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A REINVESTIGATION OF THE MECHANISM OF Δ^5-3-KETOSTEROID ISOMERASE FROM BOVINE ADRENAL GLANDS

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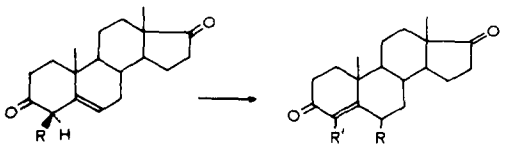
The mechanism of the isomerization of androst-5-ene 3,17-dione by the isomerase of bovine adrenals has been reinvestigated using the methodology previously developed for the study of the bacterial enzyme of *Pseudomonas testosteroni* However, owing to the lower activity of the mammalian enzyme, competitive non-enzymic reaction cannot be neglected It has been shown that even in the absence of spontaneous isomerization, epimerization and exchange of the label on C4 takes place in the buffer This prevents any quantitative discussion of the course of the reaction It is however possible to conclude that the mechanism we have proposed for the bacterial enzyme, that is, besides the classical $4\beta \rightarrow 6\beta$ transfer and exchange with the medium, a competitive abstraction of the 4α proton, accounts for the data obtained with the mammalian microsomes

Introduction

The isomerization of Δ^5 into Δ^4-3-ketosteroids (Scheme 1) by the isomerase of *Pseudomonas testosteroni* has been shown to involve a stereospecific $4\beta \rightarrow 6\beta$ hydrogen transfer [1–2]

This isomerization has also been investigated in different animal tissues [3–7] but in this case, the results concerning the stereospecificity of the removal of the protons on C4 and the occurrence of an intramolecular $4\beta \rightarrow 6\beta$ hydrogen transfer are quite conflicting

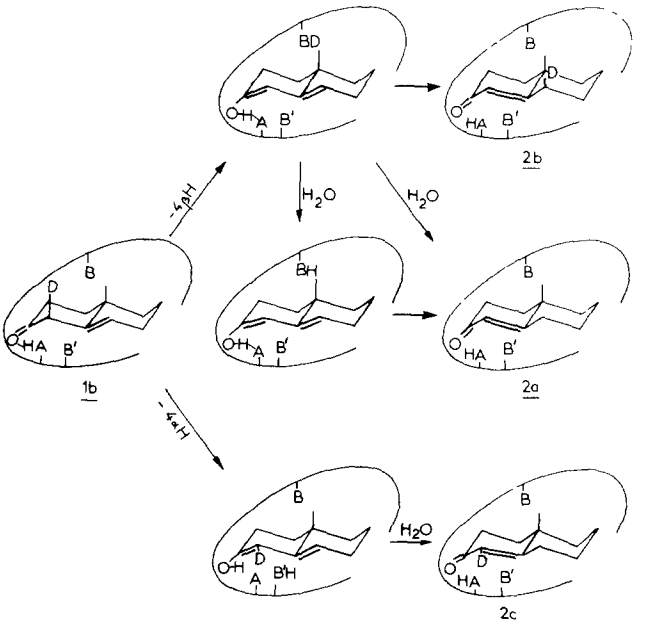
However, our recent study of the bacterial enzyme



1 a R = H
b R = D

2 a R = R' = H
b R = D R' = H
c R = H R' = D

Scheme 1 Isomerization of androst-5-ene 3,17-dione 1



Scheme 2 Reinvestigated mechanism for the bacterial isomerase of *P testosteroni* [8]

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[8] has shown that the mechanism is indeed more complex than described in addition to the classical $4\beta \rightarrow 6\beta$ transfer and some exchange with the medium, it implies a competitive abstraction of the 4α proton (Scheme 2)

These results prompted us to reinvestigate the mammalian enzyme catalysed isomerization, using the methodology developed in the study of the bacterial enzyme [8]

Materials and Methods

All chemicals were of the highest purity available ^2H_2O was purchased from C E A (Saclay-France) and contained 99 9% ^2H Compound 1b was synthesized according to the method of Malhotra and Ringold [2] and contained more than 0 95% ^2H in the 4β position (checked by ^1H-NMR [8])

