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A REINVESTIGATION OF THE MECHANISM OF *PSEUDOMONAS TESTOSTERONI* Δ^5-3-KETOSTEROID ISOMERASE

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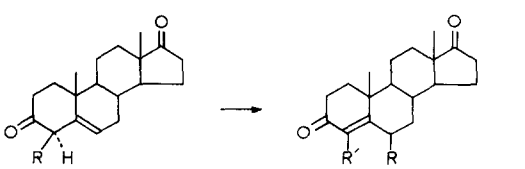
Summary

The mechanism of the isomerisation of Δ^5-3,17-androstenedione by the isomerase (3-oxosteroid Δ^4—Δ^5-isomerase, EC 5.3.3.1) of *Pseudomonas testosteroni* has been reinvestigated with Δ^5-[4-β-^{2}H]androstenedione as substrate in H_2O and Δ^5-androstenedione in ^2H_2O. A precise localisation of the label in Δ^4-androstenedione has revealed that the previously reported $4\beta \rightarrow 6\beta$ deuterium transfer accounts for only a part of the reaction. Along with this process, removal of the 4α proton is also occurring. This has already been observed with mammalian isomerases. Hence the assumed difference in mechanism between the bacterial and mammalian enzymes is very unlikely.

Introduction



Scheme 1. The ketosteroids 1 and 2.

The isomerisation of Δ^5-3-ketosteroids is carried out by an induced bacterial enzyme (3-oxosteroid Δ^4—Δ^5-isomerase, EC 5.3.3.1) of *Pseudomonas testosteroni*, which has been intensively studied [1—6]. Since the classical work of Talalay and Wang [1] and Malhotra and Ringold [2], this reaction is often quoted in the literature [7—10] as an example of a highly stereoselective intramolecular isomerisation. According to these authors, 1a (see scheme above)

is converted into 2 in ^2H_2O with negligible deuterium incorporation, whereas 1b gives stereospecifically 2b, in H_2O.

In mammals, the course of this isomerisation, the last step of progesterone and Δ^4-3,17-androstenedione biosynthesis, is not so well understood and conflicting results have been published [11—14]. The discrepancies may have several origins, as recently pointed out by Murota et al. [14]. An important one is certainly the difficulty in locating precisely the label between positions 4 and 6 in Δ^4-androstenedione. With the exception of Fukushima et al. [13] who used chemical exchange *, the other authors either did not attempt this localisation or used the infrared method which is very inaccurate because of the weak intensity of the C-^{2}H bands.

As we intended to study further the mechanism of beef adrenal isomerase reaction, in connection with Alfsen and coworkers [15], we developed a routine technique for the localisation of deuterium and checked carefully its validity.

While testing this method in the case of the bacterial enzyme isomerisation, we found results which are not in agreement with those of Malhotra and Ringold [2].

Materials and Methods

Deuterated water (99.8%) was obtained from CEA (France).

Total deuterium content in all the compounds was determined by mass spectrometry with an AEI MS-30 mass spectrometer. ^{1}H NMR spectra were obtained with a Varian HA-100 spectrometer at 100 MHz, and ^{2}H spectra with a Varian XL-100 spectrometer at 15.4 MHz.

Synthesis of 1b. The substrate 1b is prepared according to ref. 2 by oxidation of Δ^5-3β,17β-androstenediol containing 1.0 ^{2}H in 4β; but there is some deuterium loss during this oxidation and 1b contains only 0.75—0.9 ^{2}H, depending on the runs. The results given in Table I are recalculated for a compound containing 1.0 ^{2}H.

Enzymic conjugation

Isomerisation in H_2O. 20 mg of 1b dissolved in 20 ml of C_2H_5OH are added to 500 ml of phosphate buffer, pH 7, (0.03 M, kept at 25—26° C) followed by 6000 units of pure bacterial enzyme prepared as described in [16]. The reaction is monitored at 248 nm. When the A_{248} is constant and has reached its theoretical value, the product is extracted with ethylacetate, washed, dried and purified by thin layer chromatography. We checked that 2b (chemically synthesised) does not lose deuterium on silica gel.

Isomerisation in ^2H_2O. 10 mg of 1a dissolved in 10 ml of $C_2H_5O^2H$ are added to 75 ml of phosphate buffer, pH 7, in ^2H_2O (0.03 M, kept at 25—26° C), fol-

* In this work, the complete loss of tritium observed after reduction of the Δ^4 double bond and base equilibration in convincing. But the tritium retention in the exchange of Δ^4-androstenedione with HCl, diglyme is meaningless. We checked that neither 2b nor 2c loses ^{2}H under the conditions used by Fukushima et al. If the acid concentration and the temperature are increased, the exchange occurs, but ^{2}H in positions 4 and 6 is equally affected.

lowed by 3000 units of enzyme (which have been lyophilised and redissolved in ^2H_2O). The mixture is then treated in the usual way.

In both cases, the spontaneous isomerisation, evaluated by following the A_{248} of a blank solution of buffer and substrate is always below 5%.

Results

Deuterium localisation in Δ^4-3,17-androstenedione

We have recently shown that the localisation of deuterium between positions 4 and 6 by mass spectrometry is impossible on compound 2, but can be achieved on the corresponding (5α) saturated compound [17]. However, this requires a stereospecific hydrogenation occurring without loss or scrambling of the label, and well adapted to small amounts. We checked several techniques on authentic samples of 2b and 2c, chemically prepared [17].

The enzymic reduction by *Nocardia Corallina* according to [18], gave us rather low yields and occurs with loss of 33% of the C_4 deuterium *.

