Is a Schiff Base Involved in the Mechanism of the Δ^4 -3-Oxo Steroid 5α - or 5β -Reductases from Mammalian Liver?

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The saturation of the Δ^4 -double bond of Δ^4 -3-oxo steroids by the mammalian 5α - or 5β -reductases is by an enzyme mechanism that does not involve a Schiff-base intermediate.

The most important event in the metabolism of non-aromatic mammalian steroid hormones (I) (Scheme 1) is the saturation of the Δ^4 -double bond to give the corresponding dihydro steroid (II). There are present in the liver two NADPH-linked enzyme systems that catalyse this reaction, the microsomal 5α -reductase (EC 1.3.1.22) and the cytosolic 5β -reductase (EC 1.3.1.23) (Schriefers, 1967, and references cited therein). Both these reactions involve the direct transfer of a hydride ion to the C-5 position of the substrate, with proton uptake at C-4 (Berseus & Bjorkhem, 1967; Bjorkhem, 1969; Wilton & Ringold, 1968), and it is the stereochemistry of the new C-H bond at C-5 that distinguishes these two enzyme reactions.

The orientation of addition of the hydride ion and proton to the double bond is what would be predicted if reduction was assisted by the electron-withdrawing properties of the C-3 oxo group, since under these conditions the C-5 position is electron deficient.

In view of the now well-established requirement for proton-mediated substrate activation before hydride transfer (Wilton *et al.*, 1966, 1968; Watkinson *et al.*, 1971a,b; Akhtar *et al.*, 1972; Bloxham *et al.*, 1975) in the reduction of double bonds, the most reasonable mechanism for these two reactions would be one involving initial protonation of the carbonyl oxygen atom followed by hydride ion addition from the cofactor to C-5 and a resulting isomerization of the double bond to give an enol intermediate (IV). Subsequent collapse of the enol will result in an enzyme-mediated proton uptake at C-4 (Scheme 1; Mechanism 2). In this mechanism the enzyme enhances the electron-withdrawing capacity of the carbonyl group by protonation, which will facilitate hydride transfer to the electron-deficient C-5 position.

Another mechanism (Scheme 1; Mechanism 1) for the reduction of the Δ^4 -double bond that may be deduced from an examination of the biochemical literature involves the formation of a protonated Schiff-base intermediate (III) that polarizes the double bond to give a highly electron-deficient C-5

carbon atom suitable for hydride transfer. The feasibility of such a mechanism has recently been highlighted by studies with model compounds in which it was shown that synthetic dihydropyridines effectively reduced the carbon-carbon double bond of testosterone acetate if the 3-oxo compound was first condensed with an amine to give a protonated iminium salt (Pandit *et al.*, 1974).

The involvement of a Schiff base in an enzyme mechanism may be readily demonstrated by using substrate with an ^{18}O -labelled carbonyl group. Involvement of a Schiff-base intermediate will result in an obligatory loss of all the ^{18}O to the medium and the product will be unlabelled.

Experimental

Preparation of [3- ^{18}O]-testosterone and 5α -dihydro-testosterone

The method was based on that of Lawson *et al.* (1969). The steroid (20 mg) in dry propan-2-ol was placed in a glass tube and to this was added 4 mm-HCl in propan-2-ol (0.1 ml) and H_2^{18}O (0.1 ml; 12.0% in excess). The vial was sealed in air and refluxed at 62°C (boiling methanol) for 24 h. After addition of water and extraction into diethyl ether, the diethyl ether layer was washed with water, dried (over anhydrous Na_2SO_4) and evaporated to dryness. Crystallization from acetone/light petroleum (b.p. 60–80°C) gave testosterone (18 mg), having an ^{18}O content of 11.0% in excess. An ^{18}O value of 8.2% in excess was obtained for 5α -dihydrotestosterone. This lower value is due to some loss of label during the work-up procedure, because of the extreme lability of this carbonyl oxygen atom.

Preparation of the mammalian 5α -reductase and incubation procedure

Female Wistar albino rats (200 g) were killed by cervical fracture and the excised livers were homogenized in 0.25 M-sucrose by using a motor-driven

Potter–Elvehjem homogenizer. The 105 000 g_{av} . microsomal fraction was prepared from this homogenate, which after washing by re-centrifuging in 0.25 M-sucrose at 105 000 g_{av} . for 30 min was stored frozen at -15°C as a suspension (2 g equivalent of original liver/ml of 0.25 M-sucrose).