The mass spectra were recorded on a AEI MS-30 spectrometer or a Varian CH-5 at 70 eV The experimental error on each spectrum was assumed to be 3% The ^1H-NMR spectra were obtained with a Varian HA-100 spectrometer at 100 MHz

The bovine adrenal microsomes were prepared according to Geynet and coworkers [9] The enzyme used in the ^2H_2O assays were previously exchanged by resuspension in ^2H_2O buffer and centrifugation

Enzymic isomerization 10 mg compound 1b in 10 ml C_2H_5OH were added to 350 ml 0 02 M Tris-HCl buffer (pH 8 5)/5 mM $CaCl_2$ or $MgCl_2$ at 25°C A 1-ml aliquot was used to measure the spontaneous isomerization After addition of 1 ml microsomal enzyme (4 700 U/ml) the reaction was monitored at 248 nm After 1 h (A_{248} constant) the mixture was extracted with ethyl acetate, washed, dried and purified by TLC The deuterium content and localization were determined as previously described [10]

10 mg compound 1a dissolved in 10 ml $C_2H_5O^2H$ were added to 50 ml 0 02 M Tris-$^2HCl/^2H_2O$ buffer (pH 8 5)/5 mM $MgCl_2$ 1 ml of exchanged enzyme (approx 4 700 U/ml) was then added and the mixture was treated in the usual way

Incubation of compound 1b under non-isomerizing conditions Compound 1b was left for 1 h in Tris-HCl buffer, then extracted with benzene and the deuterium stereochemistry was checked as previously described [8]

The same experiment was carried out with inactivated enzyme The inactivation was achieved either by heating at 70°C for 19 h or by a 200 mM solution of the 5,10-seco estr-5-yne 3,10,17-trione [8], [11] for 24 h

Results

It is not possible to compare the bacterial and mammalian enzymes under identical conditions since the optimum pH for both reactions is different we used a phosphate buffer (pH 7) for the bacterial enzyme and a Tris-HCl buffer (pH 8 5) for the bovine microsomes Furthermore, the reaction time is longer for the mammalian enzyme due to the low activity of the microsomal fraction

TABLE I

ISOMERIZATION OF 1 WITH THE BOVINE MICROSOMES AT 25°C

Substrate	Medium	Total deuterium in the product (retained or incorporated	% of monodeuterated molecules on C4 (2c)	% of monodeuterated molecules on C6 (2b)
1b	Tris-HCl/H_2O pH (8 5)	0 28 $^2H_1$	19	9
1a	Tris $^2HCl/^2H_2O$ (pH 8 5)	0 29 $^2H_1$ 0 14 $^2H_2$ *	16	13

* The dideuterated molecules are labelled on C4 and C6 (cf Ref 22 of Ref 8)

TABLE II

INCUBATION OF 1b UNDER NON-ISOMERIZING CONDITIONS AT 25°C FOR 1 H IN TRIS-HCl BUFFER (pH 8 5)

Condition	Deuterium remaining in the substrate	Deuterium epimerised
Without microsome	0 68	0 27
With the thermally denaturated microsomes	0 56	0 27
With the microsomes inactivated by the kcat inhibitor	0 57	0 29

The total amount of deuterium in the conjugated product 2 is determined by mass spectroscopy The amount of deuterium on C6 is also determined by mass spectroscopy, after reduction of the double bond and selective exchange on C4 [8,10]

The results of the isomerization of androst-5-ene 3,17-dione 1 with the mammalian isomerase are given in Table I

The problem was then to determine if non-enzymic processes contribute to this isotopic distribution

We have already demonstrated by ^{1}H-NMR that compound 1b was stereospecifically deuterated in the 4β position [8] and we checked that the spontaneous isomerization remains almost negligible (0–6%, depending on the run) in Tris-HCl buffer (pH 8 5) in 1 h

