SUBSTRATE SPECIFICITY OF MICROBIOLOGICAL CONVERSIONS OF STEROIDS BY MEANS OF ACTINOMYCES ALBUS ATCC 3006

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Substrate specificity in the conversions of steroids by cultures of microorganisms and enzyme preparations has been studied by numerous authors; however, we have come across no systematic comparison of substrate specificity in these conversions. Only from the results published by German workers [1, 2] can it be seen that the substrate specificity of a culture of Streptomyces hydrogenans differs from the substrate specificity of the enzyme.

We have studied the conversion of steroids of various structures by a culture of Actinomyces albus ATCC 3006 and a cell-free preparation obtained from it. The microorganism was cultured under standard conditions, and the steroids were fermented for 72 hr. The reaction products were determined by thin-layer chromatography on alumina. 20β-Hydroxysteroids obtained by the reduction of the corresponding 20-ketosteroids with sodium borohydride [3] were used as reference materials (Table).

The culture of A. albus 3006 possessed specificity with respect to the structure of the steroid substrate. While compounds with a dihydroxyacetone chain were reduced to the corresponding 20β-hydroxysteroids completely after a fermentation time of from 11 to 20 hr [4, 5], the conversion of other steroids was not complete even after 72 hours fermentation.

The presence of a Δ^4-3-keto grouping is not necessary for conversion by a culture of A. albus 3006. For example, 3β, 11α-dihydroxypregn-5-ene-7, 20-dione (I) is reduced fairly readily. For the conversion of steroids by this culture, the molecule of the substrate must apparently contain, in addition to keto groups in positions 3 and 20, another free oxygen function. While 11α-hydroxyprogesterone (II), 17α-hydroxyprogesterone (III), deoxycorticosterone (IV), and Reichstein's substance S(V) give the corresponding 20β-hydroxysteroids on fermentation with a culture of A. albus 3006, reduction does not take place in fermentation with the acetates of these steroids (VI), (VII), (VIII), and (IX), and with progesterone (X). On fermentation with estradiol (XI) or testosterone (XII), a culture of A. albus 3006 oxidixes the 17β-hydroxy group; the corresponding 17-ketosteroids – estrone and androstenedione – are formed [5].

We carried out the following series of experiments in order to determine whether the substrate specificity of the action of a culture of A. albus 3006 on steroids of different structures was due to the specificity of the action of the enzyme or to other factors. In order to ascertain whether the enzyme separates out from the cells in the culture medium, the culture was grown in the presence of progesterone (X) (to induce the formation of enzyme). The mycelium was separated off, and the filtrate was fermented with substances S(V) in the presence of reduced nicotinamide-adenine dinucleotide ($NAD \cdot H_2$) and without it. An analogous experiment was carried out on fermentation with progesterone (X) with induction by substance S(V). No conversion took place in any of the experiments, and consequently the enzymatic reaction of the reduction of 20-ketosteroids by means of a culture of A. albus 3006 is localized either within the cell or on its surface.

It appeared likely that the structure of the steroid determines the possibility of its penetration to the site of the enzyme. To elucidate this, it was necessary to set aside the influence of the cell membrane. For this purpose, a suspension of the mycelium was treated with ultrasonics until 80-90% of the cells has been disrupted. The sonically irradiated suspension was centrifuged at 13 500 g and the supernatant liquid was separated off, the absence of living cells in this being confirmed by microscopic investigation and by inoculation on a nutrient medium. The total amount of proteins in the supernatant liquid was determined by Kalckar's method [6].

It was found that the cell-free preparation obtained from a culture of A. albus 3006 grown in the absence of any steroid was incapable of reducing 20-ketosteroids. Consequently, a culture of the actinomycete was grown in the presence of progesterone (X) in order to induce the formation of steroid oxidoreductase.

The velocity of the reaction was followed by periodically measuring the optical density at 340 mμ (the corresponding results are given in the Table*). As can be seen, the enzyme preparation reduces the 20-keto group of pregnenes of different structures, the rate of reduction varying within wide limits. Even such a change in the structure of the substrate as the removal of the oxygen function from position 3 does not prevent the enzymatic reaction, and Δ^2-5α-pregnen-20-one (XIII) is reduced at the same relative rate as a series of hydroxyprogesterones. However, when the configuration of

* The samples of 7β-hydroxyprogesterone were kindly provided by Dr. M. Shirasaka (Japan), of 12α-hydroxyprogesterone by Dr. A. Schubert (German Democratic Republic) and Dr. B. Camerino (Italy), and of 15β-hydroxyprogesterone by Dr. P. Jassy (U. S. A.), to whom we express our deep gratitude.

the coupling of the rings is changed, the capacity of the steroid for undergoing reduction is lost, and in the case of Δ^2-5β-pregnen-20-one (XIV) the enzymatic reaction does not take place at all.