Incubations were carried out in glass test tubes under conditions described in Table 1. The incubations were terminated by the addition of methanol (1 ml) and extracted with methylene chloride (2×3 ml). The combined extracts were washed with water (3 ml), dried (over anhydrous Na_2SO_4) and evaporated to dryness. The residue was redissolved in diethyl ether (0.1 ml) and analysed by g.l.c.–mass spectrometry.

Preparation of the Δ^4 -3-oxo steroid 5β -reductase and incubation procedure

Male Wistar albino rats (200 g) were killed by cervical fracture and the excised livers were homogenized in 2 vol. of 0.1 M-sodium phosphate buffer, pH 7.4, containing 4 mM- MgCl_2 , in a loose-fitting Potter–Elvehjem homogenizer. The 105 000 g_{av} . supernatant fraction was prepared and used for the incubations. Incubations (1.6 ml) were carried out in glass test tubes containing cytosolic 5β -reductase system (1 ml), 1.25 μmol of NADP^+ , 5 μmol of glucose 6-phosphate, 20 nkat of glucose 6-phosphate dehydrogenase and testosterone (200 μg in 0.1 ml of methanol). The rest of the procedure was identical with that for the 5α -reductase experiments described above. The major product, 5β -tetrahydrotestosterone, was analysed by combined g.l.c.–mass spectrometry. The testosterone was completely metabolized under the conditions of the experiment.

Measurement of ^{18}O content

All incubations were carried out with both ^{16}O - and ^{18}O -labelled steroids to allow calculation of the ^{18}O content of the ^{18}O products. The steroid mixtures were subjected to g.l.c.–mass spectrometry on an AEI MS30 instrument. Separations were carried out on a 152 cm 3% E30 or a 274 cm 3% OV1 column operating at 230°C or 300°C respectively.

Although the 5α -dihydro- and 5α -tetrahydrotestosterone products were readily resolved, the 5β -tetrahydro compound was incompletely resolved from the smaller amount of 5β -dihydrotestosterone and hence accurate ^{18}O mass measurements of the latter compound were not possible in view of the low concentration of ^{18}O used in these experiments.

Results

The substrate of choice for these experiments was testosterone, because as well as being one of the better substrates for both the 5α - and 5β -reductase

systems it may be readily and unambiguously labelled with ^{18}O at the C-3 position.

Studies with Δ^4 -3-oxo steroid 5α -reductase system

[3- ^{18}O]Testosterone was incubated with the washed microsomal fraction of female rat liver in the presence of an NADPH-generating system and the products were isolated and analysed by g.l.c.–mass spectrometry. The results shown in Table 1 indicate that there is a significant retention of ^{18}O in both the 5α -dihydrotestosterone product and its further metabolite 5α -androstane-3 α ,17 β -diol (tetrahydrotestosterone). The partial loss of ^{18}O label may be readily explained by the extreme lability of the carbonyl oxygen atom of 5α -dihydrotestosterone. This is clearly demonstrated when 5α -dihydro[3- ^{18}O]testosterone is incubated with the microsomal system, when there is a complete loss of ^{18}O from the tetrahydro product. It should be noted that in acid conditions the oxygen atom of the saturated ketone is labilized 280 times faster than that of the Δ^4 -3-oxo steroid (Lawson *et al.*, 1969). The loss of label from the 5α -dihydrotestosterone appears to be to a large extent protein-mediated, since incubation in buffer alone resulted in over 60% of the label being retained (Table 1).

The retention of ^{18}O at C-3 during the reduction of the Δ^4 -double bond rules out the possibility of a C-3 Schiff-base intermediate for this enzyme system.

Studies with the Δ^4 -3-oxo steroid 5β -reductase system

[3- ^{18}O]Testosterone was incubated with the Δ^4 -3-oxo steroid 5β -reductase system from the 105 000 g_{av} . supernatant of male rat liver. The analysis of the isolated 5β -tetrahydro compound indicated a retention of 81 and 77% of the ^{18}O label for two separate experiments, and this rules out the possibility of a Schiff-base intermediate during the course of the enzyme reduction. In these experiments there was a considerably greater retention of the ^{18}O with this system than with the 5α -reductase system detailed above (Table 1). This is most probably due to the fact that for the 5β system the rate of reduction to the dihydro compound is slower than the subsequent formation of the tetrahydro steroid and hence there is relatively less accumulation of the labile dihydro steroid in this system.