In our recent extensive work on the bacterial enzyme [8] we have shown that although epimerization and loss of deuterium may occur in the buffer in the absence of isomerization, the enzymic isomerization is so fast that the competitive reactions can be neglected It is however no longer possible to ignore them in the case of the mammalian enzyme 30% of the initial deuterium is lost and 27% epimerized (the deuterium stereochemistry on C4 of the non-isomerized substrate is checked as usual [8], after reduction of the 3- and 17-keto groups and thiobenzoylation of the diol followed by photolysis [12]) when compound 1b is incubated in the conditions used for the enzymic reaction (Tris-HCl buffer, pH 8 5, for 1 h, see Table II) (The problem cannot be solved by using the same buffer as for the bacterial enzyme (phosphate, pH 7) The epimerization in this buffer is of the same order of magnitude and the isomerization much slower) These reactions occurring on the substrate before isomerization have not been considered in previous studies We think that they may partly explain the discrepancies between the published results since their importance may depend on the experimental conditions and on the magnitude of the isotopic effect

In the case of microsomes, further exchange or epimerization can be catalyzed by other components of the crude system As suggested by several authors, the differences observed in the preceding studies [3–7] could be a consequence of these competitive reactions To test this point we have inactivated the isomerase by heating or by treatment with the Kcat inhibitor of Batzold and Robinson [11] (5,10-seco estr-5-yne, 3,10,17-trione) as previously done for the bacterial enzyme [8], and we have incubated the 4β-deuterated substrate with these preparations The results are given in Table II The epimerization of the label is very similar in the three experiments The exchange is slightly larger in the incubations with the inactivated enzymes, but the differences are close to the experimental error and we can conclude that non-enzymic reactions due to the microsomal nature of the system do not play a significant role

Discussion

The isomerization of compound 1b with the mammalian microsomes yields a mixture of compounds 2a (72%), 2b (9%) and 2c (19%) (Table I), that is the same compounds, although in different amounts, as after isomerization of compound 1b with the bacterial enzyme of *P testosteroni* [8] (25% of 2a, 50% of 2b, 25% of 2c)

The importance of the buffer-catalyzed loss and epimerization of deuterium in the substrate prevents a detailed discussion of the enzymic part of the reaction It is however clear that it contributes to the deuterium loss (70% with active microsomes, 30% in the blank)

With the bacterial enzyme, the presence of compound 2c was attributed to an epimerization of the label within the active site followed by the 4$\beta \rightarrow 6\beta$ transfer Here it is difficult to distinguish the relative

contributions of the enzyme and of the buffer in the epimerization

Since some product deuterated on C6 (2b) is recovered it implies that the intramolecular transfer $4\beta \rightarrow 6\beta$ encountered with the bacterial enzyme is also occurring with the mammalian microsomes It is much less important (9% instead of 50%) but significantly higher than the experimental error (9% is a minimal value since the amount of deuterium on the 4β position is lowered by the epimerization and exchange occurring before the isomerization step The error on the deuterium content, determined by mass spectroscopy is assumed to be 3%) This is an important point since, excluding the work of Murota et al [3], most of the former studies concluded that this pathway did not occur Murota et al [3], using a deuterated buffer observed a low incorporation of deuterium and thus concluded that the intramolecular migration was probably the predominant pathway We have demonstrated with the 4β-deuterated substrate that it was indeed a minor pathway But we also observe a low incorporation of deuterium when the isomerization is carried out in ^2H_2O This is very likely due to an incomplete exchange of the microsomes

Akhtar et al [7] have recently studied a placental isomerase where the intramolecular transfer has not been demonstrated As they have pointed out, this is compatible with two mechanisms The removal of the hydrogen on C4 and the protonation on C6 may be mediated by two different groups or, as in the case of the bacterial enzyme, the same group may be operating, the exchange of $B^{-2}H$ of B-H (Scheme 2) with the medium being faster than the protonation of C6 The intramolecular transfer that we have observed, although low, strongly favours the second hypothesis, at least for bovine adrenal micromes

It is tempting to conclude that all the isomerases from bacterial or mammalian origins work according to the same mechanism involving competitive processes (competitive abstraction of the 4α and 4β protons, intramolecular hydrogen migration vs hydrogen exchange), the relative importance of which varies with the system under investigation

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