The acetates of 17α-hydroxyprogesterone (VII), deoxycorticosterone (VIII), and substance S (IX) are not reduced by the enzyme preparation. At the same time, the enzyme preparation reduces steroids in which an acetoxy group is spatially remote from the 20-keto group, as was shown on the basis of the conversion of 11α-hydroxyprogesterone acetate (VI) and 11α, 17α-dihydroxyprogesterone 11-acetate (XV) by the cell-free preparation. It may be assumed that the introduction of voluminous substituents into the neighborhood of the 20-keto group sterically hinders the enzymatic reaction. An analogous phenomenon has been reported in the case of esters of 17α-hydroxyprogesterone and cortisone [2].

Substance	Conversion by the culture	Conversion by the enzyme preparation (V_{rel})	Capacity for inducing the formation of steroid oxidoreductase
Reichstein's substance S (V)	++	1.5	
Hydrocortisone (XXII)	++	0.87	
Cortisone (XXI)	++	0.75	
11α-Hydroxyprogesterone (II)	+	0.75	
11β-Hydroxyprogesterone (XIX)	+	2.5	
11-Ketoprogesterone (XX)	+	8.0	+
17α-Hydroxyprogesterone (III)	+	1.5	
Deoxycorticosterone (IV)	+	0.36	+
12α-Hydroxyprogesterone	+	0.28	
16α-Hydroxyprogesterone	+	0.46	
11α, 17α-Dihydroxyprogesterone	+	0.92	
3β, 11α-dihydroxypnegn-5-ene-7, 20-dione (I)	+	0.35	
Progesterone (X)	–	1.0	+
7β-Hydroxyprogesterone (XVI)	–	0.75	–
15α-Hydroxyprogesterone (XVII)	–	1.75	–
15β-Hydroxyprogesterone (XVIII)	–	2.8	+
11α, 16α-Dihydroxyprogesterone	–	0	
Δ^2-5α-Pregnen-20-one (XIII)	–	0.3	–
Δ^2-5β-Pregnen-20-one (XIV)	–	0	–
11α-Hydroxyprogesterone acetate (VI)	–	0.75	–
17α-Hydroxyprogesterone acetate (VII)	–	0	–
Deoxcorticosterone acetate (VIII)	–	0	
Acetate of Reichstein's substance S (IX)	–	0	
11α, 17α-Dihydroxyprogesterone 11-acetate (XV)	–	0.54	

Note. The symbol (++) denotes complete conversion, (+) partial conversion, and (–) the absence of conversion.

On the basis of the oxidation of 20β-hydroxypregn-4-en-3-one to progesterone it has been shown that the enzyme preparation brings about not only the reduction of a 20-keto group but also the oxidation of a 20β-hydroxy group.

It was impossible to effect the oxidation of the 17β-hydroxy group of testosterone (XII) by means of the cell-free preparation in the presence of NAD and NADP, which indicates the reduction of a 20-keto group and the oxidation of a 17β-hydroxy group by different enzyme systems.

The above-described experiments have established that the spectrum of the action of the enzyme is broader than the spectrum of the action of a culture of the actinomycete. By means of a preparation of steroid oxidoreductase it is possible to reduce a series of steroids that are not reduced by the microorganism (see Table). We may mention that while the relative rates of the reduction of the alcohol (XIX) and the ketone (XX) by a preparation of steroid oxidoreductase are considerably higher than the rate of reduction of cortisone (XXI) and hydrocortisone (XXII), when the live culture acts on these steroids the conversion of the steroids (XXI) and (XXII) is complete in 11 and 15 hr, and on fermentation with (XIX) and (XX) under the same conditions a considerable amount of unreduced steroid remains even after 3 days.

Since in the production of the cell-free preparation the mycelium was grown in the presence of progesterone, in studying the reduction of steroids by the cell-free preparation we were using an extract known to contain steroid oxidoreductase. In the study of the conversions of steroids by a culture of the actinomycete, the steroid under investigation was

added to the growing culture; in the first stage, the formation of steroid oxidoreductase by the microorganism must have taken place.