Hydrogenation either with $(\phi_3P)_3RhCl$ (30 bars) or 5%Pd/C occurs without any deuterium loss or scrambling, but gives a mixture of (5α) and (5β) isomers which cannot be separated by thin layer chromatography. Hence, direct determination by mass spectrometry cannot be used and we have to apply the exchange technique: when the mixture of isomers obtained by reduction over Pd/C is treated with 0.25 N methanolic NaOH for 1.5 h, the deuterium located on carbon 4 is selectively exchanged. Hence the deuterium content of C-4 is the difference between total content of deuterium in compound 2 and in the saturated product after enolization. The sequence: isomerisation, purification by thin layer chromatography, hydrogenation, exchange, purification by thin layer chromatography can be carried out on a small scale (approx. 10 mg). The error is at most 5% for the C-6 deuterium (the error on a mass spectrometric determination) and 10% for the C-4 deuterium which requires two mass spectrometric determinations.

The course of the enzymic reaction

The results in Table I represent the average of four experiments for run 1 and two experiments for run 2.

The total amount of retained or incorporated deuterium is about the same as in previous experiments: 74% retention in run 1 (Malhotra and Ringold found 65%) and 22% incorporation in run 2 (12% in Talalay's work, in a medium containing 89% D_2O).

But, contrary to the claim of Malhotra and Ringold, we find a high percentage of deuterium at position 4 in both runs. Because of this disagreement, we confirmed the presence of deuterium on carbon 4 by ^1H and ^2H NMR in compounds obtained in run 1.

According to the ^1H NMR spectrum, the amount of deuterium at C-4, esti-

* Fukushima et al. [13] have carried out this reduction, without loss of the C-4 ^3H by the (5-α) reductase of female rat microsomes but it is not a convenient technique for a systematic study, and loss of the C-4 label has been observed by Björkhem [19] with this system for a related substrate.

TABLE 1

DEUTERIUM RETENTION OR INCORPORATION DURING THE ENZYMIC ISOMERISATION

Run	Substrate and medium	Total ^2H	^2H on C-4	^2H on C-6	^2H on C-4/ Total ^2H
1	1b-H_2O	0.74	0.21	0.43	32%
2	1a-^2H_2O	0.22	0.10	0.12	43%

mated on the integral of H-4 (δ = 5.7 ppm) using the 18-methyl (δ = 0.92 ppm) as an internal standard is 0.43 D.

The presence of deuterium at C-4 was also directly observed on the ^2H NMR spectrum, which exhibits two peaks, at 5.7 and 2.0 ppm. The 5.7 ppm signal corresponds to ^2H-4 since the δ_{2H} values are identical to the corresponding δ_H values [20]. The signals of H-6α and H-6β being not readily identified on the ^1H spectrum, the 2.0 ppm peak was attributed to the D-6β by comparison with the ^2H spectrum of an authentic sample of 2b. The relative areas of the two peaks are 22 : 78.

Owing to the rather low accuracy of the NMR determination *, the results obtained by the three methods are quite in agreement.

It is clear that the 4β proton is not completely removed and transfered during the conjugation with the *P. testosteroni* isomerase.

In their study of the conversion of [4β-^3H]cholesterol into coprostanol involving a Δ^5- to Δ^4-isomerisation, Björkhem and Gustafsson [21] and Permentier and Eyssen [22] have observed that a few percent of tritium were retained in the 4 position. This is probably due to the same phenomenon.

Discussion

Several hypotheses have to be considered to explain this result.

The spontaneous isomerisation, cannot account for it: the enzymic reaction reaches completion within 5 min under the conditions we are using. We checked that during this time, the spontaneous isomerisation in the phosphate buffer, pH 7, is less than 5%.

The reversibility of the reaction, which could also have been at the origin of this result has been excluded: when 2b is submitted to the enzymic reaction, under the same conditions as 1b, and the deuterium localized as described, no loss nor migration is observed.

The presence of ^2H at 4α in 1b was also ruled out. The deuterium retention at C-4 could be easily explained by the mechanism of Malhotra and Ringold if there is some ^2H at 4α in the starting material. This is a priori possible since during the oxidation of Δ^5-3,17-[4β-^2H]androstenediol into 1b (without conjugation), there is some deuterium loss. This must happen through an enolisation, reprotonation mechanism. Some epimerisation of the 4β-^2H could also have occurred by the same process.

* In the ^1H spectrum the reference methyl peak is not well separated and in the ^2H spectrum the area of a weak resonance is frequently underestimated.

But we checked by ^1H NMR * that the samples of 1b we have used are specifically deuterated at the 4β position.

It is then necessary to conclude that along with the 4β → 6β migration found by Malhotra and Ringold, the isomerisation partly occurs, competitively, by abstraction of the 4α proton, leaving the deuterium at position 4. Several processes can still be considered for this part of the reaction.

Intramolecular 4α → 6α migration.

Removal of the 4α proton and 4β protonation of the so formed enol (or enolate), resulting in the epimerisation of deuterium, followed by intramolecular migration of the 4β proton. The deuterium incorporation pattern, in the reaction in ^2H_2O, shows that protonation at position 4 is occurring.

Removal of the 4α proton and protonation of the enol (enolate) at carbon 6.

Before attempting a complete description of this complex reaction we think that it is necessary to have the results of the enzymic isomerisation of the 4α-deuterated Δ^5-androstenedione that we are presently synthesizing.

But it may be already pointed out that the common assumption of a difference in mechanism between the bacterial and the mammalian enzymes is very probably unjustified.

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* In 1 the 4α and 4β signals are well resolved, appearing respectively at 2.85 and 3.28 ppm. The comparison of the integrals of H-4α and H-6 (δ = 5.25 ppm) shows that there is no deuterium in 4α. For a sample containing 0.90 ^2H (mass spectrometry), we observed by accumulating the signals of H-4α and H-4β a broad singlet corresponding to H-4β, the area of which is equal to 10% of the H-4α area.

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