times. Then it was washed with double-distilled water and with 0.05 M phosphate buffer prepared in a 0.001 M solution of the tetrasodium salt of EDTA and containing 10^{-5}M of β-mercaptoethanol. The washed mycelium was suspended in a phosphate buffer of the same composition (10 ml of buffer to 1 gram of washed mycelium) and was kept in dry ice for 20 hr. Then the mycelium was thawed out at room temperature and, in 10-ml portions of the suspension, it was broken down by ultrasonics (ULA-250 ultrasonic generator) at 2–6° C for 6–8 min. The degree of breakdown of the mycelium (80–90%) was checked by the microscopic observation of a preparation of the broken-down mycelium stained with Methylene Blue. The homogenate was centrifuged at 2–4° C at 1000 × g for 40 min. The resulting supernatant 1000 was centrifuged at 13 500 × g under the same conditions, and the supernatant 13 500 was used for further purification and also as a source of the enzyme in incubation with steroids.

Precipitation with ammonium sulfate. With stirring, ammonium sulfate was added to the supernatant 13 500 at 0–2° to 60% saturation. After being left to stand at the same temperature for 1 hr, the mixture was centrifuged at 2–4° C and at 13 500 × g for 30 min. The supernatant liquid was brought to 90% saturation with ammonium sulfate. The precipitated protein was collected by centrifuging at 13 500 × g and was dissolved in the minimum volume of phosphate buffer.

Dialysis. Before use, Sephadex G-25 "coarse" was kept in double-distilled water for 3 days. The swollen Sephadex was transferred to a column with a volume of 35 ml and was equilibrated for a day with the phosphate buffer. The solution of protein obtained after ammonium sulfate precipitation was deposited on the surface of the Sephadex and the protein was eluted with phosphate buffer, 2.5-ml fractions being collected.

Determination of enzymatic activities. The activity of an enzyme preparation of 20α-HOR was determined from the rate of change of the optical density (ΔD) at 340 mμ. The specific activity was measured from the change in the optical density in 1 min caused by 1 mg of protein. A change in optical density of 0.001 was taken as the unit of specific activity. The rates of the reaction were measured at 28° C in a thermostated chamber placed in an SF-4 spectrophotometer. A quartz cell with a capacity of 3 ml and a light path length of 1 cm was charged with 150 μM of phosphate buffer (pH 7.4), 0.5 ml of a solution of the enzyme preparation, 0.18 μM of NADP · H_2, and 0.12 μM of 17α-hydroxyprogesterone in 0.02 ml of ethanol. The comparison cell was charged with the same components except for the steroid solution, which was replaced by 0.02 ml of ethanol. The protein content in the enzyme preparation was

determined by Kalcar's method [11]. After the end of incubation, the existence of 20α-HOR activity was qualitatively confirmed by the extraction of the contents of the working cell with chloroform (2 × 2 ml), evaporation of the extract, and the chromatographic investigation of the fermentation products.

To determine the 16α-hydroxylase activity (substrate: progesterone) the 3β-HOR and the DH activities (substrate: 16α,17-epoxyprogesterone) and the 17β-HOR activity (substrate: estradiol), a flask was charged with 150 μM of phosphate buffer, 3 ml of a solution of the enzyme preparation, 0.15 μM of steroid in 0.02 ml of ethanol, and 0.4 μM of coenzyme (NADP for 17β-HOR, and NADP · H_2 for the others). The reaction mixture was incubated at 28° C for 18 hr, and then the contents of the flask were extracted with chloroform (2 × 2 ml) and the content of incubation products in the extract was investigated by chromatography.

Substrate specificity of a cell-free preparation. A flask was charged with 150 μM of phosphate buffer, 3 ml of supernatant 13 500, 3.7 μM of NADP · H_2, and a solution of 1 μM of steroid in 0.03 ml of ethanol. The mixture was incubated at 28° C for 18 hr, and then the contents of the flask were extracted with chloroform and the incubation products were determined chromatographically (see Table 2).

Induction of the formation of 20α-hydroxysteroid oxidoreductase. The inducing capacity of the steroids was determined from the activity of the supernatant 13 500 from a culture of A. roseochromogenus grown in the presence of the steroid under investigation (50 μg per ml of culture liquid). Combined induction with 17α-hydroxyprogesterone and cortexolone was carried out similarly at a concentration of each of the inducing agents of 50 μg per ml of culture liquid (see Table 1).

Test of the inhibiting activity of cortexolone in the incubation of 17α-hydroxyprogesterone. A cell was charged with 150 μM of phosphate buffer, 0.5 ml of supernatant 13 500, 0.18 μM of NADP · H_2, and 0.1 μM each of 17α-hydroxyprogesterone and cortexolone in 0.02 ml of ethanol. The comparison cell was charged with the same components with the exception of the steroids. The measured rate of transformation of the 17α-hydroxyprogesterone was compared with the rate obtained in the incubation of 17α-hydroxyprogesterone along with the same enzyme preparation.

Fermentation of 17α,20α-dihydroxypregn-4-en-3-one with a culture of A. roseochromogenus and with a cell-free preparation obtained from it. A flask containing 100 ml of nutrient medium [2] was inoculated with 10 ml of seed material of A. roseochromogenus, and the culture was grown for 24 hr. Then a solution of 5 mg of the steroid in 0.5 ml of ethanol was added to the flask, and fermentation was carried out at 28° C on a rotary shaking maching (200 rpm) for 72 hr.

Another flask was charged with 150 μM of phosphate buffer, 3 ml of supernatant 13 500, 3.7 μM of NADP, and 1 μM of steroid in 0.03 ml of ethanol. The mixture was fermented at 28° C for 18 hr. In both cases a chromatographic investigation carried out after the end of fermentation showed the absence of conversion products.

CONCLUSIONS

The formation of 20α-hydroxysteroid oxidoreductase with a constitutive enzyme of Actinomyces roseochromogenus is activated by certain predominantly monohydroxy derivatives of pregnane. Some steroids suppress the formation of the enzyme. Intact cells of A. roseochromogenus and a cell-free enzyme system possess identical substrate specificities which are close to the substrate specificity of the 20α-hydroxysteroid oxidoreductase of rat testes and reduce only 20-oxopregnanes with a 17-oxygen function. The specific role of the 17-oxogen function appears at the substrate-enzyme level.

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