Discussion

The fact that there is retention of label after the reduction of [3- ^{18}O]testosterone by both the liver Δ^4 -3-oxo steroid 5α - and 5β -reductases rules out the possibility of Schiff-base formation (Mechanism 1) for both these enzyme systems.

Mechanism 2 must be regarded as the preferred representative of a number of mechanisms in which

Table 1. Incubation of [3-¹⁸O]testosterone or 5 α -dihydro[3-¹⁸O]testosterone with microsomal fraction from female rat liver

Each incubation (2ml) contained the steroid (200 or 400 μ g in 0.1 ml of methanol), Tris/HCl buffer, pH 7.4 (180 μ mol), glucose 6-phosphate (5 μ mol), NADPH (1.2 μ mol), 20 nkat of glucose 6-phosphate dehydrogenase and a microsomal preparation equivalent to 200 mg of liver. Expt. 3 involved incubation of 5 α -dihydro[3-¹⁸O]testosterone with (a) microsomal fraction and (b) buffer only. Incubations were for 1 h in air at 38°C. The re-isolated testosterone or 5 α -dihydrotestosterone and the biosynthesized 5 α -dihydrotestosterone and 5 α -tetrahydrotestosterone were separated by g.l.c. and analysed by mass spectrometry. N.D., not detected.

Expt. no.	Steroid incubated	¹⁸ O content of testosterone (%)	¹⁸ O content of 5 α -dihydrotestosterone (%)	¹⁸ O content of 5 α -tetrahydrotestosterone (%)
1	Testosterone (400 μ g)	10.7	5.0	4.0
2	Testosterone (200 μ g)	*	4.9	3.6
3(a)	5 α -Dihydrotestosterone (200 μ g)	—	*	N.D.
3(b)	5 α -Dihydrotestosterone (200 μ g)	—	5.2	—

* Complete conversion into products.

the carbonyl group remains intact. Four major variations of such a mechanism are in fact possible (Scheme 2).

(a) A Michael-type addition in which hydride transfer to C-5 is followed by double-bond isomeriza-

tion and the formation of a negatively charged oxygen atom. Collapse of the enolate will give the product.

(b) Initial protonation of the carbonyl oxygen followed by double-bond migration and

hydride transfer to C-5. Collapse of the enol will give the product.

(c) Initial hydride transfer to C-5 followed by proton uptake at C-4.

(d) Initial protonation at C-4 followed by hydride transfer to C-5.

Mechanisms 2(a) and 2(b) both use the C-3 carbonyl group in the actual reaction mechanism, whereas Mechanisms 2(c) and 2(d) do not. We would argue strongly in favour of the direct involvement of the carbonyl group in the reaction, since there is an obvious mechanistic advantage in achieving the maximum possible polarization of the carbon-carbon double bond before reduction. It is noteworthy that there appears to be no recorded example of the steroidal Δ^4 -double bond that has been reduced by these systems in the absence of a functional carbonyl group at C-3; this is consistent with the involvement of this group in the enzyme mechanism. As mentioned in the introduction we have previously proposed that in the nicotinamide nucleotide-linked reduction of double-bond systems the hydride-transfer step occurs to a substrate molecule already activated by protonation. The extension of this general mechanism to the reduction of the Δ^4 -double bond would require that Mechanism 2(b) and not 2(a) must operate for this system. We therefore propose that both the Δ^4 -3-oxo steroid 5 α - and 5 β -reductase from mammalian liver operate by Mechanism 2(b), in which the initial polarization of the carbonyl group is achieved by an enzyme-mediated protonation or by an enzyme-bound bivalent metal ion acting as a Lewis acid.

Such a mechanism does not operate in the model system (Pandit *et al.*, 1974), because the very low pH

that would be required to protonate the carbonyl oxygen atom would deactivate the dihydropyridine analogue. The positive charge on the nitrogen atom of the iminium salt, however, is not affected by pH and hence this compound may be readily reduced by the dihydropyridine derivative under neutral conditions.

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