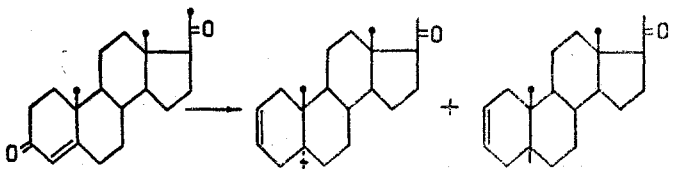
It is natural that a culture will reduce only steroids capable of inducing the formation of steroid oxidoreductase (provided that the enzyme is capable of reducing these compounds). If, however, a given steroid is incapable of inducing the formation of steroid oxidoreductase, the culture will not reduce it even if the enzyme reduces this steroid.

In order to ascertain what role is played by the inductivity factor, we checked the capacity of a number of steroids for inducing the formation of steroid oxidoreductase A. albus 3006. The inducing capacity of the steroids was determined from the capacity for converting progesterone (X) of cell-free preparations obtained from the mycelium grown in the presence of the steroid under investigation (see Table). It was found that some steroids do not induce the formation of steroid oxidoreductase, and consequently the reduction of these steroids by a culture of A. albus 3006 (at least without induction by another substance) is impossible in principle. At the same time, the enzyme system itself is capable of reducing the 20-keto group of such steroids.

The results obtained for progesterone (X) and 15β-hydroxyprogesterone (XVIII) proved to be curious. As already mentioned, these substances are reduced by an enzyme preparation obtained from the mycelium in which the formation of steroid oxidoreductase was induced by progesterone. When the actinomycete is grown in the presence of these steroids, steroid oxidoreductase is formed, since the cell-free preparation obtained from such a culture is capable of reducing 20-keto steroids. Nevertheless, the culture does not reduce progesterone (X) and 15β-hydroxyprogesterone (XVIII).

On the induction of the formation of steroid oxidoreductase, substrate specificity of the action of the permease effecting the transfer of the steroid into the cell possibly develops and, moreover, it is likely that transport systems exist possessing the specificity of action necessary for realizing the enzyme reaction of the reduction of a 20-keto group even after the formation of the enzyme.

In order to obtain steroids not containing an oxygen function in position 3, progesterone (X) was reduced with an excess of zinc dust in acetic acid by a recognized method [7]. It was possible by fractional crystallization to separate the mixture of substances into two ketones:



X XIII XIV

The absence of a Δ^4-3-keto grouping from them was confirmed by their UV and IR spectra. According to the results of the IR spectra, these substances retained the carbonyl group in position 20.

The NMR spectra indicate the presence of two vinyl protons; consequently, the double bond may be present either in the 2, 3 or in the 3, 4 position (the 1, 2 position is excluded by the very method of preparation). The chemical shifts found for the signals of the hydrogen atoms of the 19-CH_3 and the 18-CH_3 groups (in τ) found are close to those calculated from Zürcher's results [10] and indicate that compound (XIV) belongs to the A/B-cis series and (XIII) to the A/B-trans series:

5α, 14α-Androstane	19-H	18-H
Chemical shift of the signal	0.792	0.692
Increment for		
A 17β-$COCH_3$ group	−0.008	−0.083
A Δ^2 (or Δ^3) bond	0.000	0.000
Chemical shift		
Calculated for XIII	0.784	0.609
Found	0.75	0.55
5β, 14α-Androstane		
Chemical shift of the signals	0.925	0.692
Increment for a 17β-$COCH_3$ group	−0.008	−0.083
Chemical shift		
Calculated for XIV	0.917	0.609
Found	0.94	0.55

On comparing the molecular rotations with those calculated according to Klyne [11]:

	5α Series	5β Series		5α Series	5β Series
Pregnane	+ 52°	+ 58°	Pregnane	+ 52°	+ 58°
20-CO	+254	+254	20-CO	+254	+254
Δ^2	+152	− 24	Δ^3	+149	− 44
Calculated, M_D	+458	+288	Calculated, M_D	+455	+268

it becomes evident that in compound (XIV) (found M_D + 294°) the double bond must be located in the 2, 3 position. The constants of compound (XIII) obtained by us are close to the constants of the previously described 5α-pregn-2-en-20-one. By analogy, we may assume that in compound (XIII) (M_D + 432°) the double bond is in the 2, 3 position.

EXPERIMENTAL

Reduction of the steroids on fermentation with a culture of A. albus 3006. The culture was grown for 24 hr in 100-ml flasks each containing 20 ml of nutrient medium (2% of glucose, 1% of maize extract, 0.5% of $CaCO_3$, and 0.5% of NaCl in tap water) at 28° in a rocking device (2p rpm); a solution of 3 mg of the steroid in 0.2 ml of acetone was then added and fermentation was carried out for 3 days, after which the mixture was extracted with 2 × 10 ml of chloroform. The extract was evaporated at 40-45° and was chromatographed in a thin layer of neutral alumina (see Table).

Attempt to reduce 20-ketosteroids with the native liquid separated from the mycelium. The culture was grown under the same conditions, the mycelium was filtered off, the native liquid was filtered through a Zeitz filter with 20 ml in each flask, followed by 3 mg of Reichstein's substance S (V) in each case, and the flasks were shaken on the shaking machine at 28° for 2 days. To each of a number of the flasks 3 mg of NAD·H_2 was added.

The culture was grown as described above, but in the presence of progesterone (X) (5 mg per 100 ml of culture liquid). To each of the resulting filtrates, we added 3 mg of substance S (V) both with NAD·H_2 and without it.

The culture of A. albus 3006 was grown as described above, but in the presence of substance S (V) (5 mg per 100 ml of culture liquid). To each of the filtrates, 3 mg of progesterone (X), with and without the addition of NAD·H_2 was applied.

In no case did reduction take place.

Attempt to oxidize 20β-hydroxysteroids by means of a culture of A. albus 3006. A culture of A. albus 3006 was grown for 24 hr under the conditions described above and to each 100 ml of culture was added 10 mg of 20β-hydroxypregn-4-en-3-one or (to some of the flasks) 17α, 20β-21-trihydroxypregn-4-en-3-one. Fermentation was carried out for 48 hr.

The experiment was repeated in a culture grown in the presence of progesterone (X) or substance S (V) (5 mg per 100 ml of culture liquid).

In no case did oxidation of the 20β-hydroxy group take place.

Production of a cell-free preparation of A. albus 3006. The culture was grown for 6 hr in flasks each containing 100 ml of nutrient medium (0.02 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4$, 0.1 g of NaCl, 0.3 g of $CaCO_3$, 0.1 g of K_2HPO_4, and 1 g of starch in tap water), a solution of 0.5 mg of progesterone (X) in 0.5 ml of alcohol was added, and the growth of the mycelium was continued for a further 18 hr.

The mycelium was filtered off and was washed with double-distilled water, a 0.001 M solution of EDTA, and a 0.05 M phosphate buffer (pH 7.4). The washed mycelium was suspended in 20 ml of phosphate buffer prepared in a 0.001 M solution of EDTA (pH 7.4), kept for 12-20 hr at −70°, thawed, and disintegrated for 12 min in 10 ml portions at 0-6° in a ULA-250 generator of ultrasonic vibrations. The disintegrated mycelium was centrifuged at 13 500 g (5-8°, 45 min). The supernatant liquid was separated off and stored at the temperature of dry ice. The absence of living cells was checked by microscopy and culturing on a nutrient medium.

The activity of the preparation was measured by means of a SF-4 spectrophotometer with respect to the change in the intensity of adsorption (ΔD) at 340 mμ. The change in the optical density during 1 minute caused by 1 mg of protein was taken as the unit of activity:

$$A = \frac{\Delta D}{t \cdot c} .$$

The total amount of protein was determined from the formula [6]

$$c(\text{mg/ml}) = 1.45 \cdot D_{280} - 0.74 \cdot D_{260} .$$

The rate of the enzymatic reaction was measured at 26° in a thermostated chamber. A 3-ml cell with a path length for light of 1 cm was charged with 2.5 ml of phosphate buffer prepared in a 0.001 M solution of EDTA, 0.2 ml of cell-free preparation, 0.03 ml of a $7 \cdot 10^{-3}$ M solution of NAD·H_2, and 0.02 ml of a $2 \cdot 10^{-2}$ M solution of steroid in alcohol. The comparison cell was charged with the same components except for the steroid. The optical density was measured at 340 mμ at the beginning of the reaction (D_0) and after 2.5, 5, 10, and 15 min from the beginning of the reaction. The rectilinear part of the curve was used for calculating the initial velocity. The value obtained for the initial velocity of the reduction of the steroid (V_X) was related to the velocity of the conversion of progesterone (X), (V_{pr}), and V_{rel} was obtained.

Determination of the inductive capacity. A cell-free preparation of steroid oxidoreductase was obtained by the method described above. Instead of progesterone, the same amount of the steroid the inductive capacity of which was to be measured was added to the growing mycelium.

The conversion of progesterone by the cell-free preparation obtained was determined spectrophotometrically and was checked by thin-layer chromatography on alumina.

Attempt to oxidize testosterone (XII) with a cell-free preparation obtained from the mycelium of A. albus 3006. A culture of A. albus 3006 was grown under the conditions described above. After 6 hr, some of the flasks were treated with 5 mg of estradiol (XI) each, and others with 5 mg of progesterone (X); no steroid was added to some of the flasks. The culture was grown for a further 42 hr. Cell-free preparations were obtained by the method described above. The conversion of testosterone (XII) by the cell-free preparations obtained without the addition of a co-enzyme and with the addition of NAD or NADP were investigated. In no case did conversion take place.

Oxidation of 20β-hydroxypregn-4-en-3-one by a cell-free preparation. A cell-free preparation was made by the method described above. A 3-ml cell was charged with 2.5 ml of phosphate buffer prepared in a 0.001 M solution of EDTA, 0.2 ml of the cell-free preparation, 0.03 ml of a $7 \cdot 10^{-3}$ M solution of NAD, and 0.02 ml of a $2 \cdot 10^{-2}$ M solution of 20β-hydroxypregn-6-en-3-one. The comparison cell was charged with the same components with the exception of the steroid. The optical density was measured as follows: $D_0 = 0.0$; $D_2 = 0.52$; $D_3 = 0.066$; $D_4 = 0.079$; $D_5 = 0.089$; consequently, the velocity of conversion

$$V_x = \frac{0.052}{2} = 0.026.$$

Chromatography of the mixture obtained as a result of the enzymatic reaction on plates with thin fixed layers of silica gel in the benzene-acetone (4:1) system showed the presence of progesterone and the initial 20β-hydroxypregn-4-en-3-one.

Reduction of progesterone (X) with zinc in acetic acid. A solution of 600 mg of progesterone in 300 ml of glacial acetic acid was purged with carbon dioxide and then with vigorous stirring 30 g of zinc dust activated with hydrochloric acid was added to the flask in portions over 1 hr.

After the end of the addition, the mixture was shaken for a further 1 hr, after which the zinc dust was filtered off and was washed with acetic acid. The washing solution was combined with the main filtrate and 300 g of ice was added. The mixture was neutralized with caustic soda and was extracted with 4 × 500 ml of ether. The extract was evaporated to a volume of 200 ml, washed with 200 ml of 5% sodium carbonate solution and with water, and dried over $MgSO_4$. After evaporation in vacuum, 464.5 mg of colorless crystals were obtained, part of which melted at 99-103° and part at 122-126°. Fractional crystallization from methanol gave 180 mg of 5α-pregn-2-en-20-one (XIII) with mp 129-132°, $[\alpha]_D$ + 144° (5.3 mg in 0.5 ml of $CHCl_3$).

IR spectrum: 1705 cm^{-1} (20-CO).

NMR spectrum: 0.55 (18-CH_3); 0.75 (19-CH_3); 1.96 (21-CH_3); 5.07; 5.26; 5.37; 5.56 τ [CCl_4, standard $Si(CH_3)_4$].

Found %: C 84.14; H 10.90. $C_{21}H_{32}O$. Calculated %: C 83.94; H 10.73.

In addition, 82.6 mg of 5β-pregn-2-en-20-one (XIV) with mp 137-141°, $[\alpha]_D$ + 98° (5.35 mg in 0.5 ml of $CHCl_3$) was isolated.

IR spectrum: 1702 cm^{-1} (20-CO).

NMR spectrum: 0.55 (18-CH_3); 0.94 (19-CH_3); 1.95 (21-CH_3); 5.15, 5.3, 5.5 τ [in CCl_4, standard $Si(CH_3)_4$].

Found %: C 84.03; H 10.83. $C_{21}H_{32}O$. Calculated %: C 83.94; H 10.73.

Literature data for 5α-pregn-2-en-20-one: mp 125°, $[\alpha]_D$ + 141° [8]; mp 125-128°, $[\alpha]_D$ + 152° [9].

SUMMARY

In a comparison of the substrate specificity of the action of a culture of Actinomyces albus 3006 and a cell-free preparation obtained from the mycelium of A. albus 3006, it was found that the spectrum of the action of the steroid oxidoreductase of A. albus is broader than the spectrum of the action of the culture. Some of the factors responsible for the difference in the specificity of the action of the culture of the microorganism and the enzyme preparation are examined.

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